Stress Responses of Foodborne Pathogens
Tian Ding • Xinyu Liao • Jinsong Feng
Editors

Stress Responses
of Foodborne Pathogens

Springer
Preface

Bacterial foodborne pathogens are exposed to a number of stressors during food processing. Bacterial pathogens can perform a series of metabolic regulations to recognize, respond to, and adapt to stress in order to survive during food production. Accordingly, activation of the stress response in foodborne pathogens can lead to decontamination failure, posing a potential threat to food safety and public health. Deciphering the molecular basis of the bacterial response to stressors is critical for developing and optimizing food preservation technologies to effectively prevent microbial contamination and maximally preserve food quality. This book is divided into 5 parts that aim to comprehensively present and discuss recent findings on the stress response of foodborne pathogens. Part I presents the impact of the stress response of foodborne pathogens on food safety and public health. Part II summarizes recent findings on the resistance of foodborne pathogens to common stressors associated with food processing (e.g., heat, cold, acid, osmosis, and oxidation). Part III addresses the developmental adaptation of foodborne pathogens to emerging nonthermal decontamination methods (e.g., ultrasound, high-pressure processing, pulsed electric fields, cold plasma, ultraviolet light, irradiation, and phytochemicals). Part IV discusses the molecular mechanisms underlying the multiple stress responses of foodborne pathogens (e.g., viable but nonculturable state, persistence phenotype, biofilm, spores, programmed cell death, and cross-adaptation response) and their associated virulent properties. Part V highlights the prospects of strategies to efficiently control the evolution of the stress response in foodborne pathogens (e.g., biomarker discovery and hurdle strategy development). The book is a valuable resource for researchers, graduate students, food processing engineers, and product developers in the fields of food science and microbiology.
We sincerely thank all authors for their contribution to this book. We would also like to thank the publisher and editorial team of Springer Nature for their cooperation and efforts.

Hangzhou, China

Tian Ding
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Part I
Introduction
Chapter 1
The Importance of Understanding the Stress Response in Foodborne Pathogens Along the Food Production Chain

Tian Ding, Xinyu Liao, and Jinsong Feng

Abstract  Foodborne pathogenic bacteria encounter various environmental stressors along the food production chain, including production, processing, storage, distribution, and preparation. Bacteria are able to initiate a sophisticated regulatory network and alter the expression of proteins and genes to respond to external stressors. This can help bacteria survive under harsh conditions, which may lead to bacterial residue in foods. Furthermore, the development of a bacterial stress response might induce cross protection against other stresses and enhance pathogenicity, which could pose potential risks to food safety and public health. Therefore, a comprehensive and adequate understanding of the molecular mechanisms underlying the stress response of foodborne pathogens is important for the development and rational design of effective sterilization processing to ensure food safety with maximum preservation of food quality.

Keywords  Foodborne pathogenic bacteria · Food production chain · Stress response · Food safety

1.1 Introduction

During recent decades, the food industry has grown rapidly to satisfy the increase in population and contributed substantially to economic development worldwide. Food safety is considered the biggest issue in the food industry and is closely related to human health. The World Health Organization (WHO) estimates that the...
consumption of contaminated food results in approximately 600 million people getting sick and 420,000 deaths each year worldwide (WHO 2015). Foodborne pathogens can lead to diarrheal diseases or serious infections and have been regarded as major contributors to outbreaks of foodborne illnesses. A surveillance report concluded that microbial food contamination is responsible for over 98% of foodborne illnesses in the United States (Tack et al. 2020). In China, microbial food contamination is also the leading cause of foodborne illness, accounting for more than 42% of total reported cases (Ren et al. 2019). The most common foodborne pathogens include *Salmonella*, *Campylobacter*, enterohemorrhagic *Escherichia coli* (e.g., *E. coli* O157:H7), *Listeria*, and *Vibrio cholerae*. The threats of these foodborne pathogens vary, with symptoms ranging from relatively mild malaise to serious, life-threatening diseases (e.g., hemolytic uremic syndrome). It is important to apply efficient technologies to achieve microbial decontamination, ensure food safety, and extend the shelf life of food.

To date, various methods have been adopted to control microbial contamination in the food industry, including low pH (acid), heat, low temperature, and novel sterilization techniques (e.g., high hydrostatic pressure, pulsed field, ultrasound, and cold plasma) (Begley and Hill 2015). Along the food production chain, foodborne pathogens tend to encounter various stresses, which are conditions that are far from suitable parameters for bacterial growth and replication (Fig. 1.1) (Zorraquino et al. 2017). Due to the sophisticated regulatory systems, bacteria are able to initiate multiple strategies against external stressors to achieve survival in harsh environments. The development of bacterial stress responses can result in decontamination failure and the persistence of microorganisms in foods or food processing environments, presenting potential risks to food safety and public health. In recent decades, an increasing number of researchers have investigated the microbial stress responses induced by food-associated stressors and the changes in associated virulence properties (Begley and Hill 2015). However, the molecular mechanisms underlying the stress adaptations employed by foodborne pathogenic bacteria are still obscure and more explorations are required in the future. An adequate understanding of the bacterial stress response is important for the rational design and implementation of food processing to produce safe foods.

1.2 Common Foodborne Pathogens and the Associated Stress Response

1.2.1 *Salmonella Species (spp.)*

*Salmonella* spp. belong to the family *Enterobacteriaceae* and are regarded as among the most common causative agents of foodborne diseases (Scallan et al. 2011). Based on the data collected by the European Centre for Disease Prevention and Control (ECDC), almost one in three foodborne outbreaks in the European Union
The common stressors encountered by foodborne pathogens along the food production chain.

**Fig. 1.1** The common stressors encountered by foodborne pathogens along the food production chain.
(EU) were caused by Salmonella in 2018, and most of those outbreaks were highly associated with the consumption of contaminated eggs (ECDC 2019). In the United States, the Centers for Disease Control and Prevention (CDC) estimated that 1.2 million foodborne illnesses with 450 deaths are caused by Salmonella each year, mainly attributed to the contamination of chickens, cucumbers, fresh-cut melons, eggs, pistachios, raw tuna, and sprouts. Salmonella spp. are Gram-negative bacteria with a diameter of 0.5–1.5 μm and a short rod shape. Generally, these bacteria are unable to produce either spores or capsules (Agbaje et al. 2011). The abundant flagella around the envelope confer upon Salmonella strong motility (Kwon et al. 2000).

Salmonella spp. can be classified into various serotypes based on flagellar proteins (H antigens), lipopolysaccharide moieties on the cell surface (O antigens), and capsular protein antigens (Vi antigen). Currently, over 2600 serotypes have been identified, and approximately 100 serotypes can infect humans (Guibourdenche et al. 2010). Among those, Salmonella Enteritidis and Salmonella Typhimurium are the serotypes that contribute to most foodborne outbreaks (Liang et al. 2015). Salmonella infection is mainly induced through the consumption of contaminated water or food. First, Salmonella passes through the mucus layer of the intestinal wall to adhere to the epithelium (Haraga et al. 2008). Subsequently, enterocytes phagocytize Salmonella into Salmonella-containing vacuoles (SCVs), where the bacteria grow and replicate (Kubori et al. 2000). Salmonella are then released from epithelial cells to the submucosa after SCVs reach the basolateral membrane. Salmonella are engulfed by phagocytes, and then SCVs are formed again. Finally, Salmonella cells disseminate through the reticuloendothelial system, leading to the further infection of organs with life-threatening symptoms. In a study by Sun et al. (2019), it was demonstrated that Salmonella infection resulted in directional electric fields in the gut lumen, which accelerated the migration of macrophages to the anode of the electric field (Fig. 1.2).

During infection processing, Salmonella employs a series of virulence factors to efficiently invade host cells. The type III secretion system (T3SS) is one of the most
important systems possessed by *Salmonella*, contributing to the secretion of effector proteins into the host cell cytoplasm for invasion (de Jong et al. 2012). Most virulence-associated genes are collected in a specific region of chromosomes or plasmids and are called *Salmonella* pathogenicity islands (SPIs) (Marcus et al. 2000). SPIs consist of several types, among which SPI-1 is the most well-characterized. It has been reported that the genes within SPI-1 play a critical role in invasion into enterocytes. The effectors encoded by SPI-1 mainly include SipABCD and SptP. SipA and SpiC are reported to contribute to the cytoskeletal rearrangements of host cells. The roles of SipB, SipC, and SipD have been associated with the secretion of effectors by the T3SS (Collazo and Galán 1997). SptP is a phosphotyrosine phosphatase that probably contributes to the alteration of actin cytoskeletons by *Salmonella* (Fu and Galán 1998). SPI-2 mainly participates in the survival of *Salmonella* within epithelial and phagocyte cells (Fàbrega and Vila 2013). There are approximately 40 genes enclosed in SPI-2, which consists of four operons of *ssa* (encoding T3SS components), *sse* (encoding secreted effectors), *ssr* (encoding secretion regulators), and *ssc* (encoding chaperone) (Kuhle and Hensel 2002; Nikolaus et al. 2001). Compared with SPI-1 and SPI-2, the research on other kinds of SPIs is limited, and the functions of some SPIs are not yet clearly understood. Fimbriae (de Jong et al. 2012), lipopolysaccharides (LPS) (Darveau and Hancock 1983), outer membrane proteins (OMPs) (Futoma-Kołoch et al. 2019), and enterotoxins (Verbrugghe et al. 2015) are also vital contributors to the virulence of *Salmonella*. Fimbriae can enhance the adherence to epithelial cells and the colonization of the mucous membrane of the small intestine by *Salmonella* (de Jong et al. 2012). LPS is located on the surface of the outer membrane of *Salmonella* and contributes to the prevention of phagocytosis and killing by phagocytes (Darveau and Hancock 1983). OMPs (e.g., lipoproteins and porins) are the major components of *Salmonella* outer membranes and contribute to the direct interactions with host cells (Futoma-Kołoch et al. 2019).

*Salmonella* are frequently isolated from food sources, including eggs, poultry, pork, beef, dairy products, nuts, mangoes, vegetables, and water (Anastasiadou and Michailidis 2016). The illness caused by *Salmonella* infection consists of two types: nontyphoidal salmonellosis and typhoid fever. Nontyphoidal salmonellosis is generally caused by all serotypes except *S.* Typhimurium and *S.* Paratyphi A. The associated symptoms include abdominal cramps, headache, nausea, vomiting, diarrhea, and fever, the onset of which is 6–72 h after exposure. Typhoid fever caused by *S.* Typhimurium and *S.* Paratyphi A can lead to symptoms of abdominal pain, diarrhea, and headache, with serious complications such as septicemia and septic arthritis. Elderly people, children under 5 years old, and people with compromised immune systems are especially vulnerable. To date, the stress response of *Salmonella* to low pH (acidic stress), osmotic pressure, extreme temperatures, and antimicrobial compounds has been studied in previous research (Table 1.1). In a study by Finn et al. (2015), *Salmonella enterica* serovar Typhimurium was found to upregulate the gluconate metabolism and osmoprotective transporters against osmotic pressure caused by sodium chloride (NaCl), potassium chloride (KCl), and glycerol, which are commonly used food humectants.
Campylobacter jejuni, one of the most common foodborne pathogens, is generally rod- and spiral-shaped. Due to their microaerophilic characteristics, C. jejuni are preferentially cultured under microaerophilic conditions (5% oxygen, 85% nitrogen, 10% carbon dioxide) (Blaser et al. 1987). To date, the precise virulence mechanism conducted by C. jejuni is not yet well known (Neal-Mckinney and Konkel 2012). Some factors have been reported to be potentially associated with the pathogenicity of C. jejuni. The flagellum, a major contributor to the motility of C. jejuni, might promote its colonization and invasion of the intestines (Grant et al. 1993). The flagellar rod of C. jejuni is surrounded by a motor, which is made up of FlgP, PflA, PflB, and MotAB units (Beeby et al. 2016). Nachamkin et al. (1993) reported that C. jejuni lacking flagella or with a compromised flagellum failed to infect three-day-old chicks, while C. jejuni with fully motile ability caused successful colonization in the chick ceca. Additionally, it has been reported that the flagellum might also work as a protein secretion system of C. jejuni, which could export effectors to regulate interactions with host cells (Barrero-Tobon and Hendrixson 2012). In addition, some proteins of C. jejuni have been reported to be associated with adherence to host cells, including Campylobacter adhesin to fibronectin (CadF), fibronectin-like protein A (FlpA), and jejuni lipoprotein A (JlpA) (Cróinín and Backert 2012). Both CadF and FlpA are located in the outer membrane of C. jejuni and contribute to specific binding to the extracellular matrix protein fibronectin of host cells (Krause-Gruszczynska et al. 2007). JlpA, a lipoprotein with a molecular mass of 42.3 kDa, has been reported to interact with heat shock protein (HSP) 90α of HEp-2 cells by Jin et al. (2003). In a study by Jin et al. (2001), it was found that C. jejuni TGH9011 with defects in the jlpA gene resulted in a greater than 15% reduction in adherence to HEp-2 cells compared with their wild-type counterparts. Unlike other pathogenic bacteria, only one kind of toxin, known as cytolethal distending toxin (CDT), was found to be produced by C. jejuni (Pickett

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CDT belongs to the holotoxins, which consist of CdtA, CdtB, and CdtC subunits. CdtB is the major effector of CDT and can cause breaks in DNA strands of host cells, resulting in the irreversible arrest of the cell cycle in the G2/M phase and further programmed cell death (Lara-Tejero and Galán 2001). CdtA and CdtC are mainly involved in the translocation of CdtB through the cell membrane of host cells. However, the exact mechanisms underlying CdtA/CdtC-assisted transportation of CdtB are not clear. Serine protease HtrA (high-temperature requirement A) is also considered an important factor for the virulence of C. jejuni (Baek et al. 2011). In the study of Brøndsted et al. (2005), defects in the htrA gene compromised the ability of C. jejuni to infect human epithelial cells. The probable function of HtrA is to cleave the adherens junctions, tumor suppressor protein E-cadherin, and tight junction protein occludin, which could temporarily open the cell-to-cell junctions in the epithelium to allow the transmigration of CdtB. However, the exact mechanisms underlying CdtA/CdtC-assisted transportation of CdtB are not clear. Serine protease HtrA (high-temperature requirement A) is also considered an important factor for the virulence of C. jejuni (Baek et al. 2011). In the study of Brøndsted et al. (2005), defects in the htrA gene compromised the ability of C. jejuni to infect human epithelial cells. The probable function of HtrA is to cleave the adherens junctions, tumor suppressor protein E-cadherin, and tight junction protein occludin, which could temporarily open the cell-to-cell junctions in the epithelium to allow the transmigration of C. jejuni (Boehm et al. 2012; Harrer et al. 2019). Harrer et al. (2019) employed immunoblotting to detect the cleavage products of human occludin by HtrA and found that a carboxy-terminal fragment (37 kDa) was produced. Zarzecka et al. (2020) used a cryo-electron microscopy at 5.8 Å resolution to reveal the detailed structure of C. jejuni HtrA, which consisted of four trimers and was a dodecamer. Apart from the aforementioned factors, some other potential virulence factors of C. jejuni proposed by previous studies are still not fully understood and should be further investigated to reveal the exact functions in vivo.

In the United States, the Centers of Disease Control (CDC) estimated that there are approximately 1.5 million illnesses caused by C. jejuni infection each year (CDC 2019). In Europe, the reported cases of campylobacteriosis reached 246,158, with a hospitalization rate of 30.5%, in 2017 (EFSA 2018). The symptoms caused by C. jejuni include diarrhea, blood in the stool, fever, and stomach cramps. In addition, C. jejuni infections probably result in the occurrence of complications, such as arthritis, irritable bowel syndrome, and even paralysis. Dairy products, poultry, and water are the primary sources of C. jejuni infection. The responses of C. jejuni to various food-associated stressors have been widely studied and are summarized in Table 1.2. The stress response might facilitate the survival of these bacteria along the food production chain. For example, Baek et al. (2011) revealed that the HtrA

<table>
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<th>Stress response</th>
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<tbody>
<tr>
<td>Acid stress response</td>
<td>Askoura et al. (2020), Reid et al. (2008)</td>
</tr>
<tr>
<td>Antimicrobial resistance response</td>
<td>Kovač et al. (2015)</td>
</tr>
<tr>
<td>Heat stress response</td>
<td>Boehm et al. (2015), Apel et al. (2012), Baek et al. (2011), Brøndsted et al. (2005), Konkel et al. (1998), Wu et al. (1994)</td>
</tr>
<tr>
<td>Cold stress response</td>
<td>Hughes et al. (2009)</td>
</tr>
<tr>
<td>Osmotic stress response</td>
<td>Cameron et al. (2012)</td>
</tr>
<tr>
<td>Oxidative stress response</td>
<td>Baek et al. (2011)</td>
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</table>
chaperone contributed to the degradation and folding of heat-injured proteins within *C. jejuni*, leading to tolerance of heat exposure at 42 °C.

### 1.2.3 Pathogenic *Escherichia coli*

*Escherichia coli*, a Gram-negative bacterium with peripheral flagella and fimbriae, is an important facultative anaerobic bacterium in the intestines of humans and animals. Most types of *E. coli* are generally harmless and live in the intestines of humans and animals as commensal flora. However, a few strains carrying virulence factors are pathogenic. Pathogenic *E. coli* can be divided into six categories according to the types of virulent factors, including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and Shiga toxin-producing *E. coli* (STEC) (Newell et al. 2010). STEC, also known as verocytotoxin-producing *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC), is the most ubiquitous type that contributes to foodborne outbreaks, and it is also called verocytotoxin-producing *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC). The United States CDC estimated that there are over 260,000 illnesses caused by STEC annually (CDC 2011), among which STEC O157 accounts for over 37%. Shiga toxin produced by STEC, also called Vero toxin, contains A and B subunits. The B subunit of Shiga toxin binds to the globotriaosylceramide-3 receptor, resulting in the internalization of *E. coli*. Subsequently, the A subunit leads to the dissociation of the N-glucosidic bond of 28S rRNA in eukaryocyte, which inhibits the biosynthesis of proteins and causes the death of host cells (Endo et al. 1988). Additionally, the intimin produced by STEC participates in adherence to intestinal epithelial cells. Effectors, such as translocated intimin receptor (Tir) and alpha-tubulin-specific protease (EspG), are transferred into host cells through the type III secretion system, resulting in attached and affected lesions (AEs) (Caron et al. 2006). The virulence factors of STEC also include lectins, LPS, and outer membrane proteins. Infections caused by STEC generally result in severe stomach cramps, diarrhea, and vomiting (Griffin and Tauxe 1991). The stress resistance development of *E. coli*, especially pathogenic *E. coli*, to acid (low pH) (Fig. 1.3), heat, osmosis, low temperature, and antibiotics have been reported in the previous studies (Table 1.3). Apart from the common stressors, Pereira et al. (2020) made use of transcriptomic analysis to reveal the molecular mechanisms underlying the response of *E. coli* to biocides (e.g., sodium hypochlorite and peracetic acid). The zinc homeostasis was identified to be closely associated with the development of biocide resistance in *E. coli*.
The molecular mechanisms underlying the acid stress response of *Escherichia coli*. This figure is reprinted from Arcari et al. (2020), licensed under CC BY.
Listeria, which are Gram-positive bacteria, have a genome size of 2.7–3.0 Mb, encoding more than 2,000 proteins. *Listeria* can be divided into 7 species, including *L. monocytogenes*, *L. ivanvii*, *L. innocua*, *L. murrayi*, *L. grayi*, *L. seeligeri*, and *L. welshimeri*. Among these strains, *L. monocytogenes* has been identified as one of the most common foodborne pathogenic bacteria (Swaminathan and Gerner-Smidt 2007). *L. monocytogenes* is a short rod-shaped cell with blunt ends and a size of approximately 0.4–0.5 μm × 0.5–2.0 μm. *L. monocytogenes* cannot produce spores or form capsules. At temperatures ranging from 20 to 25 °C, *L. monocytogenes* can produce flagella, which confer locomotive ability. However, when the temperature is increased to 37 °C, fewer or even no flagella form, and thus, the motility of *L. monocytogenes* decreases. These bacteria can grow under 10% NaCl and 40% bile solution at pH values ranging from 3.1 to 9.6 (Mellin and Cossart 2012). *L. monocytogenes* is a facultative anaerobic bacterium that can grow under low oxygen conditions (Ollinger et al. 2008). The invasive virulence factors expressed by *L. monocytogenes*, for instance, internalin InlA can interact with the receptors of small intestinal epithelial cells, assisting *L. monocytogenes* in invading host cells. In addition, *L. monocytogenes* can produce listeriolysin O (LLO) and phosphatidylinositol phospholipase C (PI-PLC), which can disintegrate the phagosome (Fig. 1.4). Therefore, *L. monocytogenes* can escape into the cytoplasm and make use of nutrients from host cells for growth and reproduction. The ActA surface protein expressed by *L. monocytogenes* aggregates the actin of the host cell and forms an actin tail at one end of the bacterial cell, which drives the movement of the bacteria in the cytoplasm. Therefore, infection can be achieved from one cell to another through the formation of a longer comet-like tail structure to enter the next host cell during the endocytosis of neighboring cells. With the combined action of

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<th>Stress response</th>
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<tr>
<td>Biocide stress response</td>
<td>Pereira et al. (2020)</td>
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<tr>
<td>Acid stress response</td>
<td>Ávila et al. (2013), Bae and Lee (2017), Buchanan and Edelson (1999),</td>
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<td></td>
<td>Huang et al. (2007), Johnson et al. (2014), Kim et al. (2016), Leenanon</td>
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<td>et al. (2003), Montet et al. (2009), Wang et al. (2020), Orihuel et al.</td>
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<td>(2019), Olesen and Jespersen (2010), Seo et al. (2015), Tsai and Ingham</td>
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<td>(1997), Xu et al. (2019, 2020), Yang et al. (2018)</td>
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<tr>
<td>Heat stress response</td>
<td>Adhikari et al. (2016), Vidovic et al. (2012), Slanec and Schmidt (2011),</td>
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<td></td>
<td>Azizoglu and Drake (2007)</td>
</tr>
<tr>
<td>Cold stress response</td>
<td>Li et al. (2018), Lu et al. (2011), Vidovic et al. (2011), Jones et al.</td>
</tr>
<tr>
<td>Antibiotic resistance</td>
<td>Soufi et al. (2009), Khaita et al. (2008)</td>
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</tbody>
</table>

1.2.4 *Listeria*

*Listeria*, which are Gram-positive bacteria, have a genome size of 2.7–3.0 Mb, encoding more than 2,000 proteins. *Listeria* can be divided into 7 species, including *L. monocytogenes*, *L. ivanvii*, *L. innocua*, *L. murrayi*, *L. grayi*, *L. seeligeri*, and *L. welshimeri*. Among these strains, *L. monocytogenes* has been identified as one of the most common foodborne pathogenic bacteria (Swaminathan and Gerner-Smidt 2007). *L. monocytogenes* is a short rod-shaped cell with blunt ends and a size of approximately 0.4–0.5 μm × 0.5–2.0 μm. *L. monocytogenes* cannot produce spores or form capsules. At temperatures ranging from 20 to 25 °C, *L. monocytogenes* can produce flagella, which confer locomotive ability. However, when the temperature is increased to 37 °C, fewer or even no flagella form, and thus, the motility of *L. monocytogenes* decreases. These bacteria can grow under 10% NaCl and 40% bile solution at pH values ranging from 3.1 to 9.6 (Mellin and Cossart 2012). *L. monocytogenes* is a facultative anaerobic bacterium that can grow under low oxygen conditions (Ollinger et al. 2008). The invasive virulence factors expressed by *L. monocytogenes*, for instance, internalin InlA can interact with the receptors of small intestinal epithelial cells, assisting *L. monocytogenes* in invading host cells. In addition, *L. monocytogenes* can produce listeriolysin O (LLO) and phosphatidylinositol phospholipase C (PI-PLC), which can disintegrate the phagosome (Fig. 1.4). Therefore, *L. monocytogenes* can escape into the cytoplasm and make use of nutrients from host cells for growth and reproduction. The ActA surface protein expressed by *L. monocytogenes* aggregates the actin of the host cell and forms an actin tail at one end of the bacterial cell, which drives the movement of the bacteria in the cytoplasm. Therefore, infection can be achieved from one cell to another through the formation of a longer comet-like tail structure to enter the next host cell during the endocytosis of neighboring cells. With the combined action of
LLO and broad-spectrum phospholipase C (PC-PLC), *L. monocytogenes* is released from formerly infected cells and enters the cytoplasm of newly infected cells, where it continues to proliferate (Cossart 2011). Most *L. monocytogenes* strains contain Listeria pathogenicity island 1 (LPI-1), consisting of six virulence genes, including *prfA*, encoding a listeriolysin regulatory protein (Heras et al. 2011); *plcA*, encoding a 1-phosphatidylinositol phosphodiesterase; *hly*, encoding a listeriolysin O; *actA*, encoding an actin assembly inducing protein; *plcB*, encoding phospholipase C;
and two housekeeping genes: \textit{prs}, encoding ribose-phosphate pyrophosphokinase, and \textit{ldh}, encoding L-lactate dehydrogenase.

The infection caused by \textit{L. monocytogenes} is known as listeriosis and occurs through the consumption of foods contaminated with \textit{L. monocytogenes}. Listeriosis can lead to a series of symptoms, such as headache, fever, diarrhea, and gastroenteritis. Children, elderly women (>65 years old), pregnant women, and people with compromised immunity are more vulnerable to \textit{L. monocytogenes}, and a low level of \(10^2 \sim 10^4\) \textit{L. monocytogenes} cells could result in severe infection of these people (Radoshevich and Cossart 2018; Schuppler 2014). It is estimated that there are approximately 1600 cases of listeriosis with 260 deaths each year in the United States (CDC 2021a). The primary food sources of \textit{L. monocytogenes} infection include raw milk (unpasteurized), soft cheese, deli meat and meat products, smoked seafood, raw sprouts, and instant foods (Scallan et al. 2011; Pinner et al. 1992). \textit{L. monocytogenes} can express the glutamate decarboxylase A (GadA), which plays an important role in maintaining the homeostasis of the intracellular pH under acidic conditions. When \textit{L. monocytogenes} enters the human small intestine, the production of bile acid hydrolase (BSH) can decompose cholate and increase its tolerance to bile acid, thereby ensuring survival in the small intestine. Table 1.4 gives a summary of the responses of \textit{L. monocytogenes} to various food-associated stressors. Sergelidis and Abraham (2009) demonstrated that the accumulation of heat shock proteins conferred upon \textit{L. monocytogenes} increased survival by 1 to 8 times under heat treatment.

### Table 1.4 The summary of the studies about the stress response of \textit{Listeria monocytogenes}

<table>
<thead>
<tr>
<th>Stress response</th>
<th>References</th>
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<tbody>
<tr>
<td>Osmotic stress response</td>
<td>Ringus et al. (2012)</td>
</tr>
<tr>
<td>Cold stress response</td>
<td>Chan et al. (2008)</td>
</tr>
<tr>
<td>Oxidative stress response</td>
<td>Chan et al. (2008), Fisher et al. (2000), Pöntinen et al. (2017b), Oliver et al. (2013), Loepfe et al. (2010)</td>
</tr>
<tr>
<td>Acid stress response</td>
<td>Pöntinen et al. (2017a), Oliver et al. (2013)</td>
</tr>
<tr>
<td>Low oxygen/anaerobic condition</td>
<td>Mueller-Herbst et al. (2014)</td>
</tr>
<tr>
<td>Antimicrobial exposure</td>
<td>Knudsen et al. (2013), Whiteley et al. (2017), Wu et al. (2017)</td>
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\textit{1.2.5 Vibrio parahaemolyticus}

\textit{Vibrio parahaemolyticus} has a size of approximately 0.5 ~ 0.8 µm \(\times\) 1.4 ~ 2.4 µm and does not form capsules and spores. Single-ended flagella can move quickly in a liquid medium (Yaashikaa et al. 2016). \textit{V. parahaemolyticus} is a Gram-negative halophile as well as a facultative anaerobe. The optimal growth conditions are 36 °C,
pH 7.0–9.0, and the osmotic pressure equal to a NaCl concentration of 2–4%. Due to its high sensitivity to heat, it can be easily inactivated by exposure at 65 °C for 5 min or 80 °C for 1 min. Given its halophilic nature, *V. parahaemolyticus* is mainly distributed in salt-rich environments, including coastal and marine waters. The flagellum of *V. parahaemolyticus* helps it swim and attach. In addition, the lateral flagella contribute to the swarming of *V. parahaemolyticus*. Multivalent adhesion molecules (MAMs), the outer membrane proteins of *V. parahaemolyticus*, can bind to fibronectin to achieve interactions with host cells. To acquire iron, *V. parahaemolyticus* employs siderophores (e.g., ferrichrome, aerobactin, and vibrioferrin) as iron chelators, which are secreted by the PvsABCDE or FhuCDB ABC complex (Fig. 1.5). The toxins secreted by *V. parahaemolyticus* include thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH), which contribute to the formation of tetrameric pore complexes in the host cell membrane and result in cytotoxicity.

**Fig. 1.5** Summary of the virulence factors of *Vibrio parahaemolyticus*. The flagellum is sheathed to extend the bacterial membrane and contributes to the motility of *V. parahaemolyticus* in liquid environments. Flagella are produced along the lateral side of the bacterium and play a role in swarming motility. Multivalent adhesion molecule 7 (MAM7) is a prerequisite for attachment to host cells through binding with fibronectin and phosphatidic acid. *V. parahaemolyticus* uses the siderophores vibrioferrin, ferrichrome, and aerobactin, along with heme, to scavenge iron from the external environment. These iron transporters are internalized by membrane receptors on the outer membrane of *V. parahaemolyticus* and transported to the cytoplasm by different ABC complexes. This figure is reprinted from Broberg et al. (2011) with the permission of Elsevier.
V. parahaemolyticus mainly colonizes the surface of marine organisms, such as cod, sardines, mackerel, flounder, octopus, shrimp, crab, clams, lobster, crayfish, scallops, and oysters (Su and Liu 2007). If people consume uncooked seafoods contaminated by V. parahaemolyticus, they may get sick, known as vibriosis, with symptoms such as abdominal cramps, watery diarrhea, nausea, vomiting, and fever (Su and Liu 2007). These symptoms generally occur within 24 h after infection and might last for approximately 3 days. In the United States, the CDC reported that the annual cases of vibriosis reached approximately 80,000, of which 45,000 cases were V. parahaemolyticus-associated vibriosis (CDC 2021b). The stress response of this pathogenic bacterium during food processing is a potential risk to food safety and should be carefully evaluated (Table 1.5). Elvira et al. (2020) reported that the survival of V. parahaemolyticus under low temperature (7 to 14°C) was attributed to the upregulation of the ropS gene, which further improved the production of the cold shock protein encoded by the cspA gene.

### Table 1.5 The summary of the studies about the stress response of Vibrio parahaemolyticus

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<thead>
<tr>
<th>Stress response</th>
<th>References</th>
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<tr>
<td>Heat stress response</td>
<td>Urmersbach et al. (2015), Beuchat and Worthington (1976)</td>
</tr>
<tr>
<td>Acid stress response</td>
<td>Wong et al. (1998, 2018), Yeung and Boor (2004), Ming Lun Chiang et al. (2005)</td>
</tr>
<tr>
<td>Osmosis stress response</td>
<td>Naughton et al. (2009)</td>
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<tr>
<td>Ethanol stress response</td>
<td>Chiang et al. (2006)</td>
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1.2.6 Staphylococcus aureus

Staphylococcus aureus is a Gram-positive bacterium that is widely distributed in the natural environment. S. aureus can survive under harsh conditions, including low water activity, the extreme temperature fluctuation, and a high osmotic environment. The pathogenicity of S. aureus is attributed to complex regulation by various virulence factors. S. aureus enterotoxins are water-soluble, single-chain, and spherical low-molecular-weight proteins (22–29 kDa) that are stable to heat (100°C for 30 min), digestive protease (e.g., pepsin), radiation, and a wide range of pH values (Spaulding et al. 2013). In addition, S. aureus can secrete hemolysins, which are divided into α, β, γ, δ, and ? types. α-hemolysin is the most common form which can form pores in the host cell membrane, disrupting ion homeostasis and resulting in lysis of whole host cells (Wiseman 1975). Panton-valentine leukocidin (PVL) produced by S. aureus has been demonstrated to be associated with acute necrotic skin infection. The consumption of food contaminated by S. aureus enterotoxins can
result in the symptoms of vomiting, diarrhea, and abdominal pain (Murray 2005). Generally, people are cured on their own within 24 h after being infected, while a few people might experience fatal symptoms of dehydration and electrolyte imbalance (Murray 2005). Food, such as meat, eggs, and dairy products, is a common source of *S. aureus* contamination (Wattinger et al. 2012). When a cow is infected with mastitis caused by *S. aureus*, its milk and the associated products may contain *S. aureus* (Johler et al. 2011). Skin contact or sneezing during processing also easily causes *S. aureus* contamination of food (Asao et al. 2003). The development of a stress response within *S. aureus* toward heat, low temperature, oxidation, and osmosis has been investigated previously (Table 1.6). Take low temperature for instance, Wood et al. (2019) revealed that the GTPase enzyme Era and DEAD-box RNA helicase CshA contributed to the cold adaptation of *S. aureus*.

### Table 1.6 The summary of the studies about the stress response of *Staphylococcus aureus*  

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<tr>
<th>Stress response</th>
<th>References</th>
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<tr>
<td>Oxidative stress response</td>
<td>Gaupp et al. (2012)</td>
</tr>
<tr>
<td>Acid stress response</td>
<td>Zhou and Fey (2020), Bore et al. (2007)</td>
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<tr>
<td>Desiccation resistance</td>
<td>Chaibenjawong and Foster (2011)</td>
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1.2.7 *Clostridium perfringens*

*Clostridium perfringens* is a Gram-positive anaerobic bacterium without flagella (Lebrun et al. 2010). Under certain circumstances (appropriate pH, temperature, and ratio of carbon source), the spores formed are elliptical and distributed in the center or near the ends of the bacteria (Garcia et al. 2012). The capsules produced by *C. perfringens* are highly dependent on environmental conditions, and they can form capsules in the body or on culture media containing serum or sugar. Unlike other Clostridia, *C. perfringens* can release toxins while forming a capsule. The symptoms of *C. perfringens* infection are mainly caused by extracellular enzymes and toxins secreted by *C. perfringens* (Petit et al. 1999). *C. perfringens* can produce several kinds of toxins, among which alpha (α), beta (β), epsilon (ε), and iota (ι) are the
major types. According to the ability to produce $\alpha$-, $\beta$-, $\varepsilon$-, and $\iota$-type toxins, *Clostridium perfringens* can be further divided into types A to E (Songer 1996) (Table 1.7).

Alpha ($\alpha$) toxins can be produced by *Clostridium perfringens* types A to E. They exhibit the activities of both phospholipase and sphingomyelinase, which result in membrane damage and complete rupture of host cells through hydrolysis of phosphatidylcholine and sphingomyelin (Navarro et al. 2018). Beta ($\beta$) toxins, mainly produced from B- and C-*Clostridium perfringens*, can induce pore formation in the membrane. This results in the selective transportation of $\text{Ca}^{2+}$, $\text{Na}^+$, and $\text{Cl}^-$ into host cells, causing severe cell swelling. It has been demonstrated that epsilon ($\varepsilon$) toxins and pore-forming toxins can be produced by both *Clostridium perfringens* types B and D. The activation of epsilon toxins is achieved after the removal of carboxy- and amino-terminal peptides by digestive proteases (e.g., trypsin) of the host (Minami et al. 1997). In the study of Savva et al. (2019), cryo-electron microscopy was applied to reveal the double $\beta$-barrel structure of epsilon toxin-induced pore formation in the host cell membrane at 3.2 Å resolution. Generally, iota ($\iota$) toxins are secreted by *Clostridium perfringens* type E. During infection, *Clostridium perfringens* proliferates rapidly and produces large amounts of toxins (e.g., $\alpha$-toxins), which can directly react with epithelial cells or be absorbed into the blood circulation system, resulting in intestinal necrosis and bleeding (Goossens et al. 2017). The pathogenicity of *Clostridium perfringens* type A strain is mainly attributed to $\alpha$ toxins (lethal dose, 50% [LD$_{50}$] in mice, 3$\mu$g/kg) (Cooper and Songer 2009), which can cause gas gangrene, traumatic infection, necrotizing enteritis, and other intestinal diseases (Sawires and Songer 2006). In addition, *Clostridium perfringens* enterotoxins (CPEs) are the cause of gastrointestinal-associated symptoms (García and Heredia 2011). Raw meat and poultry are the major vehicles of *Clostridium perfringens*, which can lead to outbreaks of botulism. *Clostridium perfringens* has been listed as the most common cause of foodborne illness by the CDC in the United States, with a total of approximately one million cases each year (CDC 2021c). The Public Health Agency of Canada estimated 177,000 infections of *Clostridium perfringens* each year in Canada (PHAC 2021). Botulism illness is a rare but serious foodborne disease that might result in paralysis and even death. The response of *Clostridium perfringens* to stressors is summarized in Table 1.8. Charlebois et al. (2017) demonstrated that biofilm formation is a strategy employed by *Clostridium perfringens* against disinfectants, including potassium monopersulfate, quaternary ammonium chloride, hydrogen peroxide, and glutaraldehyde solutions.

**Table 1.7** The spectrum of toxins produced by various *Clostridium perfringens* strains

<table>
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<tr>
<th><em>Clostridium perfringens</em> strains</th>
<th>Toxin types</th>
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<tr>
<td><em>C. perfringens</em> type A</td>
<td>$\alpha$-type</td>
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<tr>
<td><em>C. perfringens</em> type B</td>
<td>$\alpha$, $\beta$, $\varepsilon$-type</td>
</tr>
<tr>
<td><em>C. perfringens</em> type C</td>
<td>$\alpha$, $\beta$-type</td>
</tr>
<tr>
<td><em>C. perfringens</em> type D</td>
<td>$\alpha$ and $\varepsilon$-type</td>
</tr>
<tr>
<td><em>C. perfringens</em> type E</td>
<td>$\alpha$ and $\iota$-type</td>
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Bacteria carry out multiple strategies to survive harsh environments and ensure that they can adapt to changes in environments. Stress responses might allow bacteria to develop higher resistance to various stressors as well as improved virulence, which poses potential risks to food safety and human health. For example, acid adaptation has been reported to increase the survival of bacteria in various low pH foods, including fermented dairy products (e.g., cheese and fermented milk) (Leyer and Johnson 1992; Shen et al. 2007) and orange and apple juices (Álvarez-Ordóñez et al. 2009a, b). Additionally, the acid stress response was reported to show cross protection against thermal exposure (Shen et al. 2011). In the study of Qiao et al. (2020), the minimum inhibitory concentration (MIC) of quinolones and aminoglycosides against *S. aureus* was enhanced by fourfold after the cold adaptation (4 and −20 °C). Oh et al. (2019) indicated that *C. jejuni* isolated from retail-based raw chickens already process aerotolerance and more easily survive exposure to multiple stressors, including peracetic acid, low temperature (refrigeration), and freeze-thaw cycles. Pre-exposure of *E. coli* O157:H7 to heat at 54 to 60 °C resulted in the increased resistance to high hydrostatic pressure, which might be attributed to the activation of RpoS and RpoH (Gayán et al. 2016). He et al. (2018) reported that the adaptation to 5% ethanol for 1 h could confer the resistance of *S. Enteritidis* toward the apple juice with a pH value of 3.57. In addition, the development of a resistance response to food-associated stressors could contribute to survival in host environments (Horn and Bhunia 2018). Alves et al. (2020) found that cold adaptation (11 °C) enhanced the survival of *L. monocytogenes* through the simulated gastrointestinal (GI) tract conditions, including the oral phase, stomach phase, and intestinal phase.

Additionally, the virulence has been shown to be improved in stress-adapted bacteria, which could be a concern for human health. A study conducted by Harris et al. (2012) demonstrated that pre-adaptation to 2% NaCl (the concentration usually used for meat processing) significantly enhanced the production of Shiga toxin by *E. coli* O157:H7. Conte et al. (2000) exposed *L. monocytogenes* to lactic acid (pH 5.1) for 1 h at 37 °C and found an improved invasive ability of acid-adapted *L. monocytogenes* in the enterocyte-like (Caco-2) cells. In a study by Perez et al. (2010), the acid-adapted *S. Enteritidis* and *S. Typhimurium* exhibited higher survival rates when exposed to simulated gastric fluid. Furthermore, the acid adaptation conferred upon *Salmonella* higher intestinal invasion capacity during the infection.

### Table 1.8 The summary of the studies about the stress response of *Clostridium perfringens*

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<th>Stress response</th>
<th>References</th>
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<tr>
<td>Heat stress</td>
<td>Novak et al. (2001), Heredia et al. (1998, 2009), Raju et al. (2007)</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>Charlebois et al. (2017)</td>
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<td>Cold stress</td>
<td>Limón et al. (2011)</td>
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<td>Ultraviolet irradiation</td>
<td>Raju et al. (2007)</td>
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of Rattus norvegicus. Regarding osmotic stress, Lorentzen et al. (2011) reported that 2% NaCl-adapted *L. monocytogenes* showed a higher invasion rate in Caco-2 cells, indicating the potential contamination risks of salt-cured foods. Reports on the association between foodborne outbreaks and stress resistance are limited. Muchaamba et al. (2019) discovered that the *L. monocytogenes* LL195 strain isolated from a listeriosis outbreak related to Vacherin Montd’or cheese exhibited the enhanced acid and salt tolerance compared with those isolated from salad or meat. It is speculated that such acquired tolerance could contribute to survival during cheese processing. In addition, the *L. monocytogenes* LL195 strain showed the highest infective ability in the zebrafish model. Oxygen restriction conditions (e.g., vacuum packaging) have been shown to enhance the invasive ability of *L. monocytogenes* in Caco-2 cells by approximately 100 folds (Andersen et al. 2007) (Fig. 1.6). Additionally, exposing EHEC to low oxygen stimulated the expression of Sfp fimbriae, which contributed to the adherence to human intestinal epithelial cells (Müsken et al. 2008). The anaerobic environment induced the accumulation of flagellin B, fibronectin-binding protein, and protease Htpx within *Campylobacter* (Lee et al. 2014). The overlap in molecular regulation between the stress response and virulence might contribute to the enhanced pathogenicity of stress-adapted pathogens. In a study by Sleator et al. (2001), OpuC, an ATP-binding osmolyte transporter, was demonstrated to be associated with the development of osmotic resistance in *L. monocytogenes* and contributed to the colonization of *L. monocytogenes* in the upper small intestine of mice. Hejair et al. (2017) also found that the deletion of *ompF* and *ompC* gene, which were highly associated with osmotic tolerance, impaired the ability of pathogenic *E. coli* to infect duck and mouse models,
indicating that both osmotic stress resistance and pathogenesis shared the same regulators, OmpF and OmpC.

1.4 Conclusions

Throughout the food production chain, foodborne pathogenic bacteria tend to encounter various stressors. The development of stress adaptation might facilitate the survival of pathogens in foods and lead to incomplete inactivation by common decontamination treatments, posing remarkable challenges to food safety and public health. To date, there is still a knowledge gap associated with the molecular mechanisms underlying the stress responses of foodborne pathogens against food-associated stressors. More investigation are required in the future. In addition, the potential risks of enhanced stress resistance and improved virulence resulting from the initiation of the microbial stress response should be further carefully evaluated by researchers.

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The Importance of Understanding the Stress Response in Foodborne...


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Part II
Response of Foodborne Pathogens to Various Food Processing-Related Stressors
Chapter 2
Response of Foodborne Pathogens to Thermal Processing

Xiang Wang and Jianwei Zhou

Abstract Thermal processing is considered as the principal method of microbial inactivation in food industry. The use of heat to achieve a specific lethality is one of the important critical control points in reducing the risks associated with foodborne pathogens. However, the mechanisms of bacterial thermal inactivation and the factors affecting bacterial heat resistance are still not well understood. This chapter gave a general overview for various aspects of heat processing, including the thermal inactivation kinetics, the factors affecting the microbial heat resistance, the effect of thermal exposure on the cellular sites of microbes, as well as the regulation mechanisms underlying microbial heat resistance. This could help in predicting the effects of heat on foodborne pathogens and develop hurdles to ensure efficient microbial inactivation.

Keywords Thermal processing · Heat · Foodborne pathogens · Injury · Heat shock response

2.1 Thermal Processing in Food Industry

Thermal processing is the most common food processing operation (Alvarez-Ordonez et al. 2008). The major purpose of thermal processing is to guarantee food safety by eliminating pathogens and inactivating their enzymes or other metabolites in food (Sun 2012). It also leads to the physical and chemical changes that may assist to develop taste and flavor. Thermal treatment generates color,
structure, and taste and reduces anti-nutrients, allergens, and toxins. It can also enhance the structure of food components, render them more digestible. The thermal processing can be achieved by different means, such as hot water, steam, hot hair, and so on.

Thermal processing is a general term that describes all forms of heat treatment. Thermal processing dates from when people started using fire to cook. Although the reason for this first use of thermal processing remains debated (Carmody et al. 2012), the primary reason for heating might not for food disinfection. In 1804, a commercial bottling plant for preserved foods was established by Nicolas Appert. Since then thermal processing has undergone extensive developments. In the 1850s, Louis Pasteur disproved the theory of spontaneous generation and first discovered that microorganisms were responsible for food spoilage. The invention of the pressure cooker in 1874 is considered as an important landmark of thermal processing (Hersom 1975). In the 1890s, Prescott and Underwood established the relation between time and temperature in the thermal process, which contributed to the understanding of the thermal sterilization process. In 1921, Bigelow established the logarithmic nature of the thermal death time (TDT) curve (Bigelow 1921), and the safety standards for efficient inactivation of safety for destroying Clostridium botulinum (then Bacillus botulinus) in the canning process. In 1943, the well-recognized concepts of D- and z-values were introduced (Ball 1943; Katzin et al. 1943). The D-value (or the decimal reduction time) is defined as the time at a given condition (e.g., temperature, matrix) required to reduce 90% (1-log reduction) of microorganisms being studied. The z-value is defined as the change in temperature required in order to achieve a tenfold (one-log) reduction or increase in the D-value. It can be determined from the slope of the line that results from plotting the log of D-values versus temperature and indicates the change in the death rate based on temperature. Both D- and z-values are indirectly used to establish and describe the thermal processes.

The target microorganisms concerned in heating of food depend on the food types and potential contamination microorganisms. Traditional canning aims at the destruction of all spores (C. botulinum, Bacillus cereus, etc.). Nowadays, milder heat processing to satisfy the consumers’ demand for fresher products has become a new trend. However, reducing the intensity of thermal treatments raises concerns on the microbiological food safety.

Processing times should be kept to a minimum to retain maximum quality of products, while ensuring consumer safety. Therefore, more attention has been paid to the nonspore forming pathogens such as Escherichia coli O157:H7, Listeria monocytogenes, Cronobacter sakazakii, Salmonella enterica, and Staphylococcus aureus.
2.2 Thermal Inactivation Kinetics

2.2.1 Log-Linear Model and Thermal Death Time Concept

For over one hundred years, it was assumed that the heat could penetrate in the food product uniformly (Chen et al. 2008), and the thermal inactivation could be described by the log-linear kinetics. In this model, it is assumed that all microbial cells in a population have equal heat sensitivity and that the death of an individual is dependent on the random chance that a key molecule or “target” within it receives sufficient heat (Cole et al. 1993). Thermal inactivation kinetics follows a first-order model according to Bigelow (1921) and can be expressed as

$$\log(N_t) = \log(N_0) - \frac{k_{\text{max}} t}{\ln(10)} = \log(N_0) - \frac{t}{D},$$

(2.1)

where $N_t$ is the number of survivors after heat exposure for time $t$, $N_0$ is the initial population, $k_{\text{max}}$ denotes the specific inactivation rate, and $D$-value is the time for 90% population reduction at an isothermal temperature. The $z$-value indicates the sensitivity of the microorganisms to changes in temperature and is defined as the change in temperature required in order to achieve a tenfold (one-log) reduction or increase in the $D$-value (Eq. 2.2). A larger $z$-value indicates that the rate of destruction of microorganism is less temperature sensitive. A small $z$-value indicates higher temperature sensitivity and a small change in temperature will result in a significant change in microbial population. So far, these two thermal death time parameters have been widely used to evaluate the heat resistance of microbial strains, as shown in Eq. (2.2).

$$\log(D) = \log(D_{\text{ref}}) - \frac{(T - T_{\text{ref}})}{z},$$

(2.2)

where $D_{\text{ref}}$ is the decimal reduction time at a given reference temperature $T_{\text{ref}}$ (°C).

As shown in Fig. 2.1, the $z$-value can be determined from the values of $D$ and the corresponding temperatures $T$ following the below equation:

$$z = \frac{T_2 - T_1}{\log D_1 - \log D_2}.$$

(2.3)

The $D$-values are determined at a specific and constant temperature. The $z$-value enables to calculate $D$-values at different temperatures. The heating time at a specific temperature ($T$) required to achieve an equivalent thermal treatment at a reference temperature ($T_{\text{ref}}$) is generated from Eq. (2.4).
\[ L = t \cdot 10^{(T-T_{ref})/z}. \] (2.4)

However, for a non-isothermal process, it is necessary to integrate the achieved lethal rate at the temperature ranges. For a certain microorganism, the lethality \( F \) of a non-isothermal process can be calculated according to Eq. (2.5). The thermal treatment is deemed sufficient if the time obtained from the process lethality is equal to or more than the set performance standard.

\[ F = \int_{0}^{t} 10^{(T-T_{ref})/z} \, dt. \] (2.5)

### 2.2.2 Non Log-Linear Models

Although the log-linear model is extensively used for the description of heat-induced microbial inactivation, there is growing evidence to support that the inactivation of vegetative cells does not always follow the first-order kinetics (Augustin et al. 1998; Linton et al. 1995; Valdramidis et al. 2006), especially for the mild thermal treatments. A study by Van Boekel (2002) indicated that log-linear kinetics was an exception rather than the law for the heat processing. Van Boekel (2002) examined about 120 published microbial survival curves and found that only about 4% of them could be considered as the log-linear ones. The inactivation curve possibly shows a shoulder (a delay before the beginning of a death phase) and/or a tailing (a residual subpopulation surviving), and the curvature of the curve can be either concave or convex (Fig. 2.2).

There is still controversy about the reasons for the nonlinear behavior of heat-induced microbial inactivation (Stringer et al. 2000). Possible causes include the
limitations of the experimental procedure such as use of mixed populations, treatment heterogeneity, clumping or clump separation during thermal treatment or enumeration of survivors, protective effect of dead cells or from some food components (fat, protein), method of enumeration, statistical design, poor heat transfer, among others (McKellar and Lu 2003). Some supporters of the first-order kinetics theory have claimed that the nonlinear results were attributed from the lack in the sufficient laboratory skills. However, this hypothesis is untenable (Peleg 2006). The second explanation is that the shoulders or tails of the inactivation curves are associated with heat inactivation mechanism or microbial heat resistance. In recent years, a study conducted the thermal inactivation of single cells and confirmed that the nonlinear inactivation patterns might be resulted from the development of microbial stress resistances to thermal or other non-thermal factor (Aguirre et al. 2009; Lidstrom and Konopka 2010). Two microbial subpopulations might be generated, and one is more heat sensitive than the other (Humpheson et al. 1998; Moats et al. 1971; Peleg and Cole 1998). It can be caused by either phenotypic or genotypic heterogeneity (Fig. 2.3). Phenotypic heterogeneity refers to transiently increased resistance associated with the changes in physiological or epigenetic characteristic, but the no mutations occur in the microbial genome (Metselaar et al. 2016). The heat-resistant cells are found to produce more heat shock proteins than the heat sensitive ones did (Xavier and Ingham 1997). Genotypic heterogeneity is attributed from the presence of stable stress-resistant variants with an inheritable stress-resistant phenotype caused by the genomic alterations (van Boekel 2002).

\( D \)-values should be used with caution in order to avoid the inappropriate application in some cases (Davey et al. 2001). If the isothermal survival curves are nonlinear, the generated \( D \)- and \( z \)-value might be produced in large deviations (Peleg 2006). Nonetheless, the \( D \)- and \( z \)-value from nonlinear curves in some studies were sometimes obtained from a limited number of data points that might be considered as the log-linear part of the inactivation curves.

**Fig. 2.2** Various forms of nonlinear microbial inactivation curves. (a) linear with tailing (×), sigmoidal-like (□), linear with a preceding shoulder (○), (b) biphasic (▽), concave (×), biphasic with a shoulder (□), and convex (○) (Geeraerd et al. 2005)
When the shapes of inactivation curves are different, the comparison of heat resistance can be difficult. A useful method to tackle this problem has been introduced by Van Asselt and Zwietering (2006). Taking the variation in microbial strains and food matrix into account, the concept of average $D$-value ($D_{\text{average}}$) was proposed and can be calculated according to the below equation.

$$D_{\text{average}} = \frac{5D + \lambda}{5}.$$  \hspace{1cm} (2.6)

$D$ is the heating time required to obtain one log reduction of target microorganisms and $5D$ reduction of the pathogen is generally required, $\lambda$ is the length of the shoulder. However, this equation only applies to the curves with shoulders. Another concept for a comprehensive estimation of the heat resistance was developed, namely $t_xD$ (Buchanan et al. 1993). It describes the time $t$ required for $x$ log reductions in the microbial population as a function of the treatment temperature $T$ and was first introduced by Buchanan et al. (1993). It can be used to determine the heat resistance of bacterial strains in a more accurate way in comparison with $D$-value alone (Valdramidis et al. 2005). The use of $t_xD$ rather than $D$-values for estimation of food inactivation processes has been accepted by many researchers (Heldman and Newsome 2003; Valdramidis et al. 2005).

Fig. 2.3 Diagram of heterogeneity in a microbial population. The slope of the initial phase of inactivation (green line) represents the rapid death of the heat sensitive subpopulation and the slope of the second phase (red line) represents the more heat-resistant subpopulation. This figure is printed from Ayrapetyan et al. (2015), with the permission of Elsevier.
In order to model non log-linear microbial inactivation curves, a number of primary models have been proposed for use under static temperature conditions. Most of models cannot deal with all shapes of curves, and most of them are based on log-linear inactivation. GInaFit (Geeraerd and Van Impe Inactivation Model Fitting Tool) (Geeraerd et al. 2005), a freeware add-in tool for Microsoft Office Excel, includes ten different types of microbial survival models. It covers all known survivor curve shapes for vegetative bacterial cells. The tool is user friendly and can identify (one of) these curvatures on an experimental data set provided by the end-users.

2.3 Thermal Processing Principles on Safety

Among the foodborne pathogens of concern in food industry, bacterial spores are generally the most heat-resistant forms, although the heat resistance of bacterial spores varies considerably. Nonspore forming bacteria are generally not resistant to temperature above 60 °C to 70 °C. For instance, *Listeria* is relatively resistant to heat, but a thermal treatment of two minutes at 72 °C or an equivalent thermal treatment is generally regarded as safe.

From the point of destruction of pathogens, thermal processes are designed to achieve the target lethality. Although the application of thermal processing to inactivate pathogens may cause undesirable changes to the quality of foods (e.g., sensory and nutritional attributes), improving shelf-life and inactivation of pathogens are two of the primary concerns. In general, a minimum process of 6 decimal reductions (6*D*) in the numbers of target microorganisms is recommended for pasteurized foods (FDA 2011; Roberts et al. 2005). The guidelines for thermal processing differ depending on the national or regional competent authorities and target pathogens. The United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) has implemented a 6.5*D* reduction of *Salmonella* spp. for all categories (cooked, fermented, salt-cured, dried) of meat products (USDA 2005). Canadian Food Inspection Agency (CFIA) requires a *PT*-value (pasteurization value, the minimum time of food exposure to a specific temperature *T*) of 6.5*D* in *Salmonella* spp. in the slowest heating point (usually the geometric center) of foods not containing poultry, whereas a minimum pasteurization causing 7*D* is needed if food contains poultry (CFIA 2014). For *L. monocytogenes*, it is recommended that the cooking and standing procedure during production and preparation of risk foods for listeriosis should give a 6-log reduction in the number of viable cells (Lund et al. 1989). The US National Advisory Committee on Microbiological Criteria for Foods (NACMCF) proposed a requirement that the process should be sufficient to achieve a minimum 5 log reduction of *L. monocytogenes* (NACMCF 2008).
Generally, the authority sets the safe cooking recommendations for consumers or catering based on a serving size of meat and meat product. For example, the safe cooking guidelines for poultry: first the worst-case level of *Salmonella* in poultry products was estimated in a serving size of 143 g of raw meat (about 100 g of cooked meat). Then they estimated about 6.7 log$_{10}$ of *Salmonella* contamination. Thus, a 7D reduction is considered sufficient to obtain acceptable level of protection (Bean et al. 2012). Though a 6.5 or 7D reduction in *Salmonella* would eliminate or adequately reduce vegetative pathogenic microorganisms in the cooked products, it is noted that a 6.5 or 7D reduction in *Salmonella* may be overly conservative in certain processing environments. In 2008, NACMCF recognized that it was acceptable when a 5.0 log reduction was achieved to address *Salmonella* spp. in meat and meat products rather than the previously recommended 6.5 log reduction used for cooked beef products and 7.0 log reduction used for cooked poultry products (NACMCF 2008). As for *L. monocytogenes*, 6D log reduction is also thought to be conservative. The prevalence (and numbers) of *L. monocytogenes* in meat is expected to be low. It is assumed that, in the worst case, fresh meat can contain 4 logs of *L. monocytogenes*/g, and a low temperature thermal treatment that ensures a 4 log reduction of *L. monocytogenes* can be regarded as safe (Gunvig and Tørngren 2015).

Concerning the recommendations for practical thermal processing or cooking, the guidelines depend on the national competent authorities. In the UK, Advisory Committee on the Microbiological Safety of Food (ACMSF) reported the cooking recommendations for burgers, which should be heated to an internal temperature of 70 °C for 2 min or its equivalent (ACMSF 2007). Similar recommendations have been made by the Food Safety Authority of Ireland (FSAI 1999). Thermal treatments recommended by ACMSF for UK differ from those specified by USDA-FSIS. USDA-FSIS published the consumer advice regarding the minimum temperature requirements for cooking a variety of raw products (USDA-FSIS 2011). It recommends that the burgers containing comminute and reformed beef patties should be cooked to achieve a minimum temperature of 160 °F (71.1 °C), and higher temperatures (165 °F/75.3 °C) are recommended for chicken breasts and the whole chicken. For the whole cuts of other meats (pork, beef, veal, lamb cuts), the recommended safe cooking temperature is 145 °F (62.8 °C) with the addition of a 3-min rest time after cook. Danish legislation requires food products to be heated at a minimum of 75 °C in the core, or the equivalent treatment is required (Gunvig and Tørngren 2015). Gunvig and Tørngren (2015) assessed the survival of *L. monocytogenes* in six different meat cuts at temperatures below 75 °C. From the results, they have established the recommendations for safe cooking of the whole roasts, chops, and steaks (raw and semi-processed), where the contamination normally occurs on the surface. The recommendation for these meats is that the core temperature should reach 58–74 °C without holding times. But for minced meat where the bacteria are distributed evenly in the meat, it is suggested to heat to an internal temperature of 70 °C for one min or equivalent.
2.4 The Factors Affecting the Heat Resistance Development in Foodborne Pathogens

A variety of factors are known to affect heat resistance of pathogens. So far, $D$- and $z$-values of various pathogens have been extensively reported in literature. However, a harmonized methodology for the study of heat resistance is still lacking. Due to the differences in the methodologies used and information provided by different researchers, it is difficult to compare the results in a correct way. Although the results of experiments in liquid medium are not always the same as in real food, the data could be expected to reflect the inherent heat resistance of the bacteria (Sörqvist 2003). Compared to a complex food matrix, the components of liquid media are definite and controllable. It provides useful basic knowledge of microbial heat resistance. It can also be used to investigate various factors affecting the heat resistance, which may be applied to further lead to studies to unravel molecular mechanisms of heat resistance (Fong and Wang 2016; Mohacsi-Farkas et al. 1999; Nguyen et al. 2006; Skara et al. 2011). Research articles on heat resistance and thermal inactivation kinetics of pathogens in liquid media should include sufficient description of microorganisms and culture conditions, heating methods, replicate testing, methods (or citation if standard methods were used) of data analysis and results.

2.4.1 Practical Determination of Thermal Inactivation Kinetics

The resistance characteristics of a microorganism are normally designated by a thermal death time (TDT) curve (by plotting the number of survivors versus time of exposure). It provides the rate of inactivation of a specific organism in a specific medium or food at a specific temperature.

2.4.1.1 Determination of Heat Resistance in Liquid Medium

Thermal inactivation of pathogens has been studied extensively in liquid medium, such as deionized water, physiological saline, phosphate buffer, brain heart infusion (BHI), tryptose phosphate broth and trypticase soy broth (TSB), resulting in a wide range of $D$-values. It is well known that the inactivation dynamics may be influenced by various factors including the bacterial strain of the species under investigation, the physiological state of microbial cells, heating and recovery conditions, etc. (Smelt and Brul 2014). Although the results of experiments in liquid medium are not always the same as in real food, the data could be expected to reflect the inherent heat resistance of the bacteria investigated better than those obtained in more complex heating menstrua (Sörqvist 2003).
2.4.1.2 Preparation of the Inoculum

In most cases, bacteria are grown in a nutrient-rich laboratory broth, such as BHI or TSB. For a majority of the reported studies, the bacteria were incubated at optimal conditions, i.e. they could be considered to have reached the late exponential or stationary growth phase. It is known that bacteria in the exponential phase are more sensitive to heat than those in the stationary phase (Chhabra et al. 2002). When the bacteria are subjected to stressful conditions such as higher salt concentration, sublethal heat treatment, acid or alkaline stress, osmotic stress, or starvation, before the actual thermal treatment, the cells will have responses to these stresses which may enhance the heat resistance of survivors. The phenomenon that cells which are exposed to one stress become more resistant to another stress is widely reported and may be referred to as “cross protection.” Therefore, the cells in stationary phase exhibit higher heat resistance presumably due to their exposure to stressful environmental conditions before the thermal treatment. The growth temperature affects lipid biosynthesis, membrane compositions, and protein synthesis, and thereby influences the heat resistance. Incubation at higher temperatures is found to induce higher rigidity of the cell membrane, which was considered as one of the explanations for the higher heat resistance of microorganisms when grown at higher temperatures (Alvarez-Ordonez et al. 2009).

2.4.2 Heating Medium Properties

For most studies, the heat resistance of pathogens is conducted in liquid medium around neutral pH (6–8). It has been reported that there is an optimum pH for heat survival of cells. Increasing acidity or alkalinity increased the rate of inactivation. Studies have shown that the maximum heat resistance of several pathogens is obtained at slightly acidified media (Anellis et al. 1954; Blackburn et al. 1997). Mañas et al. (2003) studied the effects of pH on the heat resistance of S. Typhimurium treated at 56 °C. They found that the heat resistance was maximum at pH 6.0 and acidification of the medium to pH 4.0 decreased the decimal reduction time significantly. Mañas et al. (2003) observed that the optimum pH for survival of S. Enteritidis was dependent on the temperature and NaCl concentration, and the optimum pH for survival of S. Enteritidis was 5.9–6.5.

It is a well-known observation that the reduction of $a_w$ of the heating menstruum in general has a protective effect on the heat resistance. At $a_w$ values between 0.2 and 0.4, cells are most resistant to thermal stress (Laroche et al. 2005). It is reported that the thermal protective effect on S. Typhimurium of reduced $a_w$ (0.98 versus 0.83) in sucrose solutions was as high as 100-fold (Sumner et al. 1991). In various thermal inactivation studies, reductions in $a_w$ of the heating menstruum have been obtained by the addition of salts (Clavero and Beuchat 1996), sugars (Bacon et al. 2003) or removal of water (Kirby and Davies 1990). The impact of reduced $a_w$ on the heat
resistance has been shown to be dependent not only on the $a_w$ values, but also on the specific nature of the solutes used to achieve such values (Mattick et al. 2000). In general, sugars showed a better protection effect than salts.

### 2.4.3 Thermal Treatment Parameters

Various methods were applied for the thermal treatment of liquid medium, e.g. heating in water baths using glass capillary tubes, sealed glass tubes, glass ampoules, or polyethylene pouches completely immersed in the water; test tubes, flasks, or cups placed under the water level and in some cases shaken; heating using pasteurizers; two-phase slug flow heat exchangers; submerged-coil heating apparatuses and thermoresistometers (examples are shown in Fig. 2.4) (Al Sakkaf and

![Fig. 2.4 Schematic diagram of common thermal treatment methods used for determining the inactivation kinetics in laboratory broth. (a) heating in test tubes in water bath (the dots are the spots where cultures were injected); (b) heating in flask in water bath; (c) heating in capillary tube or vial in water bath; (d) heating in polymerase chain reaction (PCR) tubes in thermal cycler; (e) heating in submerged-coil instrument](image-url)

Historically, inaccurate determinations have occurred particularly when methods have not been carefully assessed for possible flaws such as non-uniformities in heat distribution within a system. Teo et al. (1996) used an open system to evaluate the heat resistance of *E. coli* O157: H7 strains. Initially, the experiments were performed with flasks and the level of the water bath was 2.0 cm above the surface of the heating menstruum; the cells were mixed into the menstruum by hand (Fig. 2.4b). The resulting survivor curves had pronounced tails, while when using a magnetic stirrer in the flask and increasing the level of the water bath to 5.0 cm above the menstruum (Fig. 2.4b), the tailings were prevented. The $t_{60D}$ values at 50 °C obtained from the two methods were approximately 20 and 5 min, respectively. An additional disadvantage of this method is imprecision of sampling intervals. Another common method for obtaining kinetic data is the use of sealed glass tubes or vials (Fig. 2.4c). This method using sealed tubes or vials has advantages over open flask methods in that the entire tube may be immersed in the water bath. A disadvantage of this method is the equilibration time (come-up time, time needed to reach the target temperature) needed for each tube (except for capillary tube) or vial. This method does not work for temperatures at which survival time is less than the come-up time. The sealed tube method shares with the open flask method the same disadvantage of imprecise sampling. Another disadvantage is the labor-intensity associated with preparation of the containers (Keller et al. 2008).

The test tube method (Fig. 2.4a) is also commonly used due to the advantage of easy handling. Test tube is filled with the heating menstruum, which is preheated to the target temperature. At a certain time-interval, the culture is injected into the heating menstruum. Donnelly et al. (1987) compared the heat resistance of *L. monocytogenes* using test tube versus sealed tube methods. The results showed that the cells were rapidly inactivated using sealed tube methods. In contrast, the inactivation of *L. monocytogenes* using the test tube method could not be achieved within 30 min at 62 °C, and the extensive tailing of survivor curves was consistently observed. Similar observations for bacterial cells or spores have been reported in other studies when the test organism was heated in incompletely submerged tubes (Beckers et al. 1987; Schuman et al. 1997; Zimmermann et al. 2013). The microbial cells remaining in the tubes walls above the level of the water bath were regarded to be responsible for this tailing phenomenon, and these cells were not exposed to the intended temperature. Thus, when using the test tube method, the contamination of the vessel wall should be avoided. The methods for the heat resistance assessment pose impact on the results, including shape of survivor curves and $D$-values. Overall, an ideal method for thermal inactivation kinetics determination should have the features of avoiding the influence of temperature fluctuation, relative precision of sampling intervals, sufficient heat for all the microbial cells, and less labor intensive.
2.4.4 Recovery Media Conditions

In most of the cases, the recovery and enumeration of thermally treated bacteria were generally performed on agar medium. For the thermal inactivation of single microbial strain in the laboratory, nonselective mediums are often used due to the absence of competitive microbiota. Some selective media, containing compounds that inhibit the growth of non-target microorganisms, are also applied for the enumeration of surviving cells. A variety of studies have compared the recovery of selective and nonselective media for the thermally treated microbial cells, and found that estimated survival counts were higher on the nonselective media (Aljarallah and Adams 2007; Rowan and Anderson 1998; Suo et al. 2012). The difference might be attributed from the heat-induced injured cells. The recovery of pathogens is often performed on the medium with an incubation at optimal conditions. For examples, *Salmonella* and *L. monocytogenes* are incubated at 30–37 °C for 24 h-7 days (O’Bryan et al. 2006). In some studies, thermally treated microbial cells are recovered under anaerobic conditions (Blackburn et al. 1997; George et al. 1998). George et al. (1998) reported that incubation of heat-injured cells under anaerobic conditions increased the survival. Moreover, an incubation at lower temperatures was found to enhance the recovery of thermally injured microbial cells (George et al. 1998).

2.5 The Damaged Effect of Heat Treatment on Cellular Targets of Foodborne Pathogens

The exact cause of microbial cell inactivation upon heat treatment is still not fully understood. It is believed that multiple cellular sites have been inactivated by the thermal treatment, and as the damages accumulate, the final death of microbial cells occurs (Bevilacqua et al. 2020). The existing shoulders of thermal inactivation curves have been associated with the accumulation of sublethal injury of microbial cells. The cellular site targeted by the thermal inactivation can be the cell wall, the cytoplasmic membrane, ribosomes, DNA, RNA, and proteins (Fig. 2.5) (Cebrián et al. 2017; Wu 2008).

The effects of heat treatment on cell structure and internals have been observed by the scanning electron microscope (SEM) or transmission electron microscope (TEM). Under heat treatment, it is shown that the morphology of most microbial cells changed, showing a state of apparent shrinkage and cell wall collapses, and the agglomeration of cell contents (Ma et al. 2019). The structure of microbial cell wall was found to be affected by heat. For Gram-negative bacteria cells, it was observed that the lose of lipopolysaccharide molecules and vesicles after heat treatment (Tsuchido et al. 1995). The thermal-induced damages in the cell wall were reported to be more serious with the pretreatment of bile salts, lysozyme, or hydrophobic antibiotics (Lund et al. 2000; Mackey 1983). For the cell walls of Gram-positive bacteria, composed primarily of peptidoglycan and teichoic acids, is also affected by
heat exposure (Mackey 2000). The results demonstrated that heat treated cells were more sensitive to penicillin, which indicated that cell wall was damaged by the thermal treatment (Teixeira et al. 1997). The lose of D-alanine in the teichoic acids was found in Staphylococcus aureus after a heat exposure (Teixeira et al. 1997), which led to the chelation of Mg$^{2+}$ which preventing their function in certain essential metabolic processes.

The damages in microbial cell membranes by heat have been widely reported. Results obtained from a differential scanning calorimetry (DSC) showed that the microbial cell membrane seemed to be easily affected at relatively low temperatures (Nguyen et al. 2006; Teixeira et al. 1997). When the membrane integrity was compromised after heat treatment, it would lead to the further leak of intracytoplasmic materials, such as metal ions (e.g., Mg$^{2+}$, K$^+$) and amino acids (Skinner and Hugo 1976), the alterations in the entrance and exit of several components (Coote et al. 1994; Hurst and Hughes 1978; Kramer and Thielmann 2016; Marcén et al. 2017; Pierson et al. 1971), the loss of respiration activity (Kramer and Thielmann 2016); and the disruption of the osmotic and pH homeostasis (Baird-Parker et al. 2000; Leguériel et al. 2007; Teixeira et al. 1997). However, a clear relationship between membrane damages and cell inactivation has not been fully clarified yet. Some studies indicated that cell death might take place before the membrane damages (Marcén et al. 2017).

Both DNA and RNA of microbial cells have been reported to be affected by heat treatment. Although DNA is thermostable, it is reported that heat could provoke injuries in the DNA molecules. Compared with DNA, RNA is more heat sensitive. RNA denaturation has been regarded as one of the most evident consequences of the heat exposure (Iandolo and Ordal 1966). Ribosomal ribonucleic acid (rRNA) is the RNA component of ribosomes, and it is found that the damages to the ribosomes were also induced by the heat treatment (Tomlins and Ordal 1976). Ribosomes consist mainly of RNA and proteins, and the heat-induced denaturation of protein was also a factor for function loss of ribosomes.

**Fig. 2.5** Summary of the relevant cellular events that occur in a vegetative bacterial cell upon exposure to heat. This figure is reprinted from Cebrián et al. (2017), licensed under CC BY.
Proteins play an important role in cellular functions in the form of structural components or enzymes. Protein denaturation can lead to a loss of functionality in several ways. Detoxifying enzymes such as catalase or superoxide dismutase, chaperones, and proteases (DnaK, DnaJ, GroEl/GroES, Clpx, etc.), as well as DNA repair enzymes, contribute to the damage repair of microbial cells (Ziemienowicz et al. 1993). Transport pumps and channels are found to be susceptible to heat denaturation. It is assumed that the intracellularly heat-induced protein aggregation might be difficult to be reversed if the microbial cells already harbor other sublethal injuries, for instance, the damages in the membrane. Ribosomal subunits may change their higher structure whereas proteins may coagulate.

Disruption of the certain cellular sites by heat might contribute to the final death of microbial cells. It is speculated that the critical components of thermal inactivation could be the RNA polymerase and ribosomes (Cebrián et al. 2017). However, the amount of ribosome loss that a cell can withstand is still not clear (Niven et al. 1999). Cytoplasmic membrane is also regarded as a critical component targeted by heat, but the degree of damages should be enough to prevent the repair (Cebrián et al. 2017). Moreover, the damages in various cellular components might occur simultaneously (Cebrián et al. 2017).

It is known that not all microbial cells subjected to a thermal treatment, especially the mild heat, are completely killed, and some survivors being physiologically injured (termed as sublethal injury) might be produced (Besse 2002). Sublethal injury of microorganisms implies the transient damages to cell wall or membrane permeability, which might result in the loss of some cell components (e.g., proteins, enzymes, ribosome, or nucleic acids) (Gilbert 1983). Sublethal injured cells are more sensitive to selective antimicrobial agents in medium than the normal counterparts do (Wu 2008). Microbial cells sublethally damaged fail to form visible colonies on selective media but only nonselective culture media, and the differences in the colonies between the selective and nonselective media has been used for the quantitative analysis of sublethally injured cells (Jasson et al. 2007). Under suitable conditions, heat-induced sublethally injured cells may return to a normal state and be able to multiply through repairing the damaged components (Ariefdjohan et al. 2004; Wu 2008). It is also reported that the occurrence of sublethally injured cells may enhance the virulence or stress resistance of microbes (Lianou and Koutsoumanis 2013; Silva et al. 2015; Skandamis et al. 2008).

2.6 Regulatory Network for Heat Resistance of Foodborne Pathogens

2.6.1 Specific Heat-Shock Response Related Genes

Specific heat shock response allows microbial cells to survive through a variety of stressors, in particular heat (Abee and Wouters 1999). Heat exposure can cause
protein denaturation, which leads to misfolding and loss of function (such as enzyme activity) of proteins (Govers et al. 2017). Specific heat shock response is associated with increased production of heat shock proteins (HSPs), including highly conserved chaperones and ATP-dependent proteases, which help microbial cells survive by protecting other proteins/enzymes from damages or degradation (Juneja et al. 1998). Among these HSPs, two representative chaperones encoded by groEL and dnaK genes contribute to the protein folding during heat stress, and these proteins bind the hydrophobic surfaces of unfolded proteins together with their co-chaperones (GroES and DnaJ-GrpE) (Hu et al. 2007). Another group of HSPs are composed by ATP-dependent proteases (e.g., ClpP, HslV), which are responsible for the removal of damaged polypeptides (Missiakas et al. 1996). When these proteases assemble into complex ring-shaped structures, other members of this HSP group combine on a single polypeptide and act as both chaperone and protease activities.

When specific heat shock response is triggered by thermal stressor, numerous genes encoding HSPs are known to be upregulated. Van der Veen et al. (2007) treated *Listeria monocytogenes* EGD-e at 48 °C for 40 min, and found the upregulation in the transcription of 8 heat-inducible genes (*dnaJ, dnaK, hrcA, groEL, groES, clpE, clpB*, and *clpP*). Thermotoleranced *Salmonella* showed significant high expression levels of genes *dnaK* and *htrA* (Gaafar et al. 2019). Another transcriptomic study also identified that the heat response-related genes of *E. coli* were upregulated against the damages in proteins induced by heat stress (Li and Ganzle 2016). The effect of heat on the expression of specific heat shock response-related genes in foodborne pathogens is shown in Table 2.1. Recently, the positive effect of standalone ClpG on bacteria heat resistance has been proven (Lee et al. 2018; Mogk et al. 2021; Panagiotis et al. 2019). A strong increase of the prevalence of *clpG* in *E. coli* strains showed advantages during temporal exposure to lethal temperatures (Boll et al. 2017). ClpG was identified in various Gram-negative

### Table 2.1 The effect of heat on the expression of specific heat shock response-related genes in foodborne pathogens

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Parameters of heat treatments</th>
<th>Genes</th>
<th>Fold change in expression</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>48 °C, 40 min</td>
<td><em>dnaJ, dnaK, hrcA, groEL, groES, clpE, clpB, clpP</em></td>
<td>+(2.3–4.0)</td>
<td>Van der Veen et al. (2007)</td>
</tr>
<tr>
<td><em>Salmonella Enteritidis</em></td>
<td>55 °C, 15 min</td>
<td><em>clpB, clpX, dnaJ, ftsJ, mopA, mopB, etc.</em></td>
<td>Upregulated</td>
<td>Kobayashi et al. (2005)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>58 °C, 10 min</td>
<td><em>dnaJ, groES, ibpA, ibpB, grpE, clpB, hslJ, hslO, etc.</em></td>
<td>Upregulated</td>
<td>Guerenc et al. (2013)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>60 °C, 5 min</td>
<td><em>groEL, dnaK</em></td>
<td>Upregulated</td>
<td>Zhang et al. (2020)</td>
</tr>
<tr>
<td><em>Clostridium botulinum</em></td>
<td>45 °C, 60 min</td>
<td><em>hrcA, grpE, dnaK, dnaJ, groEL, groES</em></td>
<td>+(1.7–5.6)</td>
<td>Selby et al. (2017)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>48 °C, 10 min</td>
<td><em>ClpB, htpGX, ibpAB, clpX, degP, groL, groS, dnaK, etc.</em></td>
<td>Upregulated</td>
<td>Chueca et al. (2015)</td>
</tr>
</tbody>
</table>
bacteria including pathogenic species like *Enterobacter* spp., *Pseudomonas aeruginosa*, *Chronobacter* spp., *Salmonella enterica*, and *E. coli* (Boll et al. 2017; Lee et al. 2015, 2016; Mercer et al. 2015). Compared to the widespread ClpB disaggregase, ClpG is more potent. It exerts higher unfolding forces, enabling it to process highly stable aggregates, and thus improve heat resistance of bacteria (Panagiotis et al. 2019).

### 2.6.2 Sigma Factor Regulated Heat Shock Genes

Bacterial cells exploit alternative sigma factors to specifically direct the transcriptional machinery to a subset of selected gene promoters, which can achieve the positive transcriptional regulation of heat shock genes (Roncarati and Scarlato 2017). A dedicated alternative sigma factor is the subunit of RNA polymerase that confers promoter recognition specificity to the transcription enzyme (Roncarati and Scarlato 2017). For example, the class II stress genes encode general stress proteins in *L. monocytogenes*, of which the expression is regulated by the alternative sigma factor SigB (Soni et al. 2011). Van der Veen et al. (2007) found that 51 genes identified as being SigB-regulated were overexpressed after heat treatment, and SigB-regulated *opuC* operon (*lmo1425-lmo1428*) has been shown to be responsible for the accumulation of osmolytes in response to salt, acid, and cold stress (Sleator et al. 2001). In *E. coli* cells, the development of heat resistance has been linked to the induction of the general stress responses, which was attributed to the expression of alternative sigma factor $\sigma^{32}$ (Abee and Wouters 1999). Regulatory mechanisms controlling the transcription of heat shock genes in some foodborne pathogens are summarized in Table 2.2.

### 2.6.3 Other Functional Genes

Genes related other cellular functions, such as SOS response and DNA repair, cell division, cell wall synthesis and virulence can be also affected by heat stress. Take *L. monocytogenes* as an example, expression changes of the genes associated with the aforementioned functions provide valuable insights into survival strategies of bacterial cells under different thermal treatment conditions (Table 2.3) (Van der Veen et al. 2007).

In the family *Enterobacteriaceae*, a new identified genomic island termed as the locus of heat resistance (LHR) substantially increases the heat resistance (Mercer et al. 2015, 2017). The LHR provides heat resistance to several species, including *E. coli*, *Cronobacter* spp., and *S. enterica* (Mercer et al. 2015, 2017). LHR is a 15- to 19-kb genomic island, the complement of LHR-comprising genes for heat resistance and the stress induced or growth-phase-induced expression of LHR-comprising genes are not well unknown yet (Mercer et al. 2015). The LHR encodes several
putative heat shock proteins, proteases, and transport proteins. Fragments of the LHR were also linked to increased heat resistance in *Klebsiella pneumoniae* and *C. sakazakii* (Bojer et al. 2010; Gajdosova et al. 2011). Mercer et al. (2017) determined the contribution of the seven LHR-comprising genes. They observed LHR-encoded heat shock proteins sHSP20, ClpKGI, and sHSPGI are not sufficient for the heat resistance phenotype; YfdX1, YfdX2, and HdeD are necessary to complement the LHR heat shock proteins and to impart a high level of resistance. While, the deletion of *trxGI*, *kefB*, and *psiEGI* gene of the LHR did not significantly affect the heat resistance.

### 2.7 Conclusions

Thermal processing has a long history in food industry, and it is also still the most commonly used method of microbial inactivation for consumers at home. This chapter summarizes the microbial food safety concerns from the perspective of thermal processing, and the factors affecting the microbial heat resistance. So far, D- and z-values of various pathogens have been extensively reported in literature. However, a general methodology for the study of heat resistance is still lacking. It is recommended to standardize research methods. This envisaged standardization may aid in meaningful comparison of results obtained among various laboratories. The microbial inactivation efficacy of a thermal treatment depends on various factors.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Transcriptional regulator</th>
<th>Regulated genes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>HrcA</td>
<td>Class I: <em>groESL</em>, <em>dnaK</em>, and others</td>
<td>Van der Veen et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>σB</td>
<td>Class II: Genes coding for general stress proteins</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CtsR</td>
<td>Class III: <em>clpP</em>, <em>clpB</em>, <em>hslU</em>, and others</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>σ72 (σH, RpoH)</td>
<td><em>groESL</em>, <em>dnaK-dnaJ</em>, <em>grpE</em>, <em>ibpA</em>, and others</td>
<td>El-Samad et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>σE (σ24)</td>
<td><em>degP</em>, <em>clpX</em>, <em>lon</em>, and others</td>
<td>Carruthers and Minion (2009)</td>
</tr>
<tr>
<td></td>
<td>CtsR</td>
<td><em>clpP</em>, <em>clpC</em>, <em>clpB</em>, <em>hrcA-dnaK</em>, <em>groESL</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>σB</td>
<td>Genes coding for general stress proteins</td>
<td></td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>σ72 (RpoH)</td>
<td><em>groESL</em>, <em>dnaK-dnaJ</em>, <em>lon</em>, <em>clpB</em>, and others</td>
<td>Slamti et al. (2007)</td>
</tr>
</tbody>
</table>
One of the most important factors is the variability of heat resistance among strains of the same species. They may show distinct heat inactivation characteristics, and the reasons behind this are still not well known yet. The stress response of microbial cells to heat at the cellular and transcriptomic level is also introduced. Although various studies have performed, the clear mechanisms under thermal inactivation still remain unclear. Further studies on the specific expression of genes or proteins responsible for heat resistance may facilitate to develop novel technology in combination with heat for efficient inactivation of foodborne pathogens in the food industry.

Table 2.3 Effect of heat stress on other functional genes in *Listeria monocytogenes*

<table>
<thead>
<tr>
<th>Genes of <em>Listeria monocytogenes</em></th>
<th>Gene function</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOS response and DNA repair related genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>recA</td>
<td>Recombination protein RecA</td>
<td>+(2.6–5.5)</td>
</tr>
<tr>
<td>dinB</td>
<td>DNA polymerase IV</td>
<td></td>
</tr>
<tr>
<td>uvrA</td>
<td>Excinuclease ABC (subunit A)</td>
<td></td>
</tr>
<tr>
<td>uvrB</td>
<td>Excinuclease ABC (subunit B)</td>
<td></td>
</tr>
<tr>
<td>umuD</td>
<td>DNA polymerase V</td>
<td></td>
</tr>
<tr>
<td>umuC</td>
<td>DNA polymerase V</td>
<td></td>
</tr>
<tr>
<td>Cell division related genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>minD</td>
<td>Highly similar to septum placement protein MinD</td>
<td>−(2.4–4.3)</td>
</tr>
<tr>
<td>minC</td>
<td>Similar to septum placement protein MinC</td>
<td></td>
</tr>
<tr>
<td>mreD</td>
<td>Similar to cell-shape-determining protein MreD</td>
<td></td>
</tr>
<tr>
<td>mreC</td>
<td>Similar to cell-shape-determining protein MreC</td>
<td></td>
</tr>
<tr>
<td>divIVA</td>
<td>Similar to cell-shape-determining protein MreB</td>
<td></td>
</tr>
<tr>
<td>Cell wall synthesis related genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dltA</td>
<td>D-alanine-activating enzyme (Dae)</td>
<td>−(2.6–5.0)</td>
</tr>
<tr>
<td>dltB</td>
<td>DltB protein for D-alanine esterification</td>
<td></td>
</tr>
<tr>
<td>dltC</td>
<td>D-Alanyl carrier protein</td>
<td></td>
</tr>
<tr>
<td>dltD</td>
<td>DltD protein for D-alanine esterification</td>
<td></td>
</tr>
<tr>
<td>PrfA-controlled virulence genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>prfA</td>
<td>Listeriolysin positive regulatory protein</td>
<td>+4.35</td>
</tr>
<tr>
<td>plcA</td>
<td>Phosphatidylinositol-specific phospholipase C</td>
<td>+3.86</td>
</tr>
</tbody>
</table>

One of the most important factors is the variability of heat resistance among strains of the same species. They may show distinct heat inactivation characteristics, and the reasons behind this are still not well known yet. The stress response of microbial cells to heat at the cellular and transcriptomic level is also introduced. Although various studies have performed, the clear mechanisms under thermal inactivation still remain unclear. Further studies on the specific expression of genes or proteins responsible for heat resistance may facilitate to develop novel technology in combination with heat for efficient inactivation of foodborne pathogens in the food industry.

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X. Wang and J. Zhou


Chapter 3
Response of Foodborne Pathogens to Cold Stress

Jiahui Li, Wen Wang, and Xihong Zhao

Abstract  Low temperature is often used in food processing. However, a series of physical and biochemical modifications can be produced by foodborne pathogens in the process of transient stress and low temperature adaptation, these changes make foodborne pathogenic bacteria continue to grow at low temperature, and even increase its pathogenicity and drug resistance, causing huge damage to food safety and human health. This chapter mainly introduces the application of low temperature in food processing, the changes of physiological and biochemical characteristics of bacteria exposed to a cold environment, and the factors affecting the development of cold resistance of foodborne pathogens, with emphasis on various cold resistance mechanisms of foodborne pathogens.

Keywords  Low temperature · Cold transient change · Cold resistance factors · Cold resistance mechanism · Membrane modification · CIPs · Sigma factor

3.1 Introduction to the Application of Low Temperature in Food Processing

As the material basis of human survival, food is rich in nutrients, and it is also a good medium for microbial growth and reproduction (Ray and Bhunia 2001; Alderson and Rowland 1995). However, food can harbor a variety of food-related microorganisms that are composed of foodborne pathogens and spoilage bacteria (including Bacillus cereus, Campylobacter jejuni, Clostridium perfringens, Escherichia coli, ...
Listeria monocytogenes, Salmonella, Staphylococcus aureus, Vibrio parahaemolyticus, and Yersinia pseudotuberculosis) (Law et al. 2015; Machado et al. 2020; Karan et al. 2013). Among them, foodborne pathogens are the main causes of food safety problems and human food poisoning or large-scale infection, and are responsible for numerous infectious diseases in humans. In order to ensure food safety, various microbial inactivation methods have been applied for food decontamination, such as high hydrostatic pressure, high-pressure sterilization, radiation, thermal processing, drying, pulsed electric fields, acidification, and low temperature treatment (such as chilling, freezing, and refrigeration) (Alvarez-Ordóñez et al. 2015).

Low temperature (chilling, freezing, etc.) is one of the most widely used methods for food preservation through the growth and metabolism inhibition of foodborne pathogens (Prokopov and Tanchev 2007; Wesche et al. 2009). It has the functions of prolonging food shelf life, preventing food spoilage, and keeping food fresh (Qiao et al. 2020). Fresh products (fruits, vegetables, etc.), aquatic products, dairy products, animal carcasses (meat, poultry, etc.), and their corresponding products (dumpings, ham sausage, ice cream, etc.) usually need to be chilled to lower temperatures throughout food processing process, especially during storage, transportation, distribution, and sale (Ricke et al. 2018; Hennekinne et al. 2012; Bortolaia et al. 2016; Puah et al. 2016). The National Shellfish Sanitation Program (NSSP) stipulates that shellfish should be chilled to 10 °C for a specified period of time when harvested for raw food (Baker 2016). The cold chain logistics of fresh food have been constantly promoted and improved.

The use of ice cellars for food preservation was dated back to China as early as 1000 Before Christ (BC) (Archer 2004). The history of frozen foods can be traced back to the sixteenth century in France, when ice makers made flavored ices (Lund et al. 2000). Since 1930s (retail sales revolution of frozen foods began in 1930), frozen foods have grown in variety and quantity, changing the family lifestyles (Archer 2004). Besides, with the increasing demand for minimally processed foods and ready-to-eat foods (smoked seafood, cheese, deli meats, etc., which can be consumed without any further heat treatment (Alves et al. 2020; Desai et al. 2019), the “refrigerated processed foods of extended durability” (food that has been refrigerated for a long time but will not go bad) was proposed (Nissen et al. 2002). These products are usually refrigerated at close to freezing point to keep healthy and safe (Markland et al. 2013).

3.2 The Changes in the Morphological, Physiological, and Biological Characteristics of Bacteria Under Cold Condition

Food is a complex environment in which foodborne pathogens may face a variety of stressors, including limited nutrient availability, adverse permeability, oxidation, extreme pH, and temperatures (Alvarez-Ordóñez et al. 2015). Bacteria need a
suitable temperature range to grow, develop, and reproduce normally. Low temperature provides bacteria with a cold stressor (Ricke et al. 2018). When exposed to a low temperature, bacterial cells go through three stages (Thieringer et al. 1998; Weber and Marahiel 2003): The first stage is the instantaneous reaction after exposure, known as the acclimatization post-shock phase. The second stage is the recovery phase. Finally, the cells enter a phase of permanent adaptation to cold stressor (the third phase) through gene expression modification, at which point the bacterial cells reach stationary growth (Ricke et al. 2018). It is generally believed that during the first stage of hypothermic shock, bacteria establish complex physiological reactions and induce changes in various physiological and biochemical characteristics (Phadtare and Inouye 2004; Gualerzi et al. 2003) including the decrease in cell membrane fluidity. The changes in membrane fluidity is the primary signal for microorganisms to sense cold stimulation (Deming 2002). Generally speaking, when bacteria are transferred from the optimal culture temperature to the cold culture environment, the membrane fluidity firstly decreases and then slightly increases. It was found that the membrane fluidity was firstly decreased and then increased slightly when the optimal growth temperature of Shewanella putrefaciens was transferred from 30 °C to 4 °C. However, with the extension of the incubation time at 4 °C, the membrane fluidity of S. putrefaciens recovered to a certain extent (Yang et al. 2020). Microbial cell membrane is mainly composed of fatty acids, which can be regulated by gene expression to maintain membrane fluidity (Phadtare 2004). The sudden drop in temperature results in a decrease in membrane fluidity, leading to the compromise in the functions of membrane proteins (Palonen et al. 2010). In addition, coagulation of cytoplasmic membrane lipids impedes biomolecular transport and other membrane-related processes (Phadtare 2004; Dahlsten et al. 2014) (Fig. 3.1), reducing enzyme catalytic activity and nutrient transport capacity.

Fig. 3.1 Schematic diagram of molecular mechanisms underlying the cold adaptation of foodborne bacteria, including an increase in membrane lipid fluidity and induction of cold-related proteins (Tasara and Stephan 2006)
Under a low temperature, the rate of enzymatic reaction in microorganisms is suppressed, slowing down the synthesis, degradation, and energy generation of microorganisms (Ray et al. 1994). For example, at 4 °C, the synthesis of most proteins involved in the pentose phosphate pathway, the tricarboxylic acid cycle, glycolysis, and electron chain transporters were found to be inhibited by the cold stress (Piette et al. 2011). Cold treatment was found to excessively stabilize the secondary structure of nucleic acids, which subsequently led to the inhibition of the DNA replication, transcription, degradation, and mRNA translation, and finally reduction in growth rate (Virtanen et al. 2018; Palonen et al. 2010). It is also reported that low temperature induced the production of radical oxygen species by both slowing metabolism and increasing oxygen solubility, which could prevent the germination of mesophilic spore forming species (such as mesophilic B. cereus) (Chattopadhyay et al. 2011; Virtanen et al. 2018; Markland et al. 2013). If the temperature drops further to the freezing point of the cytoplasm, ice crystals will form inside the cell, which can cause very serious damage to the cell structure (Ray et al. 1994). Yang et al. (2009) reported the changes in the transcriptome of V. parahaemolyticus under cold stress when the temperature dropped from 37 °C to 10 °C. It was found that most metabolism-related genes (e.g., most of the genes that regulate energy metabolism, fatty acid and phospholipid metabolism, amino acid synthesis, protein synthesis, and purine, pyrimidine, nucleoside, and nucleotide metabolism) were significantly downregulated under cold stress (Yang et al. 2009). At low temperatures (such as from 37 °C to 28 °C), the expression of virulence factors (e.g., protease, hemolysin) and the cytotoxicity of some clinical V. parahaemolyticus were also decreased (Mahoney et al. 2010). In addition, the researchers found that low temperature reduced the membrane fluidity of B. cereus and led to the conversion of its glucose metabolism pathway from glycolysis to pentose phosphate (Choma et al. 2000). Moreover, low temperature affected the anoxic fermentation of B. cereus, resulting in significant reduction in its ability to produce ethanol. The intracellular redox balance was also compromised by cold stressor, resulting in a low synthesis rate of branch-chain fatty acids, and ultimately inhibiting the growth of B. cereus (de Sarrau et al. 2012). The time duration for the acclimatization post-shock phase may vary with regards to the gene expression and growth rate reduction and subsequent protein synthesis for cold survival response (Ricke et al. 2018).

Bacteria can adapt to the external low temperature by regulating the expression of a series of genes (Konkel and Tilly 2000). During the second stage, bacterial cells grow faster and gradually resume the cellular protein biosynthesis (Ricke et al. 2018). When exposed to low temperature, the membrane fluidity of most foodborne pathogens firstly decreased and then slightly increased over time. Membrane fluidity is fundamental to cell function and survival, and it is critical to membrane integrity (Thieringer et al. 1998). Bacterial cells maintain membrane fluidity by regulating the composition of fatty acids in membrane (Yang et al. 2020). Fatty acids (FAs) are the main components of cytoplasmic membrane, and also participate in the adaptation of cells to environmental stress (Alves et al. 2020). In addition, at this stage, bacterial cells can adjust their transcription and translation functions through inducing DNA
supercoiling, optimizing RNA processing, and cold-inducible proteins production (Barria et al. 2013). The accumulation of low molecular weight solutes (e.g., carnitine, glycine betaine, mannitol, and trehalose) can also protect bacteria against cold stress (Kandror et al. 2002; Ewert and Deming 2011; Krembs et al. 2011; Hoffmann and Bremer 2011). For example, *E. coli* exhibit a complex pattern of growth behaviors under low temperature stress, including the “loss of culturability and recovery” phase (King et al. 2016). At this stage, there were obvious DNA damage, reduced utilization of carbon sources, and downregulation of molecular chaperones and related proteins associated with the oxidative damage reaction (Proteins regulated by RpoS, such as SufA/ECs2391, SufC/ECs2389, KatE/ECs2438, and SufS/ECs2387, seem to be involved in responses to oxidative damage) (Kocharunchitt et al. 2012; King et al. 2016). Cold treatment can induce the generation of a large number of reactive oxygen species (ROS), including atomic oxygen (O), singlet oxygen (¹O₂), ozone (O₃), superoxide anion (O₂⁻), hydroxyl radical (•OH), hydroxyl ions (OH⁻), and hydrogen peroxide (H₂O₂), etc. (Chen et al. 2020; Jiang et al. 2020). These ROS can damage the structure and function of bacteria in a variety of ways (including DNA damage, cell leakage, protein denaturation, lipid peroxidation, etc.) and interfere with cell metabolism (Wesche et al. 2009; Cheng et al. 2020). In addition, when bacterial exogenous ROS are present, it was observed that most pathogens can reduce further injury, metabolic activities and energy requirement by reducing their surface area (Joshi et al. 2011). It has been shown that prolonged exposure of *E. coli* cells to cold stress (14 °C) resulted in downregulation of several elements involved in oxidative stress responses (King et al. 2014). However, *E. coli* at this stage also showed a transient induction of rpo-controlled envelope stress response and activated the RpoS (the master stress regulators) and Rcs (involved in colonic acid biosynthesis) phosphorylation relay system (Vidovic and Korber 2016; Hagiwara et al. 2003; Kocharunchitt et al. 2012). Increased expression of several genes or proteins associated with DNA repair, amino acid biosynthesis, protein and peptide degradation, carbohydrate catabolism, and energy production (such as the tricarboxylic acid (TAC) cycle and the glycolysis/gluconeogenesis pathway) were observed in cold-stressed bacteria (The strain of *E. coli* O157:H7 Sakai) (King et al. 2016).

Although low temperature treatment is effective in limiting bacterial growth during food processing, exposure to low temperatures might also induce the cross-protection, virulence response, or cold adaptation of food-related spoilage and pathogenic microorganisms (Ricke et al. 2005, 2018). It was reported that after prolonged cold stress, the biofilm formation ability (After 2 years of cold stress, compare the frost exposure periods of 6 months and 2 years), virulence gene expression, and drug resistance (10 °C for 24 h) of *L. isteria monocytogenes* were increased (Al-Nabulsi et al. 2015; Slama et al. 2012). Cold stress induced the expression of a large number of virulence-associated genes, encoding the type III secretion system (T3SS), the effectors for SPI-1 and SPI-2 in *Salmonella* Typhimurium, and also induced the cross resistance to acid (Ricke et al. 2018). It is reported that the overall pathogenicity of cold-stressed *Salmonella* was increased and the association between S. Typhimurium and Caco-2 epithelial cells through
adhesion and invasion was also enhanced (Shah et al. 2014; Ricke et al. 2018). Resistance to *S. aureus* CICC10201 was also reported to increase after 4 weeks of exposure at 4 °C or −20 °C (Qiao et al. 2020). Among them, the mRNA expression of some virulence factors also showed a significant increase. For example, *S. aureus* CICC10201 cells exposed to 4 °C for 4 weeks showed significantly increased gene expression levels of *fuhD, hla*, and *sstD* (Qiao et al. 2020).

### 3.3 The Factors Affecting the Cold Resistance Development in Foodborne Pathogens

There are several factors that can affect the resistance of microbial cells to cold stress. These factors mainly include bacterial characteristics (bacterial species, growth phase, water content, etc.), freezing rate, freezing temperature, cold storage time, and the recovery medium which are used to estimate the viable microbial survivors. Furthermore, when exposed to a cold shock, the concentration and pH of extracellular solutes, and the presence of compounds with cryobiological properties may also pose an effect on the microbial cold adaptation (Archer 2004).

#### 3.3.1 The Kind of Microorganism and Its Growth Phase

The growth phase of the foodborne pathogens affects their resistance or sensitivity to freezing (Archer 2004). Microbes in logarithmic growth phase are more sensitive to freezing than those in other growth phases (Hamad 2012). The difference may be related to the nutritional status and other aspects of cell physiology in various phases. In studies of *E. coli*, higher carbohydrate level has generally been found to improve its survival (King et al. 2016; Hamad 2012; Calcott and MacLeod 1974). In addition, the susceptibility or resistance of different microorganisms to the potentially lethal effects of freezing varies greatly (Archer 2004). It has been reported that the bacterial spores and Gram-positive bacteria were generally more resistant to freezing and thawing than Gram-negative bacteria did (Lund et al. 2000; Archer 2004; Hamad 2012). And the differences between the various genera of Gram-negative (or Gram-positive) bacteria are significant. When frozen to −70 °C, about 90% of *Bacillus mesentericus* spores survived (Hamad 2012). However, under the same treatment, the survival rate of *E. coli*, *Pseudomonas aeruginosa* and *S. aureus* were 58%, 18%, and 96%, respectively. Lu et al. (2011) also observed that when *C. jejuni*, *E. coli* O157:H7 and *P. aeruginosa* were incubated at −18 °C for 20 days, the survival rate of *C. jejuni* was low, while the levels of *E. coli* O157:H7 and *P. aeruginosa* remained high (10⁴ CFU/ml) (Lu et al. 2011). Based on the differences in cold/heat tolerance, microorganisms can be divided into five groups: psychrophilic microbes with the optimum growth temperature of 15 °C or lower, psychrotrophic
microbes with the optimum temperatures of 20–40 °C, mesophilic microbes with the optimum growth temperature of 30–40 °C, thermoduric microbes which can survive at relatively high temperatures and grow within the temperature range of mesophiles and thermophilic microbes with the optimum growth temperature of 45–65 °C (Hamad 2012) (Table 3.1). Even strains from the same species may respond differently toward cold stress. Studies have found that the resistance of different clinical and poultry-derived C. jejuni strains to chilling at 4 °C and freezing at −20 °C varied greatly. Clinical strains were significantly more tolerant to chilling than poultry-derived strains, while the tolerance to freeze-thawing was lower than that of poultry-derived strains (Archer 2004; Chan et al. 2001). Phillips et al. (1998) investigated the effects of cold stress at 4 °C on Salmonella Enteritidis PT4 I and E. E strain is more tolerant and pathogenic than PT4 I. It was found that at the end of chilling treatment, sublethal damages of strain I was 29%, while that of strain E was 93%. And strain E showed a significant decrease after 12 days of treatment at 4 °C (Phillips et al. 1998). In addition, when exposing S. aureus CICC10201 and S. aureus W3 to cold stress (4 °C and −20 °C) for 4 weeks, it was found that the MIC value of S. aureus CICC10201 to quinolones and aminoglycosides

Table 3.1 Cardinal growth temperatures of some common foodborne pathogens (Source: Hamad 2012)

<table>
<thead>
<tr>
<th>G+/G−</th>
<th>Organisms</th>
<th>Categories</th>
<th>Minimum (°C)</th>
<th>Optimum (°C)</th>
<th>Maximum (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram-negative</td>
<td><em>Aeromonas hydrophila</em></td>
<td>Psychrotrophs</td>
<td>0–4</td>
<td>28–35</td>
<td>42–45</td>
</tr>
<tr>
<td></td>
<td><em>Campylobacter jejuni</em></td>
<td>Mesophiles</td>
<td>30</td>
<td>42–45</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em></td>
<td>Mesophiles</td>
<td>7</td>
<td>35–40</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella spp.</em></td>
<td>Mesophiles</td>
<td>5–10</td>
<td>35–37</td>
<td>45–49</td>
</tr>
<tr>
<td></td>
<td><em>Shigella</em></td>
<td>Mesophiles</td>
<td>7</td>
<td>37</td>
<td>45–47</td>
</tr>
<tr>
<td></td>
<td><em>Vibrio cholerae</em></td>
<td>Mesophiles</td>
<td>10</td>
<td>37</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td><em>Vibrio parahaemolyticus</em></td>
<td>Mesophilic strains</td>
<td>13</td>
<td>35–37</td>
<td>42–44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Psychrotrophic strains</td>
<td>3–5</td>
<td>30–37</td>
<td>40–42</td>
</tr>
<tr>
<td></td>
<td><em>Yersinia enterocolitica</em></td>
<td>Psychrotrophs</td>
<td>−1–4</td>
<td>28–30</td>
<td>37–42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Psychrotrophic strains</td>
<td>4–5</td>
<td>28–35</td>
<td>30–35</td>
</tr>
<tr>
<td></td>
<td><em>Clostridium perfringens</em></td>
<td>Mesophiles</td>
<td>12–20</td>
<td>30–47</td>
<td>45–51</td>
</tr>
<tr>
<td></td>
<td><em>Listeria monocytogenes</em></td>
<td>Psychrotrophs</td>
<td>0–4</td>
<td>30–37</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td><em>Staphylococcus aureus</em></td>
<td>Mesophiles</td>
<td>5–10</td>
<td>35–40</td>
<td>44–48</td>
</tr>
</tbody>
</table>
was increased by more than 4 times, while the resistance of *S. aureus* W3 to the same antibiotics did not change significantly, but the cell activity would be enhanced (Qiao et al. 2020).

### 3.3.2 The Freezing Rate

The freezing rate and post-cold stress time also affect the cold resistance of foodborne pathogens. When *E. coli* was frozen to $-70 \, ^\circ C$ at a rate of $6 \, ^\circ C/min$, the survival rate was about 70% (Hamad 2012; Jay 2000). However, when the cooling rate was 1 $/^\circ C/min$, the survival level of *E. coli* dropped to about 40%, and when the cooling rate was enhanced to 100 $/^\circ C/min$, the bacteria survival rate was reduced to about 20% (Hamad 2012). Generally, the faster the food is cooled above freezing temperature, the faster the microbes die. Because the coordination of various biochemical reactions required for the metabolism in microbial cells is rapidly disrupted during a rapid cooling (Dumont et al. 2004; Storey and Storey 2009). However, regarding the frozen food, slow freezing is more likely to cause microbial death than fast freezing does, which is due to the formation of ice crystals with small amounts and large sizes by slow freezing to cause mechanical damage to microbial cells and promote protein denaturation (Archer 2004). In addition, it was found that the biofilm formation of *S. aureus* CICC10201 cells gradually weakened with the prolongation of post-cold stress time. *S. aureus* CICC10201 and W3 were treated at $4 \, ^\circ C$ or $-20 \, ^\circ C$ for 4 weeks, and the cells showed uniform binary division with compact cell walls and intact cell contents. However, after 8 weeks of cold stress, irregular deformation, cytoplasmic wall separation, and contents leakage were observed (Qiao et al. 2020). Dykes and Moorhead (2001) conducted a commercial freezing simulated study of three *Salmonella* serotypes using beef products. They were quickly frozen ($-35 \, ^\circ C$) and slowly frozen ($-18 \, ^\circ C$) to ensure a final freezing temperature between $-17 \, ^\circ C$ and $-22 \, ^\circ C$ within 24 h incubation. After a series of treatments, (All processed beef trimmings were stored at $-18 \, ^\circ C$ for 9 months. Then a portion of the samples was thawed and refrozen at $-18 \, ^\circ C$ for 24 h to assess procedures that might be stressful for *salmonella* serotypes), no significant differences in survival rates were found for all strains during storage. This may be because, in contrast to meat, the inoculated strains freeze quickly (Dykes and Moorhead 2001). When Chen et al. (2002) studied *S. Enteritidis* in rapidly and slowly cooled eggs, they found that the growth of *S. Enteritidis* in egg yolk and albumen was inhibited in rapidly cooled eggs, but the bacterial population in egg yolk multiplied rapidly in the slowly cooled eggs (Chen et al. 2002). Moreover, it has been reported that refrigerating eggs immediately to $7 \, ^\circ C$ prevented *S. Enteritidis* that is already internalized on the yolk membrane to penetrate and contaminate the yolk, whereas *S. Enteritidis* could invade the yolk if stored at higher temperatures prior to the refrigeration (Gast et al. 2006).
### 3.3.3 The Composition of Frozen Medium

The difference in food composition may be a protective factor for the growth of foodborne pathogens under other stressors such as acid or thermal (Jarvis et al. 2016; Adhikari et al. 2018; Chung et al. 2006). If the microbial cells are cooled in distilled water, the addition of extra solutes during the freezing process minimizes the damage to the cells. The existence of NaCl (≥0.85%) in the freezing medium has been reported to promote the inhibition of *C. jejuni* and *E. coli* (Archer 2004; Abram and Potter 1984). Studies have reported that when *E. coli* was frozen to −70 °C in distilled water, the cell survival rate was about 20–80% at different cooling rates, while when the same bacterium was frozen in 0.85% saline (other conditions unchanged), the survival rate was reduced to below 10% (Hamad 2012). Moreover, it had been found that 3% NaCl could increase the freezing mortality rate of *C. jejuni* by nearly 50 times (Archer 2004; Abram and Potter 1984). Cryoprotectants (e.g., fat, glycerol, proteins, and sugars) can protect microbial cells from freezing damage in water and saline (Hamad 2012; Margesin et al. 2007). When 3% glycerol was added to water or 0.85% saline, the survival rate of *E. coli* cells frozen to −70 °C was increased to over 90%, regardless of the freezing rates (Hamad 2012). In addition, the recovery method of foodborne pathogens after freezing and thawing and the growth medium also have a significant impact on the cold resistance of bacteria (Archer 2004; Jarvis et al. 2016). For example, Smadi et al. (2012) demonstrated statistically significant differences in the use of laboratory media and chicken meat matrix to evaluate *Salmonella* growth at refrigeration temperatures. The estimated variance ratio between the two media (chicken divided by laboratory) was 6.79. This means that *Salmonella* growth on chicken substrates was 6.79 times more variable than on laboratory media (Smadi et al. 2012). Yuk and Schneider (2006) also pointed out that acid also affected the survival rate of *Salmonella* at refrigerated temperatures. *Salmonella* in stored juice showed a higher survival rate in simulated gastric juice (Yuk and Schneider 2006). The existence of other microbial strains in the foods also leads to differences in the survival rates under a cold shock. For example, Aldsworth et al. (1998) demonstrated that when the viable non-*Salmonella* microbiota was present in a food product, the *S. Typhimurium* in the food was protected from freezing injury by a competitive action. The role of the competitive microflora was to bring the resistance level of the cells in the exponential phase to that of stationary phase cells (Aldsworth et al. 1998; Ricke et al. 2018).

### 3.3.4 The Freezing Temperature

The freezing temperature also has a significant effect on the viability of foodborne pathogens. When the freezing temperature is higher than the freezing point, microorganisms that can adapt to low temperatures grow and reproduce gradually (Archer 2004). Because of the differences in biological characteristics of various
microorganisms, freezing temperature has different effects on the cold tolerance of various microorganisms. Previous studies have shown that the inactivation rates of most microorganisms at $-1$ to $-10 \, ^\circ\text{C}$ are higher than those at $-15$ to $-30 \, ^\circ\text{C}$, and the decrease in viability is usually small at temperatures of $-60 \, ^\circ\text{C}$ and below (Hamad 2012; Jay 2000). The survival rate of *B. mesentericus* spores storage in frozen water at $-1 \, ^\circ\text{C}$ for 133 days was about 50%, while the survival rate was almost 100% when they were stored in $-20 \, ^\circ\text{C}$ frozen water for the same period time (Hamad 2012; Farkas 2007). A similar phenomenon was observed in *E. coli*, *P. aeruginosa*, and *S. aureus* (Hamad 2012). In addition, studies have shown that different morphological changes were observed when *Clostridium psychrophilum* (a psychrophilic anaerobic bacterium) was exposed to a temperature ranging from $+5$ to $-15 \, ^\circ\text{C}$ (Perfumo et al. 2014). At $-5 \, ^\circ\text{C}$, the metabolism of bacterial population was retained. However, when the freezing temperature was below $-10 \, ^\circ\text{C}$, cell division was inhibited. Additionally, bacteria cells were observed to be surrounded by very thick cell walls and a capsule made of long exopolysaccharide fibers, and most cells were metabolically inactive or had slow metabolic flux (Perfumo et al. 2014; Sadiq et al. 2017).

The specific effects of the above factors on the cold resistance development of foodborne pathogens are still unclear. In order to better understand these complex relationships, it is necessary to conduct in-depth research on specific pathogens in various food systems. These results can provide important theoretical basis for the effective control of harmful bacteria in refrigerated food.

### 3.4 The Mechanisms Underlying Cold Resistance of Foodborne Pathogens

Refrigeration might be unable to completely inhibit the growth of foodborne pathogens, and even preserve the vitality of some pathogenic microorganisms. The growth and reproduction of microorganisms can lead to the deterioration of refrigerated food (Qian et al. 2013a, b), and the outbreaks of diseases associated with frozen food (Brackett 1994; Rawat 2015). It has been reported that consumption of contaminated frozen coconut milk may cause infection with *V. cholerae* (Taylor et al. 1993). In addition, frozen beef patties contaminated before cooking was reported to lead to an outbreak of *E. coli* O157:H7 infection (Taylor et al. 1993; Archer 2004). The cold adaptability of microorganisms is attributed to multiple mechanisms, including the maintenance of membrane fluidity, DNA superhelix modification, low temperature catalytic activity of enzymes, induction of cold-related proteins, and antifreeze protectants (Fig. 3.1), which contribute to the survival of bacteria at low temperatures (Ricke et al. 2018).
3.4.1 Membrane Modification

A sudden drop in temperature can result in a decrease in membrane fluidity of microbial cells (Cao-Hoang et al. 2008; Casadei et al. 2002; Chu-Ky et al. 2005; Fonseca et al. 2019). The maintenance of cell membrane fluidity is an important factor for microorganisms to adapt to low temperatures (Vigh et al. 1998). Foodborne pathogens regulate membrane fluidity and permeability through the following regulating mechanisms, including: (1) changing the composition of fatty acids in the cell membrane through increasing the content of unsaturated fatty acids, desaturating fatty acids, shortening the average length of acyl chain of fatty acids, adjusting the proportion of cis to trans fatty acids, increasing the proportion of branched fatty acids and isometric branched fatty acids, adjusting anteiso to iso fatty acid proportionalities, and reducing the content of cyclic fatty acids (Shivaji and Prakash 2010). Among them, increasing the content of unsaturated fatty acids in the membrane is the most important regulation, which contributes to reducing the melting point of membrane lipids and maintaining the liquid crystal state at low temperature (Annous et al. 1997). Studies have shown that membranes composed of short-chain fatty acids and branched or unsaturated fatty acids are more loosely structured and less tightly packed, leading to the increase in the fluidity of membranes and the absorption capacity of nutrients (Mendoza 2014; Parsons and Rock 2011). The researchers found that the synthesis of unsaturated fatty acids in E. coli cell membranes increased with the modification of fatty acid isomerization at low temperatures (citations), while the content of palmitic acid C\textsubscript{16:0} decreased (Shivaji and Prakash 2010; Marr and Ingraham 1962). In addition, when the temperature decreased from 30 to 10 °C, the ratio of short- to long-chain fatty acids and branched fatty acids to straight-chain fatty acids of L. monocytogenes was found to be increased (Li et al. 2002). It was reported that Bacillus subtilis also adapted to low temperature by increasing the proportion of anteiso to iso branched-chain fatty acids (Beranová et al. 2010). And unsaturated fatty acids play an important role in the cold adaptation of Pseudomonas spp. (Garba et al. 2016). (2) Increasing the content of some extracellular proteins and transporters. Proteomics and transcriptome studies have shown that the addition of some extracellular proteins (associated with transcription, translation, cell division, ribosomes, and fatty acid biosynthesis, etc.) and transporters (e.g., Peptide transport correlation) can increase the rate of nutrient transport in the cell membrane (Piette et al. 2011; Durack et al. 2013). (3) Changing the level of carotenoids in membrane. Temperature-dependent synthesis of specific types of carotenoids (non-polar and polar) may be a strategy for regulating membrane fluidity (Shivaji and Prakash 2010; Chattopadhyay et al. 1997). It has been reported that the content of staphyloxanthin and other carotenoids increased after Staphylococcus xylosus was grown at 10 °C, but no carotenoids were detected after growing at 30 °C (Seel et al. 2020). Enrichment of carotenoids in cells increased resistance to freeze-thaw stress, and thus had a protective effect on cells at low temperatures (Dieser et al. 2010). In addition, previous studies have found that polar carotenoids stabilize membranes to a greater extent than nonpolar carotenoids.
In two psychrophilic bacteria, Micrococcus roseus (MTCC 678) and Sphingobacterium antarcticum (MTCC 675), the levels of polar carotenoids increased while the levels of less polar carotenoids decreased (Chattopadhyay and Jagannadham 2001; Ricke et al. 2018; Kiran et al. 2004).

In general, bacteria adapt to cold temperatures by increasing the proportion of fatty acids with lower melting points (e.g., unsaturated fatty acids and hetero-branched fatty acids) to maintain membrane functions (Brillard et al. 2010; de Sarrau et al. 2012; Haque and Russell 2004; Mansilla et al. 2004). For example, B. cereus can adapt to cold by significantly increasing the ratio of unsaturated/saturated fatty acids (Brillard et al. 2010; de Sarrau et al. 2012; Haque and Russell 2004). Studies had reported that the main fatty acid composition changes caused by the low temperature adaptation process of B. cereus were the decrease of branched, saturated fatty acids iso-C_{15} and iso-C_{17}, with a concomitant increase of branched saturated fatty acids iso-C_{13} and branched, monounsaturated fatty acids iso-C_{17:1} (Alvarez-Ordóñez et al. 2015; Brillard et al. 2010; de Sarrau et al. 2012). In addition, B. cereus can produce DesA (Δ5 desaturase) and DesB (Δ10 desaturase), which form double bonds at the 5 and 10 positions of the fatty acid chain, respectively (Diomandé et al. 2015a). The consensus motifs of DesA and DesB acyl-lipid desaturases strongly suggest that they are membrane-bound associated proteins (Diomandé et al. 2014). Since most of the unsaturated fatty acids were accumulated in position 5 at low temperatures, it was speculated that DesA played a major role in the cold adaptation of B. cereus (Cifré et al. 2013) (Fig. 3.2c).

E. coli regulates the content of unsaturated fatty acids through three enzymes, FabA (β-hydroxydecanoyl dehydrase), FabB, and FabF. FabB and FabF are 3-ketoacyl-acyl carrier protein (ACP) synthase (KAS) enzymes (often referred to as condensing enzymes) (Virtanen et al. 2018; Heath and Rock 2002) in which FabF [3-oxoacyl-(acyl-carrier protein) synthase II] encoded by fabF and found to be overexpressed at low temperatures (Mansilla et al. 2004). In order to change the membrane fluidity (increase the membrane fluidity) to adapt to lower temperatures, dodecanoic acids and hexadecanoic acids were decreased and tetradecanoic acid was increased during the transition of Salmonella culture from 37 °C to 12 °C (Ricke et al. 2018; Wollenweber et al. 1983; Kropinski et al. 1987; Juárez-Rodríguez et al. 2020), accompanied by the emergence of palmitoleic acid in lipid A, an alternative to dodecanoic acid (Ricke et al. 2018). In addition, alternative Sigma factor RpoS of Salmonella has been used as a stress-response gene activator induced at stationary phase, affecting the production of cyclopropane fatty acids and involved in the survival of Salmonella during refrigeration (McMeechan et al. 2007). Moreover, in gram-negative bacteria, the phosphorylation or/and acetylation of some lipooligosaccharides (LOS), which make up the cell wall lipopolysaccharide (LPS) (located on the periphery of the external cell membrane), might also contribute to the growth of bacteria under the low temperature environment (Corsaro et al. 2001).

Recently, the two-component systems (TCSs, is commonly referred to as the phosphor-transfer pathway) related to fatty acid (FA) metabolism have been found in a variety of foodborne bacteria (such as CasKR in B. cereus, CheAY in Y. pseudotuberculosis, DesKR in B. subtilis, CorSR in Pseudomonas syringae,
Fig. 3.2 Cold adaptation of foodborne pathogens through cell membrane modification. (a) In *Escherichia coli*, at 37 °C, LpxL was responsible for the adhesion of lauric acid to lipid A (lipopolysaccharide in membrane). At low temperatures (15 °C), reduced lauric acid is balanced by palmitoleic acid. LpxP is a cold-induced acyltransferase responsible for the attachment of palmitoleic acid to lipid A. (b) A temperature-sensing two-component membrane cooling mechanism of *Bacillus subtilis*. (c) Changes in membrane fatty acids of *Bacillus cereus* during cold
PhoPQ in *Edwardsiella*, CBO0366/CBO0365 in *C. botulinum*, Fp1516/Fp1517 in *Flavobacterium psychrophilum*, YhcSR in *S. aureus*, and LisRK in *L. monocytogenes*), which may serve as a mechanism of membrane adaptation to cold (Diomandé et al. 2014; Phadtare 2004). This typical two-component signal transduction system consists of a histidine kinase sensing factor and a DNA-bound response regulator protein (Virtanen et al. 2018; Sinetova and Los 2016). CaskR (a two-component sensor-regulator system) has been observed to contribute to the adaption of *B. cereus* sensu lato (including *B. thuringiensis*, *B. weihenstephanensis* and *B. anthracis*) under low temperatures (Diomandé et al. 2014; Alvarez-Ordóñez et al. 2015). At a low temperature (12 °C), compared to the *B. cereus* ATCC 14579 WT strain, CaskR deficiency (isogenic casK/R mutant ΔcasK/R) led to a decreased expression of *desA* gene and decreased synthesis of unsaturated fatty acids at position 5 (Alvarez-Ordóñez et al. 2015; Diomandé et al. 2015b). It is speculated that CasKR participates in the cold adaptation of *B. cereus* through increasing the membrane fluidity (Fig. 3.2c) (Diomandé et al. 2014, 2015a). In *B. subtilis*, there is also a two-component system, DesKR, which can sense the temperature drop. The system consists of a sensor kinase DesK inserted in the membrane and a response regulator DesR. DesK includes five transmembrane segments that define the sensor domain and a long cytoplasmic C-terminal tail containing histidine kinases. This C-terminal kinase of DesK undergoes autophosphorylation in the presence of ATP and transfers a phosphate to DesR (Virtanen et al. 2018; Diomandé et al. 2015b; Alvarez-Ordóñez et al. 2015; Phadtare 2004). DesK mediates the phosphorylation of DesR, which activates the transcription of the membrane-bound phospholipid desaturase (the phosphorylated form of DesR is a transcriptional regulator directly involved in the activation of *des* genes at low temperature), thereby modifying the existing fatty acids in the membrane into unsaturated fatty acids (Fig. 3.2b) (Cybulski et al. 2002). Compared with Desk, the sensor protein CasK of *B. cereus* does not have the predicted transmembrane domain, so *B. cereus* senses cold through other mechanisms (Alvarez-Ordóñez et al. 2015). The role of two-component system in cold adaptation has been studied extensively in *Listeria* (Chan and Wiedmann 2008; Pöntinen et al. 2017). Through mutation analysis, Chan et al. (2008) found that the regulatory genes *lmo1060*, *lmo1377* (*lisR*), and *lmo1172* were extremely important for the low temperature adaptation of *L. monocytogenes* at

![Fig. 3.2](continued) adaptation (under aerobic condition). (1) Cold causes the membrane fluidity to decrease. (2) Protein sensor CasK senses cold through an unknown mechanism. CasK activates the regulatory protein CasR, which directly or indirectly activates the transcription of *desA* and *desB* of fatty acid desaturase genes. (3) DesA contributes to the production of unsaturated membrane fatty acid is located at position 5, mainly isoC17:1 (n = 5). The proportion of unsaturated fatty acids produced by DesB at low temperatures did not increase. (4) The proportion of isoC13 fatty acids increased while that of isoC15 fatty acids decreased. A GDSL (Gly-Asp-Ser-Leu)-Lipas is necessary for both this ratio change and cold adaptation. (5) The unsaturated fatty acids in the growth medium were incorporated into the membrane of *B. cereus*, thereby increasing the membrane fluidity at low temperatures. (6) Increased membrane fluidity compensates for the effects of cold and allows bacteria to grow (Alvarez-Ordóñez et al. 2015; Barria et al. 2013; Sinetova and Los 2016)
Studies have also shown that the expressions of histidine kinases-encoding gene \textit{yycG} and \textit{lisK} in \textit{Listeria} were induced by low temperature exposure (3 °C), and contribute to the cold stress response of \textit{Listeria} (Pöntinen et al. 2015; Chan et al. 2008).

\subsection{DNA Supercoiling Modification}

The structure and shape of DNA have an impact on its function (Kool et al. 2000). DNA supercoiling is the shape of DNA that is wrapped in living cells in prokaryotes with a very high DNA helix (Ricke et al. 2018). DNA supercoiling states, which can be either positive “overtwist” or negative “unwind,” have been shown to play a variety of roles in genome function, ensuring the integration of DNA strands during replication, initiation, transcription, and recombination (Cameron et al. 2011; Mirkin 2001). The positive DNA superhelix state is regulated by topoisomerase I and III, while the negative DNA superhelix state is regulated by topoisomerase IV and DNA gyrase (Terekhova et al. 2012). DNA topoisomerases are enzymes that play a role in all DNA-related topological states, including DNA superhelices (Ghilarov and Shkundina 2012). Temperature-induced conformational or physicochemical changes in DNA, RNA, and proteins can form the basis of temperature sensing (Eriksson et al. 2002). DNA conformation depends on the temperature-dependent changes of DNA superhelix (Hurme and Rhen 1998). Cold stress could increase the negative supercoiling of DNA, which has been shown to contribute to cold adaptation. It has been proved that cold shock can induce and accumulate DNA gyrase subunits in \textit{E. coli} (Jones et al. 1992). At low temperatures, enzymes or physical distortions in DNA make the diplex structure unstable, thus promoting the formation of open complexes with DNA polymerases (Prakash et al. 2009). Prakash et al. have found that the cold-induced changes in negative DNA supercoiling can affect the expression of some cold-induced genes in \textit{Synechocystis} (such as \textit{crhR}, \textit{desB}, \textit{hliABC}, \textit{ndhD2}, \textit{rhpA1}, and \textit{rlpA}) (Cameron et al. 2011; Terekhova et al. 2012; Shapiro and Cowen 2012; Prakash et al. 2009). Moreover, topoisomerases I and II and “nucleoid-associated” proteins (such as H-NS) also regulate the superhelix (Shivaji and Prakash 2010).

\subsection{Induction of Cold-Related Proteins}

Since 1987, the cold-induced proteins of microorganisms have been studied (Jones et al. 1987). When exposed to low temperatures, a set of proteins is produced by microorganisms to adapt to cold stress, including cold shock proteins (CSPs), cold acclimatization proteins (CAPs), and cold-induced proteins (CIPs) (Beckering et al. 2002; Sanghera et al. 2011). CSPs and CIPs are associated with the suppression of most housekeeping genes after low temperature exposure (Ricke et al. 2018). The
number of CIPs varies with the species of bacteria (Wouters et al. 2000). CSPs are the small expressed proteins with a mass of below 10 kDa, and CSPs larger than 10 kDa should belong to the CIP group (Ricke et al. 2018). CAPs are proteins characterized by very high synthesis (continuously synthesized at low temperatures corresponding to the growth rate of temperature), which mainly occurs during prolonged exposure and subsequent growth at cold temperature (Struvay and Feller 2012; Hébraud and Potier 1999). Under a low temperature condition, CAPs are synthesized at a faster rate than CSPs did (Ricke et al. 2018; Phadtare 2004). Upon a cold shock, the cell senses changes in temperature and sends a signal to the responsible component (mainly the transcriptional and translational devices, but also possibly structural elements). Thus, something can be observed in Fig. 3.3 (Barria et al. 2013).

CSPs contribute to microbial cells resistance to low temperature environments, and are also considered as one of the first proteins synthesized by microorganisms after cold shock (Keto-Timonen et al. 2016; Schmid et al. 2009; Chattopadhyay 2006). It is mainly induced after rapid cooling to regulate the adaptation to cold stress (Horn et al. 2007). Up to now, researchers have found CSPs in cold-adapted

![Fig. 3.3 Schematic diagram of protein expression pattern during cold shock adaptation. Immediately after cold shock, cell growth (green line) is temporarily halted (3 to 6 h) and non-cold shock proteins are inhibited, *Escherichia coli* cells reportedly stop growing for about 4 h at this stage. During acclimation, a large number of protein synthesis stops (non-CIPs yellow line), however, the expression of a small number of cold-induced proteins (CIPs) (black line) that play an important role in adaptation (including cold shock proteins (CSPs)) is increased, accompanied by corresponding mRNA attenuation. After the acclimation phase, cells began to adapt to low temperature and resumed growth, and a large number of protein production resumed, most of the CIPs were inhibited and their expression decreased. In the case of *E. coli*, the expression of a large number of proteins in the cells was inhibited and the expression of cold shock proteins was increased in a short time after the sudden drop of temperature. However, when the cooling continues for a period of time, the suppressed protein will be restored to a new level of expression (Barria et al. 2013; Horn et al. 2007)]
bacteria, mesophilic bacteria, extreme thermophilic bacteria, and a variety of foodborne bacteria (such as *E. coli*, *S. aureus*, *Salmonella*, *L. monocytogenes*, and *B. cereus*) (Czapski and Trun 2014; Jin et al. 2014). CSPs are produced with the regulation by the cold shock associated genes (e.g., *cspABE*), which was first found in *E. coli* and rapidly synthesized in large quantities under cold stimulation (Bae et al. 2000; Giuliodori et al. 2010; Yamanaka et al. 1998). And it was found that the larger the temperature fluctuation range, the greater the amount of CSPs were produced (Ermolenko and Makhatadze 2002). The amino acid length of CSPs is around 65–75, consisting of five antiparallel β folding β barrel lamellae (Czapski and Trun 2014; Jin et al. 2014), containing nucleic acid binding cold shock domain (CSD) (Keto-Timonen et al. 2016; Beckering et al. 2002). These sequences for CSPs are highly conserved, which are determined by two typical nucleic acid binding motifs: RNP1 (K/R-G-F/Y-G/A-F-V/I-X-F/Y) and RNP2 (L/I-F/Y-V/I-G/K-N/G-L (Behl et al. 2020). However, in *E. coli*, *S. Typhimurium*, *P. aeruginosa*, *L. monocytogenes*, and *Yersinia pestis*, etc. RNP1 and RNP2 sequences were modified as K/S-G-F/K-YG-F/L-I-X-X and L/I-V-F/Q-V/A/L-H-X-S/T/R, respectively (Horn et al. 2007; Yu et al. 2019). Although CSPs consist of up to nine members in some bacteria, they have also been shown to be conserved in a variety of Gram-negative and Gram-positive bacteria, with more than 45% similarity to the CSP families (Yang et al. 2009).

The expression and regulation of cold shock genes and their proteins are the key to cold adaptation, and cold shock proteins can participate in a variety of molecular functions such as DNA replication, transcription, and translation (Golovlev 2003). The mechanism of CSPs act as nucleic acid chaperones (Barria et al. 2013), to bind to single-stranded RNA or DNA through RNP1 and RNP2, to prevent the complementary pairing of RNA or DNA at low temperatures, to inhibit the formation of a secondary RNA structure, and to promote effective transcription and translation at low temperatures (Horn et al. 2007) (Fig. 3.4). Moreover, CSPs may also cooperate with RNA helicases to interfere with RNA degradation at low temperatures (Phadtare 2016). For example, CspA produced by *E. coli* worked as both a transcription antiterminators (enhance the expression of proteins related to low temperature adaptation, such as IF2, NusA, PNP, and RbfA) and a transcriptional activator (e.g., it promoted the expression of DNA binding protein Hns and DNA isomerase GyrA) when the temperature was lowered (Bae et al. 2000; Ren et al. 2008). Most cold-induced proteins are found to be involved in RNA metabolism, such as two exonucleases RNase R and polynucleotide phosphorylase PNPase and RNA helicase DeaD (Barria et al. 2013). In addition, CSPs can also regulate membrane fluidity (Horn et al. 2007; Casanueva et al. 2010).

CSPA protein is the most widely studied and earliest described among all CSPs, and it is also the most dominant CSPs in *E. coli* (Ray et al. 2020; Hébraud and Potier 1999). CSPA is composed of 70 amino acids and can bind to single-stranded DNA or mRNA as a gene expression regulator (Ricke et al. 2018). In addition, CSPA acts as a partner to force RNA into the single-stranded form and result in subsequent RNA degradation (Barria et al. 2013). However, studies have shown that not all CSPs are associated with cold shock reactions (Graumann and Marahiel 1996).
Among the 8 CspA homologous proteins (CspB~CspI) of *E. coli*, CspB, CspG, and CspI were induced by low temperature, while CspC and CspE were all produced when the temperature increased or decreased (Yu et al. 2019). Therefore, CspA, CspB, CspG, and CspI encoding genes were considered as cold shock-induced.
protein genes (Barria et al. 2013; Hankins et al. 2010). Of the five identified cold shock proteins (CspA~CspE) in B. cereus, only two (CspA and CspE) have been shown to be elevated after cold shock (Alvarez-Ordóñez et al. 2015).

The activation of CSP gene expression depends on a series of complex transcriptional regulatory genes (Wu et al. 2010). When the temperature changed from 37 °C to 10 °C, the expression levels of CSPs, such as CSPA, in E. coli were increased (Wu et al. 2010; Phadtare et al. 1999). The regulatory region in cspA gene has the common characteristics of CSPs-encoding genes (Fig. 3.5). It has been shown that the upstream box of E. coli regulatory elements can promote the formation of translation complex and improve the efficiency of CSP translation (Ermolenko and Makhatadze 2002; Thieringer et al. 1998). Besides, the cold box is highly conserved in the cold shock gene, which is a putative transcriptional suspension site and is involved in the inhibition of CspA expression. Studies have shown that the deletion of cold box leads to decrease in the half-life of CspAEC mRNA (Phadtare et al. 1999).

In addition, there are other CSPs in bacterial cells, such as PNP, CsdA, NusA, RbfA, and GyrA (Table 3.2). Studies have shown that RbfA, CsdA, and IF-2 accumulated in E. coli cells after the onset of cold shock, and over time, they bind with free ribosome subunits to form biologically active cold-adapted ribosome polysomes that enhanced the mRNA translation of non-cold-induced protein genes (Jones and Inouye 1996; Hankins et al. 2010). In addition, we found that CspA, CspC, and CspE induced the transcription of genes (pnp, nusA, rbf, infB, etc.) through anti-termination in E. coli, suggesting that the cold shock activation of these genes occurred through anti-transcriptional termination (Bae et al. 2000). In Salmonella, in addition to cold shock proteins such as CspA and CspE, Cold-shock DEAD box protein A (CsdA) (previously known as “DeaD”), the NusA protein, DnaA protein (encoded by dnaA), RecA protein, the trigger factor (TF) protein (tig gene), The RbfA (ribosome-binding factor A) Protein, polynucleotide
Table 3.2  Summary of some genes and their functions related to cold stress adaptation mechanisms in foodborne pathogens (with definite or putative involvement). (Sources: Barria et al. 2013; Tasara and Stephan 2006)

<table>
<thead>
<tr>
<th>Type</th>
<th>Gene</th>
<th>Description/function in cold stress adaptation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell membrane-associated protein genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>betL</td>
<td></td>
<td>Encoding betaine transporters</td>
</tr>
<tr>
<td>fbp</td>
<td></td>
<td>Encoding the fibronectin binding protein</td>
</tr>
<tr>
<td>flmA</td>
<td></td>
<td>function unknown</td>
</tr>
<tr>
<td>gbuABC</td>
<td></td>
<td>Involve in Glycine betaine absorption</td>
</tr>
<tr>
<td>oppA</td>
<td></td>
<td>Essential for growth at low temperatures</td>
</tr>
<tr>
<td>oppC</td>
<td></td>
<td>Encoding carnitine transporters</td>
</tr>
<tr>
<td><strong>Cold stress-associated protein genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aceE</td>
<td></td>
<td>Decarboxylase, pyruvate dehydrogenase</td>
</tr>
<tr>
<td>aceF</td>
<td></td>
<td>Pyruvate dehydrogenase, dihydrolipoamide acetyltransferase</td>
</tr>
<tr>
<td>aroA</td>
<td></td>
<td>Encode amino acid biosynthetic enzymes</td>
</tr>
<tr>
<td>clpB</td>
<td></td>
<td>General stress resistance proteins</td>
</tr>
<tr>
<td>clpP</td>
<td></td>
<td>General stress resistance proteins</td>
</tr>
<tr>
<td>cspA</td>
<td></td>
<td>Cold-induced RNA chaperone and anti-termination factor; transcription enhancer</td>
</tr>
<tr>
<td>cspB</td>
<td></td>
<td>Cold shock-inducible; the exact biological function is unknown</td>
</tr>
<tr>
<td>cspE</td>
<td></td>
<td>RNA chaperone; transcription antitermination</td>
</tr>
<tr>
<td>cspG</td>
<td></td>
<td>Cold shock protein homologue, cold-inducible</td>
</tr>
<tr>
<td>cspI</td>
<td></td>
<td>Cold shock protein, cold shock-inducible</td>
</tr>
<tr>
<td>cysS</td>
<td></td>
<td>Encode amino acid biosynthetic enzymes</td>
</tr>
<tr>
<td>deaD</td>
<td></td>
<td>ATP-dependent RNA helicase, involved in ribosome biosynthesis, translation initiation, and mRNA decay</td>
</tr>
<tr>
<td>degU</td>
<td></td>
<td>Involved in temperature-dependent transcriptional regulation</td>
</tr>
<tr>
<td>dnaA</td>
<td></td>
<td>DNA binding and replication initiator, global regulator of transcription</td>
</tr>
<tr>
<td>groEL</td>
<td></td>
<td>Maintain protein solubility</td>
</tr>
<tr>
<td>gyrA</td>
<td></td>
<td>DNA gyrase, subunit A; involved in DNA binding/cleaving/rejoining</td>
</tr>
<tr>
<td>hfq</td>
<td></td>
<td>Cold stress adaptive regulatory protein genes</td>
</tr>
<tr>
<td>hns</td>
<td></td>
<td>Nucleoid protein, transcriptional suppressor, repressor supercoiling</td>
</tr>
<tr>
<td>hscA</td>
<td></td>
<td>DnaK-like chaperone</td>
</tr>
<tr>
<td>hscB</td>
<td></td>
<td>DnaJ-like co-chaperone for HscA</td>
</tr>
<tr>
<td>hupB</td>
<td></td>
<td>Nucleoid protein, DNA supercoiling</td>
</tr>
<tr>
<td>infA</td>
<td></td>
<td>Protein chain initiation factor 1 (IF1), translation initiation</td>
</tr>
<tr>
<td>infB</td>
<td></td>
<td>Protein chain initiation factor 2 (IF2), translation initiation, fMet-tRNA binding, protein chaperone</td>
</tr>
<tr>
<td>infC</td>
<td></td>
<td>Protein chain initiation factor 3 (IF3), translation initiation, stimulates mRNA translation</td>
</tr>
<tr>
<td>ksgA</td>
<td></td>
<td>Regulator of ribosome biogenesis; critical for cell growth rate at low temperatures</td>
</tr>
</tbody>
</table>

(continued)
phosphorylase (PNPase) (encoded by \textit{pnp}), KsgA protein (encoded by \textit{ksgA}, involving in ribosome biogenesis), SrmB protein, and The Initiation Factors (IFs) (initiation factor 2) (encoded by \textit{infB} also affect its cold resistance (Ricke et al. 2018). Moreover, the CSP CspE in \textit{S. Typhimurium} was found to be important for biofilm formation and movement (Fig. 3.6) (Ray et al. 2020).

In conclusion, both the expression regulation of cold shock protein itself and its mechanism in cell cold adaptation are very complex. The research on the mechanism of cold shock proteins can provide a good theoretical basis for the elimination of foodborne pathogens under low temperature treatment.

**Table 3.2** (continued)

<table>
<thead>
<tr>
<th>Type</th>
<th>Gene</th>
<th>Description/function in cold stress adaptation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textit{lhkA}</td>
<td>Encoding histidine kinase sensor</td>
</tr>
<tr>
<td></td>
<td>\textit{lpxP}</td>
<td>Lipid A synthesis; cold-inducible</td>
</tr>
<tr>
<td></td>
<td>\textit{ltrA/B/C}</td>
<td>Low temperature requirement genes; function unknown</td>
</tr>
<tr>
<td></td>
<td>\textit{nusA}</td>
<td>Transcription termination/antitermination/elongation L factor</td>
</tr>
<tr>
<td></td>
<td>\textit{orfX}</td>
<td>Related to cold stress adaptation</td>
</tr>
<tr>
<td></td>
<td>\textit{otsA}</td>
<td>Trehalose phosphate synthase; critical for viability at low temperatures</td>
</tr>
<tr>
<td></td>
<td>\textit{otsB}</td>
<td>Trehalose phosphate phosphatase; cold- and heat-induced, critical for viability at low temperatures</td>
</tr>
<tr>
<td></td>
<td>\textit{pnp}</td>
<td>3′–5′ exoribonuclease, cold shock protein; critical for growth at low temperatures; involved in RNA metabolism</td>
</tr>
<tr>
<td></td>
<td>\textit{psr}</td>
<td>Cold stress adaptive regulatory protein genes</td>
</tr>
<tr>
<td></td>
<td>\textit{rnr}</td>
<td>3′-5′ exonucleases</td>
</tr>
<tr>
<td></td>
<td>\textit{rbfA}</td>
<td>Ribosome-binding factor needed for efficient processing of 16S rRNA; cold shock adaptation protein</td>
</tr>
<tr>
<td></td>
<td>\textit{recA}</td>
<td>Involved in the recombination and SOS response for DNA repair</td>
</tr>
<tr>
<td></td>
<td>\textit{rho}</td>
<td>Involved in clearing bacterial DNA of frozen ribosomes and polymerases at low temperatures, and coordinating magnesium homeostasis required by ribosomes</td>
</tr>
<tr>
<td></td>
<td>\textit{tig}</td>
<td>Protein-folding chaperone, multiple stress protein, involved in ribosome-binding</td>
</tr>
<tr>
<td></td>
<td>\textit{trpG}</td>
<td>Encode amino acid biosynthetic enzymes</td>
</tr>
<tr>
<td></td>
<td>\textit{trxB}</td>
<td>Cold stress protein genes; associated with growth at low temperatures</td>
</tr>
<tr>
<td></td>
<td>\textit{ves}</td>
<td>Cold- and stress-inducible protein, function unknown</td>
</tr>
<tr>
<td></td>
<td>\textit{yfiA}</td>
<td>Protein Y, associated with 30S ribosomal subunit, inhibits translation</td>
</tr>
</tbody>
</table>
3.4.4 Sigma Factor

Sigma factor is a subunit of RNA polymerase, which binds to the core enzyme of RNA polymerase and is responsible for the initiation of transcription (Nagai and Shimamoto 1997; Sutherland and Murakami 2018). It plays an important role in the regulation of bacterial gene expression and can promote the rapid adaptation of bacteria to the changes in environmental conditions (Francez-Charlot et al. 2009; Tuchscherr et al. 2015; Guldimann et al. 2016). Sigma factors involved in stress response mainly included Sigma B (sigB) in some Gram-positive bacteria (including the genera *Staphylococcus*, *Bacillus*, *Listeria*, etc.) and Sigma S (RpoS) in Gram-negative bacteria (Chaturongakul et al. 2008; Cebrián et al. 2009). Sigma B factor can regulate the response of Gram-positive bacteria to various environmental stimuli (Guldimann et al. 2016; van Schaik and Abe 2005). Under low temperature stress, the expression of some genes (such as *gbuA*) regulated by sigB in *L. monocytogenes* was overexpressed (Becker et al. 2000). Recently, Utratna et al. (2014) found that the expression pattern of sigB factor was similar at both low temperature and the optimal temperature. However, when the anti-sigma factor antagonist RsbV was absent, sigB activity could be only detected at low temperature, but not at the optimal temperature (Hughes and Mathee 1998; Rodríguez Ayala et al. 2020; Sinha et al. 2020; Utratna et al. 2014). Therefore, it was speculated that sigB had a unique mode of action at low temperature (Hecker et al. 2007). Although RpoS is not a cold-induced proteins, it is involved in the expression of many genes involved in adaptation to cold stress (Li et al. 2018; Vidovic et al. 2011). As the main regulator of the general stress response of *E. coli* O157:H7, the regulation of RpoS in its cold adaptation involves

Fig. 3.6  Schematic diagram of relationship between CspE and the biofilm formation in *Salmonella* Typhimurium. CspE regulates biofilm formation by downregulating another cold shock protein, CspA. However, its regulation of swarming motility is independent of CspA (Ray et al. 2020)
both positive and negative regulation (Vidovic and Korber 2016). The most significant role of RpoS is to regulate enzymes involved in adhesion adaptation. The strategy to maintain membrane fluidity at low temperature in *E. coli* O157:H7 is achieved through two positively regulating genes, *fabD* and *cfa* (encoding malonyl-coenzyme a-acyl carrier protein [ACP) transacylase and cyclopropane aliphatic acyl phospholipid synthase [cfa synthase), respectively. In addition, it was found that under low temperature and water activity stress, genes and proteins related to chemotaxis and motility were significantly downregulated under the effect of RpoS and Rcs (Kocharunchitt et al. 2012). Although no special sigma factor was found to be involved in the cold shock response in *E. coli*, existing studies have proved that 40% of genes differentially expressed in *E. coli* cells under low temperature were regulated by RpoS (Barria et al. 2013; White-Ziegler et al. 2008). In addition, it was found that RpoS factor and extracytoplasmic function RpoE factor in *S. Typhimurium* both played a role in response to cold stress. And the growth and survival ability of the rpoS/rpoE double mutant strain was weaker than that of the single mutant strain (Knudsen et al. 2014; McMeechan et al. 2007). Extracytoplasmic function (ECF) sigma factors are a subfamily of σ^{70} Sigma factors that activate genes related to stress response function. It is responsible for transmitting extracytoplasmic signals into the cytoplasm (Woods and McBride 2017). Currently, genomic sequence analysis has shown that extracytoplasmic Sigma factors exist in some foodborne bacteria, but their role in low temperature adaptation remains unclear (Carlin et al. 2010; Helmann 2002). Therefore, it is necessary to further study the regulation of Sigma factor on the cold resistance mechanism of foodborne pathogens.

### 3.4.5 Adaptation of Metabolism

In order to adapt to the low temperature environment, foodborne pathogens increase their metabolic level in various ways (Wesche et al. 2009; Cordero et al. 2016). Studies have found that *L. monocytogenes* adapted to cold stress by increasing the production of some enzymes involved in glucose metabolism and Pta-Ack A pathway, such as acetic kinase and phosphotransacetylase (De Maayer et al. 2014). RNA metabolism is crucial for cold adaptation, and low temperature over stabilizes the secondary structure of mRNA, thus reducing translation efficiency (Alvarez-Ordóñez et al. 2015). Many bacteria (such as *B. cereus* and *Y. pseudotuberculosis*) develop cold adaptation by synthesizing RNA helicases with the consumption of ATP as energy and developing adverse secondary mRNA structures in vitro and participating in ribosome biogenesis (Cordin et al. 2006; Iost and Dreyfus 2006; Hardwick and Luisi 2013). Several RNA helicases commonly exist in bacteria and have different roles in cold adaptation (Alvarez-Ordóñez et al. 2015). There are five RNA helicases in *B. cereus*, CshA, CshB, CshC, CshD, and CshE, which are all overexpressed at low temperatures, but only CshA, B, and C are necessary for cold adaptation (Pandiani et al. 2010). It has been shown that helicase
DeaD melted RNA secondary structure and promoted its degradation by cold shock exonuclease PNPase and RNase R in *E. coli* (Barria et al. 2013).

### 3.4.6 Cryoprotector

At low temperatures, some bacteria synthesize compounds called cryoprotector to prevent enzymes from freezing and crystallizing (Methé et al. 2005). These compounds include glycine betaine, trehalose, mannitol, and exopolysaccharides (EPS) (Ricke et al. 2018). Kandror et al. (2002) found that when the temperature dropped from 37°C to 16°C, *E. coli* promoted the conversion of glucose into trehalose in order to prevent protein polymerization and denaturation. EPS formed by some pathogenic and spoilage microorganisms in food at low temperature contributes to not only microbial growth, but also the reduction of food quality, leading to sticky surface of food, cross-contamination of food, and bringing risks to food processing, storage, and safety (Kandror et al. 2002).

### 3.4.7 Other Cold-Adaptation Mechanisms

Current studies have also shown that different microorganisms have different strategies to cope with cold stress (Suyal et al. 2021). For example, Halophilic microorganisms produce cold-tolerant enzymes in response to cold stress, including amylase, lipase, cellulase, pectinase, phytase, lactose dehydrogenase, and β-galactosidase (Hamad 2012; Kour et al. 2019). According to the adaptation of temperature, Halophilic microorganisms can be divided into two types: psychrotrophs and psychrophiles (Margesin and Schinner 1994). The former can grow and reproduce at 0°C, the optimum growth temperature is 20–40°C, and the maximum temperature for psychrotrophs to resist is about 43°C (Hamad 2012). Many important food poisoning and spoilage microorganisms fall into this category, mainly including *A. hydrophila*, *L. monocytogenes*, *Pseudomonas fluorescens*, *P. aeruginosa*, and *Y. enterocolitica* (Hamad 2012; Hamamoto et al. 1994). The minimum growth temperature of the latter is 0°C or lower, and the maximum temperature for psychrophiles to survive is not more than 20°C. Most of the psychrophiles include the genera *Aeromonas*, *Alcaligenes*, *Flavobacterium*, *Pseudomonas*, and *Vibrio* (Hamad 2012). Low temperature enzymes can make up for the adverse damages of low temperature on cells to a certain extent (Park and Cho 2011; Karan et al. 2013; Sahay et al. 2013). ATP-dependent, ClpP, pyruvate kinase, and a putative glycoprotein endopeptidase were found to involve in the cold adaptation of *Lactobacillus acidophilus* (Amato and Christner 2009). In addition, the antifreeze protein (AFP) reduced the freezing point of intracellular fluid to enable microorganisms to survive at low temperatures (Hashim et al. 2013; Tomalty and Walker 2014;
More investigations on the cold resistance mechanism of foodborne pathogens should be further conducted.

### 3.5 Conclusions

Foods are often treated at low temperatures during manufacturing, transportation, and storage. Foodborne pathogens respond to sudden temperature drops by maintaining cell membrane fluidity, DNA superhelix modification, CIP production, sigma factor regulation, metabolic level adaptation, and other mechanisms. Microbial cold stress resistance might induce the cross-protection against other virulence factors and stressors (e.g., acid), the expression of virulence factors, drug resistance, and biofilm formation of foodborne pathogens. In addition, the complex morphological changes of bacteria under low temperature stress may cause some foodborne pathogens to enter into VBNC state. All these make a huge threat to food safety and human health, and may cause a major infectious epidemic. Recently, it is reported that novel coronavirus was detected in a variety of frozen food (frozen fish, frozen chicken, frozen beef, pork, frozen seafood, etc.), which should be considered seriously. Further in-depth research on the cold resistance mechanism of foodborne pathogens should be conducted for the efficient elimination of pathogens in food exposed to low temperature environments.

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Chapter 4
Response of Foodborne Pathogens to Acid Stress

Yizhi Xu, Daniel Amund, Ricardo A. Wu, and Tian Ding

Abstract Acids have been widely used in food processing to enhance flavour, prevent the deterioration of food and eliminate or retard the growth of foodborne pathogens. The inhibition of foodborne pathogens can be due to disruption of outer structure of the microbial cell, inhibition of metabolic processes, or damage to the macro-cellular components such as nucleic acids and enzymes. However, some foodborne pathogens might survive, adapt, and develop tolerance to acid stress via a wide range of molecular mechanisms. Various intrinsic and extrinsic factors can affect the development of acid tolerance responses. In this chapter, we reviewed the acids commonly used in the food industry. We also discussed different modes of action of acids, the tolerance response induced in foodborne pathogens after being exposed to acid, and the various factors affecting the development of acid tolerance response.

Keywords Foodborne pathogen · Acid · Acid Tolerance Response (ATR)

4.1 Introduction

Foodborne pathogens, which are mainly bacteria, viruses, and parasites, are biological agents that are present in food and are able to cause human illness through virulence mechanisms (Abdelhamid and El-Dougdoug 2020; Bintsis 2017; Safavieh et al. 2015). Acid stress is one of the common stresses that foodborne pathogens are exposed to (Chen 2017; Gaviiri et al. 2020). The acid present in the food might come
from the intentional addition during food processing. For example, phosphoric, fumaric, and succinic acids are added to a wide range of foods to increase acidity and provide a sour taste (Das et al. 2016; Gurtler and Mai 2014; Zeikus et al. 1999). Citric, ascorbic, and tartaric acid are listed as antioxidants, which are able to bind to oxygen compounds and prevent food deterioration (Silva and Lidon 2016a). The acids in food might also come from the acid naturally occurring in the food itself. Citric and malic acids are naturally present in passion fruit, cranberry, and papaya at their natural ripeness; while tartaric acid is a natural component of mango and grape (Gurtler and Mai 2014). Acid might be the product or by-product of a microbiological fermentation process over time (e.g., lactic and acetic acid) (Guan and Liu 2020).

Most of the common foodborne pathogens, such as *Campylobacter jejuni*, *Salmonella*, *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus*, are not able to grow when the pH is below 4 (Ray 2004). The acidification of food, either by fermentation or by intentional addition is important for controlling foodborne pathogens in a diversity of food products (Miller et al. 2009). The acid might damage the cell wall and cell membrane, affect the cell’s ability to maintain pH homeostasis, influence the nutrient transport system, damage the macro-cellular components, inhibit the growth of the microorganisms, or cause cell death (Carpenter and Broadbent 2009).

Several bacteria have been shown to have an acid tolerance response (ATR), commonly developed upon prior exposure to mildly acidic conditions (Álvarez-Ordóñez et al. 2012). This allows such bacteria to survive under harsher acidic conditions that they may encounter during food processing and in the gastrointestinal tract. Enhanced survival can present consequences for food safety. The ATR is mediated by a variety of molecular mechanisms, such as proton efflux pump, ferric uptake regulator, changes in the membrane composition, and alkalinization of the cytoplasm. However, ATR development can be affected by various factors, such as intrinsic and environmental factors including the types of acid, the composition of the adaptation medium, and the growth temperature.

In this chapter, the common acids used in the food industry are introduced. The antimicrobial mechanisms of some acids are discussed with a specific focus on bacteria. Additionally, the acid tolerance response mechanisms and influencing factors are also discussed.

### 4.2 Acids Commonly Used in the Food Industry

#### 4.2.1 Phosphoric Acid

Phosphoric acid (H₃PO₄), also referred to as orthophosphoric acid, is an inorganic mineral acid, the pure compound of which is solid at room temperature and pressure (Speight 2017). Phosphoric acid aqueous solution at 85% exists as a colourless, non-volatile, and corrosive liquid (Speight 2017). It has a flat sour taste similar to
citric acid and can readily acidify beverages to a low pH value. Its relative cheapness allows it to be widely used as an acidulant in the manufacture of carbonated beverages such as cola (Guarnotta et al. 2017; Gurtler and Mai 2014). It also has been used as a sequestrant and synergistic antioxidant in food (EFSA 2019a; Silva and Lidon 2016a). Due to its compromised effect on health, addition of phosphoric acid to food is controversial (Fernando et al. 1999; Guarnotta et al. 2017), although its safety as a food additive has been re-evaluated and approved by the European Food Safety Authority (EFSA) panel on food additives and flavourings (EFSA 2019a).

4.2.2 Hydrochloric Acid

Hydrochloric acid (HCl) is a colourless corrosive solution with a sharp, and irritating odour (National Center for Biotechnology Information (NCBI) 2021a). It has a less acidic taste compared with other acids such as citric acid, hence it can be used as a food additive (Emerton and Choi 2008) (Table 4.1). Hydrochloric acid has been authorized by EFSA for application in various food categories including processed cereal-based foods, baby foods, and other foods for young children (EFSA 2019b) (Table 4.1).

<table>
<thead>
<tr>
<th>Acid</th>
<th>Functional class</th>
<th>Food examples</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoric acid</td>
<td>Acidulant, Sequestrant, Synergistic antioxidant</td>
<td>Soft drinks, jams, meat, meat products, dairy products</td>
<td>EFSA (2019a), Gurtler and Mai (2014), Silva and Lidon (2016a)</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>Acid, Acidity regulator</td>
<td>Invert (golden) syrup, glucose syrup, hydrolyzed vegetable proteins, processed cereal-based foods and baby foods, carbonated and non-carbonated drinks</td>
<td>EFSA (2019b), Emerton and Choi (2008), Miller et al. (2009), NCBI (2021a)</td>
</tr>
</tbody>
</table>
4.2.3 Sulphuric Acid

Sulphuric acid (H₂SO₄) is a colourless and highly corrosive acid (Behera et al. 2016). Because it is cheap and the salt formed from it has little flavour (Emerton and Choi 2008), sulphuric acid is applied to various foods and drinks as an acidity regulator (EFSA 2019c) (Table 4.1).

4.2.4 Acetic Acid

Acetic acid (C₂H₄O₂), also known as ethanoic acid, ethylic acid, or colloquially as vinegar, is a colourless carboxylic acid with a characteristic strong odour (Emerton and Choi 2008; NCBI 2021b). Like lactic acid, it can occur as a consequence of desirable fermentation to preserve foods such as salami, or it can be used for washing salads to reduce microbial contamination, thus acting as a preservative in ready-to-eat salads (e.g., coleslaw and lettuce) (EFSA 2018; Nascimento et al. 2003) (Table 4.2). It is more inhibitory towards bacteria and yeast than moulds (Søltoft-Jensen and Hansen 2005). The global demand for acetic acid is estimated to be more than 6.5 million metric tons with an increasing rate of 3–4% every year (Xu et al. 2011).

4.2.5 Lactic Acid

Lactic acid, or 2-hydroxypropanoic acid (C₃H₆O₃), is a colourless or yellowish odourless hydroxycarboxylic acid (NCBI 2021c). It exists in the form of two stereoisomers, which are L-(+) or S lactic acid and D-(−) or R lactic acid. Because L-(+) lactic acid is the only one that can be metabolized by the human body, its use is preferred in the food industry (Martinez et al. 2013).

Lactic acid is classified as generally recognized as safe (GRAS) by the United States Food and Drug Administration (FDA) and has been widely used in the food industry (Miller et al. 2017). It serves as an acidulant and provides a mildly acidic taste in prepared salads, pickled vegetables, baked foods, and beverages. As a food preservative, it inhibits the growth of spoilage bacteria and extends the shelf life (Krishna et al. 2018). In addition, it is used as an acidity regulator in foods such as sterilized and UHT cream, whey protein cheese, and frozen vegetables (Ameen and Caruso 2017) (Table 4.2). Moreover, like acetic acid, it develops in foods such as fermented milk and fermented meats, as a consequence of the metabolic activity of indigenous or inoculated lactic acid bacteria, thus preserving the food and contributing to the flavour. The global annual production of lactic acid is around 490,000 metric tonnes (MT) (Miller et al. 2017). It is estimated that 70% of lactic acid
Table 4.2 Application of organic acids in food and beverage industry

<table>
<thead>
<tr>
<th>Acid</th>
<th>Molecular structure</th>
<th>Functional class</th>
<th>Food examples</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td><img src="image1" alt="Molecular structure of acetic acid" /></td>
<td>Processing aid Acidity regulator Preservative</td>
<td>Meat products, ready-to-eat salads, fruits, vegetables, mustard, canned fish, bread, mozzarella cheese, cream cheese, instant puddings, baby food, pickling liquids, marinades, sauces, salad dressings, and mayonnaise</td>
<td>EFSA (2018), Emerton and Choi (2008), Nascimento et al. (2003), NCBI (2021b), Silva and Lidon (2016b)</td>
</tr>
<tr>
<td>Lactic acid</td>
<td><img src="image2" alt="Molecular structure of lactic acid" /></td>
<td>Processing aid Acidulant pH regulator Preservative</td>
<td>Salads, pickled vegetables, baked foods, beverages, chocolates, sweets, sterilized and UHT cream, whey protein cheese, frozen vegetables, nuts and seeds, salt substitutes; formulae for special medical purposes for infants</td>
<td>Ameen and Caruso (2017), Bhilwadikar et al. (2019), Krishna et al. (2018), NCBI (2021c)</td>
</tr>
<tr>
<td>Propionic acid</td>
<td><img src="image3" alt="Molecular structure of propionic acid" /></td>
<td>Flavouring agent Preservative</td>
<td>Bread and cake, meat, fruit, vegetables, cheese and cheese products, pie fillings, tomato purée, canned frankfurters, non-emulsified sauces, artificially sweetened jams, and jellies</td>
<td>Emerton and Choi (2008), NCBI (2021d)</td>
</tr>
<tr>
<td>Sorbic acid</td>
<td><img src="image4" alt="Molecular structure of sorbic acid" /></td>
<td>Flavouring agent Preservative</td>
<td>Baked goods, non-alcoholic beverages, cheese, dairy products, delicatessen products, prepared salad, and meat products</td>
<td>Emerton and Choi (2008), NCBI (2021e), Silva and Lidon (2016b)</td>
</tr>
<tr>
<td>Acid</td>
<td>Molecular structure</td>
<td>Functional class</td>
<td>Food examples</td>
<td>References</td>
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</tr>
<tr>
<td>Benzoic acid</td>
<td><img src="image" alt="Molecular structure" /></td>
<td>Antioxidant Flavouring agent preservative</td>
<td>Beverages, non-alcoholic beer, fruit products, chemically leavened baked goods, and condiments, preferably at pH below 4.5</td>
<td>NCBI (2021f), Silva and Lidon (2016b), WHO (2000)</td>
</tr>
<tr>
<td>Citric acid</td>
<td><img src="image" alt="Molecular structure" /></td>
<td>Acidity regulator Acidulant Antioxidant, Synergist Preservative Processing aid</td>
<td>Alcoholic beverages, carbonated soft drinks, syrups, juice drinks, tea and coffee, ice-cream, sports and energy drinks, confectionary, biscuits, jams, jellies, snacks, instant foods, and sauces</td>
<td>Bhilwadikar et al. (2019), Kanse et al. (2017), Kirimura and Yoshioka (2019), NCBI (2021g)</td>
</tr>
<tr>
<td>Acidulant</td>
<td>Flavoured agent</td>
<td>Acidity regulator</td>
<td>Processing aid</td>
<td>Fruit and vegetable juices, carbonated soft drinks, wines, confectionary, sports drinks, chewing gum, sorbets, jams, sweet and sour sauces, and peeled potatoes</td>
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<tr>
<td>Malic acid</td>
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<td></td>
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</tr>
<tr>
<td><img src="image" alt="Malic acid" /></td>
<td>Acidulant</td>
<td>Flavoured agent</td>
<td>Acidity regulator</td>
<td>Processing aid</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td></td>
<td></td>
<td></td>
<td>Corn and wheat tortillas, gelatin desserts, fruit juice, nutraceutical drinks and wine</td>
</tr>
<tr>
<td><img src="image" alt="Fumaric acid" /></td>
<td>Food acidulant</td>
<td>Flavoured agent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tartaric acid</td>
<td></td>
<td></td>
<td>Acidulant Antioxidant Synergist</td>
<td>Confectionary, fruit jelly, fresh pasta, jams, sugarless gum, fruit, vegetable, canned foods for children, baking powder, and biscuits</td>
</tr>
<tr>
<td><img src="image" alt="Tartaric acid" /></td>
<td>Acidulant</td>
<td>Antioxidant Synergist</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid</td>
<td>Molecular structure</td>
<td>Functional class</td>
<td>Food examples</td>
<td>References</td>
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</tr>
</tbody>
</table>
| Succinic acid | ![Molecular structure](image1) | Acidulant
Flavouring agent
| Adipic acid   | ![Molecular structure](image2) | Acidulant
Flavouring agent
| Ascorbic acid | ![Molecular structure](image3) | Antioxidant | Charcuterie and salted products, fish, some types of bread, fresh pasta, beer, peeled potatoes, and some foods for infants and children | NCBI (2021m), Silva and Lidon (2016a) |
production is used in the food industry (Martinez et al. 2013) with an increasing demand every year at a rate of 5–8% (Rodrigues et al. 2017).

### 4.2.6 Propionic Acid

Propionic acid (C\(_3\)H\(_6\)O\(_2\)) is a naturally occurring colourless and oily carboxylic acid with a slightly pungent and rancid odour. Propanoic acid, carboxyethane, ethylformic acid, ethanecarboxylic acid, and methyacetic acid are alternative names for propionic acid (EFSA 2014; NCBI 2021d). It is an FDA approved GRAS chemical with no deleterious effect on flavour. It serves as a food preservative to inhibit the growth of mould and “rope” (caused by some Bacillus spp.) in bread, cake, meat, fruit, and vegetables, and to avoid blowing of canned frankfurters (Erkmen and Bozoglu 2016). In addition, since the synergy between propionic, lactic, and acetic acids controls growth of L. monocytogenes, a combination of these acids for food preservation has been suggested (Boyaval and Corre 1995) (Table 4.2). The annual world production of propionic acid is estimated at around 450,000 tons with a 2.7% per annum rate of increase (Gonzalez-Garcia et al. 2017) and approximately 21% of production is used as a food preservative (Quitmann et al. 2014; Xu et al. 2011).

### 4.2.7 Sorbic Acid

Sorbic acid (C\(_6\)H\(_8\)O\(_2\)), also referred to as 2,4-hexadienoic acid, is a relatively odourless unsaturated linear chain carboxylic acid with a slightly acidic and astringent taste. Because of its effective antimicrobial activity against yeast, fungi, and bacteria (to some extent), sorbic acid has been widely used as a food preservative in various products, such as fruit juice, wine, pastries, cheese, nuts, meat products, and prepared salads (NCBI 2021e; Silva and Lidon 2016b) (Table 4.2).

### 4.2.8 Benzoic Acid

Benzoic acid (C\(_7\)H\(_6\)O\(_2\)) is a white crystalline powder with extremely low water solubility. Alternative names include dracylic acid and benzenecarboxylic acid (NCBI 2021f). It is the simplest aromatic carboxylic acid and occurs naturally in many foods, such as peaches, strawberries, peanuts, and various spices (Gurtler and Mai 2014; Olmo et al. 2017).

Benzoic acid was the first chemical preservative authorized for use in food in the USA (Søltoft-Jensen and Hansen 2005) and is widely applied in various foods and beverages, such as fruit, pickled vegetable, fruit juice, soft drinks, and jams to
prevent growth of yeast and other fungi (Silva and Lidon 2016b; WHO 2000). Because of its extremely low solubility, benzoic acid is used less frequently than its associated salt (Gurtler and Mai 2014).

4.2.9 Citric Acid

Citric acid (2-hydroxypropane1,2,3-tricarboxylic acid, or anhydrous citric acid) (C₆H₈O₇) is a weak tricarboxylic acid, existing as colourless and odourless crystals with an acidic taste (EFSA 2015; NCBI 2021g). It occurs naturally in vegetables and citrus fruits, such as lemons and limes, in which 8% of the dry weight is citric acid (Kanse et al. 2017; Sweise and Cressey 2018).

Because of the acidic pH, low cost, abundant supply, and light fruity taste of citric acid, it has been extensively used in the food industry with no side effects (Gurtler and Mai 2014; Silva and Lidon 2016a). It acts as an acidity regulator in alcoholic beverages, carbonated soft drinks, and syrups (Kanse et al. 2017). Because it has a pleasant taste and enhances existing flavours, it is also used as acidulant in the confectionary and drink industries (Kirimura and Yoshioka 2019). It is an antioxidant, which chelates metal ions and retards oxidation of lipids (Pop and Mihalescu 2017). It is a preservative in confectionary, biscuits, jams, snacks, instant foods, and sauces (Kanse et al. 2017) (Table 4.2). Similar to acetic, lactic, and malic acids, it is also used to wash fresh fruits and vegetables, such as apple, lettuce, cabbage, and tomato, to reduce numbers of microorganisms (Bhilwadikar et al. 2019). In 2016, the manufacturing of citric acid achieved 2.1 million tons worldwide (Kirimura and Yoshioka 2019). Approximately 70% of the production is used in the food and beverage industries (Sweise and Cressey 2018).

4.2.10 Malic Acid

Malic acid, also referred to as 2-Hydroxybutanedioic acid (C₄H₆O₅), obtained its name from the Latin word malum, which means apple, where it was first identified (Lee et al. 2019; NCBI 2021h). It is an odourless and white crystalline powder with a tart acidic taste (NCBI 2021h). Similar to lactic acid, it has D- and L-isomers. Use of the L-malic acid isomer is preferred in the food industry as it is naturally produced and can be metabolized safely (Lee et al. 2019).

Malic acid is recognized as GRAS and serves as an acidulant in food and beverages contributing a less bitter taste than citric acid (West 2017). As a preservative, malic acid can control microbial growth in foods (Kesava et al. 2016). In addition, malic acid is found to enhance the sweet and tart taste in confectionary, fruit juice, and wine (Lee et al. 2019) (Table 4.2). The global malic acid market is estimated to be between 40,000 and 60,000 MT with an increasing rate of production.
around 4% every year (West 2017), with about 85%–90% used in food and beverage production (Kövilein et al. 2019).

4.2.11 Fumaric Acid

The odourless and colourless crystalline solid, fumaric acid (C₄H₄O₄), is a naturally occurring dicarboxylic acid. Its name is derived from the plant *Fumaria officinalis*, from which it was first isolated (Roa Engel et al. 2008). Other names for fumaric acid are 2-butenedioic acid, trans-butenedioic acid, and allomaleic acid (Roa Engel et al. 2008; NCBI 2021i). As the least expensive food grade acid and strongest tasting food acidulant, fumaric acid has been used in foods and beverages since 1946. For example, it can control microbial growth in tortilla dough, impart greater relative sourness to fruit juice and acidify wine with no negative effect on flavour (Das et al. 2016; Yang et al. 2019) (Table 4.2).

The numerous applications of fumaric acid have resulted in steadily increasing production. In 2018, the global production of fumaric acid was estimated as 300 kilo-tons (KT) and about 346 KT in 2020 (Martin-Dominguez et al. 2018). Thirty three percent of the world’s consumption of fumaric acid is used in food and beverage manufacture (Das et al. 2016).

4.2.12 Tartaric Acid

Tartaric acid is a colourless or white crystalline dicarboxylic acid (NCBI 2021j), which occurs naturally in fruits, including apples and tamarinds (Carullo et al. 2020). It has two stereoisomers, which are L- and D-tartaric acid. D(-)-tartaric acid is rare, while L(+)-tartaric acid is widely present in many fruits (Comuzzo and Battistutta 2019; JECFA 2020; Ukaji and Soeta 2012), such as grapes and bananas (Izawa et al. 2010).

As in the case of many of the acids discussed above, L(+)-tartaric acid has been recognized as GRAS by FDA and is commonly used as an acidulant in confectionary, such as jam, to increase the acidity in order to aid preservation and enhance the flavour (Dziezak 2003). Due to its strong sharp taste, tartaric acid is less useful as a food additive compared with citric and malic acids. Sometimes a combination of these three organic acids may be used in food (such as jam and fruit juice) to create a unique enhanced level of taste (Kesava et al. 2016). Besides being an acidulant, tartaric acid is also used as an antioxidant, synergist, and sequestrant in various food products such as fresh pasta, jam, jelly, sugarless gum, fruit, vegetables, and canned food for children (Silva and Lidon 2016a) (Table 4.2).
4.2.13 Succinic Acid

Succinic acid is a colourless and odourless crystal four-carbon dicarboxylic acid (C₄H₆O₄) (NCBI 2021k). The name derives from the Latin *succinum*, meaning amber, from where the organic acid was first discovered. Other common names of succinic acid are butanedioic acid and dihydrofumaric acid (Saxena et al. 2017; Lee et al. 2019). In the food industry, succinic acid is used as an acidulant, flavouring, and antimicrobial agent and has been approved for use in various food categories including flavoured fermented milk products, soup, and broth, both at the maximum permitted level (Leszczewicz and Walczak 2014; The European Parliament and the council of the European union 2008; Zeikus et al. 1999) (Table 4.2). About 20,000 to 30,000 tonnes of succinic acid have been used globally every year with an approximately 10% annual rate of increase. About 13.07% of consumption has been employed in food and beverage production (Saxena et al. 2017).

4.2.14 Adipic Acid

Adipic acid, also known as hexanedioic acid, adipinic acid, and 1,4-Butanedicarboxylic acid, is a colourless and odourless straight-chain dicarboxylic acid with an acidic taste (C₆H₁₀O₄) (Deng and Mao 2015; NCBI 2021l). The global production of adipic acid is approximately three million tons every year with a 3–5% compound annual growth rate (CAGR) (Skoog et al. 2018). Although most adipic acid is used as a raw material in the nylon manufacturing industry, it is also recognized as GRAS by FDA (FDA 2020). It is regularly used as an acidulant, flavouring, and pH control agent in various food and beverage products such as fruit flavoured drinks and jams (Gurtler and Mai 2014; Skoog et al. 2018; NCBI 2021l) (Table 4.2).

4.2.15 Ascorbic Acid

Ascorbic acid (C₆H₈O₆), also referred to as vitamin C or L(+) -Ascorbic acid, derives its name from the Latin *a* (no) and *scorbutus* (scurvy, a disease attributable to deficiency of vitamin C) (NCBI 2021m; Varvara et al. 2016). It is a white or slightly yellow, odourless crystalline powder with sharp acidic taste and naturally occurs in citrus fruits and green vegetables (NCBI 2021m). Because it is a scavenger of oxidized species and inhibits oxidation of food, together with its value as vitamin C, it is an excellent antioxidant and nutraceutical, which has been applied in various foods without any side effects. Examples include fish, fruit, and some types of bread and pasta (FDA 2019; Silva and Lidon 2016a) (Table 4.2). Every year, approximately 110 KT of ascorbic acid is produced, 40% of which is used in...
the food (25%) and beverage (15%) sectors as an antioxidant (Pappenberger and Hohmann 2014).

4.3 Modes of Action of Acid Treatment on Microorganisms

Acidiﬁcation is a common method to prevent food deterioration and extend shelf life. Weak organic acids, such as acetic and citric acids, are known to possess more potent antimicrobial activity than strong inorganic acids (Gurtler and Mai 2014; Rode et al. 2010). The antimicrobial mechanisms that acids possess are still not completely understood (Carpenter and Broadbent 2009; Ricke 2003). Some potential modes of action have been proposed and summarized as follows.

4.3.1 Direct Attack on Outer Structure of Microbial Cells

The pH range of most foods is from 5.0 to 8.0. The addition of acids, either strong acids or weak organic acids, usually involves increasing the proton concentration in the matrix (e.g., medium, food) (Beales 2004; Ray 2004) (Table 4.3). The acid can act directly on the microbial cell wall, the cytoplasmic membrane and cause leakage of the cell contents (Bhilwadikar et al. 2019; Gurtler and Mai 2014; Lund et al. 2014). Royce et al. (2013) studied the effect of octanoic acid on *E. coli* membrane and reported that the membrane fluidity and membrane leakage of un-adapted *E. coli* increased as the octanoic acid concentration increased. The protons can also bind to proteins embedded on the cell surface, such as enzymes and transport permeases, and affect their three-dimensional structure, stability, and function (Bhilwadikar et al. 2019; Gurtler and Mai 2014; Lund et al. 2014).

Table 4.3 The dissociation of weak organic acids at various pH values (Modified from Lücke 2003, Republished with permission of [Elsevier Science & Technology Journals], permission conveyed through Copyright Clearance Center, Inc; Ray 2004, Copyright (2004) by Imprint. Reproduced by permission of Taylor & Francis Group)

<table>
<thead>
<tr>
<th>Acid</th>
<th>Lipophilicity</th>
<th>pKₐ</th>
<th>Amount of dissociated ion (%) at pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>+</td>
<td>4.75</td>
<td>15.5</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>±</td>
<td>3.86</td>
<td>60.8</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>++</td>
<td>4.86</td>
<td>12.4</td>
</tr>
<tr>
<td>Sorbic acid</td>
<td>+++</td>
<td>4.76</td>
<td>18.0</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>++++</td>
<td>4.19</td>
<td>40.7</td>
</tr>
<tr>
<td>Critic acid</td>
<td>−</td>
<td>3.13, 4.76, 6.40</td>
<td>81.1</td>
</tr>
</tbody>
</table>

aLipophilicity: Lipophilicity of undissociated acid
bpKₐ: Dissociation constant of organic acids
4.3.2 Metal Ion Chelation

Divalent metals such as iron, manganese, and zinc, are essential for microbial metabolism, growth, and pathogenicity. They serve as a cofactor for many enzymes, which are involved in several essential cellular functions such as respiration, translation, transcription, and cell division (Weiss and Carver 2018). Graham and Lund (1986) investigated the effect of citric acid on the growth of *Clostridium botulinum* and found that citric acid at a concentration of 50 mmol/l reduced the Ca\(^{2+}\) concentration in the culture medium by a factor between 10 and 100, while increasing the number of vegetative cells needed to produce a visible growth by a factor of around 10\(^6\). The addition of Ca\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), and Fe\(^{2+}\) ions into the medium restored the microbial growth. The results suggested that citric acid inhibits the growth of *C. botulinum* by binding Ca\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), and Fe\(^{2+}\) ions in the culture medium.

In addition, the divalent cations, especially Ca\(^{2+}\) and Mg\(^{2+}\), also play an important role in strengthening the outer membrane integrity of Gram-negative bacteria by forming salt bridge between anionic phosphate groups in lipopolysaccharide (LPS) inner core oligosaccharide region (Clifton et al. 2015). Bruel et al. (2020) studied the antimicrobial effect of citric acid against *E. coli* over a wide range of pH (4.5, 6.5, and 9.5) and found that citric acid at higher pH is completely dissociated to tribasic form causing the highest viability loss (more than 4 log CFU/ml reduction) and membrane damage. The results showing that the higher pH, the more membrane damage and the more negatively charged bacterial surface, indicate that the bactericidal effect of citric acid might also involve chelation of the divalent ions in the membrane.

4.3.3 Decrease in Cytoplasmic pH

Because of the amphipathic nature of lipid membranes, charged molecules are generally not able to penetrate the cell membrane. However, lipophilic undissociated acids, which are uncharged molecules, are able to diffuse rapidly through the membranes (Gurtler and Mai 2014; Trček et al. 2015). The near neutral cytoplasmic pH inside the cell causes the dissociation of acids to release anions and protons, both of which are not able to diffuse out through the lipid bilayer and so accumulate inside the microbial cells (Fig. 4.1) (Stratford and Anslow 1998).

Strong acids that completely ionize in a solution, do not affect the internal pH to the same extent as weak organic acids, because they are not able to pass through the cell membranes due to its impermeable nature (Rode et al. 2010; Gurtler and Mai 2014). However, when the concentration of protons in the environment is high enough, the energy-requiring proton pumps transport protons into the cell, which results in reduction of cytoplasmic pH, depletion of energy and inhibition of microbial growth (Ray 2004).
If the dissociation rate of intracellular acid is greater than the cytoplasmic buffering capacity or the rate of proton efflux, the cytoplasmic pH starts to reduce (Carpenter and Broadbent 2009). The reduction of cytoplasmic pH will affect the transmembrane pH gradient and prevent active transport, damage nucleic acids and enzymes (such as the enzymes involved in glycolysis), inhibit metabolism, and eventually prevent cell division (Fig. 4.1) (Gurtler and Mai 2014; Guan and Liu 2020; Ray 2004; Abdelhamid and El-Dougdoug 2020; Warnecke and Gill 2005). For example, glycolysis of anaerobic E. coli culture is substantially inhibited when the cytoplasm is acidified to pH < 7.0 (Lund et al. 2014).

The acid anions accumulated inside the cell increase the potassium ion concentration, elevate osmolality inside the cell, and possibly result in lethal turgor pressure. In order to maintain constant turgor pressure, the cell pumps out acid anions or other cellular anions (mainly glutamate) (Berterame et al. 2016; Carpenter and Broadbent 2009). This simultaneously disrupts the osmolality of the cytoplasm, which consequently affects the microbial cell viability (Warnecke and Gill 2005). Accumulation of intracellular anions may also affect some essential metabolic processes, which is detrimental to growth (Fig. 4.1) (Carpenter and Broadbent 2009). For example, the intracellular accumulation of acetate anions in E. coli has been shown to inhibit methionine biosynthesis. It is reported that the growth of E. coli is restored by adding methionine to the medium (Roe et al. 2002).

At a lower pH, a greater amount of weak organic acid in the food is present in the undissociated form. Hence, weak organic acids are more effective antimicrobial agents in food at pH values below their pKa values. This is known as the classical weak acid theory (Beales 2004; van Beilen et al. 2014). Lipophilic acids such as acetic, sorbic, benzoic, and propionic acids demonstrate an antimicrobial effect

![Fig. 4.1 Overview of antimicrobial mechanism of organic acid via affecting cytoplasmic pH and anion accumulation (adapted from Abdelhamid and El-dougdoug (2020) and Warnecke and Gill (2005))](image-url)
based on the classical weak acid theory (Table 4.3) (Ahmadi et al. 2017; Gurtler and Mai 2014; Ray 2004; van Beilen et al. 2014). Lactic acid has limited lipid solubility (Table 4.3). However, the inhibitory effect of lactic acid against various microorganisms such as *L. monocytogenes* and *E. coli* O157:H7 might also involve lactic acid entering the cell and affecting the internal pH because of its small molecular weight (Eswaranandam et al. 2004).

Some organic acids, such as citric acid, are lipophobic, hence they are not able to diffuse through the lipid-rich cell membrane and exert any intrinsic antimicrobial effect (Gonzalez-Fandos et al. 2020). The antimicrobial activity of citric acid is more likely a consequence of lowering the external pH and chelation of the metal ions, either in the environment or on the cell membrane as mentioned in Sect. 4.3.2.

### 4.4 Factors Affecting the Acid Resistance Development in Foodborne Pathogens

#### 4.4.1 Acid Stress Resistance

During food processing environments, foodborne pathogens might be injured upon exposure to acid sanitizers. It is documented that some pathogens are able to survive and adapt to minor changes in environmental pH and develop tolerance to acid stress (Guan and Liu, 2020). Several foodborne pathogens such as *Salmonella* spp. have developed an acid tolerance response (ATR), which assists this bacterium to survive under extreme acid stress conditions (pH 3.0–2.5) (Álvarez-Ordóñez et al. 2012). Shiga toxin-producing *E. coli* (STEC) strains possess acid resistance systems, including *rpoS* regulation factor and citrate cycle, allowing this pathogen to adapt under low pH conditions (Yu et al. 2021). O157 is the most common serogroup of STEC responsible for the most outbreaks due to its acid tolerance (Wang et al. 2020). *L. monocytogenes* has also developed an ATR, contributing to survival under lethal pH conditions (pH 3.0) (Liu et al. 2020). *Cronobacter sakazakii* has been frequently isolated from acid foods such as fruits, yogurt, and cheese, and this pathogen is resistant to acid stress similar to *Salmonella* spp. and less than *E. coli* (Zhou et al. 2020). *Vibrio vulnificus* has shown to possess an ATR, which allows this pathogen to survive at pH 4.0 after adaptation at pH 5.0 (D’Souza et al. 2019). *C. jejuni* and *E. coli* have been shown to retain the viability upon the acid conditions (pH 4.5), which indicates that these pathogens may survive in the human stomach with a low pH (Gomes et al. 2018).

#### 4.4.2 Intrinsic Factors

Previous studies have shown a wide variation in acid resistance among serovars, strains, and a given pathogen with various phases (Álvarez-Ordóñez et al. 2012; Ye
et al. 2019). Thus, it is suggested that the intrinsic characteristics of pathogens affect acid resistance. For instance, Al-Nabulsi et al. (2015) found that *L. monocytogenes* strain isolated from dairy products was more resistant to acid (pH 5.0, 30 min) compared with a strain isolated from meat. Berk et al. (2005) found that *Salmonella* Typhimurium isolates from human patients were more resistant to acid (pH 2.5 for 2 h) after acid adaptation (pH 5.0) for 18 h compared with nonhuman (food) isolates. In the study of Zhang et al. (2014), it is reported that *L. monocytogenes* cells at early stationary phase were more resistant to acid than mid-log phase cells when challenged for 1 h at pH 1.5–3.0 adjusted with hydrochloric, L-lactic, levulinic, and acetic acids. Similarly, Ye et al. (2019) reported that *Salmonella* Enteritidis cells at stationary phase exhibited higher resistance to the acid challenge of pH 3.0 adjusted with hydrochloric for the first 2 h when compared to their counterparts at log phase.

### 4.4.3 Environmental Factors

It has been widely reported that tolerance of foodborne pathogen to acid stress varies depending on environmental factors before or during lethal treatments (Álvarez-Ordóñez et al. 2012). Thus, these factors should be given special attention.

#### 4.4.3.1 Acid Types

One of the most influential environmental conditions that impacts bacterial growth and survival are the concentration of protons, which is measured as pH. Acidic or low pH can occur through natural geochemical processes or through microbial fermentation that often generates organic acid by-products (Lund et al. 2020). Previous studies have demonstrated the effect of different acids on the acid resistance of foodborne pathogens such as *S. Typhimurium*, *Pseudomonas aeruginosa*, and *E. coli* O157:H7 (Álvarez-Ordóñez et al. 2010; Bushell et al. 2019; Jones et al. 2020). The ability of foodborne pathogens to grow under low pH conditions is affected by the type of acid in the medium in which they are growing. The antimicrobial effects of organic acids have been widely reported to be caused by their undissociated form. Unlike inorganic acids, organic acids are able to pass through bacterial cell membranes as undissociated molecules, which reduces cytoplasmic pH affecting metabolic activities within the cell (Álvarez-Ordóñez et al. 2010). These acids are protonated at low pH, which makes them uncharged and more lipophilic. Under these low pH conditions, organic acids can permeate the microbial lipid bilayer and release their protons in the intracellular environment (Lund et al. 2020). Foodborne pathogens typically respond to the effect of acids by preventing a lethal drop in intracellular pH, modifying membrane integrity and fluidity, metabolic regulation, and macromolecular repair (Guan and Liu 2020).
Strong Acids (Inorganic Acids) Versus Weak Permeable Acids (Organic Acids)

Foodborne pathogens may encounter inorganic strong acids such as hydrochloric acid in the stomach and different weak organic acids in foods (Álvarez-Ordóñez et al. 2009; Zhou and Fey 2020). It has been reported that strong acids and weak acids affect bacterial cells in different ways (De Biase and Lund 2015). For instance, King et al. (2010) discovered that *E. coli* O157:H7 showed an acid- and strain-specific ATR to acetic, lactic, and hydrochloric acids. Approximately 70% of the genes (e.g., *glnA*, *lpxP*, *hemF*, *ygfA*, *trxC*, *exbD*, among others) upregulated in response to lactic acid were also upregulated by hydrochloric acid, while the response to acetic acid was different, resulting in more susceptibility to this latter one. Similarly, Álvarez-Ordóñez et al. (2010) found that acetic acid caused the highest inhibition or delay in *S. Typhimurium* growth compared to lactic, citric, and hydrochloric acid; the latter, displayed the lowest inhibition growth. Rode et al. (2010) found that lactic and acetic acids introduced more severe stress to *S. aureus* compared to hydrochloric acid. Zhou et al. (2020) reported that growth of *C. sakazakii* in hydrochloric and citric acids was significantly different at the same pH (4.0–4.2) Whole transcriptomics analysis revealed that expression patterns under the stresses of hydrochloric and citric acid were different. Gavriil et al. (2020) found that undissociated acetic acid affected the acid adaptation responses of *S. Enteritidis* Phage Type 4 in a concentration- and pH-dependent manner. These studies suggest that organic acids or weak permeable acids, possess higher antimicrobial effect compared to inorganic acids or strong acids.

### 4.4.3.2 Adaptation pH and Time

Several studies have investigated the acid resistance of foodborne pathogens following adaptation to a range of acid pH values for short or long time. Chiang et al. (2012) investigated the effects of pH ranging 5.0 to 6.0 on acid adaptation of *Vibrio parahaemolyticus* strain 690. The results indicated that *V. parahaemolyticus* exposed to pH 5.5 for 90 min exhibited the highest acid tolerance. Cells. Burin et al. (2014) inoculated *Salmonella* strains at pH 4.0, 5.0, 6.0, or 7.0 for exposure of 6, 12, 24, and 48 h, respectively. Cells grown at pH 5.0 and 6.0 presented similar survival rates as the control ones at pH 7.0. Gene expression assay demonstrated the overexpression of *rpoS* at pH 5.0 and 6.0, suggesting that *rpoS* plays an important role in the ATR of *Salmonella*. Álvarez-Ordóñez et al. (2009) tested the acid adaptation of *S. Typhimurium* at pH 6.4, 5.4, and 4.5 using different acids (acetic, ascorbic, citric, lactic, malic, and hydrochloric acids). The authors found that *S. Typhimurium* cells adapted at pH 6.4 and 5.4 for 24 h developed a higher ATR against the challenge of pH 3.0 compared with the cells adapted at pH 4.5. Gavriil et al. (2020) investigated several combinations of acetic acid concentrations (0, 15, 25, 35, and 45 mM) and pH values (4.0, 4.5, 5.0, 5.5, 6.0) on the ATR of *S. Enteritidis* to the following treatment of pH 2.5. Increased survival was observed
when cells were acid-adapted at pH 5.5 and 6.0, while no effect was observed at lower pH values. These studies suggest that the effective output of acid-adaptation is greatly influenced by adaptation pH and time.

4.4.3.3 Composition of the Adaptation Medium

It has been reported that nutrient composition influences the acid resistance of foodborne pathogens during the acid adaptation stage (Álvarez-Ordóñez et al. 2012). Gavriil et al. (2020) investigated the effect of undissociated acetic acid in inducing acid resistance of S. Enteritidis Phase Type 4 both in laboratory media (tryptone soy broth) and in acid food matrix (tarama salad). It is reported that it is easier for S. Enteritidis Phase Type 4 to develop the acid resistance in the laboratory medium than in the tarama salad. However, in the food matrix, none of the acid adaptation treatments (0, 15, 25, 35, 45 mM acetic acid and pH 4.0, 4.5, 5.0, 5.5, 6.0) enhanced the survival of S. Enteritidis. The authors concluded that other intrinsic factors (e.g. pH and temperature) in the food might affect the acid resistance of S. Enteritidis. Álvarez-Ordóñez et al. (2009) investigated the inactivation of S. Typhimurium at pH 3.0 in brain heart infusion and meat serum after acid adaptation in the same mediums at different pH (6.4, 5.4, and 4.5). Acid-adapted cells obtained in both mediums were more resistant to pH 3.0 when meat serum was used as challenge medium compared to brain heart infusion. Amino acids in the acid challenge media could provide protection to foodborne pathogens against low pH (Álvarez-Ordóñez et al. 2009).

4.4.3.4 Growth Temperature

Growth temperature may also act as an important factor that affects the acid resistance of foodborne pathogens, although it has received little attention. Álvarez-Ordóñez et al. (2010) studied the effect of growth temperature (10, 25, 37, and 45 °C, for the time needed to reach late stationary phase) on the survivals of S. Typhimurium CETC 443 under the acid conditions up to pH 4.25 with hydrochloric, acetic, citric, and lactic acid. Results showed that the acid resistance of S. Typhimurium CETC 443 to pH 3.0 was highest at temperature growth of 37 °C and lowest at 10 °C measured by D-value. On the other hand, cells grown at temperatures 25 and 45 °C displayed similar D-values at pH 3.0. In another study, Álvarez-Ordóñez et al. (2013) investigated the survival of S. Typhimurium in yogurt (pH 4.1) and orange (pH 3.6) during the storage at 4, 10, 25, and 37 °C. S. Typhimurium survived in both foodstuffs, especially in yogurt. The results showed that refrigeration temperatures protected S. Typhimurium from inactivation in yogurt and orange juice.
4.5 Acid Stress Defence Systems in Foodborne Pathogens

4.5.1 Ferric Uptake Regulator

Iron is important for various cell functions such as DNA synthesis, protein synthesis, and metabolism (Troxell and Hassan 2013). Under acidic conditions, high amount of soluble iron Fe^{2+} is available. This abundance of iron can, however, result in the generation of reactive oxygen species (ROS) via the Fenton reaction leading to oxidative stress, which is toxic. Hence, the homeostasis of free iron is important to assure the normal cellular activities within the microbial cells. The ferric uptake regulator (Fur) protein controls the expression of genes associated with iron acquisition and storage (Reid et al. 2008).

In some bacteria, the Fur protein has been shown to be involved in the acid stress response. The role of Fur in acid stress response has been demonstrated in S. Typhimurium, whereby mutants lacking fur showed less survival at pH 3 using hydrochloric acid (Hall and Foster 1996). It was reported that acid-stressed C. jejuni (hydrochloric acid at pH 5.2 and acetic acid at pH 5.7) overexpressed an iron-regulated Fur-like protein, indicating that controlling intracellular iron level might contribute to acid resistance (Birk et al. 2012). Upregulation of genes (exbD, fepD, ydiE, hemF) involved in iron uptake were also observed in E. coli K-12 and E. coli O157:H7 exposed to hydrochloric, acetic, and lactic acids at pH 5.5 (King et al. 2010).

4.5.2 Proton Efflux Pumps

The enzyme F_{1}F_{0}-ATPase (ATP synthase) found in bacterial cell membranes, plays the function of expelling protons from within the cytoplasm to the external environment, thereby maintaining cytoplasmic pH homeostasis. It consists of two parts, the water-soluble F_{1} portion and the transmembrane F_{0} portion. The enzyme possesses dual functions: ATP synthesis by utilizing the electrochemical gradient of protons and as an ATPase in the hydrolysis of ATP to pump protons out of the cell under acidic conditions. Pumping protons across the cytoplasmic membrane is a consequence of ATP hydrolysis by the F_{1} subunit (Ferguson et al. 2006; Sun 2015).

Sun et al. (2012) demonstrated that deletion of the F_{1}F_{0}-ATPase subunit genes atpD and atpE resulted in low survival of E. coli at pH 2.5 using hydrochloric acid. The role of F_{1}F_{0}-ATPase in acid tolerance of L. monocytogenes was demonstrated in the study by Cotter et al. (2000). In this study, cells treated with an ATPase inhibitor (N,N'-dicyclohexylcarbodiimide) showed greater sensitivity to acidic stress conditions (pH 3.5 using lactic acid). In contrast, F_{1}F_{0}-ATPase was shown to not be involved in the ATR of B. cereus (Mols et al. 2010).
4.5.3 Changes to Membrane Composition

The cell membrane is a major target of various stress conditions encountered by bacteria. Alterations to the membrane composition could be a means to minimize the permeation of harmful molecules into the cell, thereby enhancing survival (Beales 2004; Álvarez-Ordóñez et al. 2012). Acid stress may induce the modification of the fatty acid composition of the cell membrane, which alters membrane fluidity (Álvarez-Ordóñez et al. 2010).

The study by Álvarez-Ordonez et al. (2008) on *S. Typhimurium* identified a reduction in the ratio of unsaturated to saturated fatty acid and an increase in the cyclopropane fatty acid content in response to acidification, compared to non-acid-adapted cells. These changes were associated with a decrease in membrane fluidity. Similar findings have been reported in studies on *E. coli* O157:H7 and *L. monocytogenes*. Yuk and Marshall (2005) observed that acid-adaptation of *E. coli* O157:H7 with citric and lactic acid resulted in a decrease in the ratio of cis-vaccenic to palmitic acids (unsaturated: saturated fatty acid ratio) as pH decreased, thus representing a decrease in membrane fluidity. Mastronicolis et al. (2010) reported that adaptation of *L. monocytogenes* to hydrochloric, lactic, and acetic acid at pH 5.5 resulted in similar changes to membrane fatty acid profiles leading to decreased membrane fluidity.

4.5.4 Urease

Enzyme systems that generate alkaline products, such as ammonia, are another acid tolerance mechanism developed by bacteria to consume excess protons in the cytoplasm and maintain pH homeostasis. One of such is urease, which hydrolyses urea into ammonia and CO\textsubscript{2}. The ammonia consumes protons and is ionized into ammonium, which raises the intracellular pH (Guan and Liu 2020). Bacteria can acquire urea from the environment and also generate urea through arginine catabolism (Zhou and Fey 2020). The role of urease in the acid stress response has been shown in *S. aureus* (Zhou et al. 2019). In contrast, however, the study by Mols and Abee (2008) demonstrated that ureolytic strains of *B. cereus* did not show improved growth rate under acidic conditions (pH 5) or improved survival after acid shock (pH 2.5–4.8 for up to 30 min) in the presence of urea. This was explained by the low urease activity of the strains and the absence of upregulation of urease genes under acidic conditions.
4.5.5 Arginine Deiminase

Another pathway for generating alkaline compounds to counteract low intracellular pH is the metabolism of the amino acid arginine via the arginine deiminase (ADI) pathway. The ADI system consists of three enzymes: arginine deiminase, ornithine carbamoyltransferase, and carbamate kinase (Shabayek and Spellerberg 2017). Arginine is first converted to citrulline and ammonia by arginine deiminase. Ornithine carbamoyltransferase then catalyzes the phosphorolysis of citrulline to ornithine and carbamoyl phosphate. The ornithine is then transported out of the cell, while the carbamoyl-phosphate is converted to CO₂ and ammonia by carbamate kinase, which generates ATP from ADP in the process. As a result, protons are neutralized by ammonia and CO₂ formed by ADI system, and the ATP produced is available to extrude protons via ATPase (Guan and Liu 2020).

Some studies have demonstrated that L. monocytogenes and B. cereus possess ADI system genes (arc genes) which contribute to acid resistance. In L. monocytogenes, a qualitative analysis indicated increased relative expression of arcA, arcB, arcC, and arcD after exposure to low pH using hydrochloric acid (pH 4.8). Furthermore, the deletion of arcA resulted in decreased growth rate at pH 4.8 using hydrochloric acid and decreased survival at pH 3.5 using lactic acid (Ryan et al. 2009). In B. cereus strains, the ADI gene arcA was upregulated significantly under sublethal acid stress with hydrochloric acid (pH 5.4 up to 1 h), but was not induced after more severe acid stress (pH 4.5 up to 1 h) (Mols et al. 2010).

4.5.6 Amino Acid Decarboxylation Systems

Amino acid decarboxylation systems in Gram-negative and Gram-positive bacteria, such as lysine decarboxylase, arginine decarboxylase, ornithine decarboxylase, and glutamate decarboxylase, have been shown to contribute to pH homeostasis. Decarboxylation of amino acids (e.g., lysine, arginine, ornithine, and glutamate), are the reactions that consume protons and produce alkaline end products, i.e., amines, and as such, are strategies to prevent a critical drop in intracellular pH (Lund et al. 2020). Decarboxylases are inducible at low pH. Lysine decarboxylase converts lysine to cadaverine; arginine decarboxylase converts arginine to agmatine; glutamate decarboxylase converts glutamate to gamma-aminobutyric acid (GABA); ornithine decarboxylase converts ornithine to putrescine (Ryan et al. 2008; Álvarez-Ordóñez et al. 2012; Arcari et al. 2020).

The glutamate decarboxylase (GAD) system is considered to play a major role in the resistance of L. monocytogenes to low pH (Ryan et al. 2008). The GAD system is also present in E. coli, in addition to lysine decarboxylase and ornithine decarboxylase systems (Arcari et al. 2020). S. Typhimurium has been demonstrated to possess
lysine decarboxylase and arginine decarboxylase systems for pH homeostasis (Álva rez-Ordóñez et al. 2012).

4.6 Conclusions

This chapter demonstrates the importance of acids in the food industry and describes the possible mechanisms they possess to inhibit microbial growth or kill microbes. It also highlights that the adverse environmental conditions have obligated foodborne pathogens to evolve acid resistance response via various molecular mechanisms. The great variability of intrinsic and environmental factors, makes it difficult to obtain a complete descriptive picture of this response. Further studies should be focused on predicting the effect of these factors with a high degree of precision.

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Chapter 5
Response of Foodborne Pathogens to Osmotic Stress

Pradeep K. Malakar, Jing Liu, Qian Wu, Zhaohuan Zhang, and Yong Zhao

Abstract Osmotic solutes (salt and sugar) are predominantly used to inhibit foodborne pathogens in the food industry. Osmotic stress created by these solutes triggers morphological and physiological changes in the foodborne pathogens, reducing or retarding their growth. These changes include modifications in membrane proteins and transportation systems to mitigate the adverse effects of osmotic stress. The emergence of transcriptomics, proteomics, and structural biology should lead to improved hurdle technology to control foodborne pathogens and subsequently provide safe food throughout the food chains from farm to fork.

Keywords Osmoprotectants · Osmotic stress · Biofilms · Adaptive growth · Morphology

5.1 Introduction to Common Osmotic Solutes Applied in Foods

Osmotic solutions can be ionic and nonionic polar (Sahylin et al. 2017). Table 5.1 shows the common osmotic solutes applied in foods. The most commonly used ionic compounds are sodium chloride (NaCl) and calcium salts, which are used mainly in vegetables and tubers (Nishadh and Mathai 2014; Pereira da Silva et al. 2015). Nonionic polar substances, such as sucrose and sweeteners such as stevia, have been commonly used in fruit (Ferrari et al. 2011) and some vegetables (Bhupinder and Bahadur 2016).

The removal of water from food materials is one of the oldest techniques to make food safe and available (Janiszewska-Turak et al. 2021). The technical term
describing one of these processes is osmosis. Osmosis involves the movement of water molecules or other solvent molecules from a low-concentration solution through a semipermeable membrane to a high-concentration solution (Metris et al. 2017). The semipermeable membrane promotes a pressure gradient or osmotic pressure, facilitating the movement of these solvent molecules.

Immersion of a food material in aqueous concentrated solutions containing one or more solutes is a common process in the food industry (Sahylin et al. 2017). The most common solutes used are salt and sugar. These osmotic solutes, when combined with an additional bacterial hurdle, further inhibit bacterial growth (Sleator and Hill 2002). In 2021, a data-independent acquisition proteomics method was employed to analyze the response of *Lactiplantibacillus plantarum* NMGL2 to combinational cold and acid stresses during the storage of fermented milk made with the strain at 4 °C for 21 days (Zhang et al. 2021). This inhibition is particularly important in ready-to-eat (RTE) meat, fruits, vegetables, seafood, and fermented foods such as salami and cheese (Desmond 2006).

Specifically, osmotic dehydration (OD) is a procedure to remove water (Kaymak-Ertekin and Sultanoglu 2000). In contrast to normal evaporative drying, osmotic dehydration of fruits and vegetables is achieved by placing the solid/semisolid food material into a hypertonic solution (sugar or salt) where simultaneous counter diffusion of solutes and water between the osmotic solution and vegetable tissues occurs (Chandra and Kumari 2015). Kumari et al. (2020) showed that sucrose, maltodextrins, and stevia are useful osmotic agents to process sweet lime peel. Furthermore, sucrose was observed to be the best osmotic agent for osmo-convective drying of sweet lime peel. After the ultrasound pretreatment (10 min, 33 kHz) and sucrose-osmosed sweet lime peel treatment when subjected to air drying, the moisture content of sweet lime peel decreased from 28.70 to 1.91%.

Microorganisms lack resistance to high osmotic environments. Under osmotic stress, the bacterial cell walls appear hollow and damaged, and the osmotic pressure of the cell is altered, resulting in cell contraction, damage and impairment of metabolic activities. Rothe et al. (2013) showed that, under hyperosmotic stress (400 mM on glucuronate, 700 mM on galacturonate medium), galacturonate or glucoronate metabolism was impaired, and the maximal cell density of

<table>
<thead>
<tr>
<th>Types</th>
<th>Common osmotic solutes</th>
<th>Foods</th>
<th>Concentration</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>Ionic polar</td>
<td>NaCl</td>
<td>Vegetables</td>
<td>3%, 6% and 9%</td>
<td>Nishadh and Mathai (2014)</td>
</tr>
<tr>
<td></td>
<td>Calcium salts</td>
<td>Tubers</td>
<td>20% and 26.5%</td>
<td>Pereira da Silva et al. (2015)</td>
</tr>
<tr>
<td>Nonionic polar</td>
<td>Sucrose</td>
<td>Fruit</td>
<td>0.5%</td>
<td>Ferrari et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>Sweeteners</td>
<td>Vegetables</td>
<td>30°brix, 45°brix And 60°brix</td>
<td>Bhupinder and Bahadur (2016)</td>
</tr>
</tbody>
</table>
kduID-deficient *Escherichia coli* was reduced by 30% to 80% with a resultant 1.5- to 2-fold longer doubling time than that of wild-type *E. coli*.

### 5.2 Effect of Osmotic Stress on Foodborne Pathogens

All cells have a stable turgor pressure, and single-cell microorganisms must maintain normal growth (Rojas et al. 2017). Once microorganisms are exposed to high osmotic pressure conditions, irreversible damage can occur, with rapid outflow of cell water along the osmotic gradient (Clarke and Lilly 1962). This strategy is used as a bacteriostatic or bactericidal means in the food industry. Sofos (1984) thoroughly reviewed the antimicrobial properties of NaCl in foods and prospected the application prospects of chloride salts of other ions (i.e., KCl, MgCl2, and CaCl2) in the food industry. Taormina Peter (2010) also discussed the antimicrobial properties of sodium chloride in foods and addressed the impact of salt and sodium reduction or replacement on microbiological food safety and quality. Adding NaCl contributes to the microbiological stability of many foods, including deli meat, canned foods, dry and semidry sausages, and smoked fish. Although excess sodium consumption is a primary cause of hypertension and cardiovascular diseases, its important contribution to microbiological safety in food production warrants further attention. Additionally, some sugars, including those commonly used in food production—glucose, fructose, sucrose, and maltose have exhibited significant antimicrobial effects at high concentrations. However, the extent of inhibition differs from one pathogen to the other. In the case of *S. abony* and *E. coli*, the highest inhibition was observed in the case of sucrose, while maltose showed the lowest inhibition at the corresponding concentrations. Furthermore, in the case of *Staphylococcus aureus*, the lowest inhibition was observed for fructose, while the highest inhibition was observed for glucose and sucrose (Mizzi et al. 2020).

Generally, bacterial growth is divided into four stages: lag, logarithmic, stationary, and decline phases. Changes in the osmotic environment of cells driven by sucrose, KCL and NaCl-mediated decrease in a_w induce additional lag phases which impact bacterial growth. Mellefont et al. (2005) studied the lag time responses for *Salmonella Typhimurium* M48 subjected to abrupt osmotic shifts (0.5% to 9% NaCl of BHI media). Immediately after transfer from optimal a_w to low a_w, inactivation of a portion of the population occurred for all the conditions tested. The degree of inactivation became progressively larger with larger shifts in a_w, and the initial decline in population was followed by a period during which no change in numbers occurred, followed by growth that appeared, in most cases, to be exponential.

The influence of osmotic stress on bacteria also manifests in the cell wall. Turgor pressure drives mechanical expansion of the cell wall in *Bacillus subtilis*. Hypoosmotic shock transiently inhibits growth and slows cell wall synthesis, and membrane tension induces growth arrest via electrical depolarization (Rojas et al. 2017). In *E. coli*, a sudden increase in the external concentration causes a pressure
drop across the cell envelope; if the external osmolality remains high, cells grow more slowly, smaller, and at reduced turgor pressure (Pilizota and Shaevitz 2014).

5.3 Response of Foodborne Pathogens to Osmotic Stress

Hypertonic stresses on foodborne pathogens are routinely exploited by the food industry to deliver safe foods. For adaptation and survival in such hypertonic environments, cellular responses to hypertonic stress include increasing cellular persistence or adaptive cellular states, changes in morphology, adaptation of growth dynamics, and enhancing biofilm formation (Fig. 5.1) (Boons et al. 2013; Hazeleger et al. 2006).

5.3.1 Adaptive Cellular State

Survival mechanisms of bacteria under a hostile environment are achieved by detecting molecular signaling molecules and activating stress responses. One stress response is tolerance, which allows survival under suboptimal or even sublethal conditions (Alvarez-Ordonez et al. 2015). These stress tolerance responses are mainly transient and include both structural and physiological modifications in the microbial cell, but they can become permanent if the adverse environment persists.

Fig. 5.1 Potential response of foodborne pathogens to hypertonic solution treatment
These responses are monitored by a complex genetic regulatory machinery (Alvarez-Ordonez et al. 2015). Stressed microbial cells in various physiological states are generated, including sublethally injured or dormant cells known as persisters and viable but nonculturable (VBNC) cells. Persisters refer to a small proportion of a resistant microbial subpopulation with low metabolic activity and the same genotype surviving after all microorganisms have succumbed to the effect of stressor. The viable but nonculturable (VBNC) state is a dormant state of nonspore forming bacteria under environmental stress. Cells in the VBNC state are still alive with low levels of metabolic activity but lose their ability to form colonies on growth medium.

The VBNC state has been extensively studied in nonsporulating foodborne bacteria. This physiological state is characterized by an inability of cells to grow on general culture media (Alvarez-Ordonez et al. 2015). However, these VBNC cells are still viable, and their metabolic activity can still be detected (Li et al. 2014; Pienaar et al. 2019). In a VBNC state, microbial cells may maintain virulence and have been associated with outbreaks of foodborne diseases (Asakura et al. 2002; Li et al. 2017). Entry into the VBNC state is accompanied by key cellular changes, a reduction in metabolic activity, differential gene expression, and adaptive morphological changes (Pinto et al. 2015). These physiological adaptations occur over a range of timescales, depending on the intensity and abruptness of exposure to the stress-inducing factor(s).

The VBNC state is induced by osmotic stress (Rodrigues et al. 2015). Rodrigues et al. (2015) demonstrated that the decline in culturability became noticeable for all Salmonella strains when 0.6 M NaCl was added to the growth media, and the time period for the decline in cell viability (from $10^6$ CFU mL$^{-1}$ to less than $10^0$ CFU mL$^{-1}$) was 140 days. Song and Lee (2021) further explored the induction of the VBNC state of E. coli O157:H7 and Salmonella enterica serovar Enteritidis when incubated in LBWS with a maximum of 30% NaCl for 35 and 7 days, respectively. The VBNC state was triggered in media containing higher salt concentrations, and these cells reverted to normal growth when the stressor was removed. Additionally, Yoon et al. (2017) analyzed the factors that influence the induction of the VBNC state in Vibrio spp. The increasing levels of NaCl in the artificial sea water (ASW) microcosms resulted in the drastic loss of culturability in Vibrio parahaemolyticus ATCC 27969, V. parahaemolyticus ATCC 33844, and V. vulnificus ATCC 33815, although these strains are halophilic (salt-requiring).

### 5.3.2 Changes in Morphology

The foodborne pathogens E. coli and S. aureus, in common with all prokaryotic cells, have a membrane structure comprising a cell wall and plasma membrane. These barriers regulate the balance between intracellular and external environments and maximize the potential of growth for microbial cells (Clarke and Lilly 1962). Both the cell wall and the plasma membrane of bacteria have semipermeable
membrane properties, allowing H₂O and nutrients for bacterial growth to pass through these barriers.

An isotonic or neutral solution is an ideal environment for bacterial growth and division (Rojas et al. 2017). By contrast, a hypotonic environment creates an imbalance in the ion concentrations between the outside and inside of cells. The resultant osmotic pressure provides a driving force for the rapid influx of water into microbial cells, leading to swelling and rupture of the cells. However, under a hypertonic environment, the inverse occurs, and water within the microbial cells oozes out, resulting in plasmolysis (Fig. 5.2).

Bacteria have evolved several strategies to tolerate a range of osmotic pressures, but a finite limit exists regarding the resistance to osmotic pressure. Exceeding this limit inhibits bacterial growth, eventually leading to cell death. Most foodborne bacteria cannot grow and reproduce in hypertonic solutions such as a concentrated salt solution or concentrated sugar solution (Brul and Coote 1999). Therefore, the osmotic solutions containing appropriate sugar or NaCl concentrations are used as bacteriostatic agents in food processing and storage.

Many studies have documented the changes in the cell membrane of pathogenic bacteria after treatment with a hypertonic solution. Hajmeer et al. (2006) used transmission electron microscopy (TEM) to observe the morphological changes in *E. coli* O157:H7, and *S. aureus* with the incubation at 35 °C for 12 and 24 h in 5%–10% NaCl solution. TEM micrographs showed evidence of the magnitude of morphological damage to *E. coli* O157:H7 cells, and these changes were significantly more severe than those in *S. aureus* cells. The severity of cell injury also increased as the NaCl concentrations increased from 5% to 10%. At 10% NaCl, the damage to *E. coli* O157:H7 cells was fatal, resulting in the loss of cellular integrity. Salive et al. (2020) studied the cellular morphology of VNBC *Salmonella* induced by 1.2 M NaCl and observed these cells for up to 180 days. Using atomic force microscopy, they showed that the surfaces of VNBC cells became irregular and rough and the *Salmonella* cells slowly transformed to a coccoid shape. The coccoid form presented by *Salmonella enterica* in VBNCS was also reported by Rodrigues.
et al. (2015), and these morphological changes in cells in the VBNC state form a part of the adaptations to osmotic stress.

5.3.3 Adaptive Growth

Osmotic stress is a known inhibitory factor of bacterial growth (Salive et al. 2020). Paramasivam et al. (2007) found that a basal medium (broth) augmented with 10% NaCl significantly deceased the growth of V. parahaemolyticus, Bacillus cereus, Pseudomonas aeruginosa, and Proteus mirabilis. Kim and Chong (2017) evaluated the effects of salinity on the growth rates of Vibrio sp. B2. Their results showed that the specific growth rate was decreased by 28%, 85%, and 90% as the concentration of NaCl was increased in marine broth media to 0.6 M (3.5%), 1.2 M (7.0%) and 2.4 M (14%). Tolerance to salt concentrations varies with the lifestyle of the bacterial cells, which exists either in the planktonic form or attached to a surface. Smet et al. (2015) studied the impact of osmotic stress on planktonic and surface growth. While both forms of growth had similar characteristics under unstressed conditions, under high salt concentrations, the growth rate, $\mu_{\text{max}}$ (h$^{-1}$), of planktonic cells was higher. S. Typhimurium planktonic cells incubated at pH 5.5 and 6% (w/v) NaCl grew at 0.183 h$^{-1}$, while the $\mu_{\text{max}}$ of the surface colonies was only 0.142 h$^{-1}$. The growth of planktonic L. monocytogenes at pH 6.0 and 8% (w/v) NaCl was 0.145 h$^{-1}$ compared with the $\mu_{\text{max}}$ of the surface colonies at 0.113 h$^{-1}$.

5.3.4 Biofilm Formation

Biofilms are environments created by microorganisms to maintain their survival in certain niches (Flemming and Wingender 2010). More than 80% of bacterial infections in the human population are associated with biofilms, and approximately 60% of foodborne outbreaks are caused by biofilms (Bridier et al. 2015). These biofilms are built up initially as a monolayer of extracellular matrix on a surface and comprise mainly extracellular polysaccharides (EPS), structural proteins, cell debris, and nucleic acids. These monolayers are then developed into the pliable three-dimensional, complex and self-assembled structures to support the bacterial communities (Costerton et al. 1999).

Biofilms are isotropic biogels comprising bacteria, extracellular polymers, and nutrient aqueous solutions (Kalisk et al. 2009). These biogels absorb and remove solvents under the action of osmotic pressure with the resultant expansion and contraction. When the concentration of the solution outside these membranous biofilms is greater than that of the components inside the membranes, the components and nutrients in the biofilm permeate out of the biofilm. Therefore, hypertonic solutions may inhibit the growth of biofilms by affecting the important components
of biofilms. Kim and Chong (2017) demonstrated that the protein and eDNA concentrations of salinity-stressed bacteria were increased at 1.2 M and 2.4 M NaCl.

Osmotic solutes have been shown to inhibit the growth of biofilms. Mizan et al. (2018) evaluated the promotive and/or inhibitory effects of the combination of NaCl and glucose on the biofilm formation for *V. parahaemolyticus*. The authors showed that the addition of NaCl and glucose above a threshold level (2% salt and 0.015% glucose at 2% salt) induced the progressive stepwise inhibition of *Vibrio* growth and biofilm formation after 24 h of incubation. Bazire et al. (2007) demonstrated the suppression of *P. aeruginosa* biofilms due to the hyperosmotic stress, and the biofilm amount was reduced by approximately two-thirds under the osmotic stress conditions (0.5 M NaCl for 24 h). Michon et al. (2014) studied the effect of hypertonic saline (6% NaCl for 24 h incubation) on *P. aeruginosa* strains isolated from cystic fibrosis (CF) patients. Sodium chloride at 3% or higher inhibited the biofilm formation of CF patient isolates.

## 5.4 Circumventing Osmotic Stress

The cytoplasm of bacterial cells contains a reservoir of osmotic potential to control osmotic stress because of the semipermeable nature of the cytoplasmic membrane and semielastic properties of the cell wall (Erhard and Reinhard 2019). Additionally, the dedicated water channels called aquaporins are embedded in the bacterial cell membrane for the movement of water along an osmotic gradient (Wu et al. 2021). The perception of water or solvent movements across the semipermeable cytoplasmic membrane can trigger the physiological and genetic changes to resist osmotic stress (Wu et al. 2020). During food processing or in the food matrix, the bacterial cells routinely encounter various osmotic stressors.

One defense mechanism for bacterial cells to osmotic pressure is the accumulation or release of solutes from the cytoplasm (Zhan et al. 2021). These solutes include inorganic ions such as potassium ions and organic molecules known as osmoprotectants (Omari 2021). Some halophiles, such as *V. parahaemolyticus*, accumulate KCl to molar concentrations (0.5%), and most bacterial proteins function only in high salt environments (Beuchat 1974). Osmoprotectants assist the tolerance of bacterial cells to osmotic stress, and the osmoregulatory mechanisms for the accumulation and release of osmoprotectants are sophisticated (Cuenca et al. 2021). In some instances, this ability synergistically confers the tolerance of bacterial cells to heat treatment. Table 5.2 provides some examples of these organic molecules that help regulate osmotic stress resistance in several foodborne pathogens.

The synthesis, accumulation and import of these organic compounds are tightly regulated by some small regulatory RNAs (sRNAs) within bacterial cells (Bojanovic et al. 2017). Bazire et al. (2007) demonstrated that Ssr54 has a broad range of actions in the *S. flexneri* response to hyperosmotic environmental stresses and in controlling its virulence to adapt to environmental stresses encountered during host infection (Yang et al. 2020).
### Table 5.2  Response of foodborne pathogens to osmotic stress caused by the osmotic solutes, salt and sugar

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Food</th>
<th>Osmoprotectants</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>Deer jerky, dry fermented sausage, shell hazelnuts</td>
<td>Trehalose, glycine-betaine, proline</td>
<td>Regulator of RpoS during growth at all stress conditions</td>
<td>Kocharunchitt et al. (2012)</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>Beef, pork, eggs, poultry, fruits vegetables</td>
<td>Proline, glycine betaine, ectoine, and trehalose</td>
<td>Stress responses in <em>Salmonella</em> are controlled by an assortment of these regulators</td>
<td>Spector and Kenyon (2012)</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>Ready-to-eat (RTF) food</td>
<td>Proline betaine, acetyl carnitine, gamma-butyrobetaine and 3-dimethylsulfoliopropionate</td>
<td>The control of osmolyte accumulation is achieved at the transcriptional, translational, and posttranslational levels</td>
<td>Sleator et al. (2003)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Peanut butter, chocolate, milk powder and egg powder</td>
<td>Proline betaine</td>
<td>Proline betaine was taken up by the proline transport systems of <em>S. aureus</em></td>
<td>Amin et al. (1995)</td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td>Fish, shellfish and oysters</td>
<td>Trehalose, proline, glutamate, betaine, glycine betaine, ectoine</td>
<td><em>V. parahaemolyticus</em> is capable of de novo synthesis of ectoine at high salinity</td>
<td>Naughton et al. (2009)</td>
</tr>
</tbody>
</table>
5.4.1 Osmoprotectants

Exposure of bacteria to environments with high concentrations of osmotic solutes triggers rapid fluxes of water out of the cells, leading to reduced turgor and dehydration of the cytoplasm (Erhard and Reinhard 2019). Under high osmolality environments, bacteria synthesize or accumulate large amounts of organic osmolytes in the intracellular solute pool (Sleator and Hill 2002). These osmoprotectants are highly compatible with the physiology of the cell and minimally disrupt cellular functions (Weber and Jung 2002). Osmoprotectants comprise a few organic solutes, including the disaccharide trehalose (Kim et al. 2009), amino acid proline (Milner et al. 1987), trimethylammonium compound, and glycine betaine (Landfald and Strom 1986). The intracellular accumulation of compatible solutes as an adaptive strategy to high osmolality environments is evolutionarily well conserved in bacteria.

Osmoprotectants also maintain the growth rate of various members of Enterobacteriaceae, including *E. coli*, in media with high salt concentrations (Le Rudulier and Bouillard 1983). Perroud and Le Rudulier (1985) demonstrated that high levels of glycine betaine transport occurred when the cells are grown in media with elevated osmotic strength. Glycine betaine uptake is increased sixfold after adding NaCl to the growth media for 30 min. Dinnbier et al. (1988) exposed *E. coli* cells to 0.5 M NaCl and the authors found that a large amount of glutamate and trehalose were transported by these cells. Regarding osmoadaptation of growing *E. coli* cells, the uptake of proline has priority over trehalose synthesis, which is preferred over K⁺ and glutamate as osmoprotectants.

Adaptation to high osmolality environments can also trigger resistance to other stresses (Reed 1990) but is dependent on the osmoprotectant type. Fletcher et al. (2001) investigated the effects of ten homologs of betaines on the induction of thermotolerance in *S. Typhimurium*. The high osmolality-dependent growth at 45 °C was decreased partially by γ-aminobutyrate betaine (1 mM) and 3,4-dehydroproline betaine (1 mM), and blocked completely by pipecolate betaine and homarine.

Amin et al. (1995) used nuclear magnetic resonance (NMR) to detect the protective effect of proline betaine on *S. aureus* under osmotic stress. Proline betaine accumulated to high levels in osmotically stressed *S. aureus* cells, indicating that proline betaine is an efficient osmoprotectant for *S. aureus*.

Osmoprotectants also play an important role in protecting other foodborne pathogens, and these osmoprotectants include betaine for *V. parahaemolyticus* (Naughton et al. 2009) and glycine betaine and L-carnitine for *L. barotolerance* (Smiddy et al. 2004).
5.4.2 Cellular Response Systems

Cellular adaptation to rapidly changing osmotic environments is critical for the survival of foodborne pathogens in extremely osmotic environments. Under an environment with changing osmolality, the cellular response systems within bacterial cells compensate for these adverse effects (Chen et al. 2004).

First, bacterial cells should be able to sense osmosis-induced stressors, including either the loss of turgor pressure or an increase in intracellular ionic strength (Beuchat 1974). Subsequently, the stress response systems associated with osmotic stress are initiated by bacterial cells. A key step in this cellular response is the generation of an appropriate cellular signal and transduction of the signal from the exterior to the interior (Bazire et al. 2007). Typical examples of such signals are conformational or structural changes in signal-transducing components, the phosphorylation of proteins and/or a conformational switch of these components.

The cellular signal is then transduced to an appropriate target system in the interior of the cell. Because of signal transduction, the target system changes its functional state by switching its enzymatic activity or affinity for binding to further components of the cell, such as specific DNA motifs or target proteins (van der Heide et al. 2001). Functional alterations of the target system will finally cause appropriate cellular responses to the primary osmotic event—gene expression, metabolite synthesis, protein synthesis, or activation of an enzyme or a transport protein (Burgess et al. 2016).

5.4.3 Regulatory Genes in Osmotic Stress Adaptation

When foodborne pathogens are exposed to various stresses in a food processing environment, several survival mechanisms are initiated, including surface structure modifications and the modulation of genes responsible for stresses (Esbelin et al. 2018; Orihuel et al. 2019). Many studies are available on the fundamental aspects of bacterial survival, including cell membrane modification, DNA modification, transcriptional and translational responses, trigger factors, and initiation factors (Begley and Hill 2015; Burgess et al. 2016). Additionally, many studies are available on the fundamental aspects of bacterial survival.

Romeo et al. (2007) reported that BusR, a repressor protein for an ATP binding cassette import system (BusAB/OpuA) of glycine betaine in Lactococcus lactis, is activated by the ionic strength of the cytoplasm. This activation is dictated by the osmolality of the exterior of the cell. Wang et al. (2020) studied the resistance profiles of Salmonella isolates to hyperosmotic stress (salt contents more than 8.0%) and demonstrated that CyaR and InvR, which are involved in the general stress response and stress adaptation, are induced at high expression levels (more than sevenfold). Chassaing and Auvray (2007) used the transposon insertion site mutagenesis to identify a gene of L. monocytogenes, lmo1078, which encodes a
putative UDP-glucose pyrophosphorylase that catalyzes the formation of UDP-glucose. Glucose is a precursor of membrane glycolipids and cell envelope teichoic acids, which are important components for cold and salt tolerance. Furthermore, DP-glucose pyrophosphorylase activity is important for cold and salt tolerance. Utratna et al. (2011) observed that the expression of sigma B-dependent genes, opuCA, lmo2230, lmo2085, and sigB, is correlated with the magnitude of the osmotic stress applied. Ito et al. (2009) proved that the gene YggT, a homolog of fkuB of the marine bacterium Vibrio alginolyticus, endows E. coli cells with the tolerance to osmotic shock (salinity stress). YggT alters the metabolic pathways of E. coli cells to produce osmolites under hyperosmotic conditions.

5.5 Conclusions

Producing safe foods is complicated by the presence of pathogenic microorganism. The osmotic environment designed into food processing can inhibit the growth of some pathogens; thus, this method is used as a mild bacteriostatic or bactericidal means in the food industry. However in the presence of osmotic pressure, some pathogenic bacteria in food have evolved strategies to evade this stress. These strategies include changes in cell morphology, the upregulation of regulatory genes, and the expression of proteins in cellular response systems. The intake of osmoprotectants also provides a strategy for bacteria to control osmotic pressure. These evolutionary methods complicate the use of osmotic stress against foodborne pathogens for the food industry. Future studies should focus on strategies to overcome the resistance to osmotic pressure at the regulatory level in pathogenic bacteria.

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Chapter 6
Response of Foodborne Pathogens to Oxidative Stress

Nadira Naznin Rakhi, Latiful Bari, and Md. Mizanur Rahaman

Abstract The term “stress” refers to any extracellular influence that threatens the ability of microorganisms to perform their living functions. In nature, microorganisms are constantly exposed to diverse changes in temperature, oxygen, moisture, light, pH, and chemical composition. Thanks to their wide array of molecular responses that make them survive by providing cellular protection against stresses. This protection from such inevitable stresses in microbial systems is ensured by sophisticated genetic regulatory systems and molecular stress responses specific to individual chemical or physical threats. This chapter will summarize and discuss current knowledge about the oxidative stress response evolved in food pathogens following the oxidative stress encountered in food and food processing environments (including acid, osmotic and oxidative stress, starvation, detergents and disinfectants, chilling, heat, and other nonthermal technologies) and from the food preservation technologies designed to rapidly inactivate microbial cells including thermal processes such as low-temperature storage (refrigeration and freezing), irradiation, high-pressure processing, use of strong oxidant compounds, reduction of moisture content (concentration and drying), control of redox potential (use of controlled atmospheres and vacuum packaging), and acidification (fermentation and addition of organic acids) certainly with special emphasis on virulence and growth fitness.

Keywords Reactive oxygen species (ROS) · ROS detoxification · Food pathogens · Oxidative stress · Protection and repair

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T. Ding et al. (eds.), Stress Responses of Foodborne Pathogens, https://doi.org/10.1007/978-3-030-90578-1_6
6.1 Introduction

Despite the current effort to ensure safe food from farm-to-fork, foodborne illness is still a major global health concern in both developing and developed countries affecting trade and economics along with public health (Jahan 2012). Foodborne pathogens are responsible for different gastrointestinal diseases followed by chronic sequelae and disability, and even death (Lindsay 1997). The foodborne pathogens involved in major outbreaks with significant morbidity and mortality in recent times include *Listeria monocytogenes* in cantaloupe, *Salmonella* spp. in chicken eggs and ground turkey, and *Escherichia coli* in fenugreek seeds (Mack et al. 2012). While multiple host factors including age, immunity, and eating habits contribute to the prevalence of foodborne diseases (Newell et al. 2010), microbial factors including stress adaptation is also an important factor. Pathogenicity of foodborne pathogens may depend on the environmental challenges or stresses present (Begley and Hill 2015).

On the other hand, aerobic respiration in organisms ranging from human to microorganisms yields reactive oxygen species (ROS), which lead to oxidative stress, a condition characterized by an elevated level of reactive free radicals occurring from the excessive production of oxidant species (Mandelker 2011) or the decline of antioxidant defense (Suzuki et al. 2011). The physiological or pathological conditions may produce oxidative stress which may lead to diverse diseases directly (Suzuki et al. 2010) through damaging cellular proteins and other macromolecules. Also, the stress may cause the ultimate disruption of the gastrointestinal tract barrier leading to gastric ulcers, inflammation, etc. along with other noninfectious diseases including cancers, hypertension, diabetes mellitus, and ischemic heart diseases in humans (Suzuki et al. 2010). Besides, the endogenous and the exogenous oxidative stress poses effects on the pathogenesis and survival of foodborne pathogens (Vergara-Irigaray et al. 2014). Beginning from food production, processing, storage, distribution, and preparation to food digestion, foodborne pathogens encounter diverse stress conditions, among which oxidative stress is the most common stress encountered during the adaptation phase of these pathogens (Fig. 6.1) (Johansson and Freitag 2019). Also, this is one of the major factors that pathogens need to overcome in order to survive inside/outside the host cell (Fig. 6.1) ( Attack and Kelly 2008). As a result, pathogens evolved sophisticated mechanisms to sense and adapt to oxidative stress by producing different enzymes (Whiteley et al. 2017) such as superoxide dismutases (Pesci et al. 1994), catalases (Chelikani et al. 2004), thioredoxins, cytochrome c peroxidases (Attack and Kelly 2008), peroxiredoxins such as alkyl-hydroperoxide reductase and thiol peroxidase (Poole et al. 2000), and bacterial globins (Poole and Hughes 2000) to protect themselves from oxidative damages to cells or cellular macromolecules (e.g., membrane, proteins, and DNA). Also, responses to oxidative stress may lead to cross-protection or cross adaptation to other stresses including heat, salinity, and heavy metals (Davi and Minc 2015). Besides, oxidative stress has been implied in antibiotics-mediated killing of pathogens (Kohanski et al. 2007). So, elucidating the complete
mechanisms underlying the responses of foodborne pathogens to oxidative stress may lead to a significant development in food processing and preserving techniques as well as reducing the prevalence of global foodborne diseases.

### 6.2 Generation of Oxidative Stress

Even though atmospheric oxygen is crucial for the growth of aerobic bacteria, using oxygen as an electron carrier in oxidative phosphorylation leads to oxidative stress (Mols et al. 2009). Oxidative stress arises from the production of toxic ROS, such as the superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$) through incomplete reduction of oxygen, as the complete reduction of oxygen forms water, which is a four-step reaction:

$$O_2 \rightarrow O_2^- \rightarrow H_2O_2 \rightarrow HO \rightarrow H_2O$$

So, bacteria possess different mechanisms to maintain the homeostasis of ROS. To maintain cytosolic redox status, bacteria have redox regulating thiol molecules such as glutathione and the dicysteine proteins, thioredoxin and glutaredoxin, which are maintained in reduced states by glutathione reductase and thioredoxin reductase, respectively (Larsson et al. 2007). Thioredoxin plays a major role in maintaining the cellular reducing environment. Along with thioredoxin and low-molecular-weight thiols, many Gram-positive bacteria use alternative thioredoxin-based enzymes as antioxidant systems (Lu and Holmgren 2014).
But the rate of ROS generation may increase depending on the environmental oxygen concentration (Lushchak 2011). As a result, pathogens conduct mechanisms not only to regulate ROS homeostasis, but also have pathways for removing stress sources and repairing the cellular damages caused by that stress (Guo and Gross 2014). A DNA microarray assay with C. jejuni showed that increased environmental oxygen concentration stimulated the expression of different genes related to oxidative phosphorylation, antioxidation, and nucleic acid metabolism (Kaakoush et al. 2009).

6.2.1 Primary Generation Through Respiration

Primary oxidative stress can arise from the exposure of oxidizing agents produced endogenously through aerobic respiration, intracellular redox reactions, autooxidation reactions, etc. or exogenously due to the action of host innate immune cells (Fig. 6.2).

6.2.1.1 Endogenous Oxidative Stress

While the complete reduction of oxygen during aerobic respiration results in the formation of H₂O using oxygen as the final electron acceptor, the incomplete reduction upon the interaction with flavoproteins (e.g., oxidases and monooxygenases) could result in ROS (Messner and Imlay 1999). Because while

![Fig. 6.2 Schematic representation of the bacterial response against different oxidative stress. The white box indicates the effects of the different oxidative stress (both exogenous and endogenous); yellow color indicates the damages that can be caused directly and indirectly by oxidative stress and the orange colored box indicates bacterial responses against these damages.](image)
interacting with the reduced FAD (flavin adenine dinucleotide) cofactor of flavoenzymes, electrons get transferred to molecular oxygen that converts it into endogenous superoxide anions (O\(^{-2}\)) and hydrogen peroxide (H\(_2\)O\(_2\)) (Korshunov and Imlay 2010). Apart from these flavoenzyme catalyzed reactions, Fenton chemistry may lead to the generation of ROS, especially the highly reactive hydroxyl radicals (HO\(^{-}\)), when Fe reacts with H\(_2\)O\(_2\). In a biological system, the overall reaction through which (Fe\(^{3+}\)) is reduced by O\(_2\)\(^{-}\) to yield ferrous iron (Fe\(^{2+}\)) is called the Haber-Weiss reaction followed by the Fenton reaction below to produce \(\cdot\)OH:

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \cdot\text{OH}
\]

So, the concentration of Fe in bacterial cells plays an important role in the regulation of oxidative stress (Van Vliet et al. 2002). Along with Fe, other metals such as Cu and Cd are also hypothesized to react in this manner through Fenton chemistry, although the significance of nonferrous metals catalyzing this chemistry has not yet been resolved (Macomber and Imlay 2009).

### 6.2.1.2 Exogenous Oxidative Stress

Exogenous oxidative stress is most commonly encountered by the pathogenic bacteria to survive through overcoming the defense of host immune cells such as macrophages, monocytes, and neutrophils. Because these immune cells have NADPH oxidase (NOX), which generates superoxide (O\(^{-2}\)) during an oxidative burst, when NOX transfers electrons from NADPH to oxygen and finally dismutation of O\(^{-2}\) produces H\(_2\)O\(_2\) (Nauseef 2004). This H\(_2\)O\(_2\) can be used by myeloperoxidase (MPO) to produce hypochlorite (OCl\(^{-}\)), another bactericidal compound. During phagocytosis, upon engulfing the bacteria by the phagocytic cells, MPO in the phagosome binds to bacteria leading to MPO-mediated killing through H\(_2\)O\(_2\)-dependent conversion of Cl\(^{-}\) to OCl\(^{-}\) (Klebanoff 2005). Nitric oxide (\(\cdot\)NO) is also produced by all immune cells, which is a reactive oxidant reducing oxygen and oxidizing NADPH showing potent cytotoxic properties against bacteria. When a macrophage encounters a pathogen, it produces nitric oxide synthase (iNOS or NOS2) which catalyzes the conversion of L-arginine to L-citrulline and \(\cdot\)NO (Woodmansee and Imlay 2003). Moreover, \(\cdot\)NO and O\(^{-2}\) can react to form another highly reactive intermediate peroxynitrite (OONO\(^{-}\)) (Huie and Padmaja 1993).

### 6.2.2 Secondary Generation Through Other Stresses

The secondary oxidative stress arises from exposure to other stressful conditions including low pH and high temperature, when actively respiring bacteria encounter...
conditions that affect the electron transport chain (Fig. 6.3). Probably these stressful conditions hamper the electron transfer chain in a way that causes the premature leakage of electrons to oxygen, resulting in the formation of $O_2^-$ (Imlay 2003). To a certain limit, bacteria can convert $O_2^-$ to water through the action of normal response mechanisms, while above this limit, $O_2^-$ participates in Fenton reaction or can rapidly react with NO to produce $ONOO^-$ (Beckman and Koppenol 1996). Heat and acid stresses have been reported to cause the production of $OH^-$ and/or $ONOO^-$ (Mols et al. 2009). However, high salt concentration has a limited effect on the formation of $OH^-$ and/or $ONOO^-$ (Mols et al. 2009). On the other hand, low pH exposure also causes the formation of NO in Bacillus cereus (Shatalin et al. 2008). Antibiotics are also reported to cause the formation of OH- radicals in E. coli and Staphylococcus aureus (Kohanski et al. 2007). Antibiotics such as kanamycin cause a burst of free radicals in Bacillus subtilis (Mols and Abee 2011), while the peptidoglycan synthesis inhibitors enduracidin and bacitracin induce thiol-oxidative stress (Rukmana et al. 2009).

6.3 Targets of Oxidative Damage

The targets of oxidative damage and its consequences have been determined from the studies with the mutant bacteria unable to detoxify/neutralize the ROS such as bacteria lacking the genes encoding for superoxide dismutase, catalase, and peroxidase (Park et al. 2005). ROS can damage any oxidizable moiety in biological macromolecules. So, the mutants lacking the antioxidant proteins encoding genes become highly susceptible to oxidative damages upon being exposed to oxidants such as paraquat and $H_2O_2$.

Superoxide and $H_2O_2$ can lead to the inactivation of enzymes through interacting with the Fe-S centers of the enzymes (Flint et al. 1993). They can release Fe from Fe-S cluster containing proteins, which can be further oxidized to disrupt the activity of enzymes (Flint et al. 1993). Also, the reaction of $H_2O_2$ and the $Fe^{2+}$ of an iron-containing protein, possibly through Fenton chemistry cause irreversible protein carbonylation and the formation of protein aggregates (Dukan et al. 1999). On the other hand, $H_2O_2$, $HO^-$, and $ONOO^-$ may also oxidize cysteine, methionine, and tryptophan, which may ultimately inactivate enzymes reversibly or irreversibly. For example, cysteine may be oxidized in reversible (sulfenic acid or S-thiolation) or irreversible manner (i.e., sulfenic acid and sulfonic acid) (Chouchani et al. 2011). Likewise, oxidation of methionine to methionine sulfoxides can be reversed by the action of methionine sulfoxide reductase (Gaupp et al. 2012).

However, Fe released from Fe-S cluster in presence of $H_2O_2$ creates an intracellular environment generating the highly reactive $HO^-$ through Fenton chemistry (Keyer and Imlay 1996). Hydroxyl radicals are highly reactive reacting with virtually any molecule it encounters. So, considering the close proximity, the charge–charge interaction between positively charged $Fe^{2+}$ molecules liberated and the negatively charged DNA phosphodiester backbone may facilitate the lethal/
Fig. 6.3 Schematic representation of the secondary generation of ROS in *Bacillus* spp. The stresses other than oxidative stress including heat, acid, antibiotics, and high salt concentrations, may disrupt the electron transfer chain which results in the secondary origin of ROS such as superoxide ($O_2^-$). $O_2^-$ leads to a similar response in the bacterial system followed by the conversion into hydrogen peroxide ($H_2O_2$). The nitrosative stress through the production of nitric oxide (NO) has a crucial role in oxidative stress maintenance, as NO inhibits the production of OH from Fenton reaction by inhibiting the formation of $Fe^{3+}$ to $Fe^{2+}$ recycling reduced thiols (R-SH). Also, NO can activate catalase degrading $H_2O_2$ to oxygen and water ($H_2O$). Contrarily, NO itself may get converted into the highly reactive $ONOO^-$ from its reaction with $O_2^-$ (The figure was adapted from Mols and Abee (2011) with permission)
nonlethal DNA damages induced by HO-produced from Fenton chemistry (Keyer and Imlay 1996).

Also, oxidative stress leads to lipid peroxidation of the fatty acids in the cell membrane, which decreases the membrane fluidity, and disrupt the functions of membrane-bound proteins significantly (Humphries and Szweda 1998). Besides, lipid peroxidation works as an amplification step resulting in the formation of more radicals and polyunsaturated fatty acids, which are degraded to a range of products including aldehydes, which in turn is very reactive and can damage other macromolecules like proteins (Humphries and Szweda 1998).

So, if not detoxified, oxidative stress can damage cellular macromolecules including proteins, DNA, and membranes. Studies with *E. coli* showed that increased concentration of superoxide causes a reduction of dehydratase activity by twofold, and impaired growth by fourfold and significant DNA damage by fivefold compared with the unstressed counterparts (Gort and Imlay 1998). Also, significant DNA damage and metabolic disruption were noticed in mutant *E. coli* unable to scavenge H$_2$O$_2$. As the mutant had defective isopropyl malate isomerase belonging to a family of [4Fe-4S] dehydratases, the major reason behind metabolic disruption is mediated through the damage of iron-sulfur clusters by increasing H$_2$O$_2$ level (Jang and Imlay 2007).

6.4 Downstream Effects of Oxidative Stress

While oxidative stress can damage the macromolecules of cells, bacteria have evolved mechanisms to resist and repair oxidative damages. Food pathogens have dedicated mechanisms for the detoxification of ROS as well as the general stress response. However, the stress response and resistance mechanisms and their regulations may vary from one foodborne pathogen to another. Complete characterization of the mechanisms for oxidative stress resistance and regulation will ensure food safety concerns more effectively.

6.4.1 General Stress Response

As different stress conditions affect bacteria in a similar fashion, a common set of genes is expressed while bacterial pathogens encounter stressed conditions including oxidative and other stresses like heat or acid. These general stress response genes encode for Clp proteases and chaperones protein, GroES, DnaK, etc. for protein protection, refolding and turnover indicate the common malfunction/damage to the crucial biomolecules of foodborne pathogens (Mols and Abee 2011). Another effective way to control the general stress response genes against multiple stressed conditions is to regulate the genes through alternative sigma factors. While the consecutive sigma factor is responsible for the expression of housekeeping genes
under normal conditions, an alternative sigma factor can induce the expression of genes, specific for the stressed conditions (Begley and Hill 2015). The alternative sigma factor RpoS plays role in modulating stress responses in Gram-negative bacteria (E. coli, Salmonella, and Vibrio), while in Gram-positive bacteria (L. monocytogenes, Bacillus subtilis, and S. aureus), the key stress responses regulating alternative sigma factor is SigB (σB) (Begley and Hill 2015). These regulators are responsible for coordinating the expression of genes involved in tolerance of acid, high osmolarity, temperature, antibiotics/bacteriocins, ethanol, and even prolonged starvation as well as in biofilm formation and sporulation (Abram et al. 2008; Becker et al. 1998; Begley et al. 2006; Van Schaik and Abee 2005; Cebrián et al. 2009; Hengge 2008; Pané-Farré et al. 2006; Schellhorn 2014).

However, the regulation of genes mediated by these alternative sigma factors is strain specific. For example, SigB mediated regulation of stress response genes in four different L. monocytogenes strains included a total of 63 genes, but the genes upregulated through SigB in a single strain differed from each other (Oliver et al. 2010). So, the outcome of SigB regulation in stress management may be phenotypically different among different strains.

### 6.4.2 ROS Detoxification

#### 6.4.2.1 Hydrogen Peroxide Detoxification

Hydrogen peroxide is converted to water and oxygen by catalase enzyme, which plays a crucial role in oxidative defense (Imlay 2008). Catalase enzymes can be classified into monofunctional or typical catalases, bifunctional catalase-peroxidases, and manganese-containing catalases (Chelikani et al. 2004). All species of Staphylococcus except for S. saccharolyticus and S. aureus subspecies anaerobius are catalase-positive (Götz et al. 2006). The catalase present in S. aureus is a monofunctional heme-containing tetrameric catalase, which is encoded by the monocistronic katA gene (Sanz et al. 2000), while S. xylosus, S. equorum, and S. saprophyticus have two different catalase genes, katA and katB (Blaiotta et al. 2010). Another foodborne pathogen, C. jejuni also has katA gene for detoxifying hydrogen peroxide and is crucial for the survival of the pathogen within macrophages (Grant and Park 1995). Also, downstream of katA, there is a heme-trafficking protein Cj1386, which is needed for the complete catalase activity in C. jejuni as well as contributes to the colonization of chicken intestines (Flint et al. 2012). Apart from katA, pathogens have several peroxiredoxins that are expressed upon the presence of H$_2$O$_2$, such as Tpx, Ohr-like protein, and AhpC (Wolf et al. 2008). Peroxiredoxins convert alkyl hydroperoxides into their corresponding alcohols and use NADH or NADPH as the reducing equivalents for this reaction. Among different classes of peroxiredoxins, the alkyl hydroperoxide reductase gene (ahpC) is a part of an operon with ahpF, which encodes a homodimeric flavoenzyme functioning as a dedicated disulfide reductase and this enzyme transfers electrons from NAD(P)H to AhpC.
which is the peroxide reducing part of the operon (Poole 2005). This type of alkyl hydroperoxide reductase is present in *Salmonella enterica* and *E. coli*, but the alkyl hydroperoxide reductase is found in *C. jejuni* lacking in *ahpH* (Baillon et al. 1999). *C. jejuni* has three different classes of peroxiredoxins, which are alkyl hydroperoxide reductase (AhpC), and two putative peroxidases: thiol peroxidase (Tpx) and bacterioferritin comigratory protein (Bcp) (Baillon et al. 1999). However, *C. jejuni* mutant having no *ahpC* is highly susceptible to cumene hydroperoxide, an organic peroxide, but not to H$_2$O$_2$ (Baillon et al. 1999). Besides, another periplasmic protein class, cytochrome c peroxidases (CCP) reduces H$_2$O$_2$ to water, while *C. jejuni* has two putative CCP genes; *docA* and *Cjj0382*, among which the latter functions in oxidative stress defense strain-dependently (Parkhill et al. 2000). Moreover, in *C. jejuni*, *luxS* mutation showed differential effects on the resistance to oxidative stress (Elvers and Park 2002). The rubredoxin oxidoreductase/rubrerythrin chimeric protein Rrc can also impart resistance to oxidative stress caused by menadione (i.e., superoxide) and H$_2$O$_2$ by interacting with exogenous and endogenous H$_2$O$_2$ (Flint et al. 2014).

### 6.4.2.2 Superoxide Detoxification

Superoxide dismutase (SOD) is the primary detoxifying enzyme responsible for the detoxification of superoxide (Winterbourn et al. 1975). SOD is a metalloenzyme that catalyzes the conversion of superoxide (O$^-_2$) to oxygen and H$_2$O$_2$ (Fig. 6.4), among which the latter gets further reduced to water and oxygen by the function of catalase (Sect. 6.4.2.1) or alkyl hydroperoxide reductase. So, by converting O$^-_2$ into nontoxic molecules, SODs plays crucial roles in protecting bacteria from damages directly caused by O$^-_2$, but also indirectly protects the cells by preventing the formation of other toxic molecules causing oxidative and/non-oxidative (i.e., nitrosative) stress derived from the reactions with O$^-_2$, such as OONO$^-$. SODs can be classified into four types depending on the metal ion cofactor (Fridavich 1995):

(i) The copper-zinc type (Cu/Zn-SOD)
(ii) The manganese type (Mn-SOD)
(iii) The iron type (Fe-SOD)
(iv) The nickel type (Ni-SOD)

![Fig. 6.4 Detoxification of reactive oxygen species (ROS) by sequential reactions by superoxide dismutase (SOD) and catalase, peroxidase](image)
Different foodborne pathogens possess different SODs to defend against oxidative stress caused by $O^{−2}$. For example, *E. coli* has three different *sod* genes including *sodA* (encoding Mn-SOD), *sodB* (encoding Fe-SOD), and *sodC* (encoding Cu/Zn-SOD) (Imlay 2008), while *C. jejuni* has only the Fe-SOD SodB (Pesci et al. 1994). And two monocistronic SODs, SodA and SodM (Mn/Fe-SOD) are present in *S. aureus* (Poyart et al. 1995) and only SodA is present in coagulase-negative staphylococci lacking the SodM (Barrière et al. 2001). As *C. jejuni* has only one SOD for superoxide detoxification, SodB, and the mutation in *sodB* was found to make the bacterium susceptible to both superoxide (e.g., menadione) and peroxide stress (e.g., $H_2O_2$ and cumene hydroperoxide) (Palyada et al. 2009). Also, paraquat (i.e., superoxide) exposure upregulates the expression of Cj1371, a putative periplasmic protein, which is a homolog of virulence gene in *Shigella flexneri*, VacJ (virulence-associated chromosome locus J), and mutation of *cj1371* increases *C. jejuni* susceptibility to the oxidative stress of paraquat (Suzuki et al. 1994; Garénaux et al. 2008). However, bacterial motility may have roles in the resistance of *C. jejuni* resistance to superoxide stress, as the reports showed that mutations of genes for flagellar biosynthesis and modification (e.g., *motAB*, *flgR*, *flhB*, *flgD*, and *pseB*) makes *C. jejuni* significantly susceptible to the superoxide generator-menadione and also to $H_2O_2$ to a lesser degree (Flint et al. 2014). Presumably, mutations in flagellar genes change the proton motive force required for the flagellar rotation and such disturbance causes electron leakage leading to the generation of ROS, and affecting the response to oxidative stress in *C. jejuni* (Flint et al. 2014).

However, in vitro experiments showed that expression level and activity of SOD can be increased by the presence of different oxidants. For example, *sodA* in *S. aureus* is induced by endogenous or internal oxidative stressors and *sodM* by exogenous $O^{−2}$ stress (Gaupp et al. 2012). While the general stress response genes are transcribed by the σB, both SODs in *S. aureus* are transcribed from σA-type promoters, although one of the two promoters for the expression of *sodA* is negatively regulated by σB. On the other hand, while the effect of σB on the expression of *sodM* is not clearly elucidated yet, *sodB* gets upregulated in the in σB-deficient strains (Karavolos et al. 2003). Also, the staphylococcal accessory regulator (SarA) acts as a repressor of *sodM* at the level of transcription independent of σB (Ballal and Manna 2009).

### 6.4.2.3 Hemoglobin-Like Protein

Hemoglobin-like protein (Hmp) was first discovered in *E. coli* (Vasudevan et al. 1991) and later found in wide varieties of bacteria. It is a flavohemoglobin with an N-terminal heme-containing globin domain and C-terminal NAD- and FAD-binding domains functioning as a ferredoxin-NADP$^+$ oxidoreductase-like domain in combination (Ermler et al. 1995). However, this protein can function as NO-reductase, NO-dioxygenase, and alkylhydroperoxide reductase (Bonamore and Boffi 2008). In aerobic condition, the Hmp from *E. coli* converts $O_2$ to $\cdot$NO and to nitrate using NAD (P)H; but under anaerobic conditions, the reaction is efficient converting $O_2$ to $\cdot$NO.
and to N₂O (Gardner et al. 1998). In *S. aureus*, Hmp activity is highest during microaerobic/anaerobic growth or in presence of nitrosative stress (Gonçalves et al. 2006). It probably works as a transcription repressor to sense the presence of nitrosative stress (NO⁻₂) (Richardson et al. 2006).

### 6.4.2.4 Detoxification of Other Oxidative Agents

Apart from hydrogen peroxide and superoxide, foodborne pathogens encounter other oxidants such as peracetic acid and sodium hypochlorite, especially during the cleaning and disinfecting of food-contacting surfaces. The response to these oxidants, mainly peracetic acid is almost similar to the response observed in the case of hydrogen peroxide (Ceragioli et al. 2010). This is probably because of the chemical nature of peracetic acid, which is a mixture of hydrogen peroxide and acetic acid. On the other hand, Sodium hypochlorite, a commonly used ingredient in disinfectants may also exert oxidative stress. For example, in *B. cereus*, exposure to sodium hypochlorite causes the expression of genes functioning in the metabolism of sulfur-containing amino acids such as cysteine and methionine (Ceragioli et al. 2010) and consequently, the oxidation of sulfhydryl-group is found at high level following the exposure. Another oxidative compound, diamide causes thiol oxidation and disulfide stress in *B. subtilis* and activates the genes of vegetative catalase and alkyl hydroperoxide reductase (Ole Leichert et al. 2003). Besides, as disulfides stress may lead to irreversible thiolation of proteins, S-cysteinylilation happens to protect the proteins from damage (Hochgräfe et al. 2007; Huyen et al. 2009). Low molecular weight thiol bacillithiol may be working to resist the effect of both diamide and other electrophile-associated thiol stresses (Antelmann et al. 2008; Gaballa et al. 2010).

Also, different virulence and stress response genes are expressed, when pathogens get exposed to O₂⁻-producing paraquat such as siderophore production and iron transport-associated genes in *B. anthracis*, genes associated with cell envelope stress in *B. subtilis* (Passalacqua et al. 2007; Cao et al. 2005).

### 6.4.3 Regulation of Stress Defense Genes

Oxidative stress may reversibly or irreversibly inactivate proteins through the rapid inactivation of Fe-S cluster or oxidation of some cysteine and methionine-containing proteins. So, oxidative stress can alter enzymatic activity leading to changes in metabolite concentration and cellular redox potential. These changes ultimately signal the redox/metabolite-responsive regulators regulating components of the oxidative stress response.
6.4.3.1 Ferric Uptake Regulator

The ferric uptake regulator (Fur) is a transcriptional regulator protein playing crucial roles in iron homeostasis in many bacteria, including different foodborne pathogens such as *C. jejuni* and *Vibrio cholera* (Fig. 6.4) (Van Vliet et al. 1998). Fur is a homodimeric metalloprotein, which has DNA binding domain at N-terminal and zinc at C-terminal (Sheikh and Taylor 2009). So, when Fur binds with Fe, it can regulate the transcription of genes by binding to a specific DNA sequence known as the Fur box in the promoter characterized by a 19-bp inverted repeat sequence (GATAATGATAATCATTATC) (Baichoo and Helmann 2002). Primarily Fur represses the genes; therefore, fur activation in an iron-limited medium produces similar results as those observed in a fur mutant (Johnson et al. 2011). However, in case of oxidative stress, it acts as a transcriptional activator for *katA* leading to increased catalase activity (Horsburgh et al. 2001b). It is still not clear how two-iron bound Fur acts as a transcriptional activator for *katA* expression, while holo-Fur causes the repression. Nevertheless, PerR is more significant in maintaining redox potential than Fur (Van Vliet et al. 1998).

6.4.3.2 PerR

PerR is a homolog of Fur and plays an important role in peroxide stress resistance (Fig. 6.5) (Van Vliet et al. 1999). PerR is rare among Gram-negative bacteria, while the first PerR reported in Gram-negative bacterium was in *C. jejuni* (Van Vliet et al. 1999). Mutations in perR make bacteria hyper-resistant to peroxides, such as H$_2$O$_2$ and cumene hydroperoxide through upregulation of antioxidant proteins, such as KatA and AhpC (Bsat et al. 1998; Van Vliet et al. 1999).

As a Fur homolog, the activity of PerR depends on metals. PerR has structural zinc, but DNA binding activity of PerR increases when PerR binds to either Fe or Mn (Lee and Helmann 2006). PerR binds to the promoter of its own gene (PerR box) and blocks the expression of PerR. So, PerR bound with Fe or Mn will act as a transcriptional repressor by binding to the PerR box (AAGTATTATTTATTATTATTA), a consensus DNA sequence (Horsburgh et al. 2001a). The PerR box overlaps with -35 sequence of the upstream promoter and a part of the downstream promoter (Kim et al. 2011). As a result, the regulation of perR transcription is mediated through these two overlapping promoters. In the presence of H$_2$O$_2$, the iron in PerR causes HO· production, HO· oxidizes the amino acid histidines, which coordinate iron leading to the loss of iron followed by DNA binding activity of PerR (Lee and Helmann 2006). When PerR binds to Mn, there is less probability of HO· metal ion loss and its inactivated by H$_2$O$_2$, because Mn is a poor mediator of the above-mentioned HO· producing Fenton reaction. So, in the absence of HO·, the metal coordinating histidines is not oxidized and thus PerR can bind to DNA. So, this can easily explain why the presence of high Mn$^{2+}$ and low Fe$^{2+}$ repress PerR regulon in the presence of H$_2$O$_2$ (Horsburgh et al. 2001b).
The metal-binding effect of PerR involves manganese in *B. subtilis* (Fuangthong et al. 2002), but in *C. jejuni*, iron does the same instead of manganese (Kim et al. 2011).

### 6.4.3.3 CosR

CosR (*Campylobacter* oxidative stress regulator) is an important regulator of oxidative stress resistance in *C. jejuni* and an essential response regulator (Fig. 6.5) (Hwang et al. 2011b). So, this was an obstacle to determining the function of CosR by producing knockout mutants until the use of antisense-mediated gene knockdown. The expression of 32 proteins is regulated by CosR, while it positively controls AhpC and KatA, and negatively SodB, Dps, and Rrc along with strain-specific oxidative stress resistance mediator LuxS (Hwang et al. 2011b). CosR is downregulated by the presence of paraquat, but not by H₂O₂ (Hwang et al. 2011b). So, CosR can specifically sense superoxide stress, while it also affects the resistance genes against both peroxide and superoxide. However, as superoxide is the first toxic by-product produced during the oxygen reduction cycle, sensing superoxide probably makes CosR more effective in resisting oxidative stress than peroxide sensing in *C. jejuni*. Such CosR homologs are also found in bacteria belonging to

![Fig. 6.5 Schematic diagram of bacterial peroxide resistance regulator (PerR) (Van Vliet et al. 1999), ferric uptake regulator (Fur) (Palyada et al. 2009), multiple antibiotic resistance regulator (MarR) (Pomposiello et al. 2001) and oxidative stress regulator (CosR) (Hwang et al. 2011b). Black solid line indicates upregulation, black dotline indicates downregulation and red dotline indicates transcriptional downregulation](image)
e-Proteobacteria, such as Campylobacter, Helicobacter, Arcobacter, and Wolinella (Hwang et al. 2011b).

### 6.4.3.4 Regulators of MarR Family and Others

MarR regulator family includes MgrA (multiple gene regulator) and SarZ involved in sensing oxidative stress mainly in B. subtilis, E. coli, S. aureus, rather major foodborne pathogens (Fig. 6.5). However, Cj1556 is a MarR family transcriptional regulator, which upregulates peroxide stress defense genes \((\text{perR, katA, ahpC, etc.})\) and conducts negative regulation of \(\text{sodB}\) (Gundogdu et al. 2011).

MarR family proteins contain a single cysteine in their active sites and have a DNA binding helix-turn-helix motif and a dimerization domain (Beggs et al. 2020). The dimerization domain can access the oxidative agents, and oxidation of two cysteine residues of the dimer causes dissociation of the MgrA/SarZ proteins from DNA thus affects the expression of MgrA/SarZ responsive genes. The downstream genes regulated by MgrA/SarZ include genes for biosynthesis of capsule, nuclease accumulation, \(\alpha\)-toxin, coagulase, protein A synthesis, autolysis as well as efflux pumps associated with different antibiotics (Ingavale et al. 2003). SarA is a similar DNA binding protein with a single cysteine at the interface of dimerization which may sense oxidative stress. The DNA binding activity may be influenced by redox potential of the cell cytoplasm. Probably that is how SarA represses superoxide dismutase and thioredoxin reductase transcription (Ballal and Manna 2009).

Several other regulators are found in \(C.\ jejuni\) including CprRS two-component system, which is composed of an essential response regulator element (CprR) and the sensor kinase CprS (Svensson et al. 2009). However, \(C.\ jejuni\) does not have the stress-related sigma factors found in other bacteria (e.g., RpoS). It has only three sigma factors: RpoD \((\sigma^{70})\), RpoN \((\sigma^{54})\), and FliA \((\sigma^{28})\) (Parkhill et al. 2000), among which RpoD and FliA cause transcription of housekeeping genes and flagella biosynthesis genes, respectively (Hendrixson et al. 2001). On the other hand, apart from regulating transcription of other genes, RpoN has also contributed to the oxidative stress response in \(C.\ jejuni\) (Hwang et al. 2011a).

Along with these above-mentioned regulators, the most known regulatory systems of oxidative stress defense include SoxRS for the regulation of superoxide stress and OxyR for peroxide defense in \(E.\ coli\) and \(Salmonella\) (Imlay 2008). After exposure to superoxide-generating agents, such as paraquat, SoxR (superoxide response regulator) induces SoxS protein expression, which is involved in oxidative stress defense (Greenberg et al. 1990). However, redox cycling drugs producing superoxide can directly activate SoxR (Gu and Imlay 2011).

On the other hand, OxyR upregulates the peroxide stress defense genes, such as \(\text{ahpCF, dps, and katG}\) upon binding to the promoters of those genes co-operatively with the RNA polymerase in \(E.\ coli\) (Tao et al. 1993), while the expression of those genes is also induced by the presence of 1 mM \(\text{H}_2\text{O}_2\) (Zheng et al. 2001). More interestingly, OxyR itself is activated by the presence of \(\text{H}_2\text{O}_2\) through the formation
of a disulfide bond upon the oxidation of two cysteine residues (Cys 199 and Cys 208) (Zheng et al. 1998; Imlay 2008).

### 6.4.4 Pigmentation

Bacterial pigmentation is another oxidative stress resistance mechanism like carotenoid pigments of *S. aureus*, known as staphyloxanthin, which can quench toxic singlet oxygens (Gaupp et al. 2012). So, they act as potent antioxidants for detoxifying ROS. Even when the oxidative burst in phagocytes is inhibited or in the case of NOX deficiency, pigments can resist phagocytic killing (Liu et al. 2005).

### 6.4.5 Metal Homeostasis

Transition metal ions (i.e., Fe, Cu, Mn, and Zn) act as cofactors to enzymes, hence crucial for reactions and the biological system (Gutteridge et al. 1982). These transition metals as cofactors are capable of transferring electrons during reactions, but this same ability leads to ROS production through Fenton chemistry (Gutteridge et al. 1982). That is why the reactions mediated by these cofactors containing enzymes need to be tightly regulated to avoid overproduction of ROS to toxic levels (Fig. 6.2). So, metal homeostasis plays role in maintaining the redox potential of the cellular system. For example, oxidative stress regulator, PerR can regulate the genes (e.g., *bcp*, *ftnA*, and *mrgA*) for iron sequestration and homeostasis, indicating the importance of iron sequestration during the periods of oxidative stress (Wolf et al. 2008). Manganese is another essential cofactor involved in diverse metabolic reactions as well as oxidative stress resistance (Kehres and Maguire 2003). In contrast to iron, which is in the insoluble form in physiological pH, Mn$^{2+}$ is soluble in the physiological pH range (Gaupp et al. 2012). Also, the reduction potential of Mn$^{2+}$ is higher than Fe$^{2+}$, thus Mn$^{2+}$ may not lead to ROS generation. The activity of SodA and SodM (discussed in Sect. 6.4.2.2) requires Mn$^{2+}$ for enzymatic activity (Clements et al. 1999). Additionally, host phagocytes transport Mn$^{2+}$ out of the phagosome, indicating its importance in phagosome-mediated killing and oxidative burst. So, Mn$^{2+}$ plays an important role in the bacterial detoxification of ROS.

Zinc is another essential nutrient, although the overdose of Zn is toxic due to its competition with other metals for binding to the active centers of enzymes (Xiong and Jayaswal 1998). So, to avoid toxicity, Zn homeostasis is necessary along with its role in different physiological functions and oxidative stress resistance. Cellular Zn transport genes such as *znuABC* and *zosA* are regulated by Zn-responsive Fur homolog known as Zur in case of *znuABC*, and PerR in case of *zosA* in *B. subtilis* (Lee and Helmann 2006) and some of those transporter genes are activated in presence of H$_2$O$_2$, consistent probably working in oxidative stress resistance (Gaballa and Helmann 2002).
Copper (Cu) is an essential trace element and works as a cofactor to enzymes (e.g., Cu/Zn-SOD) functioning in oxidative stress resistance along with bacterial respiration and biosynthesis (Puig and Thiele 2002). But similar to Fe, the enzymatic activity of Cu through electron transfer between Cu$^{2+}$ to Cu$^{1+}$ oxidation states also allows ROS generation (Baker et al. 2010). Also, like Mn, Cu is one of the contributors to the bactericidal activity of the phagosome (Wagner et al. 2005).

### 6.4.6 DNA Protection and Repair

The secondary defense against oxidative damage in bacterial cells includes repairing these DNA damages induced by oxidative stress (Farr and Kogoma 1991). Different DNA repairing enzymes are induced by oxidative stress, which are also overexpressed under other stressed conditions like in the stationary phase and nutrient starvation. Different architectural proteins act in organizing DNA supercoiling in a nucleoid structure (Dame 2005). The DNA Binding Proteins from starved cells (Dps) or Dps homolog for the conformation changes of DNA from a relaxed state to a compacted state are regulated by oxidative stress regulator, PerR, and induced by H$_2$O$_2$ and iron (Kim et al. 2004). So, this process ultimately regulates the compact nature of the nucleoid, and decreases the susceptibility of DNA to oxidative damage through DNA condensation and repression of Fe$^{2+}$ generated HO$^·$. Oxidative stress may damage DNA through altering bases or sugar of the nucleotide causing base alterations or strand breakage (Demple and Harrison 1994). These DNA repair mechanisms can belong to two broad categories: (1) Excision repair which includes base excision repair (BER) and mismatch repair (MMR) and nucleotide excision repair (NER); (2) Recombinational repair.

In the multistep BER pathway, damage-specific DNA glycosylases work to scan the damaged area of DNA (i.e., base lesions) and this base lesion is corrected by removing the base from the sugar of the nucleotide forming apurinic/apyrimidinic sites (AP site) (Verly and Paquette 1972; Lindahl 1979). In the following step, injured DNA is restored by any of the two ways, either via short-patch in which patch size is 1-nucleotide or multiple nucleotide patches containing long-patch pathways (Sung and Mosbaugh 2003). On the other hand, in NER pathway, a patch of 12–13 nucleotides is excised followed by synthesis of the excised patch using the intact strand as a template and ligation of two ends. During oxidative stress, common lesions found in DNA found include 8-oxoguanine (8-oxoG) or GO lesion indicating the oxidized base 7,8-dihydro-8-oxoguanine (Shibutani et al. 1991). Besides, HO$^·$ can react with the 5,6-double bond of pyrimidines producing various oxidatively damaged products, including thymine glycol (Demple and Linn 1982). These oxidative damages get repaired through the aforementioned pathways followed upon the exposure of oxidative stress. However, DNA strand breakage is the significant oxidative damage that may even lead to microbial cell death and such damage can be repaired by recombination repair system (Eisen and Hanawalt 1999). So, during the oxidative stress, Rec proteins mediate the recombination repair of
DNA to revert such severe consequences (Ambur et al. 2009). In *E. coli*, RecBCD binds to the blunt end of a double-stranded DNA break, while the helicase activity of RecB and RecD unwinds the DNA for initiating the repair process. In case of Gram-positive bacteria lacking RecBCD exonuclease/helicase complex, Gram-positive bacteria starts the recombinational repair of DNA using the AddAB nuclease/helicase complex, which is a functional homolog of RecBCD (Alonso et al. 1993; Eisen and Hanawalt 1999; Ambur et al. 2009; Yeeles and Dillingham 2010). After unwinding the DNA, RecA binds to the single-stranded DNA and pairs with the complementary strand followed by strand invasion. Later, RuvAB causes branch migration and in combination with RuvC, RuvAB resolves the Holiday junction by cutting the DNA. Later processes of branch migration and resolution can be catalyzed by other proteins such as RecG along with RuvC and RuvAB in *S. aureus* (Ambur et al. 2009; Niga et al. 1997).

### 6.4.7 SOS Response

If the ROS cannot be detoxified enough making the general stress response systems (e.g., σ^{B}-system) exhausted, the SOS response becomes activated (Chang et al. 2006; Wolf et al. 2008). Because SOS (Save our souls) response is a global response to massive DNA damage. However, as oxidative stress can cause DNA damage, that may lead to the LexA regulated SOS response, which is a highly conserved repair system for global DNA damage caused by numerous DNA damaging agents (Chang et al. 2006; Wolf et al. 2008). During an SOS response, single-stranded DNA that is derived from damage-induced recombinational repair or stalled replication activates the sensor protein RecA. Activated RecA cleaves the SOS transcriptional repressor LexA leading to the expression of SOS genes. The SOS repair system is deactivated, when the sensor RecA no longer finds ssDNA.

### 6.4.8 Protein Damage Repair

#### 6.4.8.1 Thioredoxin

To maintain protein thiols in their functional (reduced) form, the cell cytoplasm of bacteria needs to be maintained in a reduced state. The thioredoxin and glutaredoxin systems and the low-molecular-weight thiol reductants including coenzyme A (CoASH) and bacillithiol (BSH) maintain this cellular reduced form (Di Simplicio et al. 2003). While both the thioredoxin and the glutaredoxin systems are present in many bacteria to maintain redox potential, most Gram-positive bacteria have only thioredoxin (Vido et al. 2005). The thioredoxin system is comprised of small disulfide reductase proteins named thioredoxin (trxA) and the thioredoxin reductase (trxB) using electrons from NADPH to maintain thioredoxin in a reduced state. Thioredoxins resist oxidative stress by reducing H_{2}O_{2}, removing HO-, as well as
providing reducing equivalents to peroxiredoxins and peroxidase (Arnér and Holmgren 2000). The expression of thioredoxin increases in the presence of oxidative stress such as diamide, H$_2$O$_2$, heat, salt, or ethanol (Mostertz et al. 2004).

As these redox potential maintaining proteins are cysteine containing proteins, biosynthesis of cysteine is important to maintain cellular stability. So, under oxidative stress, cysteine biosynthesis and uptake are increased in presence of diamide or H$_2$O$_2$-induced oxidative stress (Shaw et al. 2002). Under normal circumstances, the cytoplasmic concentration of cysteine is kept low due to its ability to reduce Fe$^{3+}$ to Fe$^{2+}$, which can initiate the ROS generated from Fenton chemistry (Park and Imlay 2003). So, the balance of cysteine concentration is critical to ensure the reducing cellular environment.

### 6.4.8.2 CoA Reductase

Apart from the function of Coenzyme A as a metabolic reactant, CoA and other low molecular weight free thiols like bacillithiol are important to maintain the reducing environment of the cytoplasm (Fahey 2001). To maintain a supply of reduced CoA (CoASH), coenzyme A disulfide reductase reduces (CoASSCoA) to CoASH and protein-SH utilizing NADPH (Coulter et al. 1998).

### 6.4.8.3 Methionine Sulfoxide Reductase

Methionine in protein can readily be oxidized (Brot and Weissbach 2000). Methionine in protein is oxidized to produce diastereomeric S and R forms of methionine sulfoxide, which compromise the function and/or structure of the methionine containing protein and make it highly susceptible to oxidation (Fig. 6.6) (Dean et al. 1997). To revert the damage caused by methionine oxidation, methionine sulfoxide reductases (MsrA and MsrB) work to reduce the oxidized form (Moskovitz et al. 1996). Figure 6.6 exhibits the repair process of methionine sulfoxide damage in bacterial cell envelope protein in detail with particular examples of *E. coli*, *Neisseria* spp., and *S. pneumoniae*. Mutations of msrA and msrB, especially an msrA/B double mutation in *C. jejuni* make them highly susceptible to peroxide and superoxide stress upon the exposure of different dilutions of H$_2$O$_2$, diamide, sodium nitroprusside, and spermine NONOate and also to nitrosative stress (Atack and Kelly 2008). Mutations in Msr proteins render *H. pylori* with highly susceptibility to superoxide stress, although it is probably because of the dismutation to hydrogen peroxide followed by the formation of hydroxyl radical, rather than the superoxide directly (Vogt 1995). Msr enzymes are crucial for colonization and persistence in host in case of *Lactobacillus reuteri* and *H. pylori* considering its role in biofilm formation, adhesins expression along with oxidative stress management (Alamuri and Maier 2004).

Here, Mo–MPT represents molybdenum–molybdopterin. This figure is reprinted from Ezraty et al. 2017 with the permission of Nature Research.
Protein repair from the damage due to the oxidation of methionine sulfoxide in bacterial cell envelope. (a) In *Escherichia coli*, MsrPQ is composed of MsrP and MsrQ proteins. A molybdopterin-containing oxidoreductase (MsrP) that is located in the periplasm reduces both (R-) and (S)-diastereomeric forms of Met-O, while the second component of this system is a hem-containing membrane-bound protein (MsrQ). Met-O is reduced by the MsrPQ system using electrons from the quinone pool (represented by Q in the figure). (b) In case of *Neisseria* species, an outer membrane lipoprotein (PilB) that is composed of MsrA (tandem methionine sulfoxide reductase A) and MsrB domains fused to an amino-terminal thioredoxin (Trx) domain. The cytoplasmic thioredoxin (Trx) -
Fig. 6.6 thioredoxin reductase (TrxR)–nicotinamide adenine dinucleotide phosphate (NADPH) system provides MsrA and MsrB domains with electrons through the three-domain (the α-, β-, and γ-domain) containing inner membrane protein DsbD and the Trx domain of PilB. (c) In case of Met-O reduction in Streptococcus pneumonia, MsrAB2 is the fusion protein that is surface-exposed and anchored to the cell membrane. On the other hand, the cytoplasmic Trx system which is the electron supplier for the reduction of MsrAB2 is composed of the DsbD homologues CcdA1 and CcdA2 and the surface-exposed thioredoxin-like lipoproteins Etrx1 and Etrx2.
6.4.8.4 Fe-S Cluster Repair

Iron–sulfur clusters are prosthetic groups involved in diverse reactions including electron transfer, different enzymatic activity, sensing environmental changes, and gene regulation, while Fe–S cluster is highly susceptible to oxidation as previously described. So, in aerobic system, to maintain viability and functionality, Fe-S clusters need to be repaired. In some bacteria, Fe-S cluster assembly is maintained by the suf system, while the oxidative stress-induced damage of Fe–S can be repaired by IscS (Nachin et al. 2003). Besides, oxidative damage of the Fe–S cluster containing proteins can be repaired by YtfE in E. coli and ScdA in S. aureus (Justino et al. 2006). ScdA is also induced by the presence of peroxide stress and can be regulated by another oxidative stress regulator, MgrA (Chang et al. 2006).

6.4.9 Effect of Oxidative Stress on Diverse Survival Mechanisms

As a part of common stress response mechanisms, oxidative stress defense has an impact on diverse bacterial survival mechanisms such as biofilm formation, aerotolerance, and induction of a viable-but-non-culturable (VBNC) state (Kim et al. 2015).

Oxidative stress induces biofilm formation in foodborne pathogens, which is a major survival strategy for pathogenic bacteria under stressed environments (Parsek and Singh 2003). So, biofilm formation on food processing equipment is a serious concern to the food industry, as biofilms are hard to clean and bacteria in biofilm achieve resistance to chemical disinfectants and antibiotics (Hall-Stoodley and Stoodley 2005). So, biofilm can act as a persistent source of microbial contamination in the food industry. Also, E. coli O157:H7 biofilm expresses oxidative stress defense gene, peroxidase, and SodC, making the bacterial population resistant to oxidative stress produced from the presence of different concentrations of oxidative stress agents such as paraquat, menadione, and H2O2 (Kim et al. 2006). Likewise, overexpression of oxidative stress defense proteins such as AhpC and Tpx was observed in C. jejuni biofilms (Kalmokoff et al. 2006). So, it has been estimated that oxidative stress induces biofilm formation in pathogens, especially while vigorous biofilm formation in aerobic conditions compared to microaerophilic conditions was noticed in case of microaerophilic C. jejuni, which could be possibly due to increased oxidative stress in aerobic conditions (Reuter et al. 2010).

Oxidative stress was found associated with another survival strategy, VBNC state. V. vulnificus ΔoxyR mutants with defective catalase activity promptly enters into a VBNC state (Kong et al. 2004). Supplementation of H2O2-degrading enzyme and/or ROS scavenging compounds including catalase and sodium pyruvate can revert the VBNC state in E. coli O157 and V. vulnificus (Mizunoe et al. 1999).
Lastly, aerotolerance is the survival strategy strongly affected by oxidative stress. Several studies reported confirmed the effect of oxidative stress resistance mechanism on the aerotolerance of the oxygen-sensitive microaerophilic or anaerobic bacteria to survive in oxygen-rich environment (Chiang and Schellhorn 2012). For example, two thiol peroxidases, Tpx and Bcp play role in aerotolerance of *C. jejuni*, while mutations in *ahpC* and its upstream gene *fdxA* diminishes its aerotolerance (Van Vliet et al. 2001; Atack et al. 2008).

### 6.5 Interaction Between Oxidative Stress and Other Environmental Stresses

While different stressed conditions create oxidative stress in pathogens as discussed in Sect. 6.2.2, surviving mechanisms against these stressed conditions other than oxidative stress such as low pH, high temperature, heavy metals, lactic acid bacteria, and high salt concentration can protect the pathogens against oxidative stress and vice versa (Imlay 2015). So, the interaction between oxidative stress and other stressed conditions, which may be bidirectional should be investigated thoroughly to ensure effective food preservation and food safety.

Foodborne pathogens mostly originate from the gastrointestinal tracts of humans and animals. As a result, their optimum temperature for growth is close to the natural temperature of the host animals. So, during food processing, packaging and storage, these foodborne pathogens may undergo dramatic changes in temperature (Capozzi et al. 2009) and resist the effect of temperature changes through the production of different heat- and cold-shock proteins (Wouters et al. 2000). Also, oxidative stress is dependent on the pathogens’ response to temperature stress. For example, catalase activity in *C. jejuni* increases with increasing temperature (Hazeleger et al. 1998).

While low pH is commonly utilized in food preservation techniques, foodborne pathogens may get exposed to acid stress in foods (Hill et al. 1995) causing a similar environmental condition like passing through the stomach (Chowdhury et al. 1996). These acid stress conditions influence the expression profile of oxidative stress genes. For example, in *E. coli* O157:H7, acid exposure changes the expression of *oxyR* and *soxS* which play key roles in oxidative stress defense (Allen et al. 2008).

On the other hand, concentrated sugar syrup or such osmolytes which are used to preserve foods by decreasing water availability (Beales 2004), this osmotic stress such as 1% NaCl increases resistance to oxidative stress (H₂O₂) in *B. cereus* (Browne and Dowds 2001). Both mild (2.5% NaCl) and severe (5% NaCl) salt stress induces higher expression of oxidative stress defense genes such as *ahpC*, *katA*, and *katE* in *B. cereus* (Den Besten et al. 2009).

However, there have been reports that mutations of *katA* and *sodB* reduce the minimal inhibitory concentrations (MICs) of ciprofloxacin and rifampicin by about twofold (Hwang et al. 2013). So, oxidative stress can also increase the susceptibility of bacterial pathogens to antimicrobials. Besides, surviving the nutrient scant
condition enables the bacteria to get resistance against multiple stressed conditions including heat, oxidative, and osmotic stress in *E. coli* (Jenkins et al. 1988, 1990).

So, oxidative stress is associated with pathogens’ response to different stressed conditions, although the exact mechanism behind this association is still undetermined. Possibly the stressed conditions affect metabolic pathways and electron transport chain leading to the generation of ROS, while oxidative stress defense systems are required to ensure ROS homeostasis under the stress conditions.

### 6.6 Food Processing Induced Oxidative Stress and Its Effect on Pathogenicity

The current food processing environment and host innate defense strategies share striking similarities. These food processing, packaging, or preserving conditions create a sublethal environment for the pathogens, which alters the gene expression profile of the pathogens to withstand this sublethal condition. So, despite reducing/eliminating the load of pathogens or toxins, these processes make the pathogens not only more virulent or pathogenic, but also make them more resistant to different stress conditions and antimicrobials (Esbelin et al. 2018).

One of the common food preservation or storage practices is modified oxygen packaging (MAP), in which an optimal mixture of oxygen, carbon dioxide, and nitrogen is present within a high barrier or permeable package to maintain the minimal level of oxygen (Caleb et al. 2013). While the purpose of such packaging is to suppress the growth of aerobic pathogenic or spoilage bacteria by inhibiting aerobic metabolism, this process has been reported to create a stress response in aerobic bacteria (Poole 2012). Also, this condition resembles the anaerobic condition in the human gastrointestinal system, as the level of oxygen becomes gradually decreased from proximal small intestine to distal large intestine favorable for the growth of anaerobic microbiota. While many aerobic and facultative anaerobic foodborne pathogens remain close to the mucosal epithelial cells of the host where they can get access to oxygen from host cells during colonization, foodborne pathogens devise different strategies to survive this condition, which may even lead to higher pathogenicity.

For example, type 1 fimbriae, the virulence factor of *Salmonella* spp. for attaching to the host cell is expressed in high quantity in aerobic environment but not in anaerobic environment. But in anaerobic environment, *Salmonella* uses tetrathionate as the terminal electron acceptor for anaerobic growth, which is produced from the reaction between ROS produced from epithelial cells and thiosulfate produced by the gut microbiota (Winter et al. 2010).

In case of *Listeria monocytogenes*, cytochrome aa3-type menaquinol oxidase (QoxAB) is used for respiration under reduced oxygen levels possibly during host infection instead of cytochrome *bd*-type (CydAB) terminal oxidase which is used in aerobic condition (Corbett et al. 2017). Also, it is reported that 28 different genes get
upregulated including lmo0355 encoding fumarate reductase along with the generation of a proton motive force using $F_1F_0$-ATPase in anaerobic conditions (Müller-Herbst et al. 2014). More importantly, in anaerobic condition, *L. cytogenes* show enhanced adhesion and invasion (Andersen et al. 2007) of cultured cells possibly due to the high expression of *Listeria* adhesion protein (LAP) also known as alcohol acetaldehyde dehydrogenase (Adh) (Müller-Herbst et al. 2014) and internalin B (InlB), respectively, although they produce less listeriolysin O (LLO) (Wallace et al. 2017) in anaerobic environment. Besides, the oxygen-limiting condition also increases acid tolerance in *L. monocytogenes*, which may also help in the invasion of the bacteria from the stomach to the intestine (Sewell et al. 2015).

Similarly in Enterohemorrhagic *E. coli* (EHEC) (West et al. 2008) and *S. flexneri* (Vergara-Irigaray et al. 2014), anaerobic condition promotes virulence and adhesion through the upregulation of genes encoding type III secretion system (TTSS), leading to enhanced invasion and pathogenesis. Also, under anaerobic condition, different toxin-producing genes get upregulated, including cholera toxin (CT), toxin-coregulated pili (TCP), and AphB, a transcriptional activator of TcpP in *Vibrio cholerae* (Liu et al. 2011).

Not only these aerobic or facultative anaerobic bacteria, but also microaerophilic *Campylobacter* having a strict requirement for oxygen ($O_2$), hydrogen ($H_2$), and carbon dioxide ($CO_2$), shows increased virulence under anaerobic condition through the expression of putative virulence genes responsible for mobility and epithelial cell invasion (Lee et al. 2014). So, the anaerobic foodborne pathogens like *Clostridium botulinum*, *C. perfringens*, and *C. difficile* find the anaerobic environment in the packaging and/or the host system suitable for colonization and expression of virulence genes upon entry with contaminated food (Freedman et al. 2016; Rossetto et al. 2014).

So, these finding clearly indicate the oxygen-limiting MAP-induced stress adaptation make the foodborne pathogens more prepared to survive in the host gastrointestinal tract as well as to invade and colonize more efficiently. Apart from, direct MAP, foodborne pathogens may encounter oxidative stress in both food/food processing environments due to the use of different $H_2O_2$-based disinfectants used in the food processing and packaging units.

### 6.7 Conclusions

Foodborne pathogens are exposed to different oxidative stresses both endogenously and exogenously, especially due to different processes foods encounter before and after ingestion. So, these pathogens evolved specific defense strategies to survive through the stressed conditions along with general stress responses. These defense mechanisms are diverse, interconnected, and not clearly elucidated yet and also have bidirectional relationships with the responses of other types of stresses. However, considering the effect of oxidative stress response on survival and pathogenicity of foodborne pathogens, these stress response mechanisms need to be investigated.
thoroughly to develop an effective food preservation strategy and to ensure food safety.

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Part III

Response of Foodborne Pathogens to Emerging Nonthermal Technologies
Chapter 7
Response of Food-Borne Pathogens to Ultrasound

Hongmei Liao

Abstract  As one of the rapidly developing emerging nonthermal techniques, ultrasound and/or combination with other strategies have been applied to enhance food quality and ensure food safety, especially in food with heat-sensitive characteristics. Ultrasound has received more and more attention of researchers and food manufacturers in recent years. However, the responses of microorganisms to ultrasound treatments arise challenges to the development of this technology in food industry. Food-borne pathogens may be unaffected, be sublethally injured, survive in a viable but non-culturable state, or be inactivated when exposed to ultrasound treatments. The effects of ultrasound in the morphology, structure, and function of membranes, intracellular structure, enzymes, proteins, and DNA might be varied and relevant with the microbial state. Till now, limited study has explored the regulator mechanisms underlying food-borne pathogens response to ultrasound treatment, though several important genes have been found to contribute to the microbial ultrasound resistance. This chapter mainly focused on the cutting-edge advances and challenges in the application of ultrasound for controlling food-borne pathogens in food, with the consideration of studies in the last 10 years.

Keywords  Ultrasound · Food-borne pathogens · Stress responses · Inactivation · Resistance · Mechanisms · Sublethal effects · Oxidative stress · Dormancy

7.1 General Introduction of Ultrasound and Its Effects on Food-Borne Pathogens

Ultrasound-based technologies have been widely explored and applied as the toothbrush, sonar carrier, etc., nowadays. The understandings of ultrasound can be traced back to 1794, when the Italian scientist Lazzaro Spallanzani discovered that bats

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T. Ding et al. (eds.), Stress Responses of Foodborne Pathogens, https://doi.org/10.1007/978-3-030-90578-1_7
emitted unperceivable sound waves in their navigation at night (Kehinde et al. 2020; Suslick 1989). Since the development of techniques for generating ultrasound waves in liquid dates back to the 1920s, it has reported that ultrasound waves produced destructive effects on the living cells, including a variety of plant, animal, and microbial cells (Beckwith and Olson 1932). The biological effects of ultrasound were first thoroughly studied by Wood and Loomis (1927), in which study stimulating ultrasound waves and their destructive effects on virus, bacteria, potato shoots, and animal tissues in vitro and in vivo had been reported. A relatively early literature about using ultrasound in biology goes back to 1942, when an efficient ultrasound generator had been designed, built, and successfully operated (Lynn et al. 1942).

The history of ultrasound application targeting microorganisms can be traced back to 1929 (Fig. 7.1), when a paper was published entitled “The destruction of luminous bacteria by high frequency sound waves” (Harvey and Loomis 1929). In 1946, it was found that ultrasonic irradiation could disintegrate 12 species of bacterium when preparing cell-free enzyme extracts of bacteria (Stumpf et al. 1946). For instance, the degree of disintegration of *Escherichia coli* was achieved up to 63% when exposed to a ultrasonic irradiation for 15 min (Stumpf et al. 1946), which was calculated according to the release of nitrogen from bacterial cells to cell-free juice. By the 1960s, the uses of ultrasound in the processing industries were well accepted and this interest has continued to develop later. The number of the publications on the application of ultrasound and its hurdles for food decontamination has increased rapidly in the last two decades (Fig. 7.2).

Ultrasound refers to the sound waves with frequencies of above the threshold for human hearing (> 16 kHz) (Suslick 1989). Ultrasound has been a subject of research in the areas of radiology nuclear medicine medical imaging, cardiology, surgery, oncology, pathology, medicine, neuroscience and neurology, physiology, acoustics, engineering, etc. According to the input power, the intensity, and the frequency applied, ultrasound is classified in two different categories (Dolas et al. 2019). One is the low power-high frequency ultrasound with the intensities of less than 1 W/cm² and frequencies of over 100 kHz (Dolas et al. 2019). It is generally used for monitoring the composition and physicochemical properties of food components during processing and storage, which is crucial to retain food properties or improve food quality. The other one is named as high power-low frequency ultrasound with intensities of over 1 W/cm² and finite frequency ranging 20–500 kHz, which cause the phenomenon of cavitation, induce mechanical, physical, and chemical/biochemical changes through cavitation, supports many food processing operations, such as extraction, freezing, drying, emulsification, and microbial inactivation on the food contact surfaces (Kehinde et al. 2020; Awad et al. 2012). Generally, ultrasound with the frequencies of 20 kHz to 10 MHz is considered as the power ultrasound, in other words “higher-power ultrasound at lower frequencies,” mainly due to they have the ability to cause cavitation and produce injury or complete destruction of living cells, especially microorganisms (Demirdöven and Baysal 2008). Therefore, the ultrasound mentioned in this chapter mainly refers to “power ultrasound,” using ultrasound in short.
Fig. 7.1 The history of the application of ultrasound or the ultrasound-based hurdles for disrupting microorganisms, especially in food.
As a mechanical wave, ultrasound wave requires a material medium for its propagation and as such cannot travel through a vacuum (Dolas et al. 2019). This characteristic facilitates ultrasound wave transfer through most of foods, especially in liquid foods, such as fruit juices, milk, and other beverages, etc. Thus, as versatile and simple method for the disruption of cells and the production of extracts, ultrasound and its combination with other strategies had been wildly applied in food industry. Ultrasound induces mechanical, chemical, and biochemical effects in liquids via the production and subsequent collapse of cavitation bubbles (Suslick 1989). This emerging technology has been used as alternative to conventional food processing operations for controlling microstructure and modifying textural characteristics of fat products (sonocrystallization), emulsification, de-foaming, modifying the functional properties of proteins, inactivation or enhancement of enzymatic activity to extend shelf life and quality of food products, microbial inactivation, freezing, thawing, freeze drying and concentration, drying and facilitating the extraction of various food and bioactive components (Awad et al. 2012).

Ultrasound using sonication baths or ultrasonic immersion probes with different lengths, diameters, and tip geometries has been widely applied for microbial inactivation (Table 7.1). Generally, the use of individual ultrasound treatment results in limited microbial inactivation. However, the combination of ultrasound and pressure

Fig. 7.2 The number of publications on the application of ultrasound and ultrasound-based hurdles for food decontamination in the last two decades
## Table 7.1 Application of ultrasound and ultrasound+ technologies in the aim of microbial inactivation

<table>
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<th>Food-borne pathogens</th>
<th>Ultrasound processing parameters</th>
<th>Food matrix</th>
<th>Results</th>
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<tr>
<td>1 Escherichia coli, Listeria innocua</td>
<td>TS at 60 °C and 400 W</td>
<td>Green juice</td>
<td>7.42-log reduction of <em>E. coli</em> was achieved after 6 min; combining with 100 mg/L of nisin, 7-log reduction of <em>L. innocua</em> was detected after 3 min.</td>
<td>Oner (2020)</td>
</tr>
<tr>
<td>2 Escherichia coli, Staphylococcus aureus</td>
<td>US (24 kHz and 0.34 W/g of acoustic energy density) during 10 min. Vacuum (8.46, 11, and 16.93 kPa), heat (40, 45, and 50 °C), 1–3 intermittent vacuum pulses</td>
<td>Soursop puree</td>
<td>≥7 log₁₀ CFU/g reduction of both bacteria</td>
<td>Martínez-Moreno et al. (2020)</td>
</tr>
<tr>
<td>3 Escherichia coli, Staphylococcus aureus</td>
<td>TS at 60–62 °C for 0.38–0.55 min</td>
<td>Peptone water</td>
<td>This was the first reported study that reached a 5-log reduction of <em>S. aureus</em> using ultrasonic processing</td>
<td>Baboli et al. (2020)</td>
</tr>
<tr>
<td>4 Escherichia coli</td>
<td>US (120 μm, 24 kHz) at 25–55 °C up to 10 min in continuous pulsing mode.</td>
<td>Strawberry juice</td>
<td>5.69–5.75 log reduction</td>
<td>Yildiz et al. (2019)</td>
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<tr>
<td>5 Escherichia coli, Saccharomyces cerevisiae</td>
<td>TS (20 kHz), at 40–60 °C and exposure time for 9–31 min</td>
<td>Khoonphal (<em>Haematocarpus validus</em>) juice</td>
<td>Led up to 5 log cycles reduction of them. Thermosonicated juice had extended shelf life up to 10 days at 4 °C with desirable physiochemical quality than conventional treatment.</td>
<td>Sasikumar et al. (2019)</td>
</tr>
<tr>
<td>6 Escherichia coli K-12</td>
<td>TS (37 kHz) at 40–60 °C and 150 W for 30 min</td>
<td>Pumpkin juice</td>
<td>For batch TS, maximum inactivation of <em>Escherichia coli</em> K-12 was 6.62 ± 0.00 log CFU/mL.</td>
<td>Demir and Kilinc (2019)</td>
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Table 7.1 (continued)

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<th>Food matrix</th>
<th>Results</th>
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<td>7 <em>Escherichia coli, Staphylococcus aureus</em></td>
<td>US (30 °C, 100 kPa), TS (50 °C, 100 kPa), MS (30 °C, 400 kPa), and MTS (50 °C, 400 kPa)</td>
<td>Phosphate buffer (10 mM, pH 7)</td>
<td>All experiments indicated that Gram-positive <em>S. aureus</em> was more resistant to sonication than Gram-negative <em>E. coli</em>, but the lethality rate became more comparable with more violent cavitation.</td>
<td>Chantapakul et al. (2019)</td>
</tr>
<tr>
<td>8 <em>Escherichia coli O157:H7</em></td>
<td>TS, MS, and MTS (30–80 °C) and power intensities (280, 420, 560, and 700 W) for 10 min.</td>
<td>Blueberry juice</td>
<td><em>E. coli</em> O157:H7 was slightly inactivated by TS after 5 min (0.17-log reduction), <em>Escherichia coli</em> O157:H7 was rapidly inactivated by MTS (5.85-log reduction) after 5 min. The destruction of <em>E. coli</em> cells as a result of these treatments was confirmed using SEM and TEM.</td>
<td>Zhu et al. (2017)</td>
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<tr>
<td>9 <em>Escherichia coli ATCC 25922, Pichia pastoris GS115, Aureobasidium pullulans 2012</em></td>
<td>US (37 kHz), 80 W</td>
<td>On stainless steel coupons</td>
<td>2.2 and 3.1 log CFU/coupon reductions within 0.2 min for <em>E. coli</em> and <em>P. pastoris</em>, respectively and 1.0 log CFU/coupon reductions within 0.1 min for <em>A. pullulans</em>.</td>
<td>Zhao et al. (2017)</td>
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<td>10 <em>Listeria monocytogenes, Shigella sonnei, Byssochlamys fulva, Saccharomyces cerevisiae</em></td>
<td>TS, 17.3, 32.7, and 49.6 W for cases with 25, 50, and 75% amplitude levels. 60, 70, and 80 °C, ~25 min</td>
<td>Tangerine juice</td>
<td>Results indicated the effect of temperature was higher than pulsed-ultrasound amplitude for <em>L. monocytogenes</em>, <em>S. sonnei</em>, and <em>S. cerevisiae</em>, while the effect of these two parameters was similar for <em>B. fulva</em>.</td>
<td>Hashemi et al. (2019)</td>
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<td>11</td>
<td><em>Staphylococcus aureus, Escherichia coli</em></td>
<td>US at 600 W for 5 min, combining with heating at 63 °C</td>
<td>Milk</td>
<td>Results indicated US treatment (600 W, 5 min) with postheating (63 °C 5 min) reduced <em>S. aureus</em> and <em>E. coli</em> by 1.58 and 2.02 log CFU/mL, respectively, but yielded 0.26 and 0.19 log CFU/mL sublethal <em>S. aureus</em> and <em>E. coli</em>. Milk preheated to 63 °C (5 min) followed by US (600 W, 5 min) showed 1.17- and 1.68-log reductions for <em>S. aureus</em> and <em>E. coli</em>, respectively, and the sublethal <em>S. aureus</em> and <em>E. coli</em> were reduced to 0.16 and 0.09 log CFU/mL.</td>
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<td>12</td>
<td><em>Staphylococcus aureus</em></td>
<td>US and mild heat 55 °C for 3, 5, 7, 10, and 15 min.</td>
<td>0.85% sterile saline solution</td>
<td>The results showed the membrane damage was synchronous with esterase inhibition during the exposure to sonication, leading to the immediate lethal effect.</td>
</tr>
<tr>
<td>13</td>
<td><em>Escherichia coli</em></td>
<td>US</td>
<td>Sterile water and synthetic waste water</td>
<td><em>E. coli</em> K-12 DoxyR appeared to be more resistant to the treatment together with <em>gadW, gadX, gabT</em>, and <em>gabD</em>, whereas the mutant K-12 <em>DnaK</em> was more sensitive with 0.25-log (CFU/mL) reduction in comparison to their isogenic wild-type <em>E. coli</em> K-12.</td>
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<td>14</td>
<td><em>Escherichia coli</em>, <em>Staphylococcus aureus</em></td>
<td>TS at 400 W, 24 kHz, 50–51 °C, acoustic energy density of 1.3–1.4 W/mL for 10 min</td>
<td>Soursop nectar</td>
<td>TS treated nectar retained 85% of ascorbic acid, polyphenol oxidase inactivation, and microbiological and physico-chemical stability during 30 days at 4 °C.</td>
<td>Anaya-Esparza et al. (2017a)</td>
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<td>15</td>
<td><em>Listeria monocytogenes</em>, <em>Salmonella enterica</em> serovar Typhimurium</td>
<td>US and mild heat 40 kHz, 23, 40, 50, and 60 °C + slightly acidic electrolyzed water</td>
<td>Fresh-cut bell pepper</td>
<td>When compared to the control, there was no significant change (p &gt; 0.05) in the texture (color and hardness) of the samples that were treated by 1 min of the combined treatment during storage at 4 °C for 7 days.</td>
<td>Luo and Oh (2016)</td>
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<td>16</td>
<td><em>Escherichia coli</em> ATCC 35218, <em>Salmonella enteritidis</em> MA44, and <em>Saccharomyces cerevisiae KE 162</em> and indigenous flora</td>
<td>US at 25 ± 1 °C combing with a continuous flow-through pulsed light system</td>
<td>Apple juice</td>
<td>US + pulsed light system led up to 3.7–6.3 log reductions of inoculated microorganisms, the combined treatment delayed yeast and mold recovery and prevented from browning development during storage.</td>
<td>Mariana Ferrario and Guerrero (2016)</td>
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<td>17</td>
<td><em>Escherichia coli</em></td>
<td>US (20 kHz), 90% amplitude for 5 min, 2 s on and 4 s off.</td>
<td>Cactus pear juice</td>
<td>Total inactivation was observed in both fruit juices after 5 min of US treatment at most amplitude levels (with the exception of 60% and 80%). After 1 and 2 days of storage, the recovery of bacteria counts was observed in all cactus pear juices.</td>
<td>Cruz-Cansino Ndel et al. (2016)</td>
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<td>Page</td>
<td>Organism(s)</td>
<td>Methodology</td>
<td>Medium</td>
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<td>18</td>
<td><em>Escherichia coli</em> ATCC 25922, <em>Saccharomyces cerevisiae</em> ATCC 2366</td>
<td>US amplitudes (50, 75, and 100%) and times (0, 6, 12, 18, 24, and 30 min)</td>
<td>Pomegranate juice</td>
<td>US-pasteurization at 65 °C for 10 min showed the best results in retention of ascorbic acid and other phenolic compounds with significant reduction in enzyme activities and complete inactivation of microbes.</td>
<td>Pala et al. (2015)</td>
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<td>19</td>
<td><em>Escherichia coli</em></td>
<td>US at 120 mm and temperature from 50 to 60 °C (0–30 min).</td>
<td>Apple juice</td>
<td>US also showed positive results (&gt;6 log reduction) after 5 min when temperature was kept at 60 °C regardless of the operation mode.</td>
<td>Moody et al. (2014)</td>
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<td>20</td>
<td><em>Escherichia coli</em>, <em>Staphylococcus aureus</em>, <em>Pseudomonas fluorescens</em></td>
<td>MTS (20–52 °C), acoustic intensity (60–120 W/cm²) and treatment time (40–240 s) at a constant pressure (225 kPa)</td>
<td>Raw milk</td>
<td>Reductions of up to averaging 1.6 log CFU/mL were achieved for <em>E. coli</em> and <em>P. fluorescens</em>. Lower inactivation values were reported for <em>S. aureus</em> (1.05 log CFU/mL).</td>
<td>Cregenzán-Alberti et al. (2014)</td>
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<td>21</td>
<td><em>Escherichia coli</em> 3014, <em>Staphylococcus aureus</em> 3048, <em>Salmonella</em> sp. 3064, <em>Listeria monocytogenes</em> ATCC 23074 and <em>Bacillus cereus</em> 30</td>
<td>US treatment with 12.7-mm probe operated at 600 W nominal power (20 kHz) and at amplitudes of 60, 90, and 120 mm. Also, treatment time of 3, 6 and 9 min and temperature of 20, 40, and 60 °C were used.</td>
<td>Pure culture</td>
<td>After ultrasonic treatment at 60 °C, 9 min, and 120 mm, the viability of cells was not confirmed for <em>E. coli</em> 3014, <em>S. aureus</em> 3048, <em>Salmonella</em> sp. 3064, and <em>L. monocytogenes</em> ATCC 23074. Under the mentioned conditions the highest inactivation (3.48 log CFU/mL) of <em>B. cereus</em> 30 was obtained.</td>
<td>Zoran Herceg et al. (2013)</td>
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<tr>
<td>22</td>
<td><em>Escherichia coli</em> K12</td>
<td>MTS (400 kPa/59 °C), TS (100 kPa/59 °C), and MS (400 kPa/55 °C) for up to 4 min</td>
<td>Apple cider</td>
<td>Achieve a 5-log reduction in pathogenic microbial counts in juice products</td>
<td>Lee et al. (2013)</td>
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<td>Food-borne pathogens</td>
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<td><strong>23</strong> <em>Escherichia coli</em></td>
<td>Ultrasonic+TiO$_2$ particles at</td>
<td>Phosphate buffer solution</td>
<td>Synergistically enhanced the cell inactivation rate</td>
<td>Ninomiya et al. (2013)</td>
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<td></td>
<td>36 kHz, 0.28 W, 20 °C</td>
<td>100 mM pH = 7.0</td>
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<td><strong>24</strong> <em>Staphylococcus aureus</em></td>
<td>US amplitude levels (0.4, 7.5,</td>
<td>TSB broth</td>
<td>In addition, genetic algorithm-artificial neural network (GA-ANN) and adaptive neuro-fuzzy inference system (ANFIS) models were used to predict inactivation of <em>S. aureus</em>. The high inactivation of <em>S. aureus</em> was achieved under a duty cycle of 0.7:0.3 s with reduction of the population to 1.49 CFU/mL.</td>
<td>Soleimanzadeh et al. (2015)</td>
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<td>and 37.5), duty cycles (0.3: 0.7</td>
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<td>s, 0.7:0.3 s, and 0.9: 0.1 s,</td>
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<td>and time (0, 2, 4, 6, 8, 10, 12</td>
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<td>, and 14 days)</td>
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<td>kHz at 1 ms time interval.</td>
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<td>Without temperature control</td>
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<td><strong>26</strong> <em>Salmonella enterica ATCC 14023</em></td>
<td>US + super critical carbon</td>
<td>Coconut water</td>
<td>The synergistic effect of SCCD + HPU was evident and a higher microbial reduction was achieved compared to SCCD alone: at 12 MPa and 40 °C about 5-log reductions were achieved for natural microbial flora in about 15 min while about 30 min were needed for SCCD treatment.</td>
<td>Cappelletti et al. (2014)</td>
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<td>dioxide (SCCD) at 40 kHz, 10 W,</td>
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<td>25–45 °C, 60 min</td>
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<td><strong>27</strong> <em>Streptococcus thermophilus</em>,</td>
<td>TS at 60, 70, and 80 °C, and</td>
<td>Ayran</td>
<td>The yeast and mold levels in samples treated with TS were &lt;1 log CFU/mL during storage period. Other bacteria decreased a lot.</td>
<td>Erkaya et al. (2015)</td>
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<td>*Lactobacillus delbrueckii subsp.</td>
<td>times (1, 3, and 5 min), 35 kHz,</td>
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<td><em>bulgaricus</em></td>
<td>90 ± 1 °C for 1 min</td>
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<td>No.</td>
<td>Organism(s)</td>
<td>Treatment Details</td>
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<td>28</td>
<td><strong>Bacillus coagulans</strong>, Anoxybacillus flavithermus, Bacillus sporothermodurans, Bacillus licheniformis, Geobacillus stearothermophilus**</td>
<td>US at 63.5 °C for 30 min</td>
<td>Skim milk</td>
<td>US at 80% amplitude for 10 min however, inactivated the vegetative cells of <em>B. coagulans</em> and <em>A. flavithermus</em> in skim milk by 4.53 and 4.26 logs, respectively.</td>
<td>Khanal et al. (2014)</td>
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<td>29</td>
<td><strong>Enterobacter aerogenes, Bacillus subtilis, Staphylococcus epidermidis, S. epidermidis SK and staphylococcus pseudintermedius</strong></td>
<td>US (20 kHz), 13 W, 20 min, temperature below 30 °C</td>
<td>Fresh nutrient broth or PSS</td>
<td>US treatment resulted in lethal damage to <em>E. aerogenes</em> and <em>B. subtilis</em> (up to 4.5-log reduction), whereas staphylococcus spp. were not affected noticeably.</td>
<td>Gao et al. (2014b)</td>
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<tr>
<td>30</td>
<td><strong>Enterobacter aerogenes, Bacillus subtilis, Staphylococcus epidermidis</strong></td>
<td>High-frequency (850 kHz) US at 2 °C</td>
<td>Physiological salt solution (0.9% NaCl, PSS)</td>
<td>For both <em>E. aerogenes</em> and <em>A. pullulans</em>, the log of the inactivation ratio decreased linearly with sonication time, and the rate of inactivation increased (<em>D</em>-value decreased) with the increase in sonication power. The rate of inactivation was also found, for both microorganisms, to increase with a decrease in the initial cell number.</td>
<td>Gao et al. (2014a)</td>
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<tr>
<td>31</td>
<td><strong>Cronobacter sakazakii</strong></td>
<td>US (37 kHz), at 380 W for 5–100 min, combining with sodium hypochlorite</td>
<td>Head lettuce</td>
<td>Synergistic reductions on <em>C. sakazakii</em> were observed in most combined treatments, although the most synergistic reduction values were &lt;1.0 log_{10} CFU/g.</td>
<td>Park et al. (2016)</td>
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<th>Food-borne pathogens</th>
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<tr>
<td>32 <em>Geobacillus</em> <em>stearothermophilus</em>, <em>Anoxybacillus flavithermus</em>, <em>Bacillus subtilis</em></td>
<td>TS at 72 and 73 °C, 120 s.</td>
<td>Tryptic soy broth and 2% fat milk</td>
<td>1-log for vegetative bacteria in 30–45 s; ≤0.2 log for spores in 120 s</td>
<td>Deshpande and Walsh (2020)</td>
</tr>
<tr>
<td>33 <em>Bacillus subtilis</em></td>
<td>US, 55 °C-400 W-23.06 min, 63 °C-200 W-14.18 min, 63 °C-400 W-9.59 min.</td>
<td>Chinese bayberry juice</td>
<td>A 5-log reduction was achieved in 36.91 min by TP, by UH 55°C 400 W, 14.18 min by UH 63°C 200 W, 9.59 min by UH 63°C 400 W, and the inactivation date had a high goodness of fit with Weibull model, exhibiting a fast drop in first and a slow decline at last. Compared to the TP, UH treatment was not only able to cause lethal effect by compromising membrane integrity, inactivating esterase activity, but also destroying nucleic acids through the flow cytometry analysis.</td>
<td>Li et al. (2019)</td>
</tr>
<tr>
<td>34 <em>Bacillus cereus</em> spores</td>
<td>TS (20–60 kHz), at 50–70 °C, for 10–20 min</td>
<td>Pork, sterilized distilled water</td>
<td>0.47–0.79 log_{10} CFU/g</td>
<td>Owusu-Ansah et al. (2020b)</td>
</tr>
<tr>
<td>35 <em>Bacillus cereus</em> spores</td>
<td>US (20 kHz) and TS, at single frequency (20 kHz, 70 °C) and multifrequency (20/40/60 kHz, 70 °C), at 300 W for 25 min</td>
<td>Pork</td>
<td>4.16 log_{10} CFU/mL spore reduction within 5 min; US and TS methods enhanced the physicochemical properties (pH, texture, and color) of pork.</td>
<td>Owusu-Ansah et al. (2020a, b)</td>
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<td>36</td>
<td><em>Alicyclobacillus acidoterrestris</em> spores</td>
<td>TS AT 0.3–20.2 W/mL, at 75 °C</td>
<td>Orange juice</td>
<td>To conclude, HPP-assisted TS provided the best method for spore inactivation, indicating the benefit of high pressure and power ultrasound technology in addition to heat.</td>
</tr>
<tr>
<td>37</td>
<td><em>Neosartorya fischeri</em> ascospores</td>
<td>US (24 kHz), 0.33 W/mL, 75 °C</td>
<td>Apple juice</td>
<td>No inactivation for TS processing</td>
</tr>
<tr>
<td>38</td>
<td><em>Spores Bacillus mycoides, Bacillus weihenstephanensis, Psychrobacillus psychrodurans</em></td>
<td>MS and MTS at 35–95 °C, 2000 W, 20 kHz</td>
<td>McIlvaine citrate-phosphate buffer (pH 6.8)</td>
<td>The application of an ultrasonic field reduced the shoulder length, MTS treatment is capable of inactivating spore-forming bacteria and that the inactivation efficiency of the combined treatment is correlated with the thermal resistance of the spore species.</td>
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<tr>
<td>39</td>
<td><em>Alicyclobacillus acidoterrestris</em> ATCC 49025 spores, <em>Saccharomyces cerevisiae</em> KE162</td>
<td>US (600 W, 20 kHz and 95.2 mm) wave amplitude; 10 or 30 min at 20, 30, or 44 ± 1 °C</td>
<td>Commercial (pH: 3.5; 12.5°Brix) and natural squeezed (pH: 3.4; 11.8°Brix) apple juices</td>
<td>In natural apple juice, the combination of US + 60 s PL at the highest temperature build-up (56 ± 1 °C) was the most effective treatment for both strains.</td>
</tr>
<tr>
<td>40</td>
<td><em>Byssochlamys nivea</em> ascospores</td>
<td>TS (24 kHz), 0.33 W/mL, 80 °C, 200 W</td>
<td>Strawberry puree</td>
<td>Faster inactivation was achieved at higher temperatures for all the technologies tested, indicating the significant role of temperature in spore inactivation, alone or combined with other physical processes.</td>
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<td>Table 7.1  (continued)</td>
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<td>41  <em>Clostridium perfringens spores</em></td>
<td>TS (24 kHz), at 75 °C, a 60 min, 0.33 W/g, 200 W</td>
<td>Beef slurry</td>
<td>At 75 °C, a 60-min TS process resulted in a less than 1.5-log reduction for both <em>C. perfringens</em> NZRM898 and NZRM2621 spores. This heat shock + US pretreatment was able to double the spore thermal inactivation rate in beef slurry.</td>
<td>Evelyn and Silva (2015a)</td>
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*US ultrasound; TS thermostonication; MS manosonication; MTS manothermosonication*
and/or heat has showed considerable promise (Dolas et al. 2019; Paniwnyk 2017). Ultrasound-based hurdle technologies, including thermosonation (TS), manosonication (MS), and manothermosonication (MTS), have been explored and widely used, as summarized in Fig. 7.3. Based on literature searching, a summary of application of ultrasound-based hurdles, such as TS, MS, and MTS, is showed in Fig. 7.4, which insight us lots of concerns have been given to efficiently control microorganisms in food using ultrasound-based hurdles.

In food processing, ultrasound is currently used either as a single processing unit or in combination with other emerging technologies (Fig. 7.5). Ultrasound has been reported to treat the pathogenic microorganisms, including *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, *Staphylococcus aureus*, *Shigella* spp., *Cronobacter sakazakii*, and *Bacillus* spp., as shown in Table 7.1.

Food-borne pathogens (and other microbes) that exposed to ultrasound or the associated hurdles may manifest several types of altered characteristics as a
consequence, including sublethal injury, viable but non-culturable (VBNC), cross adaptation, and cell death (Fig. 7.3).

Following are some terms to describe them:

1. **Stress adaptation (Increased resistance)**. Stress adaptation refers to the phenomenon in which the pretreatment of a stressor enhances bacteria to resist the subsequent exposure of the same or other stressor (Ray 2003).

**Fig. 7.4** A summary of publications on the application of thermsonication (TS), manosonication (MS), and manothermosonication (MTS) in Food science and technology, and microbiology

**Fig. 7.5** The forms of ultrasound-based hurdles for microbial reduction in food

consequence, including sublethal injury, viable but non-culturable (VBNC), cross adaptation, and cell death (Fig. 7.3).

Following are some terms to describe them:

1. **Stress adaptation (Increased resistance)**. Stress adaptation refers to the phenomenon in which the pretreatment of a stressor enhances bacteria to resist the subsequent exposure of the same or other stressor (Ray 2003).
2. **Sublethal injured cells (they can be easily repaired).** Sublethal injury occurs following exposure of bacterial cells to unfavorable physical and chemical environments (beyond the growth range but not in the lethal range) that cause reversible alterations in the functional and structural organizations of the cells (Ray 2003). Generally, sublethal injured cells can be easily repaired under proper environments.

3. **VBNC state cells.** Under unfavorable environments, some microbial cells in a bacterial population remain viable but are not able to multiply in many recommended bacteriological media, unless they are subjected to a prior resuscitation treatment. This subpopulation is stated as a VBNC state.

4. **Dead (Non-culturable in any circumstance).** These are irreversibly injured cells. They are unable to divide or grow in any environments (Li et al. 2018a; Ray 2003).

General principles of ultrasound and its application for microbial inactivation in food have been introduced systematically by several reviews, as summarized in Table 7.2. Besides that, lots of research had employed ultrasound and its associated hurdles to inactivate microorganisms and to ensure food safety or obtain effective disinfection treatments (Alvarado-Morales et al. 2019; Krizanovic et al. 2020; Ma et al. 2020; Neto et al. 2019; Yin et al. 2019). In order to achieve efficient inactivation of food-borne pathogens, the understanding of the stress response of microorganisms to ultrasound and its associated hurdles is required. The advances of microbial responses to ultrasonic stresses in the last ten years are available in this chapter.

### 7.2 Factors Affecting the Sensitivity of Food-Borne Pathogens to Ultrasonic Stresses

Ultrasound has a strong antimicrobial capacity against a spectrum of microorganisms and has been recognized as a promising technique to meet the FDA requirement of safe fruits and vegetable products (Dolas et al. 2019). As mentioned above, high intensity-low frequency ultrasound has been widely used to inactivate food-borne microorganisms and decontaminate surfaces of devices (Dolas et al. 2019). The basic principle of the high power ultrasound is the phenomenon of acoustic cavitation (Suslick 1989). This phenomenon results in alternating compression and expansion create regions of a pressure change, with a process of the generation, growth, and collapse of large bubbles, which release strong energies. For instance, the bubbles implode violently in less than a microsecond, heating the contents inside the bubbles to 5500 °C, and creating regions with pressure change of 50 MPa (Suslick 1989). The acoustic energies bring about physical, mechanical, and chemical changes in the contacted materials, including food-borne pathogens both in food and on surface of manufacture devices (Kehinde et al. 2020). The effects of moderate heat and/or high pressure were involved when TS, MS, or MTS is applied. When
Table 7.2 Important reviews concerning ultrasound and ultrasound +, and their applications in control microorganisms in food, published in the last 10 years under the search domain of *Food Science and Technology, and Microbiology*

<table>
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<th>Source</th>
<th>Main contribution</th>
<th>Author’s conclusion</th>
<th>References</th>
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<td>1</td>
<td>A comprehensive survey on the latest and more recent applications of ultrasound on technological properties and bioactivity of food. Special emphasis has been placed on ultrasound assisted extraction</td>
<td>Ultrasonics not only represents a rapid, efficient, and reliable alternative to improve the quality of food, but it also has the potential to develop new products with a unique functionality. UAE is considered nowadays as the most feasible and economically profitable large-scale application of ultrasound in the food field. Like most innovative food processing technologies, high power ultrasonics is not a standardized technology and therefore needs to be developed and scaled up for every new application.</td>
<td>Soria and Villamiel (2010)</td>
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<td>2</td>
<td>This review presents a complete picture of current knowledge on application of ultrasound in food technology including processing, preservation, and extraction</td>
<td>It provides the necessary theoretical background and some details about ultrasound technology, the technique, and safety precautions. We will also discuss some of the factors which make the combination of food processing and ultrasound.</td>
<td>Chemat et al. (2011)</td>
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<tr>
<td>3</td>
<td>This review summarizes the major applications of low and high power ultrasound in food science and technology</td>
<td>The basic principles of low and high power ultrasound will be highlighted, and their methods and applications including important research results will be presented. These applications include meat products, vegetables and fruits, cereal products, aerated foods, honey, food gels, food proteins, food enzymes, microbial inactivation, freezing, drying, and extraction.</td>
<td>Awad et al. (2012)</td>
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<td>4</td>
<td>The main focus of this review is to summarize and synthesize the results of studies and articles about ultrasonic processing which can be adapted to the wash water decontamination process for fruits and vegetables.</td>
<td>Findings from different studies are also difficult to compare because they use different parameters such as ultrasound frequency, efficiency, acoustic energy density, time of treatment, temperature, water/sample ratios, agitation-washing protocol, species and strains of test organisms. As a result, finding the best conditions, doses, and combination of treatments for different hurdle decontamination technologies is a further challenge for the</td>
<td>Bilek and Turantas (2013)</td>
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<th>Source</th>
<th>Main contribution</th>
<th>Author’s conclusion</th>
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<td>5</td>
<td>This article reviews the advantages and limitations offered by the application of TS on fruit and vegetable juices. Additionally, its effects on bioactive compounds, physicochemical, microbiological, enzymatic, and sensory parameters in fruit and vegetable juices are also discussed</td>
<td>Scientific evidence shows that TS is a viable technology for processing of fruit and vegetable juices that preserve the quality of fruit and vegetable juices, compared with conventional thermal processing (60 °C for 30 min). An additive effect between ultrasound and heat has the potential to ensure product stability and also is effective for inactivation of enzymes present in juices.</td>
<td>Anaya-Esparza et al. (2017b)</td>
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<td>6</td>
<td>This short review covers the effect of sonication on liquids and beverages with a specific focus toward dairy and fruit juices and covers emulsification, fractionation, sterilization, and some pilot scale initiatives.</td>
<td>Enhanced emulsification and sterilization can be achieved at much lower temperatures than conventional processing, thus producing a more stable product while preventing deterioration and retaining many beneficial bioactive ingredients.</td>
<td>Paniwnyk (2017)</td>
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<td>7</td>
<td>This review enlightens knowledge in understanding the basic concept of ultrasonic’s with the principle, mechanism, and application in the food sector.</td>
<td>It specifically reviews the explored effect of ultrasonication on different juices. It explores the influence of technology in enzyme inactivation, microbial inactivation, and different quality attributes of fruit juices. The basic principles of ultrasound were summarized and graphical represented.</td>
<td>Dolas et al. (2019)</td>
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<td>8</td>
<td>This review is a supplementation to the pedagogical awareness to scholars on the suitability of ultrasonication for research procedures, and a call to industrial food brands on the adoption of this technique for the development of foods with optimally sustained nutrient profiles.</td>
<td>In all scales of food processing, viz nanotechnological, microtechnological, and macrotechnological, ultrasonication has proven to be suitable or even more efficacious relative to the conventional processing methods used in these regards. As an emerging technology, it can be used in solitude or as a composite with other techniques for the actualization of food processing objectives.</td>
<td>Kehinde et al. (2020)</td>
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ultrasound wave propagates through a material, a loss of energy in the ultrasonic wave, namely attenuation, would happen as a result of fluctuation produced by ultrasonic wave, which is associated with the microscopic structure of the material (Kehinde et al. 2020). Attenuation indicates a reduction in the amplitude of wave. Some factors such as ultrasonic beam spreading, energy absorption, dispersion, nonlinearity, transmission at interfaces, scattering by inclusions and defects, affect the amplitude and waveform of the ultrasonic wave (Soltani Firouz et al. 2019).

Acoustic cavitation is divided into stable and transient cavitation (Morey et al. 1999). Stable cavitation is characterized by bubbles which oscillate in regular fashion for many acoustic cycles, resulting in a microstreaming phenomenon to induce stress on the microbes (Chemat et al. 2011). Transient cavitation is produced from bubbles which form undergo irregular oscillations, creating regions with temperature and pressure change and resulting in the chemical, thermal, and mechanical effects (Fig. 7.3). Chemical changes include the formation of free radicals (H, OH-) due to the breakdown of the water molecule in aqueous solutions (Riesz and Kondo 1992). H- and OH- can be recombined to form hydrogen peroxide (H2O2), which also exhibited bactericidal property (Lee and Feng 2011). Part of ultrasonic energy is converted into thermal energy. Mechanical effects are caused due to the generation of mechanical shocks, resulting in the cell destruction and cell lysis (Feng et al. 2009). That’s what possible pressures and competitions food-borne pathogens should face. These above-mentioned complicate effects of ultrasound and in combination with other chemical factors, biological agents, physical conditions, and other emerging technologies result in multiple stresses to microbial cells, followed with different responses of food-borne pathogens.

Factors affecting the inactivation effects on microorganisms also inversely affect the resistance to ultrasound stresses. Generally, there are two categories of factors that may affect the sensitivity of food-borne pathogens. One is the external factors, such as treatment parameters (e.g., input power intensity/frequency, temperature, treatment time, ultrasonic amplitude), surrounding environments (e.g., pH, a_w, medium compositions, heat treatment, acid) (Dolas et al. 2019; Feng et al. 2009; Martínez-Moreno et al. 2020; Yin et al. 2019). Internal factors are associated with microbial characteristics, such as microorganism strains, growth phase, and growth temperature (Chantapakul et al. 2019; Guerrouj et al. 2016; Li et al. 2017, 2018a, b). The following subsections will describe some of the important factors that affect the resistance of food-borne pathogens to ultrasonic stresses.

### 7.2.1 External Factors

#### 7.2.1.1 Ultrasound Treatment Parameters

It is reported that the primary antimicrobial effects of ultrasonication are attributed to the intracellular acoustic cavitations, which increase the microbial cell membrane permeability, the production of localized heat and pressure, and the generation of
free radicals (Bilek and Turantas 2013; Maritza Gutierrez 1991; Suslick 1989). Therefore, the factors, such as ultrasound frequency, wave amplitude, the surrounding temperature and pressure, associated with chemical, thermal, and mechanical effects of acoustic cavitation could affect the survival of food-borne pathogens upon an ultrasonic stress.

Lower frequencies generate larger bubbles and thus a more violent cavitation collapse with higher localized temperatures and pressures (Suslick 1989). However, as frequency is increased, more collapsed events occur per unit time to provide a more uniform but less intense acoustic field (Kentish and Feng 2014). A frequency of about 20 kHz is usually applied for microbial inactivation, because this frequency range has been reported to make food-borne pathogens be sensitive to ultrasound (Condón et al. 2005; Piyasena et al. 2003; Suslick 1989). According to the study of Alliger (1975), the frequency should be applied at a value of <2.5 MHz since cavitation will not occur above that level. As shown in Table 7.1, ultrasound is generated by electrical energy supplied to a piezoelectric material referred to as a transducer in most laboratory and industrial applications. The amount of energy dissipated is a function of the amplitude and frequency, indicating that higher frequencies result in lower amplitude (Suslick 1989). So, these two factors, the amplitude and the frequency, can be considered together. Mechanical vibrations applied at frequencies of over 15 kHz are required to generate ultrasound waves (Raso et al. 1998).

7.2.1.2 The Surrounding Temperature and Pressure

As subjected to ultrasound stresses, microbial cells suffered of thermal pressure. The ultrasonic energy was transferred into the surrounding solution that induces mechanical effects but is ultimately lost as heat. In other words, the treating process usually results in a temperature increase. However, temperature control is usually be applied when ultrasound was used in combination with moderate heat (in the form of TS). According to literature, moderately elevated temperatures would cause a weakening effect on cell envelopes, and great mechanical disruption to thermal damaged cells would further increase the bacterial lethality. It is reported that heat and ultrasound act independently (Raso et al. 1998). Temperature may affect vapor pressure, gas solubility, surface tension, and solution viscosity of solutes (Vercet et al. 1999; Mason et al. 2005), so it poses a complicated effect on sonochemical reactions and also free radical generation. An increase in temperature is found to decrease the surface tension of solutes and make it easier for the production of cavitation, which may explain the enhanced intensity of free radicals spin adducts (Chemat et al. 2011; Condón et al. 2005; Feng et al. 2009). Additionally, elevated temperature induces lifting of vapor pressure and solution viscosity, and decreases gas solubility conversely, which retards the collapse event and leads to a reduction of cavitation efficiency with more bubbles are formed (Chemat et al. 2011; Condón et al. 2005; Feng et al. 2009). It is reported that moderately elevated temperatures damaged microbial cells, which exhibited increasing sensitivity to ultrasonic exposure and the
inactivation efficacy of ultrasound was, therefore, enhanced (Alvarez et al. 2003; Wordon et al. 2012). TS can be conducted under either a sublethal temperature (<45 °C) or lethal one (>45 °C) (Tiwari et al. 2009; Ugarte-Romero et al. 2007). The surrounding temperature affects the sensitivity of food-borne pathogens to ultrasound within a certain range. For example, the inactivation of *L. monocytogenes* by ultrasound was enhanced with increasing temperature from at 35 to 50 °C, though no additional inactivation was achieved at 65 °C (Ugarte-Romero et al. 2007). Another study reported 5-log reduction of *E. coli* K12 in apple cider (pH 4.1) by ultrasound combined with 60 °C exposure within 4 min. While when the temperature of below 60 °C, over 10 min was required to achieve a 5-log reduction of *E. coli* K12 (Ugarte-Romero et al. 2006). Salleh-Mack and Roberts (2007) applied TS to inactivate *E. coli* ATCC 25922 in citric and malic acids at pH of 2.5 and 4 and observed that viable *E. coli* ATCC 25922 was reduced by 5.4 to 4 logs in 9 min (Salleh-Mack and Roberts 2007). In addition, ultrasound treatment at a sublethal temperature (55 °C) exhibited better cavitation activity than that under a lethal temperature (63 °C), resulting in the lowest proportion of PI-stained cells (5.59%) (Li et al. 2019). This results were in accordance with previous study, which indicated there was a temperature threshold of cavitation activity (Kentish and Feng 2014). It has been indicated that temperature contributed more to the ultrasound-induced inactivation of most microorganisms (e.g., *E. coli*, *Salmonella* spp., *L. monocytogenes*, *S. sonnei*, *B. subtilis*, *S. aureus*) than pulsed-ultrasound amplitude did, while the effect of these two parameters on the inactivation of *B. fulva* by ultrasonic treatment was insignificant different (Hashemi et al. 2019). The application of a heat shock was found to result in no protection of bacteria to a subsequent MS treatment (Pagán et al. 1999). This indicates that heat-induced changes in cell envelopes might be not relevant to ultrasound resistance.

Pressure is another important factor. In industrial applications, the ultrasound is usually applied with a moderate positive pressure (overpressure or backpressure) over the sonicating fluid (Lee et al. 2009a, b; Pagán et al. 1999). Pressurization is reported to increase the implosion power of cavitating bubbles and result in an enhanced microbial inactivation (Feng et al. 2009). Increasing the external pressure enhances the cavitation threshold, and hence fewer bubbles are formed (Arroyo et al. 2011a; Chantapakul et al. 2019; Lee et al. 2009b; Pagán et al. 1999). However, the collapse of these bubbles is more violent to generate high shear fields (Kentish and Feng 2014; Maritza Gutierrez 1991).

In addition, treatment time, volume of bacterial suspension, surrounding pH, other chemical and biological additives are also the contributors affecting the sensitivities of food-borne pathogens to ultrasonic stress (Bermúdez-Aguirre and Corradini 2012; Luo and Oh 2016). Longer exposure times of ultrasound are usually more effective for microbial inactivation. Lower volume of bacterial suspension results in higher ultrasonic intensity (W/cm²), leading to higher inactivation of food-borne pathogens (Al Bsoul et al. 2010; Herceg et al. 2015). The processing efficiency should be considered primarily in industrial application; therefore, volume optimization for enhancing the microbial inactivation might be impractical.
7.2.1.3 pH of Medium

It is reported that lower pH resulted in higher inactivation effect of ultrasound, TS, MS, and MTS (Lee et al. 2009b). The bactericidal effect of MTS on *E. coli* K12 inactivation was found to be enhanced at low pH (Lee et al. 2009a). Under a nonlethal temperature of 40 °C, no increase in the inactivation efficacy of ultrasound on *E. coli* K12 was observed until the pH was lowered to 3 for MS treatment. For individual ultrasound, the lethal effect was decreased as acidity of the medium increased. Salleh-Mack and Roberts (2007) reported that the inactivation of *E. coli* ATCC 25922 by ultrasound at pH 2.5 was significantly higher than that at pH 4 at 30 °C (Salleh-Mack and Roberts 2007). The effect of acidity on sonication-induced inactivation of *L. monocytogenes* was found to be negligible at nonlethal temperatures (20, 30, and 40 °C), while at lethal temperatures (50, 55, and 60 °C), more than 1 log additional reduction in *L. monocytogenes* was reported when pH was as low as 3.4, comparing to at pH 7.0 (Baumann et al. 2005). Besides that, Gabriel (2012) explored the potential of a dynamic shock wave power ultrasound against pathogenic and spoilage microorganisms in cloudy apple juice (Gabriel 2012). The study revealed that acid adaptation increased the resistance of *E. coli* O157: H7 and *Salmonella* spp. but decreased that of *L. monocytogenes* toward the ultrasonic treatment.

The synthesis of acid shock proteins as postulated by Foster (2001) and Huang et al. (2007) might contribute to protecting microbial cells from the damages caused by ultrasound stresses (Huang et al. 2007; Foster 2001). The exact response of a bacterial cell to pH changes in an ultrasound treatment under different temperature and pressure still remains a topic for further studies.

7.2.1.4 The Addition of Antimicrobials

In the purpose of inactivate microorganisms, especially food-borne pathogens and spoilage ones, some chemical or biological additives have been combined with ultrasound to enhance the microbial decontamination (Demirdöven and Baysal 2008; Ferrante et al. 2007). Hurdle technology has been widely accepted for food disinfection and is defined as the simultaneous or the sequential application of factors and/or treatments to inhibit/inactivate microbial cells (Ray 2003). The combination of ultrasound with some antimicrobials, as the hurdle treatments, has been demonstrated for microbial decontamination of different foods, such as plum fruit, cherry tomato, strawberries, alfalfa seeds, fruit and vegetable juices, apples, and lettuce (Brilhante São and Dantas Vanetti 2012; Ferrante et al. 2007; Luo and Oh 2016; Park et al. 2016; Sagong et al. 2011). For instance, the combined treatment of ultrasound (45 kHz, 10 min) and 40 mg/L peracetic acid resulted in the highest reduction of the natural contaminant population on cherry tomatoes, and a reduction of adherent *S. Typhimurium* ATCC 14028 by 3.9 log_{10} CFU/g on them (Brilhante São and Dantas Vanetti 2012).
7.2.1.5 The Carrier of Microbial Cells

Generally, liquid-phase system is more homogeneous than liquid system with solid particles. As two kinds of carrier of microbial cells, these systems would affect their sensitivity to ultrasound stress. Unlike cavitation bubble collapse in the bulk liquid, collapse of a cavitation bubble on or near to a solid surface is asymmetrical because the surface provides resistance to liquid flow from that side. Surface imperfections or trapped gas acts as the nuclei for cavitation bubble formation on the surface of a particle and subsequent surface collapse then leads to shock waves, which break the particle apart (Mason et al. 2005). Cavitation bubble collapse in the liquid phase near to a particle can force it into rapid motion (Mason et al. 2005). Seymour et al. (2002) attributed the mechanical effect generated by cavitation bubbles to the microbial inactivation of ultrasound on fresh produce (Seymour et al. 2002). The authors stated that cavitation enhanced the mechanical removal of bacteria attached or entrapped on the surfaces of fresh produce by displacing or loosening particles through a shearing or scrubbing action, which achieved an additional log reduction when combined with a chlorinated water wash.

7.2.2 Internal Factors

7.2.2.1 Bacterial Characteristics

The physiological state of microbial cells has been reported to affect their resistance to ultrasound. Generally, bacteria spores are much more resistant than vegetative cells to various treatments. In addition, Gram-negative (G-) bacteria are more susceptible to injury than Gram-positive (G+) bacteria, and bacterial spores are much more resistant than vegetative cells to a particular stress. With some exceptions, it is generally accepted that bigger cells are more sensitive than smaller ones and that coccal forms more resistant than rod-shaped bacteria. Further, aerobes are also more resistant than anaerobic bacteria.

As summarized in Table 7.1, *E. coli*, *Salmonella* spp., *S. aureus*, *L. monocytogenes*, *Shigella* spp., *Cronobacter sakazakii*, and *Bacillus* spp. were the most widely studied food-borne pathogens that were subjected to ultrasound treatment. It is difficult to make comparison between the ultrasound resistance of different species or even within the same species due to the different devices used for ultrasonic treatments and the conditions used, especially the temperature and food matrix of tests (Mason et al. 2005). The sensitivity of microorganisms to ultrasound stresses varies in the order of G- bacteria > G+ bacteria > yeasts > fungi > spores (Wong et al. 2010). It is reported that *E. coli* (G- bacteria) was reduced by more than 99% after ultrasonic treatment (20 kHz, 750 W, 10 min), whereas *Lactobacillus acidophilus* (G+ bacteria) was reduced by 72% ~ 84% depending on the media used (Cameron et al. 2008). G+ bacteria have a thicker and a more tightly adherent layer of
peptidoglycans than G− bacteria (Scherba et al. 1991), which is considered as the case for the different resistance of G− and G+ bacteria toward ultrasound exposure. However, it has reported no significant differences in the ultrasound resistance between G− and G+ bacteria in some studies (Scherba et al. 1991). The outer membrane of a G− cell, consisting of lipopolysaccharide and phospholipid, might contribute to protective effects of G− bacteria toward ultrasound (Rojas et al. 2018).

7.3 The Response Sites and Nature of Injury of Food-Borne Pathogens to Ultrasound

Food-borne pathogens that exposed to different levels of ultrasound stresses may derive various response modes, of which some are reversible while other are usually irreversible (Ray 2003). As mentioned above, the mechanism underlying microbial inactivation by ultrasound is associated with acoustic cavitation. Both transient and stable cavitation result to severe damages to cells wall, membrane, intracellular compounds, such as endogenous enzymes and nucleic acid (Dolas et al. 2019). As responding to the mechanical effects of ultrasound processing, high velocity microjets generated during the collapse of cavitation bubbles could induce cellular structure disruption, such as pitting on cell walls surface and eroding bacteria cells (Zinoviadou et al. 2015). The morphology of E. coli K12 cells sonicated at 40 °C for 3 min presented significant changes as compared with control sample or cells heated alone at 40 °C for 3 min, as observed with a scanning electron microscopy (SEM) (Ugarte-Romero et al. 2006). These may due to localized extreme high temperature and pressure generated from the collapse of cavitation bubbles (Suslick 1989). The following disruption of critical components of microbial cells leads to cell lysis and final death.

The chemical effects of ultrasound result from the sonolysis of water in liquids, and the formation of free radicals (e.g., H+, O−, OH−, HO2−) and H2O2 (Suslick 1989). All of these mentioned compounds belong to reactive oxygen species (ROS) and have antimicrobial properties through oxidizing lipids in cellular membrane, intracellular proteins, and nucleic acids (Chemat et al. 2011; Feng et al. 2009; Furuta et al. 2004; Suslick 1989). Subsequently, a wide range of secondary reactions occur, including generation of H2O2 and other ROS, and extracellular and intracellular organic compounds are then oxidized (Suslick 1989). The amount of ROS increases after a successive and spontaneously happened reactions, and may induce more severe damages to bacterial cells. The hydroxyl radical (OH·) is able to react with the sugar-phosphate backbones of DNA chain, giving rise to the elimination of hydrogen atoms from the sugar (Manas and Pagan 2005). This causes the scission of the phosphate ester bonds and subsequent breaks of single strand. The breaks in double strand occur when two single strand breaks take place in each chain of the double helix at a close distance (Dolas et al. 2019). Bases are also attacked by ROS generated by radiolysis, but it is not clear whether this is relevant to microbial cell
death (Moseley 1989). A summary of the various applications including the mechanisms, parameters, and effects of ultrasound is shown in Fig. 7.3. The following sections mainly describe the response modes of food-borne pathogens to ultrasound, which is generally in an order of outside-in.

### 7.3.1 Cell Wall

Cell wall is located in the outermost of a microbial cell, which plays as a barrier when exposure of external stresses. On the basis of outer structure characteristics of bacterial cells, they as grouped as G+ and G− (Ray 2003). The later one have a complex cell wall containing an outer membrane (OM), middle membrane (MM), a thin layer of peptidoglycan or mucopentide embedded in the periplasmic materials that contain several types of proteins, and the plasma or inner membrane (IM). In OM, lipopolysaccharides (LPS), lipoprotein (LP), and phospholipids are the main compounds (Ray 2003). Phospholipid molecules are arranged in a bilayer, with the hydrophobic part (fatty acids) inside and hydrophilic part (glycerol and phosphate) outside. LPS and LP molecules are embedded in the phospholipids layer. Beneath the periplasmic materials is the plasma or the IM, composed of a phospholipid bilayer in which many types of proteins are embedded. The OM has limited transport and barrier functions, though new study indicates that OM was very important to the resistance toward environmental stresses (Rojas et al. 2018). G+ cells have a thick cell wall composed of several layers of mucopeptide (responsible for thick rigid structure) and two types of teichoic acids (wall teichoic acid and lipoteichoic acid) (Ray 2003). The wall teichoic acid molecules are linked to mucopeptide layers, and the lipoteichoic acid molecules are linked to both mucopeptide and cytoplasmic membrane. Teichoic acids are negatively charged (because of phosphate groups) and may bind to or regulate the movement of cationic molecules in and out of the bacterial cells (Ray 2003). Various changes on the OM and IM have been reported when bacterial cells were exposed to ultrasound stresses (Li et al. 2017, 2018a, b; Luo and Oh 2016; Martínez-Moreno et al. 2020). It has been reported that ultrasound seems to selectively destabilize the OM of *E. coli* without severely affecting the cytoplasmic membrane (Ananta et al. 2005). As subjected to ultrasound treatment at 20 kHz and 17.6 W for 5–18 min, an increasing population of *E. coli* with disintegrated OM, which allowed penetration of carboxyfluorescein diacetate (cFDA), was observed (Ananta et al. 2005). As shown by flow cytometry analysis of *E. coli*, an increase of processing time enhanced ultrasound-induced damages on the OM of this G− bacteria, facilitating the penetration of antimicrobial compounds into the cells (Li et al. 2016). Under MS treatment, the blurry outer cell membranes were observed, thus signifying membrane surface thinning and increased membrane permeability (Li et al. 2016). The development of pores and modifications on microbial cell membranes (*E. coli, L. innocua*) during TS were reported by several studies (Bermúdez-Aguirre et al. 2011; Moody et al. 2014). Upon disruption of IM, the release of intracellular components, such as proteins, lipids, and other
compounds may occur (Li et al. 2016). Partings, shrinkages, ruptures, and deformations on the outer surface of both bacteria (*E. coli* and *S. aureus*) were observed as a consequence of physical disruption by the shear force-induced micro-bubbles explosion (Chantapakul et al. 2019). A previous report has also demonstrated that G+ cells have not been evenly disrupted due to the lack of sonication exposure time (Lee et al. 2009a). For MTS treatment, both heat and pressurized sonication were shown to individually impact bacterial structures with decrease in the cytoplasmic electron densities, indicating the denaturation of proteins and deformation of the outer membrane (Lee et al. 2009a, b). Results illustrated that MTS posed a more severe damage on the OM of bacteria when compared to MS, indicating that the application of mild heat further enhanced the ability of pressurized sonication to break chemical bonds in membranes (Lee et al. 2009a, b). The most noticeable cellular damage occurred in *S. aureus* treated by MTS (50 °C, 100 kPa) where the majority of cell components were missing with only traces of the outer cell membrane visible (Chantapakul et al. 2019). However, major damages on microbial cells were detected when treated by pressurized sonication, such as MS and MTS (Chantapakul et al. 2019).

### 7.3.2 Nucleic Acids

Seldom studies have involved responses of nucleic acids to ultrasound stress. Generally, DNA can undergo single- and double-strand breaks. Dolas et al. (2019) reported that breakdown of DNA structure by the attack of free radicals was one of the important mechanisms of microbial inactivation by ultrasound treatment, though no direct evidence of this process (Dolas et al. 2019). Back tracing in 1989, a study of Moseley had proposed that the OH- generated from ultrasound treatments was able to react with the sugar-phosphate backbone of the DNA chain, giving rise to the elimination of hydrogen atoms from the sugar (Moseley 1989).

### 7.3.3 Intracellular Enzymes

Enzymes are a summary of special protein with specific metabolism activities. They are targeted by ultrasound waves, especially when the structure of IM is compromised (Li et al. 2016, 2017; Manas and Pagan 2005). Among them, the intracellular esterase was estimated before and after ultrasound treatment with flow cytometry (FCM) in combination with carboxyfluorescein diacetate (cFDA) (Li et al. 2016, 2017, 2019). FCM is a powerful tool for real-time data acquisition and multiparameter analysis of cell populations at the single-cell level. cFDA is a lipophilic nonfluorescent precursor that diffuses readily across cell membranes, which is widely used for the assessment of nonspecific enzymatic activity in microbial cells (Li et al. 2016, 2017, 2019). Once inside the cell, cFDA is converted
by nonspecific esterases into a polar, membrane-impermeant green fluorescent compound, carboxyfluorescein (cF). The activity of intracellular esterase in *S. aureus* and *E. coli* has been evaluated in several studies (Li et al. 2016, 2017, 2019). The intracellular esterase inhibition was highly correlated throughout the individual ultrasound treatment; while the TS treatment radically influenced the ability of *S. aureus* cells to revive by compromising membrane integrity (indicated by the penetration of PI into cells) and inactivating intracellular esterase severely (Li et al. 2017). For thermoacidophilic *Bacillus subtilis*, the proportion of cF-stained cells made up 99.96% without treatment, while it was decreased to 4.39, 0.13, 0.36, and 0.24% after the thermal pasteurized at 63 °C, TS at 55 °C and 400 W, at 63 °C and 200 W, and at 63 °C and 400 W, respectively, indicated that the intracellular esterase in TS treated *B. subtilis* decreased significantly (Li et al. 2019).

### 7.3.4 Intracellular Compounds Leakage

The leakage of intracellular compounds usually referring to leakage of protein, enzymes, nucleic acids, and cytoplasm. Protein is confined mostly within cytoplasm by cell membrane, and some OMPs locate at OM. Here, protein leakage includes both parts. Release of proteins from cytoplasm can be used to evaluate the extent of cell membrane damage (Guimaraes et al. 2018; Zhang et al. 2014). It was reported that 1.41 and 1.73 mg/mL of protein leakage were observed from *E. coli* ATCC 25922 and *P. pastoris* GS115, respectively, after a 3-min combination treatment of low concentration neutralized electrolyzed water (CEW) and ultrasound, while it was 6.22 mg/mL of protein leakage from *A. pullulans* 2012 after 2 min combination treatment of CEW and ultrasound (Zhao et al. 2017). In addition, a study of Releasing polysaccharide and protein from yeast cells by ultrasound applied a 20 kHz high-intensity ultrasound to treat *S. cerevisiae* cells, and it is found that the release selectivity, which was calculated by the ratio of the amount of polysaccharide released to that of proteins, was affected by sonication time, temperature, and ionic strength, among which temperature posed the greatest influence (Cameron et al. 2008; Zhang et al. 2014). For instance, the release selectivity at 85 °C was a factor of 9.3 the one at 25 °C. So the author proposed that the underlying mechanism of this selectivity is speculated to be thermal denaturation and aggregation of protein within yeast cells at elevated temperatures leading to the decrease of protein release by ultrasound. The released amount of proteins was found to be linearly related to the sonication intensity in the range from 10 to 39 W/cm². Besides, some results indicate that ultrasound combined with nisins potentiated microbial inactivation, possibly due to leakage of intracellular contents, such as ATP (Freitas et al. 2019).
7.4 The Response Modes of Pathogenic Bacteria Cells Under Ultrasonic Stresses

As exposed to different intensity of ultrasound treatments, food-borne pathogens may be stressed and altered into several complicated status with specific characteristics (Arroyo et al. 2012; Declerck et al. 2010; Li et al. 2017; Muñoz et al. 2012; Piao et al. 2019; Saeeduddin et al. 2015; Ugarte-Romero et al. 2006; Yin et al. 2019). Pathogenic bacterial cells in subpopulations develop a stress resistance, or were damaged sublethally till to lose viability permanently (Ray 2003). Herein, the main response modes of food-borne pathogens are summarized as follows:

7.4.1 Sublethal Injury

Sublethal injury occurs following exposure of bacterial cells to a sublethal stressor, which cause reversible damages in the metabolic activity or cell membranes of microbes (Ray 2003). Sublethal injury can be repaired and result in the microbial regrowth under suitable conditions. There are few reports about sublethal injury by TS on food-borne pathogens on juices (Arroyo et al. 2011b, 2012; Ugarte-Romero et al. 2006, 2007). A study showed that the amount of sublethally injured *S. aureus* cells caused by sonication was negligible because the membrane damage and intracellular esterase inhibition were highly correlated (Li et al. 2017). This suggested the *S. aureus* underwent immediate death when exposed to ultrasound. Mechanisms underlying the microbial inactivation by ultrasound and mild heat were distinct, and ultrasound and mild heat were found to exhibit a synergistic inactivation effect on microbial cells (Li et al. 2017). It was indicated that no sublethally damaged cells were generated after individual ultrasound treatment, whereas a certain amount of sublethally damaged cells (*S. aureus* and *E. coli* in milk) were observed after single heating treatment. Additionally, the amount of bacterial cells with sublethal injury induced by ultrasound (600 W, 5 min)-heat (63 °C, 5 min) combined treatment was significantly greater than individual ultrasound or heat exposure, up to 0.26 and 0.19 log CFU/ml for *S. aureus* and *E. coli*, respectively (Li et al. 2018b). The authors attributed sublethal injured cells to irreversible physical damages, which could cause lysed cells with fragmentized walls and membranes (Manas and Pagan 2005). In order to estimate the amounts of sublethally injured cells, treated samples were poured on both nonselective and selective medium (supplemented with 7% [w/w] sodium chloride) (Li et al. 2017). Both undamaged cells and sublethally damaged cells were able to grow on nonselective media, while sublethally damaged bacteria cannot grow on selective media supplemented with sodium chloride due to the permeability change of membranes (Li et al. 2017). The recovery of MS treated bacterial cells on the medium with sodium chloride is virtually identical to those recovered in a nonselective medium (Raso et al. 1999). This indicates that ultrasound results in irreversible damages in membranes, which is considered as all-or-nothing

7.4.2 VBNC State

Several studies have demonstrated that ultrasound or TS treatment could induce microorganisms into a special dormancy, known as VBNC state (Krizanovic et al. 2020; Liao et al. 2018a, b; Piao et al. 2019; Yin et al. 2019; Zhang et al. 2020). Krizanovic et al. (2020) reported that TS at 43 °C for 1 ~ 3 min resulted in the formation of VBNC <em>B. bruxellensis</em> population in dry wines (Krizanovic et al. 2020). Yin et al. (2019) reported that <em>Lactobacillus acetotolerans</em> in beer entered the VBNC state by TS at a constant frequency of 24 kHz at 50 °C with relatively low volumetric powers (0.6 ~ 1.8 W/mL) (Piao et al. 2019). In the same study, it revealed that <em>L. acetotolerans</em> cells decreased in size and gradually changed morphology from short rods to coccoids upon entering the VBNC state, and the resistance of VPNC cells to 0.025 M NaOH and 0.025 M KOH was enhanced compared with the exponential-phase cells. Additionally, the addition of 0.1% v/v Tween 80 into de Man Rogosa Sharpe agar was observed to enable the VBNC cells to regain culturability within 3 days. It is reported that ultrasound treatments at a frequency of 36 kHz for 15 min using a power intensity of 0.064 kW/L induced 7% <em>Legionella pneumophila</em> into the VBNC state, in contrast to power settings of 100% for 30 min, in that case the decrease in viable plate counts is attributed to a loss in cell viability (Declerck et al. 2010).

7.4.3 Regrowth, Repair, and Resuscitation

In a study, <em>E. coli</em> was decreased to below the detection of limit in green and purple cactus pear juices after ultrasound treatment with 90% amplitude for 5 min, while the regrowth of <em>E. coli</em> was observed after 2 days of storage at 4 °C (Cruz-Cansino Ndel et al. 2016). It may be attributed to reversible membrane permeabilization formed upon ultrasound treatment, which may enhance the transport of nutrients and other substances into the cells, alleviating cell metabolism and subsequently enhancing bacterial viability during storage (Cruz-Cansino Ndel et al. 2016). It should be noticed that the limit of microbial detection is 1 CFU/mL, and only 1 mL of juice sample was taken for microbial survival test in this study, there is a possible of leak detection of viable cells at the beginning of storage. In other words, the regrowth of bacterial cells during the storage might be resulted from the few remaining survivors or the resuscitation of VBNC cells.
7.5 The Molecular Regulatory Mechanisms of Food-Borne Pathogens in Response to Ultrasound

For bacteria, the principal stress signals include nutrient exhaustion, elevated temperature, alteration in pH and the redox state, variations in salt concentrations, increased amount of internal reactive oxygen species (ROS), external oxidants like hydrogen peroxide (H$_2$O$_2$), other toxic chemicals, etc., some of them are involved during ultrasound treatments and its combination with other properties.

Many of the microbial stress responses are regulated by the alternative sigma factors which recruit RNA polymerases to the promoters of specific subsets of stress responsive genes in the microbial cells (Campagne et al. 2015; Gruber and Gross 2003; Kim et al. 2007; Page 2015; Murata et al. 2012). There are two types of sigma factors, including $\sigma^H$ ($\sigma^{32}$, the heat shock sigma factor, encoded by rpoH) and $\sigma^E$ ($\sigma^{24}$, the extracytoplasmic sigma factor, encoded by rpoE), which have been known to regulate the resistance to the elevated temperature through initiating the transcription of two respective heat shock regulons to cope with protein misfolding in the cytoplasm and extra-cytoplasm (periplasm and outer membrane) in E. coli, respectively (Dartigalongue and Raina 1998; Raina et al. 1995). When exposed to ultrasound, microbial cells might suffer from mechanical stress, oxidative stress, and heat stress. According to literature investigation, there are several studies that had explored the molecular regulatory mechanisms of food-borne pathogens in response to ultrasound using the proteomics or transcriptomics analysis (He et al. 2021; Li et al. 2020, 2021). By taking the advantages of integrated transcriptomic and proteomic analyses, a total of 770 genes and 201 proteins that significantly differentially expressed upon ultrasound treatment had been reported (He et al. 2021). Further analysis revealed that genes and proteins involved several main pathways, including phospholipid metabolism, lipopolysaccharide biosynthesis and transport, and fatty acid metabolism, etc., as facing the ultrasonic stress. Furthermore, the differential expression of lipid metabolism-related genes (plda, mlaA, ompC, psd, lptD, pal, and clsA, etc.) detected by RT-qPCR reinforced the hypothesis of membrane lipid metabolism disorder in E. coli cells. And the authors suggested that the activation of membrane homeostasis maintenance and lipid asymmetry preserving system were shown to be one of the main action modes of E. coli in response to ultrasonic stress (He et al. 2021), as summarized in Fig. 7.6.

A further study revealed that differential expressions of 1217 genes were significant when exposed at 6.67 W/mL power ultrasonic density for 25 min, including 621 up-regulated and 596 down-regulated genes (Li et al. 2021). They concluded that the sonomechanical and sonochemical effects generated by acoustic cavitation were responsible for those gene expression changes (Fig. 7.7).

Using E. coli K-12 as a target bacterium, the roles of several genes in the protection or sensitivity against ultrasound-generated radicals have been addressed (Spiteri et al. 2017). The obtained results showed that the $\Delta$oxyR mutant appeared to be more resistant to ultrasound treatment (at 200 W for 3 min) together with gadW, gadX, gabT, and gabD, whereas the $\Delta$dnaK mutant was more sensitive with 5.42
Fig. 7.6 A proposed schematic diagram summarizing the main membrane lipid metabolic pathways of *Escherichia coli* cells upon ultrasonic exposure. Selected up-regulated genes/proteins are in red color. This figure is reprinted from He et al. (2021), with the permission of Elsevier.
log_{10} CFU/mL reduction in comparison to the wild-type E. coli K-12 with 2.50 log_{10} CFU/mL reduction. As the dnaK gene participates actively in the response to hyperosmotic shock, this result indicated that the dnaK gene contributed to the general stress response and more specifically to hyperosmotic stress. The deletion of genes soxS, rpoS, gadB, gadC, and yneL was found to be not involved in the protection of microbial cells against ultrasound (Spiteri et al. 2017).

7.6 Conclusions and Future Perspective

In the past 10 years, ultrasound as a promising food decontamination technology has been widely studied. In order to improve the application of ultrasound in the food industry, it is necessary to fully understand the possible stress response of microbial cells against the ultrasound. Due to the poor inactivation performance and potential induction of microbial stress response by individual ultrasound treatment, the combination of ultrasound with other techniques such as mild heat, ultraviolet light, high pressure, and antimicrobials has been developed as the hurdles to improve the microbial efficiency and minimize the development of microbial stress response. Future investigation efforts should be contributed to expand the knowledge of the
molecular regulation of microbial stress response induced by ultrasound and to develop the possible hurdle combining ultrasound and other techniques.

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Chapter 8
Response of Foodborne Pathogens to High-Pressure Processing

Yongtao Wang

Abstract  High-pressure processing (HPP) makes use of an elevated pressure for the microbial inactivation in foods. HPP works without generation of heat, which poses minimal impacts on food sensory characteristics. HPP treatment can retain better organoleptic attributes of foods and also prolong the shelf life of food product with free of any additive or preservative. Therefore, HPP represents a commercially valuable and practical alternative technology for preserving foods, and it may be able to replace the thermal processing. This chapter offers a comprehensive understanding of the basic knowledge about HPP technology, the primary factors affecting the response of foodborne pathogens to HPP, the mechanisms underlying the action mode of HPP on the cell structure, physiological functions, proteome, and transcriptome of foodborne microorganisms.

Keywords  High-pressure processing · Influencing factors · Foodborne pathogens · Response · Mechanisms

8.1 Introduction of HPP in the Food Industry

For a long time, thermal sterilization has been widely employed for food decontamination due to its low cost and high effectiveness. However, the deterioration of food quality, affecting such characteristics as aroma, taste, nutrition, color, and texture by thermal treatment, makes severe compromise in the original freshness, nutrition, and function of the food product (Huang et al. 2020). Nowadays, as the increasing demand for minimally processed foods by the consumers, traditional thermal
processing has been unable to fully meet consumers’ requirements for high-quality food. Therefore, developing alternative food sterilization techniques to maximally retain the quality of processed food and meet consumers’ demand for a high-quality food is an important research topic in food science (Li and Farid 2016).

In recent years, nonthermal sterilization technologies have become a heavily researched topic in the field of food science. Compared with traditional thermal sterilization technology, nonthermal sterilization has the advantages of low working temperature, better retention of food quality, and lesser environmental pollution, which has attracted widespread attention from the researchers (Huang et al. 2017a, b). At present, the major nonthermal sterilization technologies primarily include high-pressure processing (HPP), high-pressure carbon dioxide (HPCD), pulsed electric field (PEF), ultraviolet (UV) light, cold plasma, high-pulsed magnetic field (HMF), ultrasound, pulsed light, irradiation, biological antiseptics (biopreservatives), and chemical preservatives (Norton and Sun 2008). HPP technology is a typical physical sterilization technology that has been applied to food processing and has become the most intensively researched and widely industrialized nonthermal sterilization technology since the 1990s (Considine et al. 2008; Balasubramaniam et al. 2015).

### 8.1.1 Definition and Technical Characteristics of HPP

HPP technology refers to the treatment of sealed food in a flexible container with using water or other liquids as a pressure transmission medium and performing a pressure treatment of 100–1000 MPa at room temperature or slightly above room temperature (25–60 °C) for a period of time (Bull et al. 2004; Li et al. 2021).

HPP is a physical process that follows two basic principles. The first principle is Le Châtelier’s principle, which means that a balanced system will switch to a new equilibrium to partially cancel any changes caused (Balasubramaniam et al. 2015; Mújica-Paz et al. 2011). Regarding HPP, when the pressure increases, the balance moves to reduce the volume. Therefore, the phase transition phenomenon, molecular configuration, and chemical reaction will increase (Hamann 1957; Grauwet et al. 2016); in other words, the HPP treatment promotes the reaction to move in the direction of volume reduction. The second principle is Pascal’s principle, which means that the pressure applied anywhere in the confined incompressible fluid is equal in all directions of the entire fluid, so the pressure changes (initial difference) remain unchanged (Balasubramaniam et al. 2015). Generally, all fluids generate a force applied to them and can transmit this force without friction. Pascal’s principle means that the force transmitted to the surface of the fluid is transmitted equally on the contact surface (Balasubramaniam and Farkas 2008; Li et al. 2021). Therefore, the pressure can be instantly and evenly transmitted to the sample during HPP. The volume and size of the samples pose no impact on the application of Pascal’s principle, which means that the food samples can be treated uniformly with the rapid pressure transmission and no pressure gradient during HPP.
HPP technology can effectively kill the pathogens in food and ensure food safety at a low working temperature (i.e., room temperature or slightly above room temperature) (Yang et al. 2021). Compared with a traditional thermal sterilization, HPP has less effect on heat-sensitive compounds in food, such as vitamins and polyphenols, and can better retain the nutrition, color, and flavor of the food (Rastogi et al. 2007). HPP technology has been certified by the United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) and accepted by consumers, and it has been applied to fruit and vegetable products, meat products, and seafood (Mújica-Paz et al. 2011).

### 8.1.2 Development of HPP

As early as 1899, Hite applied HPP technology to treat the raw milk and published a research report, *The Effect of Pressure in the Preservation of Milk* (Hite 1899). This report indicated a 4-d extension in the shelf life of raw milk after exposed to 600 MPa high pressure for one hour at room temperature (Farkas and Hoover 2000). In the twentieth century, the research on HPP technology continued uninterrupted, and in 1946 Bridgman won the Nobel Prize in Physics for his discovery of protein denaturation and solidification under HPP (The Nobel Prize in Physics 1946).

With the breakthrough of HPP technology theory and equipment manufacturing, in the early 1990s, HPP technology began to be commercialized in food processing (Balasubramaniam et al. 2015). In 1990, Japan first commercialized HPP technology for the treatment of fruit juice, salad dressing, jam, seafood, jelly, and other foods (Norton and Sun 2008). In the following decades, universities (Ohio State University, China Agricultural University, Kyoto University, and so on), companies, and research institutions have also accelerated their research on HPP equipment and processing technology. Avure has been engaged in the manufacture of HPP equipment, with a variety of models (JBT 2021). At present, in developed countries, such as the United States, Spain, France, and Australia, HPP technology is commercially used for the processing of fruits and vegetables, meat products, dairy products, and seafood has increased notably (Rastogi et al. 2007; Li et al. 2017).

In 2001, HPP technology was approved by the U.S. Food and Drug Administration (FDA) for fruit and vegetable juice processing (FDA 2021). In 2004, the USDA-FSIS approved the application of HPP technology to ready-to-eat (RTE) foods, such as cooked meat (USDA-FSIS 2004). In 2009, the FDA approved pressure-assisted heat sterilization processing (R&DA 2009). As the consumer market’s demand for freshly goods continues to increase, HPP has become a popular research field for the development of minimally processed products in response to the aforementioned changes in food market demand.

HPP equipment vendors installed approximately 100 machines between 2017 and 2018 in more than 40 countries (HIPERBARIC 2018). The HPP machines were installed mostly in North America, Mexico, and Europe. North America is the largest market for HPP followed by Asia, Latin America, Oceania, and Africa. According to
a report by Markets and markets, it is estimated that by 2024, the market size of HPP will grow from US$5.2 billion in 2019 to US$6.4 billion, with a compound annual growth rate of 4.3% from 2019 to 2024 (Markets and Markets 2019). Globally, in the last two years, HPP-treated juices and beverages occupied the largest share of the HPP applications. Also, the applications of HPP on tolling extended notably (HIPERBARIC 2018).

8.2 Factors Affecting the Response of Foodborne Pathogens to HPP

High pressure can pose an impact on the cellular components of bacterial cells, including the intracellular enzymes and ribosomes, and cell membrane and its membrane proteins (Baptista et al. 2016). The pressure induces complex influence on microbial cells, accompanied by the simultaneous occurrence of multiple mechanisms to cause inactivation. The primary factors affecting the inactivation efficiency of HPP on microbial cells include the process parameters (pressure level, holding time, process temperature, pressurization/depressurization rate and mode), the types of microorganisms, and the pH of the medium.

8.2.1 Types of Microorganisms

The resistances of different microorganisms toward the high-pressure are distinct (Table 8.1). Among the microorganisms that are most sensitive to ultra-high pressure are protozoa and parasites. Molina-García and Sanz (2002) found that treatment at 200 MPa/10 min at a temperature of 0–15 °C killed all Anisakis larvae in the distilled water and physiological isotonic solution. And in the study of Lindsay et al. (2008), it is demonstrated that the treatment under the conditions of 340 MPa/1 min is sufficient to make the oocyst plaques on raspberries non-infectious to mice. In general, the structure of eukaryotic cells is more complex than that of prokaryotic cells, so the prokaryotic cells show higher resistance to stress than eukaryotic cells. Electron microscopy studies show that HPP treatment changes the subcellular structure of yeast (Kobori et al. 1995; Sato et al. 1996). HPP can decompose the nuclear membrane under 100 MPa. As the pressure increases, the intracellular mitochondria, vacuole, endoplasmic reticulum, and other organelles rupture or deform (Sato et al. 1996). When pressures were greater than 500 MPa, the organelles in the cell cannot be recognized (Hartmann and Delgado 2004; Baptista et al. 2016). Yeasts and molds are usually sensitive to pressure. But some molds, like Byssochlamys nivea, have ascospores, which exhibit extreme pressure resistance. The vegetative cells of B. nivea can be killed by a short treatment time of 300 MPa at 25 °C, but at least 600–700 MPa is required to completely inactivate its ascospores.
Table 8.1  Antimicrobial efficacy of HPP on different microorganisms

<table>
<thead>
<tr>
<th>Type of microorganisms</th>
<th>Matrix</th>
<th>Pressure (MPa)</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Inactivation levels (log₁₀ CFU/mL)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anisakis simplex</td>
<td>Distilled water or 0.9% NaCl water solution</td>
<td>200</td>
<td>0 – 15</td>
<td>10</td>
<td>2</td>
<td>Molina-García and Sanz (2002)</td>
</tr>
<tr>
<td>Toxoplasma gondii oocysts</td>
<td>Raspberry</td>
<td>340</td>
<td>20</td>
<td>1</td>
<td>4</td>
<td>Lindsay et al. (2008)</td>
</tr>
<tr>
<td>G+ (Gram-positive bacteria)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Milk</td>
<td>600</td>
<td>20</td>
<td>15</td>
<td>2</td>
<td>Patterson et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>Poultry</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>Poultry</td>
<td>375</td>
<td>20</td>
<td>15</td>
<td>2</td>
<td>Patterson et al. (1995)</td>
</tr>
<tr>
<td>Lactobacillus helveticus</td>
<td>Goat milk</td>
<td>500</td>
<td>10</td>
<td>10</td>
<td>3</td>
<td>Gervilla et al. (1997)</td>
</tr>
<tr>
<td>Leuconostoc mesenteroides</td>
<td>Phosphate buffer</td>
<td>500</td>
<td>35</td>
<td>10</td>
<td>8</td>
<td>Kaletunç et al. (2004)</td>
</tr>
<tr>
<td>G− (Gram-negative bacteria)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>Pork paste</td>
<td>300</td>
<td>25</td>
<td>10</td>
<td>6</td>
<td>Shigehisa et al. (1991)</td>
</tr>
<tr>
<td>Salmonella senftenberg 775W</td>
<td>Chicken</td>
<td>340</td>
<td>23</td>
<td>10</td>
<td>&lt;2</td>
<td>Metrick et al. (1989)</td>
</tr>
<tr>
<td>Escherichia coli O157:H7 NCTC 12079</td>
<td>Milk</td>
<td>600</td>
<td>20</td>
<td>15</td>
<td>&lt;2</td>
<td>Patterson et al. (1995)</td>
</tr>
<tr>
<td>Escherichia coli O157:H7 NCTC 12079</td>
<td>Poultry</td>
<td>600</td>
<td>20</td>
<td>15</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli O157:H7 ATCC 43894</td>
<td>Mango juice</td>
<td>400</td>
<td>20</td>
<td>18</td>
<td>6</td>
<td>Hiremath and Ramaswamy (2012)</td>
</tr>
<tr>
<td>Vibrio parahaemolyticus O3:K6</td>
<td>Oyster</td>
<td>300</td>
<td>10</td>
<td>3</td>
<td>5</td>
<td>Patterson et al. (1995)</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>Goat milk</td>
<td>450</td>
<td>10</td>
<td>10</td>
<td>4</td>
<td>Patterson et al. (1995)</td>
</tr>
<tr>
<td>Salmonella Typhimurium</td>
<td>Tryptone soy broth</td>
<td>350</td>
<td>45</td>
<td>20</td>
<td>7</td>
<td>Erkmen (2009)</td>
</tr>
<tr>
<td>Bacterial spores</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermoanaerobacterium thermosaccharolyticum</td>
<td>Water</td>
<td>700</td>
<td>105</td>
<td>5</td>
<td>5</td>
<td>Ahn et al. (2007)</td>
</tr>
<tr>
<td>Clostridium tyrobutyricum</td>
<td>Water</td>
<td>700</td>
<td>105</td>
<td>5</td>
<td>6</td>
<td>Ahn et al. (2007)</td>
</tr>
</tbody>
</table>

(continued)
Table 8.1 (continued)

<table>
<thead>
<tr>
<th>Type of microorganisms</th>
<th>Matrix</th>
<th>Pressure (MPa)</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Inactivation levels (log$_{10}$ CFU/mL)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium sporogenes</em></td>
<td>Water</td>
<td>700</td>
<td>105</td>
<td>5</td>
<td>7</td>
<td>Ahn et al. (2007)</td>
</tr>
<tr>
<td><em>Bacillus amyloliquefaciens</em></td>
<td>Water</td>
<td>700</td>
<td>105</td>
<td>5</td>
<td>7</td>
<td>Ahn et al. (2007)</td>
</tr>
<tr>
<td><em>Bacillus sphaericus</em></td>
<td>Water</td>
<td>700</td>
<td>121</td>
<td>2</td>
<td>7</td>
<td>Ahn et al. (2007)</td>
</tr>
<tr>
<td><em>Clostridium botulinum</em></td>
<td>Tris-His buffer</td>
<td>1400</td>
<td>100</td>
<td>2</td>
<td>4</td>
<td>Ahn et al. (2007)</td>
</tr>
<tr>
<td><em>Bacillus coagulans</em> 185A</td>
<td>Tomato juice</td>
<td>600</td>
<td>95</td>
<td>1</td>
<td>2.2</td>
<td>Daryaei and Balasubramaniam (2013)</td>
</tr>
<tr>
<td><em>Bacillus coagulans</em> ATCC 7050</td>
<td>Tomato juice</td>
<td>600</td>
<td>95</td>
<td>1</td>
<td>3.8</td>
<td>Daryaei and Balasubramaniam (2013)</td>
</tr>
</tbody>
</table>
Gram-positive bacteria, including *Bacillus*, *Clostridium*, *Staphylococcus*, and *Listeria*, possess a thicker peptidoglycan layer of the cell-wall structure, which render them with higher resistance toward pressure than Gram-negative bacteria. In brief, a 10 min high pressure exposure under 300–400 MPa at 25 °C is enough for the complete inactivation of the vegetative Gram-negative bacteria cells, but it requires a pressure of 500–600 MPa to efficiently inactivate Gram-positive bacteria (Chawla et al. 2011; Li et al. 2020a). Bacterial spores have special structures, such as a thick spore coat and cortex, which makes them extremely pressure-resistant at room temperature, and they can withstand pressure of even above 1200 MPa (Patterson et al. 2011; Margosch et al. 2006; Ahn et al. 2007). Therefore, although the pressure of 400–800 MPa can effectively kill the vegetative cells of foodborne pathogens, it is still difficult to achieve a complete microbial inactivation by an individual HPP treatment (Torres and Velazquez 2008). The decreasing storage temperature, water activity, or pH are usually used to inhibit the growth of bacterial spores in HPP-treated products in the storage (Buerman et al. 2020).

In recent years, the inactivation of spores has become an important research topic with respect to HPP. HPP inactivates spores in two stages. In the first stage, moderate high pressure induces spore germination, and the resistance decreases rapidly. Then the germinated spores inactivated as followed. Some studies have found that the moderate-pressure (100–400 MPa) and short time (<5 min) treatments can activate the nutrient-activated receptors (GerA, GerB, GerC, GerK, etc.) on the surface of the spore inner membrane and promote the germination (Aldrete-Tapia and Torres 2020; Doona et al. 2014). With the release of sodium, potassium, and hydrogen ions, and external flow of dipicolinic acid (DPA) from the spores, as well as the hydrolysis of large peptidoglycan (PG) cortex, a series of biophysical events occurred. The degradation of the PG cortex is of importance to complete spore germination, because it eliminates the physical limitation of the expansion of the spore core, and makes H$_2$O to enter the core to initiate enzyme activity, metabolism, macromolecular synthesis, and spore growth (Paredes-Sabja et al. 2007). Based on this phenomenon, the inactivation of spore by HPP pulsing treatment was proposed (Ahn et al. 2007). In addition to spores, the pressure-resistant cells (Mota et al. 2013), the subpopulation cells in the viable but nonculturable (VBN) cells or sublethally damaged states (Wu et al. 2020) may cause great risk to food safety and challenges of HPP application. The hurdle technology combining HPP and other external environmental factors may enhance the inactivation effect of microorganisms by eliminating stress-tolerant subpopulation in sublethally injured and VBN states, as well as inhibit their recovery into the normal state (See Sects. 8.3 and 8.4 for more details on the mechanisms).
8.2.2 Process Parameters

The process parameters (holding time, process temperature, pressure level, and pressurization/depressurization rate and mode) pose impacts on the HPP’s microbial inactivation efficacy (Smelt et al. 2002; Mills et al. 1998).

In general, the sterilization effect of HPP on vegetative cells of bacteria is enhanced with the increase of exposure time, the applied pressure, and treatment temperature (Chauhan et al. 2017; Wuytack et al. 2002; Wang et al. 2013; Hiremath and Ramaswamy 2012; Smelt et al. 2002; Mills et al. 1998; Serment-Moreno et al. 2017). Wuytack et al. (2002) estimated the tolerant capacity to HPP exhibited by several Gram-negative bacteria (e.g., *E. coli* MG1655, *Shigella flexneri*, *Salmonella Typhimurium*, *Yersinia enterocolitica*) and Gram-positive bacteria (e.g., *S. aureus*, *Lactobacillus plantarum*, *Listeria innocua*) and found that the pressures of below 200 MPa had less sterilization effects, but most of these bacteria were not resistant to pressures at or above 400 MPa. A 15 min-HPP treatment at 400 MPa at 25 °C could cause an approximately 5-log decrease in *L. dextranicum* and *Y. enterocolitica* (Wuytack et al. 2002). After treated at 400 MPa for 10 min, the number of *E. coli* O157:H7 in the mango juice was decreased by 6 logs, while complete inactivation was achieved after HPP treatment of 500 and 550 MPa for 1 min (Hiremath and Ramaswamy 2012). These researchers also observed that *E. coli* O157:H7 did not regrow when stored at 4, 12, or 20 °C for 28 days after 400 MPa of HPP treatment for 24 min.

For spores, due to the extreme pressure resistance, the treatment temperatures of HPP are required to be enhanced when exposed to the bacterial spores. For instance, the spores of 3 *Bacillus* strains (*B. licheniformis* IAM1341, *B. subtilis* IAM12118, and *B. stearothermophilus* IAM12043) cannot be inactivated when exposed to a 40-min HPP treatment with a pressure of 980 MPa at room temperature, and the spores of *B. coagulans* IAM1194 even were activated (Nakayama et al. 1996). Nevertheless, combing temperatures above 50 °C with pressure treatments over 400 MPa can be effective (Paredes-Sabja et al. 2007). Stewart et al. (2000) found a 15-min HPP treatment of 404 MPa at 70 °C decreased *B. subtilis* spores by 5 logs when compared with the reduction of less than 0.5 log caused by the same HPP exposure at 25 °C. Therefore, the pressure-assisted thermal processing (PATP) is considered as a promising hurdle method to inactivate spores, primarily processing samples with a high pressure of over 600 MPa combined with heat treatment at 100 °C (Valdez-Fragoso et al. 2010). Although many research reports have indicated that PATP technology is able to effectively kill the bacteria in the form of spores, other studies have also shown that PATP can significantly compromise the food quality. Under the conditions of commercial production, the technical and economic feasibility of using PATP to kill spores should be carefully considered.

Moreover, the pressurization/depressurization rate and mode employed during pressure treatment also affect the sterilization effect (Zhang et al. 2020). A study has reported that when the exposure time and applied pressure are fixed, a pressurization and depressurization at a rate of 400 MPa/min and 50 MPa/min, respectively,
resulted in higher inactivation of *Listeria innocua* than a pressurization and depressurization at a rate of 50 MPa/min and 400 MPa/min, respectively (Herdegen 1998). Chapleau et al. (2006) also demonstrated that the pressurization at 60 MPa/min and depressurization at 300 MPa/min led to a lower reduction of *L. monocytogenes* and *S. Typhimurium* in comparison to a high pressurization rate of 600 MPa/min and rapid depressurization of 15,000 MPa/min with comparable applied pressure value and exposure time. Further research proved that under a fixed pressurization rate, a faster rate for depressurization causes water to produce greater adiabatic expansion impulses and therefore lead to greater damages to microorganisms and higher inactivation (Furukawa et al. 2010). Similarly, under the same depressurization rate, it is found that HPP with a fast pressurization rate (120 MPa/min) yielded a higher reduction level of the total aerobic bacteria (TAB) than HPP treatment with a slow pressurization rate (60 MPa/min) in the purple sweet potato nectar (Wang et al. 2013). Therefore, the enhancement of pressurization/depressurization rate accelerates the HPP-induced microbial inactivation. In addition, Wang et al. (2013) also observed that compared with the stepwise pressurization under an identically applied pressure and exposure time to high pressure, the linear pressurization exhibited a significantly stronger antimicrobial performance. This result may be caused by the following reasons: the cellular structures of the microbial cells were damaged continuously and accumulatively by the linear pressurization, while the stepwise pressurization allows the microorganisms to develop the resistance to HPP, therefore resulting in lower inactivation efficacy.

### 8.2.3 Other Factors

External environmental factors can also significantly affect the inactivation effect of HPP on foodborne pathogens. These factors may include the culture temperature, pH, metal ions, and bacteriostatic agents.

The environment in which microorganisms grow also significantly affects their pressure sensitivity. For example, in the exponential period of growth at 10 °C, *E. coli* NCTC 8164 exhibited lower pressure sensitivity, but as the growth temperature increased to 45 °C, its pressure sensitivity increased accordingly. In the stable period of growth at 10 °C, *E. coli* exhibited a pressure sensitivity, but it decreased as the growth temperature increased, reaching a minimum at 30–37 °C, and it subsequently began to increase (Casadei et al. 2002). This might be attributed to the increase in the membrane fluidity of microorganisms as the temperature increased. One of the important factors for HPP to inactivate microorganisms is the pH of food. The inactivation effect of HPP becomes stronger with the decrease of pH. Moreover, the decrease in pH caused SLI cells to fail to recover during subsequent storage. In the study of Alpas et al. (2000), it is demonstrated that the HPP-induced reduction of several common foodborne pathogens (e.g., *E. coli* O157: H7, *S. aureus*, *S. enterica*, *L. monocytogenes*, and *S. Typhimurium*) was enhanced significantly by 1.2–3.9 logs when the pH of cell suspensions decreased from 6.5 to 4.5 by the addition of 2.1% citric acid or 1% lactic acid.
Another method for increasing the pressure sensitivity of foodborne pathogens is to add antibacterial agents to food, such as essential oils, lactoferrin, and lactoperoxidase systems (Yang et al. 2021). Essential oils are liquid aromatic oils, most of which are extracted from the plant residues and have good antiviral, antibacterial, antifungal, antitoxin, antiparasitic, and insecticidal properties (Mutlu-Ingok et al. 2020). Generally, it is believed that phenolic compounds are the main components of essential oils and accounted for their antibacterial functions (Burt 2004). Lactoferrin binds iron ions, and it has been reported that this function can inactivate some microorganisms (Del Olmo et al. 2012). Farnaud et al. (2004) demonstrated that the peptides of bovine lactoferrin hydrolyzed by pepsin could be used as penetrants to enhance the sensitivity of microorganisms to HPP processing. Further research of computer modeling revealed that the negative charges of lipopolysaccharide (LPS) reacted with positively charged residues of the peptides, which disrupt the structure of the outer membrane, promote the access of tryptophan residues to lipids, and accelerate hydrophobic interactions (Farnaud et al. 2004). The lactoperoxidase system is composed of lactoperoxidase, thiocyanate, and hydrogen peroxide, which can also improve the sterilization effect of pressure. Lactoperoxidase is a kind of oxidoreductase, which can catalyze the oxidation of thiocyanate induced by hydrogen peroxide and produce hypothiocyanite anion (OSCN\(^-\)) to exhibit the inactivation on microbial cells (Seifu et al. 2005). The lactoperoxidase systems can not only inhibit the reproduction of bacteria but also eliminate their metabolic activity. However, the application of these antibacterial agents in HPP is far from sufficient, and further research is warranted to optimize their utilization.

### 8.3 Cellular Alteration by HPP

HPP can effectively inactivate foodborne pathogens by influencing cell morphology and structure, changing cell membrane permeability and fluidity, denaturing enzymes or other important proteins, and even affecting the DNA of pathogens at a low temperature (Balasubramaniam et al. 2015). There are several aspects that may be associated with HPP-induced cellular alterations and physiological function damage.

#### 8.3.1 Effect of HPP on the Cell Morphology of Foodborne Pathogens

HPP can cause changes in the external morphology of foodborne pathogens, such as the formation of protrusions or depressions on the surface, as well as cell expansion, deformation and even rupture. The surface of untreated *L. monocytogenes* was
smooth, regular, and oval-shaped. However, after 400 MPa treatment for 10 min, the overall shape of the cells was destroyed, with protuberances or even ruptures being observed on the cell surface (Ritz et al. 2001). Normal \textit{L. mesenteroides} cells have regular beaded structures with smooth cell surfaces. After being treated at 35 °C and 500 MPa for 15 min, the beaded structure was destroyed. Some cell surfaces formed protuberances (Kaletunç et al. 2004). For \textit{L. monocytogenes}, the cell volume could increase by over three folds after a 600 MPa treatment (Tholozan et al. 2000). Pilavtepe-Çelik et al. (2008) found that the cell volumes of \textit{S. aureus} and \textit{E. coli} significantly increased after 400-MPa treatment. Spores of \textit{Aspergillus niger} were regular and rounded under normal conditions.

HPP can also lead to the denaturation of proteins and genetic materials within microbial cells. After 400 MPa treatment for 6 min, the cytoplasm exhibited obviously precipitated (Mohamed et al. 2012). The phenomenon of plasmolysis occurred in \textit{Lactobacillus viridescens} after 600 MPa treatment for 5 min, with the genetic material inside being fibrous (Park et al. 2001). Kaletunç et al. (2004) believed that HPP could destroy the cell membrane, leading to a decrease in Mg\textsuperscript{2+} concentration inside the cell, which would subsequently decrease the stability of genetic substances and cause the deposition of DNA.

\section*{8.3.2 Effect of HPP on the Cell Wall of Foodborne Pathogens}

HPP can also destroy the cell wall of foodborne pathogens. It is generally believed that the damages on the Gram-negative bacterial cell wall are the outer membrane (OM) through permeabilizing (Masschalck et al. 2003). The permeabilization is reversible during the process of compression, and when the treatment intensity is harsh enough, it tends to be irreversible as the structural components of OM are lost (Black et al. 2005). LPS in the OM works to inhibit the penetration of macromolecules and hydrophobic molecules (Kong et al. 2010). HPP may disrupt electrostatic interaction (EI) between negatively charged phospholipids or lipid A—the components of LPS and the divalent cations, which makes LPS, proteins, and porin in the OM to release (Gänzle and Liu 2015). There are two ways to achieve inactivation through a combination of HPP and natural antibacterial agents (NAs).

One way is breaking the shield of OM to NAs. HPP induces the release of LPS, causing the disruption of OM and failure to prevent the penetration by other molecules. NAs shielded by OM might pass through OM to reach PG, CM, or cytoplasm, thereby promoting the antibacterial activity against Gram-negative bacteria (Gayán et al. 2012). In other words, the OM permeability induced by HPP may make Gram-negative bacteria sensitive toward NAs, including the enzymes for PG degradation and bacteriocins for damaging on CM (Gänzle and Liu 2015).

The other way is that NAs might cause more serious damages on OM. Cationic natural antimicrobials (CNAs) (e.g., chitosan) may also disrupt EI by replacing divalent cations, induce OM components to be released, and make OM permeabilized (Kong et al. 2010; Jenssen and Hancock 2009). Regarding the
Gram-positive bacteria, it is indicated that HPP may trigger autolysin activity through CM disturbance and cell energy consumption, thereby hydrolyzing PG and cell lysis (Masschalck et al. 2003; Kalchayanand et al. 2002; Malone et al. 2002). Additionally, CNAs are also able to interact with teichoic acid of cell wall to induce the subsequent autolysin (Masschalck et al. 2003).

8.3.3 Effect of HPP on the Cell Membrane of Foodborne Pathogens

Membranes are an important component of microbial cells and primarily consist of a phospholipid bimolecular layer and bioactive membrane proteins (Deamer and Dworkin 2005). Membranes rely on the hydrogen bonds and hydrophobic interactions between molecules to maintain a stable structure and function to protect cells, convert energy, transport substances, deliver information, and participate in recognition (Lodish 2008). Studies have reported that HPP can destroy membrane fluidity and integrity and separate membrane proteins from the membrane, destroying selective permeability and other physiological functions of the cell membrane (Considine et al. 2008). The interference of HPP with the membrane permeability of foodborne pathogens is different, depending on the cell-wall structure. Mañas and Mackey (2004) found that 99.9% of *E. coli* died after treatment with 600 MPa for 8 min. The cell membrane was surrounded by protuberances inside and outside, and its plasmolysis ability disappeared. The intracellular substances, including nucleic acids and ions (K⁺, Mn²⁺, and Mg²⁺) subsequently flowed out of cells. Meanwhile, the propidium iodide (PI) can bind to DNA across the cell membrane, which shows the damages in the membrane damage to cause the death of microbial cells. All of these results fully indicated that the *E. coli* cell membrane was destroyed (Mañas and Mackey 2004). The concentration of K⁺ in the cytoplasm of *L. monocytogenes* and *S. Typhimurium* decreased from 300 mmol/L to less than 5 mmol/L after treatment with 400 MPa, indicating that the ability of the cell membrane to maintain ion balance was destroyed (Tholozan et al. 2000). Klotz et al. (2010) found that the *E. coli* membrane was damaged and that a large amount of proteins leaked from the cell after HPP. However, by comparing the fluorescence intensity of PI entering the cell before and after pressure treatment, some cells were observed to take up PI during the exposure to the pressure instead of post the decompression, which demonstrated the capacity of bacteria to repair the cell membrane damage caused by HPP.

Membrane proteins are an important unit in the cell membrane responsible for performing the functions of material transportation, energy conversion, and signal recognition inside and outside the cells (Chen et al. 2012; Ryu et al. 2019). However, HPP can lead to a decrease in membrane protein activity or the separation from the membrane. The Na⁺, K⁺-ATPase is a Na⁺, K⁺-pump across the lipid membrane that catalyzes the active transportation of Na⁺ outward and K⁺ inward using the energy
provided by ATP hydrolysis (Jørgensen 1982). More than 200 MPa of pressure could lead to a separation of the α and β subunits of Na+, K+-ATPase (Kato et al. 2002). The conversion of ATP to ADP in such prokaryotes as E. coli is catalyzed by F₁F₀-ATP enzymes located on the cell membrane (Lodish 2008). Wouters et al. (1998) found that the activity of F₁F₀-ATP ATPase in Lactobacillus plantarum began to decrease at 250 MPa. The activity was decreased by 80% when the pressure increased to 350-MPa for 10 min. Tholozan et al. (2000) found that the intracellular ATP concentration in L. monocytogenes and S. Typhimurium decreased by more than 90% and 50%, respectively, after treatment with 400 MPa for 10 min. Treatment with 600 MPa could change the structure of photosynthetic proteins on the cell membranes of Rhodobacter sphaeroides and Pseudomonas acidophilus, resulting in the separation of pigment and protein and the loss of photosynthetic ability (Gall et al. 2003). Ritz et al. (2000) found that 10 of 12 membrane proteins on the cell membrane, except for the major proteins LamB (44 kDa) and OmpA (35 kDa), were separated from the membrane of S. Typhimurium after 400 MPa and 600 MPa treatments.

8.3.4 Effect of HPP on the Internal Structure of Foodborne Pathogens

HPP could induce internal structural changes of foodborne pathogens, such as the ribosome dissociation and cytoskeleton depolymerization (Ishii et al. 2004; Niven et al. 1999). Specifically, 55 MPa treatment resulted in the separation of the 50S and 30S subunits of the ribosomes within B. subtilis and E. coli (Cocito 1978; Infante et al. 1982). Treatment with 70 MPa caused the E. coli polypeptide chain to separate from the ribosome and terminate polypeptide synthesis (Gross et al. 1993). Niven et al. (1999) found that the treatment with 250 MPa for 20 min resulted in E. coli ribosomal degeneration.

The cytoskeleton describes the structure of the protein fiber network in microbial cells; this structure contributes to the morphological properties of cells, sustaining the extracellular forces, facilitating the cell division, and supporting the order of cell internal structure. The cytoskeleton of E. coli and other prokaryotes consists of three kinds of proteins, namely, FtsZ, MreB, and CreS (Wang et al. 2016). FtsZ can assemble at the cell division site to form a circular structure, through which it participates in cell division. MreB can form a spiral filamentous structure to maintain the cell morphology. CreS is an intermediate filament-like protein found only in Caulobacter crescentus. CreS forms a curved filamentous or spiral filamentous structure under the membrane of the concave cell, which plays a significant role in maintaining C. crescentus cell morphology (Ritz et al. 2001). Ishii et al. (2004) found that the FtsZ synthesis in E. coli was inhibited under 40-MPa treatment for 30 min, preventing the cytoskeleton formation. E. coli cells were filamentous and unable to form colonies. After a 30 MPa treatment, the production of FtsZ in E. coli...
decreased. These proteins were partially denatured and depolymerized, exhibiting a length of less than 3 μm (the length of the FtsZ protein fiber was 5 μm at 0.1 MPa). The synthesis of FtsZ was completely inhibited when the pressure increased to 50 MPa. However, when the pressure returned to atmospheric pressure, the ability for the synthesis of FtsZ was restored in E. coli, accompanied with the recovery of cell divisions (Ishii et al. 2004).

8.3.5 Effect of HPP on Enzyme Activity in Foodborne Pathogens

HPP can reduce the activity of enzymes in microbial cells and even completely inactivate enzymes (Hendrickx et al. 1998). Esterase activity is a sign of vigorous metabolism in microbial cells. The esterase activity of L. monocytogenes decreased by 70% after treatment with 400 MPa/10 min (Ritz et al. 2001). The 23 °C/448 MPa/30 s treatment completely inactivated lipase, cystine arylamidase (Hendrickx et al. 1998), and chymotrypsin and resulted in moderate inactivation of valine arylamidase, esterase, esterase lipase, leucine arylamidase, and α-glucosidase within S. cerevisiae (Bang and Chung 2010). Under the same HPP conditions, the activities of esterase, lipase, cystine arylamidase, valine arylamidase, trypsin, α-glucosidase, and β-glucuronidase within E. coli were totally lost, while leucine arylamidase was partially passivated. HorA is a transporter in L. plantarum that is encoded by plasmids and relies on ATP to provide energy, which could improve the tolerance of cells to the ethanol and carbon dioxide in beer (Ulmer et al. 2002). Simpson and Gilmour (1997) found that HPP at 550 MPa for 15 min at ambient temperature efficiently inactivated several metabolic enzymes, such as glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, and other metabolic enzymes in L. monocytogenes. Chilukuri et al. (1997) observed that an 87-MPa treatment for 10 min clearly inhibited the DNA supercoiling activity of E. coli DNA gyrase.

8.3.6 Physiological Function Damage by HPP

Furthermore, HPP could also damage the physiological functions. HPP may change the spatial structure of proteins through breaking the non-covalent bonds such as hydrophobic or electrostatic interactions. Subsequently, the irreversible damages on the active centers of proteins probably lead to the loss of proteins functions (Silva et al. 2014). In addition, HHP could result in the subunit dissociation of ribosomes and the weakened amino acid transportation ability of protein, thus inhibiting protein synthesis and its supplements by the newborn ones (Gänzle and Liu 2015). The inactivation of related enzymes by HHP would also damage the DNA replication and transcription (Huang et al. 2014). Additionally, this damaging effects might be
accelerated and aggravated when HPP is adopted with the elevated temperature. Subsequently, the physiological activities of microbial cells are negatively affected under the exposure of HPP, indicating that HPP can be used to be combined with other techniques to strengthen the damage.

HPP can cause respiratory interference of cell and thus affect physiological functions. The enzymes with trans-membrane regions responsible for the respiratory chains may have been denatured after HPP treatment, thus inhibiting the ATP synthesis (Huang et al. 2014). Additionally, the proton motive force (PMF) may be disrupted through the CM permeabilization caused by HPP, resulting in the inhibition of ATP synthesis and energy production (Huang et al. 2014). Exogenous proton exchangers including lactate (Mani-López et al. 2012) or phenols (Rao et al. 2019) can carry protons out of the cell cytoplasm, which subsequently assist the damages on PMF and accelerate the interference in the respiratory.

HPP can break the pH homeostasis of cell to affect physiological functions. The membrane proteins denatured by HPP regulate the flow of protons through cell membrane, e.g., H+-ATPase which transports protons from the cytoplasm of bacterial cells (Garcia-Gonzalez et al. 2007). In the presence of extracellular acidifier including CO₂ and some microbial or plant-derived organic acids (docosahexaenoic acid), the microbial cells with CM permeabilization may fail to survival under the acidic environment, resulting in the cytoplasmic acidification, the key enzyme (endonucleases) inactivation as well as the cellular metabolism inhibition (Rendueles et al. 2011).

What’s more, HPP could facilitate the dissolution and dissociation of CO₂, reducing the pH within microbial cells (Wang et al. 2010). Consequently, the microbial cells can be inactivated through the disorder in the intracellular pH homeostasis.

HPP can break the redox homeostasis of cell to affect physiological functions. The HPP-induced damages in the CM could further cause the imbalance in metabolism, followed by an emergence of reactive oxygen species (ROS) burst. The ROS burst goes beyond the capacity of HPP-treated microbial cells to maintain the redox homeostasis, resulting in the oxidative stress generation (Aertsen et al. 2005). It is also found that HPP could cause iron to be released from Fe-S clusters and induce the occurrence of Fenton reaction to produce hydroxyl free radicals (Malone et al. 2006).

### 8.4 Regulatory Network for Pressure Resistance of Foodborne Pathogens

#### 8.4.1 Transcriptome

##### 8.4.1.1 Stress Resistance Related Genes

HPP affects the expression of stress resistance genes associated with heat shock proteins (HSPs), cold shock proteins (CSPs), and the ribosomal protein synthesis in
microorganisms. The aforementioned proteins can protect or repair the microbial cells by HPP and thus increase the viability (Malone et al. 2006; Fernandes et al. 2004; Bowman et al. 2008). HPP can cause oxidative damage to microbial cells. Aertsen et al. (2005) found that HPP treatment at 100–400 MPa caused oxidative stress in E. coli cells, which was positively correlated with the pressure level. Over 400 MPa is required for the enhancement of oxidation stress on the cells in stationary phase. When the pressure was higher than 200 MPa, the cells began to be killed. In contrast, regarding the microbial cells in the exponential phase, up to 150 MPa was efficient to enhance the oxidative stress, and then reduced again, and the number of cells also increases. It is found that the HPP followed by an anaerobic incubation contributed to the recovery of E. coli MG1655, while the E. coli strain defected in katE, sodAB, katF, oxyR, and soxS with the susceptible to oxidative stress were observed to exhibit higher sensitivity to pressure stress when compared with the wild-type counterparts. Charoenwong et al. (2011) found that the strains (E. coli BW25113) defected in DNA repair enzyme genes (polA, recA, and xthA) also exhibited sensitivity to HPP, strains with mutations in polA, recA, and xthA were all significantly more pressure sensitive (P < 0.05) than the parent strain, with log surviving fractions of, −4.93 to 5.48, and −4.90, respectively, compared with −3.50 for the parent. And the hydroxyl radicals generated by various reactive oxides (e.g., hydroperoxides) induced by HPP cause DNA damage which double-strand breaks in DNA were believed to be the inducing signal. The effect of HPP on the expression of stress resistance genes in microorganisms is shown in Table 8.2.

8.4.1.2 Cell Membrane and Cell Wall Related Genes

HPP can damage the cell membrane and cell wall of microorganisms and affect the expression of related genes. These genes mainly involved in the teichoic acid, peptidoglycan, lipoprotein and other extracellular membrane components, and the transport of various lipoproteins (Syed et al. 2016; Charoenwong et al. 2011; Robey et al. 2001). The effects of HPP on the expression of genes associated with the cell membrane/wall genes are shown in Table 8.3.

8.4.1.3 Information Processing and Storage Genes

HHP can impede the DNA replication and transcription process, including dissociation of the DNA replication apparatus, thereby damaging the helix structure and inhibiting the formation of DNA-RNA (Bowman et al. 2008). HPP was also observed to open the E. coli DNA double helix, triggering the SOS reaction (Schlesinger 1990). After HPP treatment, the expression of microbes and information processing and storage genes also changed. These genes included a variety of DNA polymerases, ligase synthesis genes, and many genes related to stabilizing and repairing DNA damage. These changes represented microbial responses to DNA damage. HPP also resulted in the upregulation of genes related to translation and ribosomes, which encode ribosomal and translation-related proteins, as well as
<table>
<thead>
<tr>
<th>Name</th>
<th>Gene function</th>
<th>Pressure/MPa treatment conditions</th>
<th>Fold changes in expression level</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>ibpA</em></td>
<td>Heat shock proteins (Hsps)</td>
<td>100 MPa</td>
<td>+1.9</td>
<td>Atlung and Ingmer (1997)</td>
</tr>
<tr>
<td><em>cspA</em></td>
<td>CspA, the major cold-shock protein of <em>Escherichia coli</em>, is dramatically induced during the cold-shock response</td>
<td>100 MPa</td>
<td>+3.9</td>
<td>Atlung and Ingmer (1997)</td>
</tr>
<tr>
<td><em>pphA</em></td>
<td>Serine/threonine-specific protein phosphatase 1, signals protein misfolding</td>
<td>Tryptose broth, 35 °C, 200 MPa, 15 min</td>
<td>+2</td>
<td>Malone et al. (2006)</td>
</tr>
<tr>
<td><em>dps</em></td>
<td>Stress response DNA-binding protein</td>
<td>Tryptose broth, 35 °C, 200 MPa, 15 min</td>
<td>−2.5</td>
<td>Malone et al. (2006)</td>
</tr>
<tr>
<td><em>nrd (nrdI, nrdH, nrdE, nrdF)</em></td>
<td>Stimulates ribonucleotide reduction</td>
<td>Tryptose broth, 35 °C, 200 MPa, 15 min</td>
<td>+1 ~ 2.2</td>
<td>Malone et al. (2006)</td>
</tr>
<tr>
<td><em>suf</em></td>
<td>SUF system</td>
<td>Tryptose broth, 35 °C, 200 MPa, 15 min</td>
<td>−1.2 ~ 1.6</td>
<td>Malone et al. (2006)</td>
</tr>
<tr>
<td><em>grx, trx (trxA, grxB, trxC)</em></td>
<td>Thioredoxin reductase, FAD/NAD(P) binding</td>
<td>Tryptose broth, 35 °C, 200 MPa, 15 min</td>
<td>1.4 ~ 2.2</td>
<td>Malone et al. (2006)</td>
</tr>
<tr>
<td><em>sucC, sucD</em></td>
<td>Encodes subunits of the succinyl-CoA synthetase</td>
<td>Lysogeny broth medium, 37 °C, 400 MPa, 15 min</td>
<td>Upregulated</td>
<td>Gayán et al. (2019)</td>
</tr>
<tr>
<td><em>sucA, sucB</em></td>
<td>Subunits of the 2-oxoglutarate dehydrogenase complex</td>
<td>Lysogeny broth medium, 37 °C, 400 MPa, 15 min</td>
<td>Downregulated</td>
<td>Gayán et al. (2019)</td>
</tr>
<tr>
<td><strong>Listeria. monocytogenes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L13, L19</td>
<td>50S ribosomal protein</td>
<td>Tryptone soy-yeast extract broth and TSYE agar, 25 °C, 400/600 MPa, 5/15 min</td>
<td>+2.91 ~ 4.7</td>
<td>Bowman et al. (2008)</td>
</tr>
<tr>
<td><em>cspB</em></td>
<td>Cold shock proteins. The expression of other genes encoding termination and anti-termination related proteins is also increased.</td>
<td>Tryptone soy-yeast extract broth and TSYE agar, 25 °C, 400/600 MPa, 5/15 min</td>
<td>+8.6</td>
<td>Bowman et al. (2008)</td>
</tr>
<tr>
<td><em>cspL</em></td>
<td></td>
<td>Tryptone soy-yeast extract broth and TSYE agar, 25 °C, 400/600 MPa, 5/15 min</td>
<td>+3.53</td>
<td>Bowman et al. (2008)</td>
</tr>
</tbody>
</table>

(continued)
proteins that enable the function and stability of tRNA and rRNA. These changes may be related to the depolymerization of ribosomes and the inhibition of translation caused by HPP (Aertsen et al. 2005; Bowman et al. 2008). The impact of HPP on information storage and processing gene expression is shown in Table 8.4.

8.4.1.4 Other Functional Genes

HPP can also cause changes in genes related to physiological functions, including motility, metabolism, cell division, and protein secretion (Malone et al. 2006). These changes can improve the motility of microorganisms, reduce the level of energy metabolism, maintain cell morphology, and help microorganisms survive adverse living conditions (Table 8.5) (Duru et al. 2021; Bowman et al. 2008). Additionally, Nikparvar et al. (2021) establish a HPP regulation database of *L. monocytogenes* in the transcriptional level by combining the transcriptomics results of *L. monocytogenes* (ScottA) exposed to an 8-min HPP treatment (400 MPa and 8 °C) with currently existing studies in order to describe the associated transcription factors and their corresponding target genes. The response of *L. monocytogenes* to the high pressure through three regulation phases: during the initial 10 min, they can still survive. After HPP exposure for 1 h, they initiated the repair system. And they might regrow after HPP treatment for 6 h.

8.4.2 Proteome

HPP can induce microorganisms to synthesize a series of special pressure-induced proteins (PIPs) (Welch et al. 1993). PIP mainly includes HSPs, CSPs, ribosomal
Table 8.3 Effect of HPP on microbial cell wall- and cell membrane-related genes

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene function</th>
<th>Pressure/MPa</th>
<th>Fold changes in expression level</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td><em>gcaD, glmS, murACEG, mraY</em></td>
<td>The biosynthesis of cell-wall polymers, particularly peptidoglycan.</td>
<td>Tryptone-yeast extract broth and TSYE agar, 25 °C, 400/600 MPa, 5/15 min</td>
<td>+2 ~ 4.4</td>
</tr>
<tr>
<td></td>
<td><em>Upps homologous genes</em></td>
<td>A cell-wall component carrier lipid protein required for the assembly of peptidoglycan, teichoic acid and other cell-wall components in bacteria</td>
<td>Tryptone-yeast extract broth and TSYE agar, 25 °C, 400/600 MPa, 5/15 min</td>
<td>+3 ~ 4.7</td>
</tr>
<tr>
<td></td>
<td><em>lmo2194–2196</em></td>
<td>Opp in the oligopeptide transporter system.</td>
<td>Tryptone-yeast extract broth and TSYE agar, 25 °C, 400/600 MPa, 5/15 min</td>
<td>&gt;+1</td>
</tr>
<tr>
<td></td>
<td><em>murG, murC, and pbp2A</em></td>
<td>Peptidoglycan synthesis</td>
<td>Tryptic soy broth, 37 °C, 200/400 MPa, 8 min</td>
<td>Upregulated</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em></td>
<td><em>rpoE</em></td>
<td>The folding of peptides on the outer membrane of bacterial cells and the biosynthesis and transportation of lipopolysaccharides; <em>rpoE</em> gene-deficient <em>E. coli</em> is extremely pressure sensitive.</td>
<td>TSB, 37 °C, 600 MPa, 8 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>rpoS</em></td>
<td>Regulate the expression of membrane lipoprotein genes (<em>cfa, osmB, osmC, osmE, osmY, ybaY</em>).</td>
<td>TSB, 37 °C, 600 MPa, 8 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>rbsK</em></td>
<td>Ribokinase (involved in ribose catabolism)</td>
<td>Lysogeny broth medium, 37 °C, 400 MPa, 15 min</td>
</tr>
<tr>
<td></td>
<td><em>Saccharomyces cerevisiae</em></td>
<td><em>ERG25</em></td>
<td>Ergosterol biosynthesis</td>
<td>YEPD, 28 °C, 200 MPa, 30 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>OLE1</em></td>
<td>Stearoyl-CoA desaturase</td>
<td>YEPD, 28 °C, 200 MPa, 30 min</td>
</tr>
</tbody>
</table>

(continued)

Among PIP proteins, HSPs and CSPs are relatively important and well-studied. HSPs are an important class of proteins involved in the stress response of microorganisms (Jaenicke et al. 1988). These proteins can be used as the chaperones to bind to the newly synthesized, misfolded, and damaged polypeptides to restore or maintain their normal conformation (Schlesinger 1990). CSPs are a series of proteins synthesized by microorganisms under the low-temperature conditions (Uh et al. 2010). The main function of CSPs is to bind to mRNA as the chaperones, subsequently preventing the formation of mRNA secondary structure, improving the catalytic efficiency of ribonuclease, and initiating the translation process (Kondo and Yumura 2020). E. coli could produce 55 kinds of PIPs after 55 MPa, 30 min treatment, most of which were CSPs and HSPs (Welch et al. 1993). A pressure of 50 MPa (65°C, 10 h) was observed to lead to the production of various HSPs with a molecular mass ranging from 38 to 70 kDa by Methanococcus thermolithotrophicus (Jaenicke et al. 1988). Li et al. (2020a, b) induced E. coli to produce a large amount of HSPs by heat treatment (100°C, 5 min) and found that the pressure resistance (400 MPa, 15 min) of these cells was significantly improved. Lactobacillus sanfranciscensis exhibited an increase in the production of CSPs and ribokinase, while a decrease in GMP synthase production was observed under a 150–200 MPa exposure (Drews et al. 2002). CSP1 and CSP2 synthesis in L. monocytogenes was increased by 2–3.5 times with a 10-min HPP exposure of 100–200 MPa (Wemekamp-Kamphuis et al. 2002). The synthesis of CSP1 and CSP3 in L. monocytogenes cells cultured at 10°C was increased by 3.5-fold and 10-fold, respectively, compared with that at 37°C, and a 100-fold increase in the survival rate of L. monocytogenes cells was observed after HPP treatment (Wemekamp-Kamphuis et al. 2002).

Table 8.3 (continued)

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene function</th>
<th>Pressure/MPa</th>
<th>Fold changes in expression level</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermococcus barophilus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TERMP_00819</td>
<td>Histone</td>
<td>Thermococcales-rich medium, 85°C, 70 MPa</td>
<td>−0.94</td>
<td>Vannier et al. (2015)</td>
</tr>
<tr>
<td>TERMP_01313</td>
<td>Acetylpolyamine aminohydrolase</td>
<td>Thermococcales-rich medium, 85°C, 70 MPa</td>
<td>−1.05</td>
<td>Vannier et al. (2015)</td>
</tr>
<tr>
<td>Thermococcus kodakarenensis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TK2070</td>
<td>Cytosolic NiFe-hydrogenase and delta subunit</td>
<td>Thermococcales-rich medium, 85°C, 25 MPa</td>
<td>−2.27</td>
<td>Vannier et al. (2015)</td>
</tr>
</tbody>
</table>
### Table 8.4  Effects of HPP on information storage and processing gene expression

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene function</th>
<th>Pressure/MPa</th>
<th>Fold changes in expression level</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hdfR</td>
<td>DNA-binding transcriptional dual regulator HdfR (H-NS-dependent flhDC regulator). Repressor for flhDC operon (encoding the master regulator for flagellar biosynthesis and swarming migration) and activator of the gltBDF operon (encoding the glutamate synthase)</td>
<td>Lysogeny broth medium, 37 °C, 400 MPa, 15 min</td>
<td>Upregulated</td>
<td>Gayán et al. (2019)</td>
</tr>
<tr>
<td>crl</td>
<td>σ38(rpoS) RNA polymerase holoenzyme assembly factor Crl</td>
<td>Lysogeny broth medium, 37 °C, 400 MPa, 15 min</td>
<td>Upregulated</td>
<td>Gayán et al. (2019)</td>
</tr>
<tr>
<td>rbsR</td>
<td>DNA-binding transcriptional dual regulator RbsR. Repressor for rbs operon (involved in ribose transport and catabolism)</td>
<td>Lysogeny broth medium, 37 °C, 400 MPa, 15 min</td>
<td>Upregulated</td>
<td>Gayán et al. (2019)</td>
</tr>
<tr>
<td><strong>Listeria monocytogenes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hup, flaR</td>
<td>Histone-like proteins that may affect DNA topology</td>
<td>Tryptone soy-yeast extract broth and TSYE agar, 25 °C, 400/600 MPa, 5/15 min</td>
<td>+1.7, +4.0</td>
<td>Bowman et al. (2008)</td>
</tr>
<tr>
<td>lmo2606, lmo2560</td>
<td>RNA polymerase α-subunit and Delta factor</td>
<td>Tryptone soy-yeast extract broth and TSYE agar, 25 °C, 400/600 MPa, 5/15 min</td>
<td>+2.2, +3.2</td>
<td>Bowman et al. (2008)</td>
</tr>
<tr>
<td>drm, pdp, pnp, guaABuppp, udk, smbA, lmo1939, lmo1463</td>
<td>Nucleotide interconversions (drm, pdp, pnp, guaAB) and salvage reactions (uppp, udk, smbA, lmo1939, lmo1463)</td>
<td>Tryptone soy-yeast extract broth and TSYE agar, 25 °C, 400/600 MPa, 5/15 min</td>
<td>Downregulated</td>
<td>Bowman et al. (2008)</td>
</tr>
</tbody>
</table>

(continued)
Table 8.4 (continued)

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene function</th>
<th>Pressure/MPa</th>
<th>Fold changes in expression level</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>hrcA</em></td>
<td>Heat-inducible transcription repressor HrcA</td>
<td>Tryptic soy broth, 37 °C, 200/400 MPa, 8 min</td>
<td>Upregulated</td>
<td>Duru et al. (2021)</td>
</tr>
<tr>
<td><em>rpoD</em></td>
<td>RNA polymerase sigma factor RpoD</td>
<td>Tryptic soy broth, 37 °C, 200/400 MPa, 8 min</td>
<td>Upregulated</td>
<td>Duru et al. (2021)</td>
</tr>
<tr>
<td><em>hpf</em></td>
<td>Ribosome hibernation promoting factor</td>
<td>Tryptic soy broth, 37 °C, 200/400 MPa, 8 min</td>
<td>Upregulated</td>
<td>Duru et al. (2021)</td>
</tr>
</tbody>
</table>

*Thermococcus barophilus*

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene function</th>
<th>Pressure/MPa</th>
<th>Fold changes in expression level</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TERMP_01033</td>
<td>Alpha-mannosidase</td>
<td>Thermococcales-rich medium, 85 °C, 70 MPa</td>
<td>−1.21</td>
<td>Vannier et al. (2015)</td>
</tr>
<tr>
<td>TERMP_01837</td>
<td>Sugar ABC transporter, permease</td>
<td>Thermococcales-rich medium, 85 °C, 70 MPa</td>
<td>+1.98</td>
<td>Vannier et al. (2015)</td>
</tr>
</tbody>
</table>

*Thermococcus kodakarenensis*

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene function</th>
<th>Pressure/MPa</th>
<th>Fold changes in expression level</th>
<th>References</th>
</tr>
</thead>
</table>

Jofré et al. (2007) reported that a pressure of 400 MPa can make *L. monocytogenes, Lactobacillus sakei*, and *Enterococcus faecium* to produce PIPS, such as ribosomal proteins, transcription factors, and DNA-MNA polymerase, which could promote the translation of microbial genes and stabilize the conformation of cellular proteins (Lodish 2008).

### 8.5 Conclusions

This chapter summarizes the primary factors affecting the actions of HPP on the inactivation of foodborne microorganisms and introduces the mechanism by which HPP inactivates foodborne microorganisms at the cell structure, physiological functions, proteome, and transcriptome levels. HPP can effectively achieve microbial inactivation in food at low working temperatures. However, many bacterial species have the resistance toward pressure. The hurdle treatment of HPP and other factors, including the mild heat, could lead to synergistic or additive effects on the microbial inactivation, which could reduce the formation of pressure-resistant subpopulation, inhibit the induction of microbial cells into SLI or VBNC states, and retard the recovery from the damages or resuscitation into culturable state.
**Table 8.5** Effect of HPP on other microbial functional genes

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene function</th>
<th>Pressure/MPa</th>
<th>Fold changes in expression level</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>otsA/B</td>
<td>The synthesis of trehalose</td>
<td>Tryptose broth, 35 °C, 200 MPa, 15 min</td>
<td>+2.1</td>
<td>Malone et al. (2006)</td>
</tr>
<tr>
<td><strong>Listeria monocytogenes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyoABCD</td>
<td>Cytochrome bo terminal oxidase</td>
<td>Tryptone soy-yeast extract broth and TSYE agar, 25 °C, 400/600 MPa, 5/15 min</td>
<td>Downregulated</td>
<td>Bowman et al. (2008)</td>
</tr>
<tr>
<td>lmo0088–0092, lmo2528–2530</td>
<td>F$_1$F$_0$-ATPase</td>
<td>Tryptone soy-yeast extract broth and TSYE agar, 25 °C, 400/600 MPa, 5/15 min</td>
<td>Downregulated</td>
<td>Bowman et al. (2008)</td>
</tr>
<tr>
<td>lmo2389, lmo02471</td>
<td>Synthesis of NADH oxidase/dehydrogenase</td>
<td>Tryptone soy-yeast extract broth and TSYE agar, 25 °C, 400/600 MPa, 5/15 min</td>
<td>Downregulated</td>
<td>Bowman et al. (2008)</td>
</tr>
<tr>
<td>secEG, yidC, yajC, ftsY, fjh</td>
<td>Protein secretion and trafficking</td>
<td>Tryptone soy-yeast extract broth and TSYE agar, 25 °C, 400/600 MPa, 5/15 min</td>
<td>&gt;+1</td>
<td>Bowman et al. (2008)</td>
</tr>
<tr>
<td>lmo 1269–1271</td>
<td>Tubulin-like GTPase that polymerizes to form the basis of the septal ring FtsZ (lmo2032), signal peptidase I homologs</td>
<td>Tryptone soy-yeast extract broth and TSYE agar, 25 °C, 400/600 MPa, 5/15 min</td>
<td>+4.3</td>
<td>Bowman et al. (2008)</td>
</tr>
<tr>
<td>ftsL, ftsA, ftsI, ftsQ, ftsE, ftsX; ezrA, divIVA, minC; mreB, mreD, mpl</td>
<td>The mediation of septum placement (ezrA, divIVA, minC) and cell shape (mreB, mreD, mpl)</td>
<td>Tryptone soy-yeast extract broth and TSYE agar, 25 °C, 400/600 MPa, 5/15 min</td>
<td>+1 ~ 11.9</td>
<td>Bowman et al. (2008)</td>
</tr>
<tr>
<td>clpE, clpP, groEL, groES, hrcA, dnaK, dnaJ</td>
<td>Protein folding, chaperone, and peptidases</td>
<td>Tryptic soy broth, 37 °C, 200/400 MPa, 8 min</td>
<td>Upregulated</td>
<td>Duru et al. (2021)</td>
</tr>
<tr>
<td>divIC, dicIVA, ftsE, ftsX</td>
<td>Cell divisions</td>
<td>Tryptic soy broth, 37 °C, 200/400 MPa, 8 min</td>
<td>Downregulated</td>
<td>Duru et al. (2021)</td>
</tr>
<tr>
<td>prfA</td>
<td>The master regulator of virulence genes in <em>L. monocytogenes</em></td>
<td>Tryptic soy broth, 37 °C, 200/400 MPa, 8 min</td>
<td>Upregulated</td>
<td>Duru et al. (2021)</td>
</tr>
</tbody>
</table>
Further studies are required to emphasize on the optimization of HPP process, the in-depth investigations to elucidate the microbial inactivation mechanism and the exploration of novel hurdle technologies combined with other methods to obtain safe HPP-processed foods with minimal processing. The mechanisms underlying developing the resistance of microbial cells to high pressure warrant deeper and more systematical study. Understanding the expression of key genes or proteins contributing to the tolerance to high pressure may facilitate the identification of potential methods in combination with HPP.

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Chapter 9
Response of Foodborne Pathogens to Pulse Electric Fields

Cheng Zhang, Wei Zhao, and Ruijin Yang

Abstract Pathogenic microorganisms including *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella*, are related to foodborne diseases and epidemics worldwide. Daily foods including milk, egg products, and fruit juice are easily contaminated by these foodborne pathogens. Today, consumers demand high-quality, fresh, and safe food. Although traditional heat sterilization technology can ensure the safety of food, it has extensively damaged the sensory and nutritional characteristics of foods. At present, pulsed electric field (PEF), one of the nonthermal technologies, exhibits a potentially wide application in the food industry. Therefore, this chapter aims to explain the mechanism underlying PEF on foodborne pathogens and the influencing factors. Additionally, the inactivation modes of PEF on different foodborne pathogens and the application of sterilization in different foods were described in detail. Furthermore, PEF-induced sublethal sterilization process was explained and a strategy to improve the killing effect of PEF in killing foodborne pathogens was proposed.

Keywords Foodborne pathogens · Pulse electric fields · Sterilization · Cell membrane electroporation · Sublethally injured cells

9.1 Introduction

Every year, foodborne diseases cause over 40 million people illness around the world. Foodborne disease can be defined as any disease caused by eating food contaminated by foodborne pathogens or chemical substances (Tauxe et al. 2010). In the United States, approximately 76 million cases of disease occur each year, of
which over 5.2 million are caused by foodborne pathogens (Barba et al. 2017). These infections cause 128,000 hospitalizations and about 3000 deaths each year. Because of its high incidence rate and mortality, foodborne diseases have attracted worldwide attention. *Listeria monocytogenes*, *Campylobacter jejuni*, *Salmonella*, and *Escherichia coli* O157: H7 are considered the most important bacteria related to the outbreaks of foodborne diseases (Hadler et al. 2020; Li et al. 2020; Mardones and Lopez 2017; Stout et al. 2020).

Traditionally, heating is the most commonly used method to eliminate food microbial contamination, especially pathogens. Although this method can ensure the safety of food, its sensory, nutritional, and physicochemical properties have been seriously damaged (Barba et al. 2017; Li et al. 2020). Therefore, the food industry is exploring ways to replace heat sterilization to reduce the negative effects of heat treatment (Asaithambi et al. 2021). Today’s consumers are more concerned about fresh, high-quality, and microbiologically safe food. PEF, as one of the new non-thermal food processing technologies, has gradually come into people's vision. Pulses with a pulsed electric field (PEF) intensity at 80–100 V/cm are repeatedly applied to the food between the electrodes, leading to a microbial reduction (Arshad et al. 2020). PEF can cause electroporation of microbial membrane, resulting in temporary or permanent changes in membrane permeability (Wang et al. 2020). Generally, this permeabilization is affected by various factors, such as the morphological properties of the cell, the thickness of the cell membrane, and the applied PEF intensity (Yogesh 2016). Many food industries have purchased PEF food treatment equipment and configured different treatment chambers according to different foods (Toepfl 2011). However, equipment cost is one of the main problems that restrict the industrialization of PEF technology. At present, PEF equipment is mainly used for the sterilization of foodborne pathogens in milk, eggs, juice, and wine (Fig. 9.1)}

![Schematic diagram of a general PEF-based food processing system](image-url)
(Puligundla et al. 2018; Xiang et al. 2014; Li et al. 2012; Yogesh 2016). PEF causes smaller increments in food temperature, shorter processing time, lower energy consumption, higher heat and mass transfer, and without chemical residue and environmental pollution when compared with conventional heat treatment (Gabrić et al. 2018). Additionally, PEF has the ability to maximumly retain the sensory properties and nutritional value of food (Gomez et al. 2019).

9.2 The Mechanism of PEF on Foodborne Pathogens

9.2.1 Cell Membrane Electroporation Theory

9.2.1.1 Electroporation

Currently, the PEF-induced inactivation on foodborne pathogens is mainly considered to be caused by the electroporation on the cell membrane. PEF can form some instantaneous micropores in the lipid bilayer of membrane, thereby causing cell membrane to become permeable (Zimmermann et al. 1976). The cell membrane separates the extracellular fluid from the cytoplasm. It represents a self-assembled layer filled with proteins, glycolipids, sphingolipids, glycoproteins, and cholesterol (Barba et al. 2015). PEF can cause the formation of micropores reversibly or irreversibly in dependence on the cell membrane thickness, the cell morphology, and the applied electric field intensity (Yogesh 2016). Once the irreversible micropores occur, it will cause cellular contents to escape, organelle damage, and cell death (Suchanek and Olejniczak 2018).

In detail, the PEF-induced electroporation assumes that there is a certain potential difference between the inner and outer surfaces of the membrane phospholipid bilayer. When polar charges are accumulated on both sides of the membrane, even without PEF, the cell can still produce a potential of about 10 mV across the membrane (Toepfl et al. 2005). When PEF is applied, charges with opposite polarity on both sides of the membrane begin to accumulate and increase the transmembrane potential. Then the electrostatic attraction between the opposite polarity charges makes the membrane thinner (Weaver and Chizmadzhev 1996). However, the initiation of cell membrane electroporation requires that the transmembrane potential difference reaches a certain threshold, typically 0.5–1.5 V. Once the potential difference generated by the applied PEF on the cell membrane is greater than the difference in the natural potentials across the membrane, and when the variation in potential increases to a critical value, the permeability of the cell membrane will increase sharply (Castro et al. 1993). At this moment, many pores are generated in the membrane. Moreover, PEF voltages fluctuate violently in an instant, resulting in an oscillation effect on the membrane. So, the combination of oscillation effect and cell membrane electroporation leads to cell collapse, and eventually, the cell membrane ruptures to inactivate foodborne pathogens (Hui 1995). Specifically, the cell membrane electroporation can be divided into the following three stages based on
the comparison between the electric field strength ($E_0$) and the critical electric field strength ($E_c$). (Fig. 9.2):

1. Non-cell membrane electroporation stage: $E_0 \ll E_c$, PEF cannot form nano-scale micropores in the membrane. Therefore, bacterial cell maintains an intact structure.
2. Reversible cell membrane electroporation stage: $E_0 \geq E_c$, and the substances inside and outside the cell can be freely exchanged through the broken nano-scale micropores.
3. Irreversible cell membrane electroporation stage: $E_0 \gg E_c$, bacterial cells will be destroyed. Higher electric field intensity causes more serious damages in cells.

### 9.2.1.2 Alteration to Cellular Biomolecules

For the industrial application of PEF, it is important and necessary to conduct a risk assessment with the consideration of the impacts of PEF on bacterial cells, including the compromise in cell physiological activities and changes in cell biomolecules. To date, researchers have carried out several studies to improve the cognition of PEF induced microbial damage. In the study of Garcia et al. (2006), it is explored the biosynthetic demands of *E. coli* to repair the PEF-induced sublethal damages on the
membrane. Repairing sublethal injured cells requires lipid synthesis, which indicates that the direct target of PEF on bacterial cells is the cytoplasmic membrane. For example, it has been reported the damages caused by PEF to the outer and cytoplasmic membranes of *Enterobacter sakazakii* in the studies of Arroyo et al. (2010a, b). In the study of Somolinos et al. (2010a), it is confirmed that sigB was involved in the biosynthetic requirements of *L. monocytogenes* EGD-e PEF to conduct the repairment of PEF induced sublethal damage on membranes. The results show that the membrane repair only needs energy generation, which is independent of sigB, indicating that the inactivation mechanism between PEF and heat is different due to different membrane targets. In the study of Ukuku et al. (2011), it is reported that the charge on the extracellular surface and intracellular ATP leakage were reduced with treatment of PEF, which demonstrated that the PEF-induced damage on *E. coli* was mainly associated with the outer membrane. A study has shown that PEF treatment could significantly change the production level of certain proteins associated with the cell structure and metabolism (Rivas et al. 2013).

It is well known that RNA, particularly the ribosomal RNA, has been considered as the cellular target of thermal treatment (Aljarallah and Adams 2007). Nevertheless, the impact of PEF on microbial cell nucleic acid has not been thoroughly studied. In the study of Zhao et al. (2014b), it is found an increasing ratio of unsaturated fatty acids to saturated fatty acids in membrane lipids by PEF exposure, which was attributed to the enhancement of membrane rigidity (decreased cell membrane fluidity and increased cell membrane viscosity) of *Saccharomyces cerevisiae* with the exposure of PEF (20 kV/cm, 0–500 μs). This study indicated that PEF will not cause obvious changes in DNA; nevertheless, it is found the enhancement of PEF resistance and the supplement of RNA stabilizer magnesium without damaging cell envelope, indicating that the RNA of *S. cerevisiae* was seriously damaged. The results show that the damage of cell membrane and RNA were related to the sublethal *S. cerevisiae*, indicating that RNA and cell membrane were the main targets damaged by PEF. In another study, the cellular damage induced by PEF in *S. cerevisiae* was studied by analyzing metabolic properties, including changes in the intracellular enzyme activities (Zhao et al. 2014a). After being treated with 100–500 μs PEF under 20 kV/cm, there was about 90% *S. cerevisiae* cells being sublethally damaged. The sublethal injury of *S. cerevisiae* is associated with the PEF-induced damage of mitochondria and cell membrane, and it is also confirmed to be involved in the repair process activated by glucose. After PEF treatment, the increase in the Ca^{2+}-ATPase activity of the *S. cerevisiae* plasma membrane, decrease in the cytoplasmic esterase activity, and variation in 10 cellular enzymes suggested that the sublethal damage of *S. cerevisiae* is related to the PEF-induced cell enzyme inactivation or activation and aggregation of proteins. These studies are helpful to describe the mechanism of PEF-induced microbial damage and inactivation. Nevertheless, the research on the PEF-induced modification in the molecular level is scarce. Therefore, the exact inactivation mode of PEF on microbial cells needs to be further studied in the future.
9.2.2 Influencing Factors of the Bactericidal Effect of PEF

In the practical application of PEF, many factors will affect the effectiveness of PEF treatment to inactivate foodborne pathogens, but these factors can basically be divided into three categories: PEF process parameters, food media (treatment media) parameters, and microbial characteristics.

9.2.2.1 PEF Process Parameters

The process parameters pose an effect on the inactivation performance of PEF on foodborne pathogens, including exposure time, applied electric field intensity, pulse polarity, pulse shape, and initial temperature. Among them, the exposure time and applied electric field intensity make the most vital contribution. In general, longer exposure time, higher electric field intensity, higher initial treatment temperature lead to a greater reduction of foodborne pathogens by PEF (Wouters et al. 2001a).

Higher voltage renders PEF with stronger antimicrobial capacity. McDonald et al. (2000) found that PEF at the intensities of 30 and 50 kV/cm caused the reduction levels of 4.75 log_{10} and 6.20 log_{10} CFU/mL of *L. mesenteroides* in orange juice, respectively. Saldaña et al. (2010a) reported that after 90 μs exposure of PEF at 30 kV/cm at the initial temperature of 15, 27, 38, and 50 °C, the reduction levels of *S. Typhimurium* were 1.5, 2.9, 4.0, and 5.0 log CFU/mL in the citrate-phosphate buffer (pH 3.5), respectively. The initial treatment temperature can increase the lethality of foodborne pathogens, which may be according to the enhance in the fluidity of the microbial cell membranes when the temperature rises. In addition, the exponential decay pulses are not generally considered as effective as square wave pulses because more energy is required for the exponential decay pulses compared with the square wave pulses. The critical value of electric field intensity for the complete inactivation of foodborne pathogens can only be reached in the last two-thirds of the total pulse width (Buckow et al. 2013). Haan and Willcock (2002) found that under similar peak voltages, the energy efficiency of exponentially decayed pulses was not more than 38% compared with square wave pulses. In terms of pulse polarity, there are unipolar and bipolar pulses. Unipolar pulses discharge in the same direction, while bipolar pulses alternately appear at a higher voltage, which usually exhibits a better sterilization effect (Niu et al. 2020).

9.2.2.2 Food Medium Parameters

PEF treatment has been applied for microbial decontamination in various foods, including milk, fruit juice, liquid egg whites, and liquid whole eggs (Wouters et al. 2001a). However, food is a very complex substance, and research on it can be conducted from the perspectives of agriculture, engineering, physics, and chemistry.
(Buckow et al. 2013). Therefore, the parameters of the food medium affect the bactericidal effect of PEF.

The low pH of food is a stressor for many bacteria, and the PEF tolerance of foodborne pathogens is significantly different. For instance, Aronsson and Rönner (2001) reported that when the pH values were reduced from 7.0 to 4.0, the reduction level of *E. coli* was enhanced sharply from 1.7 to 5.7 logs when exposed to PEF (30 kV/cm, 30 °C, 80 μs). The inactivation of *Saccharomyces cerevisiae* is less affected by pH. Wouters et al. (2001a) observed that the reduction rate of *Listeria* in the buffer at pH 3.8 was higher than that in the buffer at pH 7.0, and the pH value of the medium posed no obvious influence on the PEF-induced reduction of *Yersinia enterocolitica*. Therefore, the conclusions indicate that pH has an effect on the kinetics of inactivation, and this effect may relate to the type of foodborne pathogens. Moreover, the electrical conductivity of food is an essential factor affecting the sterilization effect of PEF. Many researches have shown that as the conductivity of the treated medium increases, the bactericidal effect of foodborne pathogens becomes worse under the same PEF treatment conditions (Moisescu et al. 2013). In the study of Wouters et al. (2001a), it was found that under the same PEF treatment, it was observed that *Listeria* was reduced by 4.5 log$_{10}$ and 2 log$_{10}$ in the system with a conductivity of 0.27 and 0.79 S/m, respectively. The decrease in the number of foodborne pathogens inactivated by PEF at high conductivity may be due to the fact that improving the conductivity also enhances the current, which leads to significant Joule heating and increases the energy loss during PEF treatment, which reduces the external impact on microbial cells. Disturbance, eventually showing a lower inactivation rate. Furthermore, studies have shown that water activity might also affect the bactericidal effect of PEF (Arroyo et al. 2010a). Generally speaking, at lower water activity, foodborne pathogens are more resistant to inactivation treatment or antibacterial agents. The research on the effect of water activity on the antimicrobial capacity of PEF is limited. In the study of Wouters et al. (2001a), it is found the protective effect of a low water activity on *Enterobacter cloacae* from the damages caused by PEF. In fact, food is a complex multi-component/multi-phase system, and other components may also affect the inactivation effect of PEF on foodborne pathogens, which need to be further discussed in the future.

### 9.2.2.3 Microbial Characteristics

The characteristics of foodborne pathogens mainly include cell size, shape, strain type, and growth cycle. Generally, bacteria exhibit higher resistance to PEF in comparison to yeast. Moreover, spores have a higher resistant capacity to PEF (Bermúdez-Aguirre et al. 2012). In addition, cell morphology is also an important factor affecting the inactivation mechanics. Generally speaking, larger cells are less tolerant to PEF due to the contribution of cell size to the establishment of critical membrane potential (Wouters et al. 2001a).

Wouters et al. (2001b) used flow cytometry to classify the cells of *Lactobacillus plantarum* according to different sizes and shapes, proving that cell morphology
affects cells are inactivated, and the results show a difference in membrane permeability, with larger cells being easier to permeate than smaller cells. In addition, foodborne pathogens in different growth cycles also show differences in PEF tolerance. Generally speaking, higher PEF resistance is observed in the microbial cells in the logarithmic growth phase in comparison to those in the quiescent phase (Pothakamury et al. 1996). This may be attributed to the difference in fatty acid composition and fluidity of microbial cell membranes under different growth cycles (Liu et al. 2017). Furthermore, the growth conditions of the foodborne pathogens, including the medium constituents, growth temperature, etc., will also affect the PEF resistance of the foodborne pathogens. Studies have shown that these factors may affect the cell membrane fluidity of foodborne pathogens. Generally speaking, the greater the cell membrane fluidity, the better the inactivation effect of PEF on foodborne pathogens (Wang et al. 2018).

9.3 The Bactericidal Effect of PEF on Different Kinds of Foodborne Pathogens

9.3.1 Campylobacter jejuni

Campylobacter species including Campylobacter jejuni are considered as vital pathogens in the field of veterinary medicine. However, because they appear repeatedly in some foods for human beings, this has attracted special attention. In developed countries, C. jejuni has been listed as the first foodborne pathogen causing human infections. According to surveys, there are around 2.4 million people in the United States are infected with E. coli and C. jejuni each year (Man 2011). In addition, almost 90% of cases show that C. jejuni is commonly isolated from stool specimens (Fitzgerald 2015). Haughton et al. (2012) made use of PEF under 65 kV/cm and 500 Hz for 5 μs to treat liquid samples, and the longest treatment (30 s) resulted in 4.33 to 7.22-log reduction of C. jejuni. Clemente et al. (2020) investigated that PEF under higher field strength can reduce C. jejuni cycle by 1.7–1.9 Log_{10} cycles. Moreover, when sequential PEF and oregano essential oils treatments were performed, significant increases in inactivation levels were observed at all field strengths tested (p < 0.05). The application of sequential treatments of chicken with PEF followed by immersion in oregano solutions (15.625 ppm) achieved maximum reductions in C. jejuni 1146 DF populations of close to 1.5 Log_{10} CFU/g, even when low field strengths and essential oils were used. This research confirmed that the PEF conditions used in this research were not suitable as the intervention to reduce the microbial contaminants during chicken processing. Therefore, Campylobacter should be considered for the optimization and development of PEF treatment.
9.3.2 Salmonella

Salmonella is a common pathogen second only to Campylobacter in foodborne diseases. After eating food contaminated with Salmonella, symptoms such as abdominal cramps, diarrhea, vomiting, fever, and blood in the stool will easily occur after 12-72 hours, and these symptoms usually last 4–7 days (Qi et al. 2016). In reality, most people infected with Salmonella do not need to be hospitalized, but high-risk groups (people with weakened immune systems, the elderly, children) are more likely to be ill and may have more serious illnesses (Robinson 2019). According to reports, in the United States, a total number of around 1.4 million cases of Salmonella infections occurred annually, leading to around 16,000 hospitalizations as well as 600 deaths (Cummings et al. 2010). Generally, the PEF-induced inactivation kinetics of microbial cells is described in a linear model in the initial phase followed by a tailing (Huang et al. 2012). Tailing phase exhibits an upward concavity, which indicates stress resistance enhancement of the microbial population (Delso et al. 2020). It has been reported that there was a marked tailing in the survival curves of S. Typhimurium inactivated by PEF (Saldaña et al. 2010c). The research results of Yun et al. (2017) showed that a preliminary incubation at a low temperature of 10 and 25 °C increased the susceptibility of S. Typhimurium cells toward PEF treatment in comparison with the condition under the temperatures of 37 and 45 °C. Application of PEF at 50 °C can reduce S. Typhimurium by 5 Log_{10} in the pH range of 4.2–6.7 (Saldaña et al. 2012). Furthermore, S. Typhimurium can exhibit microbial resistance to PEF (Sagarzazu et al. 2013), and its virulence to nematodes appears to be reduced when PEF is repeatedly used (Sanz-Puig et al. 2019). Wang et al. (2019) reported that the PEF tolerance of S. Typhimurium was associated with the changes in the membrane fluidity. Foodborne pathogens often reduce membrane fluidity to accommodate PEF treatment. Therefore, greater membrane fluidity might enhance the efficiency of PEF to inactivate foodborne pathogens (Yun et al. 2016). Furthermore, PEF could also be used as a pretreatment method of high-pressure carbon dioxide technology to enhance the effect of sterilization and shorten the treatment time of inactivating S. Typhimurium (Spilimbergo et al. 2014). On the other hand, PEF combined with cinnamic acid or other antibiotic agents can further enhance the bactericidal effect of PEF (Pina-Perez et al. 2009).

9.3.3 Listeria monocytogenes

Listeria monocytogenes is another major foodborne pathogen, which exhibits psychrophilic behavior to grow below 7 °C under aerobic and anaerobic conditions (Sheng et al. 2019). Furthermore, this bacterium can survive and grow under pH values ranging from 4.0 to 9.6, and a low water activity level of 0.9 (Välimaa et al. 2015). These characteristics make L. monocytogenes a major difficulty restricting the development of the food industry, requiring very effective control measures throughout the food chain (Lambertz et al. 2013).
First, the researchers studied the effects of treatment parameters on the reduction of *L. monocytogenes* by PEF. Álvarez (2003) observed a maximum reduction level of 4.77 logs with the exposure of PEF under 28 kV/cm and 3490 kJ/kg for 2000 μs. Zhao et al. (2013) found that when the PEF intensity was enhanced from 15 to 30 kV/cm, the percentage of sublethally damaged *L. monocytogenes* was raised from 18.98% to 43.64%. However, *L. monocytogenes* were more sensitive to PEF in media of low pH (Gomez et al. 2005). PEF treatment at 35 kV/cm for 500 μs resulted in maximum inactivation level of 3.3 logs for *L. monocytogenes* under a pH value of 3.5 (Saldana et al. 2010b). Golberg (2015) has found that the intermittently delivered PEF can precisely control the density of *L. monocytogenes* in contaminated milk. He designed a program for intermittent transmission of PEF including two sequences of 10 square wave pulses with a duration of 50 μs. The electric field intensity was 12.5 kV/cm, transmitted at a frequency of 0.5 Hz, with a 1-min pause between the sequences applied each 1.5 h. The final density of the samples treated by intermittently delivered PEF decreased from $(9.1 \pm 0.6) \times 10^7$ CFU/mL to 120 ± 44 CFU/ml. Similarly, Schottroff et al. (2019) used PEF to treat the whey protein formulations and it has found that the microbial reduction was decreased as the pH values and protein concentrations was increased. Noci et al. (2009) combined thermosonication and PEF treatment, and the highest field strength (40 kV/cm) and thermosonication (80 s) could cause $6.8 \log_{10}$ CFU/mL *L. innocua* inactivation. In addition, Palgan et al. (2012) combined PEF and manothermosonication technologies to significantly reduce the harmless *Lactobacillus* in smoothies ($p < 0.0001$). Moreover, the continuous application of high hydrostatic pressure and PEF treatments mainly showed an additive effect. However, when HPP and PEF were applied at the same time, a synergistic effect was observed, which also helped to enhance the bactericidal capacity of PEF on *Listeria* (Pyatkovskyy et al. 2018). Furthermore, successively applied high hydrostatic pressure and PEF treatments exhibit an additive effect. Whereas, the simultaneous application of high hydrostatic pressure and PEF result in synergistic effects. These all help to enhance the bactericidal effect of the PEF on *L. innocua* (Pyatkovskyy et al. 2018).

### 9.3.4 *Escherichia coli* O157:H7

*E. coli* O157:H7 is considered one of the major foodborne pathogens. This bacterium can generate verocytotoxin or shiga-toxin, and cause low platelet count, hemolytic anemia, bloody diarrhea, and thrombocytopenia (Karmali et al. 2010). Evrendilek and Zhang (2005) found that the PEF with single pulse decreased *E. coli* O157:H7 by 1.27 logs in skim milk, while it was a 1.96-log reduction level caused by bipolar pulses. In addition, under various pH values studied in this work, *E. coli* O157:H7 exhibited higher PEF resistance when compared with *S. Typhimurium*. However, the resistance of *E. coli* to PEF was affected by pH. It was found that after 150 μs PEF exposure with 35 kV/cm, a reduction value of 1 and 5 logs of *E. coli* O157:H7 inactivation resulted under the pH range of 3.5–4.5 and 5.5–6.5,
respectively. Higher PEF intensity and longer time result in more serious cell
damage. After treatment of 30 kV/cm at pH 3.5, the maximum cell damage levels
of E. coli O157:H7 and S. Typhimurium were observed to be 4.2 and 2.7 log_{10}
cycles, respectively (Saldaña et al. 2010c). In addition, the addition of preservatives
(2.7% citric acid, sodium benzoate, potassium sorbate) could enhance the PEF
susceptibility of E. coli O157:H7 in strawberry juice (Gurtler et al. 2010).

9.4 The Bactericidal Effect of PEF on Foodborne Pathogens
in Different Foods

The earliest applications of PEF in food processing were electric pasteurization
of milk. It was reported that from 1920 to 1930, some farms in the United States had
applied 220 V alternating current to sterilize milk (Palaniappan et al. 1990). Since
then, electric sterilization has begun to enter people's field of vision. Doevenspeck
(1961) designed different PEF equipment in his patent and evaluated the sterilization
of PEF treatment. Furthermore, the study found that as the PEF intensity reached
25 kV/cm, the microbial cell membrane would cause irreparable damage and the cell
contents leaked, leading to microbial death (Sale and Hamilton 1967). Therefore,
PEF has the potential to be applied to assure the microbiological safety of soups,
milk, juices, and other liquid products (Alirezalu et al. 2020; Yogesh 2016).

9.4.1 Milk

According to foodborne pathogens in milk, the impacts of PEF processing param-
eters on the pathogenic and contaminating foodborne pathogens in milk have been
explored, including Cronobacter sakazakii, E. coli, Bacillus cereus, Listeria spp.,
Salmonella spp., Staphylococcus aureus, etc. (Buckow et al. 2014). In addition, a lot
of studies have investigated the application of PEF or the combination with other
treatments to inactivate foodborne pathogens in milk is still the focus of many
researchers (Table 9.1).

Fleischman et al. (2004) found that under the same PEF intensity at 20 kV/cm, as
the treatment temperature was promoted to 55 °C, the L. monocytogenes in skimmed
milk was reduced the most (4.5 log_{10} cycle reduction). It is attributed to the
combined effect of PEF and mild heat rather than a direct effect of thermal inacti-
vation. In the study of Craven et al. (2008), the maximum reduction rate (3 log_{10}
cycles) of Pseudomonas spp. could be achieved when the PEF intensity of the
treatment was increased from 28 to 31 kV/cm. Walter et al. (2016) made use of
PEF (30–35 kV/cm, 30–50 °C) to treat E. coli ATCC 11775 in the whole milk. It is
shown that when the temperature was increased from 30 to 40 to 55 °C, the reduction
of viable cells was significantly decreased from 3 to 6 logs. Similarly, the temperature
and electric field strength could synergistically inactivate E. coli. It is also found that
PEF at 30 and 35 kV/cm under 30 and 40 °C resulted in 3 log₁₀ cycles reduction of *Pseudomonas fluorescens*. Samples treated at. Cregenzán-Alberti et al. (2017) reported the combination of PEF (20–42.5 kV/cm; 1 kHz; 25 kV; 68–165 mL/min; 68–130 μs) with thermal treatment (26–45 °C) exhibited a synergetic effect on the reduction of *E. coli, P. fluorescens* and *S. aureus* in the whole milk. It is found that the variations in PEF strength, temperature, and exposure time pose impact on the microbial inactivation capacity. Among them, all foodborne pathogens have the most significant reduction (around 5 log₁₀ cycles) under the best treatment conditions (32 °C, 40 kV/cm and 89 μs). The improvement of antimicrobial effect of PEF coupled with thermal treatment is associated with the enhancement in membrane fluidity. This process promoted the pore formation in the membrane, resulting in larger microbial reduction.

For *Bacillus cereus*, PEF (30–40 kV/cm, 55 °C) and gentle heating (fluid recirculation; 0–275 pulses; 1 L/min) had been studied for the sporicidal effect when treating the milk (Bermúdez-Aguirre et al. 2012). As the PEF strength was enhanced to 40 kV/cm, the reduction of spore could achieve a maximum value of 2.5 logs. In addition, Pina-Perez et al. (2009) explored the combined effect of PEF and several antibacterial agents (CocoanOX 12%) for inactivating *B. cereus* in liquid whole egg-skimmed milk beverages. Maximum deactivation was found to be generated under 200-μs PEF.

### Table 9.1 Effect of PEF on foodborne pathogens in milk

<table>
<thead>
<tr>
<th>Product</th>
<th>Target foodborne pathogens</th>
<th>Treatment parameters</th>
<th>Log reduction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk</td>
<td><em>Listeria monocytogenes</em></td>
<td>20 kV/cm, 55 °C</td>
<td>4.5</td>
<td>Fleischman et al. (2004)</td>
</tr>
<tr>
<td>Whole milk</td>
<td><em>Pseudomonas</em> spp.</td>
<td>28–31 kV/cm, 55 °C</td>
<td>3</td>
<td>Craven et al. (2008)</td>
</tr>
<tr>
<td>Whole milk</td>
<td><em>Escherichia coli</em> ATCC 11775</td>
<td>30-35 kV/cm, 55 °C</td>
<td>3</td>
<td>(Walter et al. 2016)</td>
</tr>
<tr>
<td>Whole milk</td>
<td><em>Escherichia coli</em></td>
<td>40 kV/cm, 32 °C</td>
<td>5</td>
<td>Cregenzán-Alberti et al. (2017)</td>
</tr>
<tr>
<td>Whole milk</td>
<td><em>Staphylococcus aureus</em></td>
<td>40 kV/cm, 55 °C</td>
<td>2.5</td>
<td>Bermúdez-Aguirre et al. (2012)</td>
</tr>
<tr>
<td>Whole milk</td>
<td><em>Pseudomonas</em> fluorescens</td>
<td>35 kV/cm, 20 °C</td>
<td>3.03</td>
<td>Pina-Perez et al. (2009)</td>
</tr>
<tr>
<td>Skim milk</td>
<td><em>Bacillus cereus</em> spores</td>
<td>32 kV/cm, 20 °C</td>
<td>6.5</td>
<td>Schottroff et al. (2019)</td>
</tr>
<tr>
<td>Liquid whole egg -</td>
<td><em>Bacillus cereus</em></td>
<td>35 kV/cm, 20 °C</td>
<td>3.03</td>
<td>Pina-Perez et al. (2009)</td>
</tr>
<tr>
<td>skim milk beverage</td>
<td>Mesophilic Psychrotrophic bacteria</td>
<td>35 kV/cm, 65 °C</td>
<td>Shelf life extended by 20 days (4°C)</td>
<td>Sepulveda et al. (2009)</td>
</tr>
<tr>
<td>Liquid whey protein formulation</td>
<td><em>Listeria innocua</em></td>
<td>32 kV/cm, 20 °C</td>
<td>6.5</td>
<td>Schottroff et al. (2019)</td>
</tr>
</tbody>
</table>
exposure at 35 kV/cm and 20 °C. This deactivation was increased when the PEF intensity and exposure duration was enhanced. When CocoanOX was supplemented in beverages, a synergistic antimicrobial effect could be observed, thus achieving maximum inactivation. However, since the formation of reversible pores could be maximized at lower PEF strengths, and the antimicrobial agents were more effective in deactivating cells when the pore structures are rearranged, so more synergy is observed at lower electric field strengths.

9.4.2 Egg Products

If egg products are not produced aseptically, they might be contaminated by foodborne pathogens (e.g., *Salmonella*). In addition, they are rich in nutrients, which are necessary for microbial growth. Therefore, egg products must be pasteurized to ensure their food safety and shelf life. Currently, heat pasteurization of liquid whole eggs is performed at 64 °C for 2.5 min (the United Kingdom) and 60 °C for 3.5 min (the United States), which reduces the *Salmonella* serotype count by 5–9 log_{10} cycles (Monfort et al. 2012a, b). Ultrapasteurization is a process of treating liquid whole eggs at 70 °C for a duration of 1.5 min (Herald and Smith 1989; Hamid-Samimi et al. 1984). Since the properties of soluble proteins in liquid whole egg might be affected by heat, it is particularly important to study the application of nonthermal processing PEF technology in the sterilization of liquid whole eggs (Table 9.2).

At present, most studies on PEF inactivation of foodborne pathogens have focused on the *Salmonella* serotypes in liquid whole eggs, egg yolks or egg whites. Monfort et al. (2010a) employed the PEF to inactivate *S. Typhimurium* and

| Table 9.2  Effect of PEF on foodborne pathogens in egg products |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Product         | Target foodborne pathogens | Treatment parameters | Log reduction | References      |
| Liquid whole egg | *Salmonella* Typhimurium *Staphylococcus aureus* | 40–45 kV/cm | 4 3 | Monfort et al. (2010a) |
| Liquid whole egg | *Salmonella* Enteritidis | 25 kV/cm, 55 °C | >8.0 | Monfort et al. (2010b) |
| Liquid whole egg | *Salmonella* Enteritidis | 25 kV/cm, 60 °C 10 mM EDTA or 2% Triethyl citrate | 9 | Monfort et al. (2011) |
| Liquid whole egg | *Salmonella* spp. | 25 kV/cm, 60 °C 10 mM EDTA and 2% Triethyl citrate | >5 | Monfort et al. (2012b) |
| Liquid whole egg | *Escherichia coli* O157:H7 | 9–15 kV/cm, 55 °C 9–15 kV/cm, 60 °C | 2.5 4 | Bazhal et al. (2006) |
| Liquid whole egg-skim milk | *Bacillus cereus* | 40 kV/cm, 360 μs, 20 °C | 3.3 | Pina-Perez et al. (2009) |
*S. aureus* in liquid whole eggs. The PEF treatments of 45 kV/cm for 30 μs and 419 kJ/kg, and 40 kV/cm for 15 μs and 166 kJ/kg resulted in maximum reduction of *S. Typhimurium* and *S. aureus* by 4 and 3 log\(_{10}\) cycles, respectively. With the exposure of PEF (25 kV, 48 μs) combined with heat treatment (55 °C, 2 min), the reduction of *S. Enteritidis* count in the liquid whole egg was achieved at 8 log\(_{10}\) cycles (Monfort et al. 2011).

Furthermore, if some modifiers were used, the efficiency of microbial inactivation will be improved; for example, combining 2% triethyl citrate or 10 mM EDTA and PEF exposure (25 kV/cm, 100 kJ/kg) and gentle heating (60 °C, 1 min) was found to decrease the *S. Enteritidis* count by 9 logs and *S. Dublin*, *S. Virchow*, *S. Typhimurium*, *S. Typhi*, and *S. Senftenberg* by 5 log\(_{10}\) cycles (Monfort et al. 2011, 2012a). Likely, Monfort et al. (2012b) evaluated the bactericidal capacity of PEF under static and continuous conditions, and then 7 different *Salmonella* serotypes were heated in liquid whole eggs containing additives (EDTA or triethyl citrate). First, PEF exposure at 5–100 kJ/kg and 725 kV/cm was performed on liquid whole eggs containing 2% triethyl citrate, and then heated (52 °C/3.5 min, 55 °C/20 s or 60 °C/10 s). Compared with heat treatment alone, the time for reducing the population of *Salmonella* in whole egg by 9 logs could be shortened by 92-fold at 52 °C, 3.4-fold at 60 °C, and 4.8-fold at 52 °C. This combined treatment could inactivate *S. serovars* Dublin, Typhimurium, Virchow, Enteritidis, Senftenberg, and Typhi for more than 5 log\(_{10}\) cycles under instatic and continuous conditions. Except for *Salmonella*, PEF treatment has also been used for the inactivation of other foodborne pathogens in egg products. In the study of Bazhal et al. (2006), it is found that the combination of 138 pulses of 9 kV/cm, 68 pulses of 11 kV/cm and 30 pulses of 15 kV/cm, with gentle heating (55 °C) reduced the *E. coli* count by ~2.5 log\(_{10}\) cycles. When the temperature was enhanced by 5 °C (60 °C), the reduction level of *E. coli* was promoted by about 1–1.5 log\(_{10}\) cycles under the same PEF intensity at 72, 40, and 10 corresponding pulses. Furthermore, the increase in the number of foodborne pathogens inactivation at higher temperatures could be attributed to the increase in electrical conductivity, because electrical conductivity is a function of temperature in liquid media.

### 9.4.3 Juice

Fruit juice is considered as a major source of vitamins for humans, of which apple and orange juice are the most consumed. Due to the action of foodborne and enzymes, the shelf life of juice is very short. But consumers need fruit juice throughout the year, so they should ensure that foodborne pathogens and enzymes are inhibited to prolong their shelf life. The traditional heat treatment method has a significant effect in destroying foodborne pathogens and inactivating enzymes in the juice, but it will cause the loss of nutrients in the juice and the deterioration of the physical, chemical, and sensory properties. At present, the application of PEF in fruit juice processing has attracted much attention. It can alleviate the compromise in food quality caused by heat (Table 9.3) (Espachs-Barroso et al. 2003).
Table 9.3 Effect of PEF on foodborne pathogens in fruit juice

<table>
<thead>
<tr>
<th>Fruit juice</th>
<th>Target foodborne pathogens</th>
<th>Treatment parameters</th>
<th>Log reduction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Escherichia coli</em> 8739</td>
<td></td>
<td>5.4</td>
<td></td>
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<tr>
<td>Orange juice</td>
<td><em>Salmonella Typhimurium</em> UK-1</td>
<td>22 kV/cm, 59 μs, 55 °C</td>
<td>2.8</td>
<td>Gurtler et al. (2010)</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella Typhimurium</em> 14028</td>
<td></td>
<td>3.5</td>
<td></td>
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<tr>
<td>Orange juice</td>
<td><em>Salmonella Typhimurium</em></td>
<td>90 kV/cm, 100 μs, 55 °C</td>
<td>5.9</td>
<td>Liang et al. (2002)</td>
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<tr>
<td>Orange juice</td>
<td><em>Listeria innocua</em></td>
<td>30 kV/cm, 12 μs, 50 °C</td>
<td>6.0</td>
<td>McDonald et al. (2000)</td>
</tr>
<tr>
<td>Orange juice</td>
<td><em>Listeria innocua</em></td>
<td>40 kV/cm, 100 μs, 56 °C</td>
<td>3.9</td>
<td>McNamee et al. (2010)</td>
</tr>
<tr>
<td>Cherry juice</td>
<td><em>Listeria monocytogenes</em></td>
<td>27 kV/cm, 131 μs, 20 °C</td>
<td>3</td>
<td>Altuntas et al. (2010)</td>
</tr>
<tr>
<td>Tropical fruit smoothie</td>
<td><em>Escherichia coli</em> K12</td>
<td>34 kV/cm, 150 μs, 55 °C</td>
<td>5.2</td>
<td>Walking-Ribeiro et al. (2008a)</td>
</tr>
<tr>
<td>Orange Juice</td>
<td><em>Staphylococcus aureus</em></td>
<td>40 kV/cm, 150 μs Thermosonication 55 °C 10 min, 40 kV/cm, 150 μs,</td>
<td>5.5</td>
<td>Walking-Ribeiro et al. (2008b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>Cranberry juice</td>
<td>Total aerobic count Moulds</td>
<td>40 kV/cm, 150 μs, &lt;25 °C</td>
<td>4</td>
<td>Jin and Zhang (1999)</td>
</tr>
<tr>
<td></td>
<td>Yeasts</td>
<td></td>
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<td></td>
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<tr>
<td>Apple cider</td>
<td><em>Escherichia coli</em> O157:H7</td>
<td>90 kV/cm, 20 μs, 42 °C</td>
<td>5.9</td>
<td>Ju et al. (2001)</td>
</tr>
<tr>
<td>Apple juice</td>
<td><em>Escherichia coli</em> K12 DH5α</td>
<td>34 kV/cm, 7.68 μs, 55 °C</td>
<td>6.2</td>
<td>Heinz et al. (2003)</td>
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<tr>
<td></td>
<td><em>Rhodotorula Rubra</em></td>
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<td></td>
<td><em>Aspergillus niger</em> ATCC 16404</td>
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<td><em>Lacticaseibacillus rhamnosus</em> GG ATCC 53103</td>
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<td></td>
<td><em>Listeria innocua</em> NCTC 11289</td>
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<td>4.3</td>
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</tr>
<tr>
<td>Grape juice</td>
<td><em>Escherichia coli</em> K12 DH5α</td>
<td>34 kV/cm, 7.68 μs, 55 °C</td>
<td>6.4</td>
<td>Heinz et al. (2003)</td>
</tr>
<tr>
<td></td>
<td><em>Rhodotorula Rubra</em></td>
<td></td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Aspergillus niger</em> ATCC 16404</td>
<td></td>
<td>4.6</td>
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</tr>
<tr>
<td></td>
<td><em>Lactobacillus rhamnosus</em> GG ATCC 53103</td>
<td></td>
<td>4.6</td>
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</tr>
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(continued)
Evrendilek and Zhang (2003) applied a PEF (29 kV/cm, 172 μs) at an outlet temperature of 25 °C to result in 5- and 5.4-log reduction of *E. coli* O157:H7 and *E. coli* 8739 in apple juice, respectively. Gurtler et al. (2010) detected that after 59 μs PEF exposure at 55 °C and 22 kV/cm, the concentration of *S. Typhimurium* strain UK-1 and 14028 decreased by 2.8 and 3.5 logs, respectively. Liang et al. (2002) reported that PEF at 90 kV/cm and 55 °C was performed in a coaxial chamber for 50 μs, and the 5.9-log reduction of *S. Typhimurium* was achieved in freshly squeezed orange juice. McDonald et al. (2000) found that a PEF intensity at 30 kV/cm for 12 μs at 5 °C or 5 μs at 42 °C resulted in 3.5- and 6.0-log reduction of *L. innocua* in orange juice, respectively. Contradictorily, McNamee et al. (2010) reported that a PEF treatment at a higher intensity of 40 kV/cm resulted in 3.9-log reduction of *L. innocua* in orange juice. It might be caused by different designs of PEF treatment chamber, pulse shape, orange juice properties, and culture techniques. It is reported that the reduction of *L. monocytogenes* in apple juice was 5 and 6.5 logs under PEF exposure at 25 kV/cm (outlet temperature 50 °C) for 31.5 and 37 μs, respectively. And it is found that PEF treatment at 27 kV/cm and 20 °C for 131 μs decreased *L. monocytogenes* by 3 logs (Altuntas et al. 2010). Timmermans et al. (2014) made use of a continuous-flow PEF system to inactivate foodborne pathogens in various juices. PEF treatment conditions under 20 kV/cm with varying frequencies were used to inactivate *L. monocytogenes, S. panama, S. cerevisiae*, and *E. coli* in the fruit juices. Based on the findings, kinetic data revealed that *E. coli* was the

<table>
<thead>
<tr>
<th>Fruit juice</th>
<th>Target foodborne pathogens</th>
<th>Treatment parameters</th>
<th>Log reduction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orange juice</td>
<td><em>Lactobacillus brevis</em></td>
<td>35 kV/cm, 1000 μs</td>
<td>5.8</td>
<td>Elez-Martinez et al. (2005)</td>
</tr>
<tr>
<td>Orange juice-milk mixture</td>
<td><em>Lactobacillus plantarum</em></td>
<td>35 kV/cm, 2.5–4.0 μs</td>
<td>2.5</td>
<td>Sampedro et al. (2007)</td>
</tr>
<tr>
<td>Cherry juice</td>
<td><em>Penicillium expansum</em></td>
<td>34 kV/cm, 163 μs, 21 °C</td>
<td>100% inactivation of spore germination</td>
<td>Evrendilek et al. (2008)</td>
</tr>
<tr>
<td>Orange juice</td>
<td><em>Salmonella Enteritidis</em></td>
<td>35 kV/cm, 4 μs, 40 °C</td>
<td>&gt;5, &gt;5</td>
<td>Mosqueda-Melgar et al. (2008)</td>
</tr>
<tr>
<td>Strawberry juice</td>
<td><em>Escherichia coli</em> O157:H7</td>
<td>18.6 kV/cm, 150 μs, 45 °C</td>
<td>3.09, 4.08, 4.71</td>
<td>Gurtler et al. (2011)</td>
</tr>
<tr>
<td>Apple juice</td>
<td><em>Lactobacillus brevis</em></td>
<td>35 kV/cm, 4800 μs</td>
<td>14.5</td>
<td>Aguilar-Rosas et al. (2007)</td>
</tr>
</tbody>
</table>
least sensitive followed by \textit{S. panama} and \textit{S. cerevisiae} was reported to be highly sensitive to the treatments at similar conditions. Moreover, among the studied microbes, \textit{L. monocytogenes} was reported to show the highest resistance under similar conditions of treatments. Studies with combined treatments of PEF and heat revealed a synergistic effect when the temperature was higher than 35 °C. Thus, at higher temperatures, a lower energy was needed to inactivate foodborne pathogens. In addition, the fruit juice with simple components of PEF has a better bactericidal effect than the fruit juice with complex components. It is reported that the enhancement of the proportion of carrot juice in the blending orange juice could compromise PEF’s inactivation performance on \textit{E. coli} (Rodrigo et al. 2003). Generally, Gram-positive bacteria exhibit higher resistance toward PEF in comparison to Gram-negative bacteria due to the variation in the outer structures (Geveke and Kozempel 2003). In the study of Walkling-Ribeiro et al. (2008b), PEF treatment under 40 kV/cm for 150 μs was required to reduce \textit{S. aureus} by 5.5 logs in orange juice. However, PEF under an intensity of 34 kV/cm for 150 μs was reported to cause reduction of \textit{E. coli} by 5.2 logs in tropical fruit smoothie (Walkling-Ribeiro et al. 2008a).

To date, more researches have shown that most foodborne pathogens have a certain degree of resistance to PEF. Somolinos et al. (2010b) found that the PEF resistance of \textit{E. coli} on orange juice variated by pH values. After 50 pulses of 20 kV/cm PEF treatment, the log_{10} cycle inactivation number of \textit{E. coli} at pH 7.0 was significantly higher than that at pH 4.0. Due to the PEF resistance of foodborne pathogens, pasteurization is still an effective method to control foodborne pathogens. Therefore, further investigation on the effect of the sublethal damage of foodborne pathogens resistant to PEF treatment on food safety is required.

9.5 The Sublethal Effect of PEF on Foodborne Pathogens

At present, based on the ability of PEF to inactivate foodborne pathogens, PEF technology has been applied to the storage and preservation of fruits, vegetables, meat, and fish. However, the spores in food generally exhibit high resistance toward PEF, even at higher electric field strengths. This characteristic may cause the pasteurization process to fail, leading to potential hazards (Arroyo et al. 2012). Except for the spores, it is also found that the microorganisms in the vegetative form also exhibit resistance toward PEF in some cases (Pillet et al. 2016). It has been reported that several foodborne pathogens, including \textit{S. Typhimurium}, \textit{E. coli}, and \textit{S. aureus}, exhibit resistance to the PEF exposure under the commonly applied conditions (Zhao et al. 2013). Saldaña et al. (2009) proposed the necessity to identify the most PEF resistant bacterial strain as the reference strain to ensure that PEF treatment could adequately inactivate foodborne pathogens. For example, Zhao et al. (2011) used flow cytometry combined with fluorescence staining to quantify the damages of PEF to microbial cells in phosphate buffer (pH 7.0) in real time. The authors found that \textit{S. cerevisiae} and \textit{Dekkera bruxellensis} were treated with electric fields of 12.0
kV/cm and 16.5 kV/cm for 50 pulses, respectively, and the sublethally injured population reached the maximum. It is reported that the expression of oxidative stress response genes and glutathione greatly induced by PEF rendered \textit{S. cerevisiae} with the resistance (Tanino et al. 2012). These results show that cell membrane electroporation is not an all-or-nothing event.

\textbf{9.5.1 Definition of Sublethal Damage}

The definition of sublethal damage was first proposed in 1951, that is, the sublethal state refers to a state of damage to microbial cells after being subjected to one or more external stimuli, in which the cells still have certain physiological activities, but basically lost the ability to reproduce in a selective medium that has no obvious inhibitory effect on the same kind of normal microbial cells, and the reproduction cycle is longer (McMeekin et al. 2008). Specifically, microbial sublethal damage is caused by certain chemical or physical processes, but this damage is generally not enough to kill the microorganism (Hurst 1977; Russell 1984); sometimes it is called metabolically damaged cells, because it is lacking in special nutrients. Therefore, the metabolically damaged cells cannot grow normally in the medium. In addition, sublethal damage to cells means that some functions of the cells are temporarily or permanently lost. When the appropriate conditions are encountered, they may return to the intact cells in their normal state. This process is called “resuscitation” (Hurst 1984; Noriega et al. 2013). Therefore, the sublethal damage state is regarded as an intermediate transition state in the process of microbial inactivation. A large number of researches have shown that after foodborne pathogens are subjected to sublethal adverse environmental effects, the cells will induce a series of physiological and biochemical reactions that are compatible with them, so as to obtain the tolerance to deal with more severe stresses, making the products in the sterilization process incomplete sterilization will eventually seriously affect the shelf life of the product (Deegenaars and Watson 1997).

\textbf{9.5.2 Sublethally Injured State}

Various types of microorganisms have different tolerances to electric fields, whether they are walled or non-walled cells, when treated with the same intensity of electric field, the treatment results obtained are also different. To date, a number of researches have investigated the PEF tolerance of different foodborne pathogens, and the associated influencing factors (Garcia et al. 2003). Somolinos et al. (2008) reported that with the exposure of PEF under 25 kV/cm, 50 pulses, 1 Hz and exponential waveform, over 99% of \textit{E. coli} BJ4L1 cells achieved to survive.

In the study of Cebrian et al. (2007), it is found that foodborne pathogens in the stable phase of cell growth exhibited higher resistance to PEF (26 kV/cm, 100 pulse)
in comparison to those in other phases. In addition, Sagarzazu et al. (2010) reported that decreasing the pH value of medium from 7.0 to 4.5 caused the *C. jejuni* NCTC 11351 cells to increase the resistance to PEF. The external treatment factors, including electrical field strength and medium pH, contribute to causing sublethal damage to cells (Jaeger et al. 2009; Saldaña et al. 2009). The results of the above studies point out that cells are more tolerant at low pH values, so the safety of the results of applying PEF to acidic foods is particularly worrying. Therefore, it is necessary to conduct further research on the recovery and growth of sublethal damaged cells after PEF treatment.

### 9.5.3 Protective Mechanisms Underlying Sublethal Injury

Currently, researchers are gradually focusing on the protection or repair mechanism underlying the sublethal damaged cells caused by PEF treatment. Furthermore, Garcia et al. (2006) indicated that the tolerance of *E. coli* in PEF depended on the repairment in cytoplasmic membrane—the direct target inactivated by PEF.

Further researches have shown that after PEF treatment, oxidative stress in the cells could be induced, thereby increasing the PEF resistance (Pakhomova et al. 2012). Tanino et al. (2012) stated that PEF under an intensity of 2–4 kV/cm induced the expression of oxidative stress response genes in *S. cerevisiae*, especially those that encode glutathione synthase. It is found that it is more easy for the expression of glutathione synthase-encoding gene to be induced by PEF in comparison to superoxide dismutase-encoding genes. In short, PEF stimulation can lead to increased glutathione and superoxide dismutase production, thereby reducing oxidative stress and cell damage to improve their PEF tolerance. Moreover, the same PEF treatment conditions were found to result in no changes in the heat stress reaction of *S. cerevisiae* (Tanino et al. 2012). Chen et al. (2005) also confirmed that the resistance of sublethally injured *S. cerevisiae* to PEF was highly associated with the glutathione-dependent biochemical defense system.

### 9.5.4 Influencing Factors of Sublethally Injured Cells

Recently, Wang et al. (2015) found that the shorter exposure time or lower PEF intensity at 5–10 kV/cm could generate a proportion of sublethally injured *S. cerevisiae* cells, which were determined by selective mediums containing 4.25% NaCl. Zhao et al. (2013) used non-selective and selective media to evaluate the lethal and sublethal damage behavior of pathogens (e.g., *E. coli*) in milk. The results showed that for *L. monocytogenes*, when the PEF intensity was enhanced from 15 to 30 kV/cm, the sublethal injury rate increased from 18.98% to 43.64%, while the sublethral rate was increased from 18.98% to 43.64% when applied to *E. coli* and *S. aureus*. The rate reaches the maximum under a PEF intensity of
25 kV/cm (40.74% and 36.51%, respectively), and then the sublethal rate would decrease as the PEF intensity was enhanced; at the same time, they also found that when the electric field strength is fixed at 30 kV/cm, the sublethal rate of E. coli cells reached the maximum at the treatment time of 500 μs, while it is reached the maximum for S. aureus and L. monocytogenes after 400 μs exposure, and then the sublethal rate will decrease with the extension of the treatment time. Another study evaluated the microbiological status of green tea beverages treated with PEF during the shelf life of storage at 4, 25, and 37 °C (Zhao et al. 2009). It was found that cold storage at 4 °C for 7 days can retard the self-repair process of PEF-induced sublethally injured bacteria, and finally prolong the microbial shelf life of green tea beverages to 90 days. These results from different research groups indicate that the percentages of microbial cells with sublethal damage not only depend on the PEF treatment parameters, but also on the characteristics of the treated foodborne pathogens, and probably achieve a maximum value after exposure to PEF under a specific condition. The PEF treatment time and electric field intensity continue to increase and remain constant or gradually decrease; in addition, it also shows that the changes in the living environment after PEF treatment will affect the survival of sublethal foodborne pathogens. At the same time, these research findings also make people re-understand the behavior and mechanism of PEF on the inactivation of foodborne pathogens.

9.6 Strategies to Improve the Bactericidal Effect of PEF on Foodborne Pathogens

At present, the application of PEF technology to food sterilization has certain limitations. It is difficult to achieve the expected results with the current technology and equipment level alone. In addition to actively improving technical equipment, synergizing high-voltage electric field technology with other technologies to enhance the sterilization effect is also an important trend in the development of PEF in the future. PEF technology and mild heat synergistic sterilization have been reported in liquid eggs, egg whites, and fruit and vegetable juices. Liu et al. (2020) also used this method when sterilizing animal by-products. The synergy of PEF and heat is a common method of enhanced sterilization, which can reduce the impact on the food itself while obtaining a better bactericidal effect (Siemer et al. 2014). Rezaeimotlagh et al. (2018) established a mathematical model of PEF energy input, time, and temperature on the inactivation of E. coli in cranberry juice. This study indicated that increasing the temperature of the juice under a constant PEF intensity significantly enhances the lethality of E. coli. And reduce the energy consumption of the electric field.

The study of PEF technology and antibacterial agent collaborative sterilization can help to improve the overall sterilization efficiency or reduce the energy input of
PEF and the concentration of antibacterial agents under the same bactericidal effect. Ait-Ouazzou et al. (2013) had confirmed the synergistic bactericidal effect of PEF and carvacrol on E. coli in apple juice, mango juice, orange juice, and tomato juice. The original physical and chemical properties and nutritional characteristics of the juice were also retained to the greatest extent. In addition, the by-product extracts of cauliflower and citrus can also achieve good synergistic bacteriostasis with PEF (Sanz-Puig et al. 2016). Synergizing the antibacterial agent with the PEF can not only achieve a powerful sterilization effect but also retain the original quality of the food and reduce the input requirements for the electric field strength. If natural antibacterial substances are used, it can also increase functional advantages such as anti-oxidation and anti-tumor. It is a kind of sterilization method with great research potential.

Synergistic sterilization of PEF technology and other low-temperature sterilization technologies is also an important idea to enhance the sterilization effect. It has been reported that PEF and high-pressure processing technology can only produce additive effects when successively acting on Listeria monocytogenes, while the two simultaneous actions can produce highly effective synergistic antibacterial effects (Pyatkovskyy et al. 2018). This may be due to the simultaneous action of mechanical pressure and electrical pressure on the cell membrane, causing fatal damage to the cell. Combining ultraviolet light or high-intensity pulsed light with PEF technology is also a method that researchers have explored extensively (Caminiti et al. 2011; Mahendran et al. 2019). Another study showed that ultraviolet light, pulsed light, and PEF alone can result in 1.8–6 log inactivation of E. coli and Pichia in cranberry and apple juice (Palgan et al. 2011). In addition, the synergistic effect of ultraviolet light or pulsed light and PEF can directly reduce the number of colonies by over 6 logs. Aadil et al. (2018) explored the synergistic effect of ultrasonic technology and PEF technology and found that the combination of the two can effectively inhibit the number of foodborne pathogens in grapefruit juice while protecting the active ingredients in the juice. In the future, it is necessary to optimize the synergistic technology of ultraviolet light, pulsed light, ultrasound, and PEF to compete with thermal sterilization technology.

## 9.7 Summary and Future Perspectives

As a new type of low-temperature sterilization technology, PEF technology has advantages those other technical methods do not have, and it is increasingly becoming a research hotspot in the field of food sterilization. However, there is still a long way to go before PEF technology can achieve industrialization in the food field. PEF technology has always been favored by researchers at home and abroad due to its strong sterilization effect and extremely short sterilization time. Through active equipment improvement and development of joint effects with other technologies, it can make up for the defects of high equipment requirements and high production costs. It is the key to bringing PEF technology to industrialization.
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Chapter 10
Response of Foodborne Pathogens to Cold Plasma

Xinyu Liao, Tian Ding, Qiseng Xiang, and Jinsong Feng

Abstract Cold plasma, the emerging decontamination technology, has attracted increasing attention in the last decades. Plasma generates a combination of various stressors (e.g., UV photons, charged particles, reactive oxygen/nitrogen species, and electric fields), which could act on the bacterial cells. When encountering external stresses, bacteria can initiate their complex stress response system within bacteria to achieve survival. In this chapter, the inactivation efficacy of cold plasma on foodborne pathogens, the associated influencing factors as well as the inactivation kinetics are introduced in detail. The mechanisms underlying the microbial inactivation by cold plasma are discussed in both the cellular and genetic levels. Lastly, the summary of various bacterial responses (e.g., morphological changes, detoxified enzymes, protective proteins, and repairing systems) against cold plasma exposure is also provided in this chapter, which might be a potential risk in food safety and should be considered during the utilization and optimization of cold plasma processing.

Keywords Cold plasma · Bacterial cells · Inactivation efficacy · Bactericidal mechanisms · Stress response
10.1 Introduction

According to the estimation of the World Health Organization (WHO), in 2010, there are around 600 million illness cases (or 1 in 10 people) with 420,000 deaths due to the consumption of contaminated food each year worldwide (WHO 2015a). Among those foodborne disease hazards, over 60% of foodborne diseases are caused by bacterial pathogens, such as *Campylobacter* spp., enteropathogenic/enterotoxigenic/Shiga toxin-producing *Escherichia coli* (EPEC/ETEC/STEC), non-typhoidal *Salmonella enterica*, *Shigella* spp., *Vibrio cholerae*, *Brucella* spp., *Listeria monocytogenes*, *Mycobacterium bovis*, *Salmonella Paratyphi* A, and *Salmonella Typhi* (CDC 2011). Foodborne bacterial pathogens have become the major risks to global food safety and human health. Some of those pathogens result in diarrheal diseases. And other pathogens, such as *Listeria, Brucella,* and *Cholera,* could bring out invasive infectious diseases, such as blood poisoning, meningitis, fever, muscle pain, arthritis, chronic fatigue, neurologic symptoms, depression, and even death (WHO 2015b).

In the food industry, sterilization is generally used to eliminate bacterial pathogens in foods to assure food safety (Li and Farid 2016). So far, various sterilization technologies have been developed in the food industry. Thermal processing is the most widely used sterilization technology, which mainly consists of ultra-high-temperature processing and pasteurization. High temperature can efficiently inactivate microorganisms, but it also could bring out the loss in nutrients as well as cause adverse damages in food quality (e.g., color, sensory, texture) (Berk 2018). In recent years, nonthermal technologies have attracted increasing attention from food researchers due to the low working temperature and less compromise in food properties. The major emerging nonthermal sterilization technologies include high pressure processing (Rivalain et al. 2010), ultraviolet/pulse light (Masschelein and Rice 2016), ultrasonic field (Gallego-Juárez and Graff 2014), pulsed electric field (Vega-Mercado et al. 1997), irradiation (Motarjemi et al. 2013), cold plasma (Liao et al. 2017) and the hurdle technology of the above technologies (Ross et al. 2003). However, some nonthermal technologies could induce the microbial stress response, posing potential risk to food safety.

Bacteria are capable to survive in the diverse environment through complex stress response systems. Stress is described as the conditions that are different from the suitable growth and survival conditions for the bacterial population (Zorraquino et al. 2017). Along the food processing, storage, distribution and preparation, foodborne bacterial pathogens suffer from a series of stresses, such as heat, osmotic pressure, acidic shock, cold, and oxidative agent (Begley and Hill 2015). Those stresses lead to damages to bacterial cells to different extents. In turn, bacterial cells make sense of the external stress and conduct the corresponding response toward the stresses through various cellular and molecular mechanisms in the group level (e.g., programmed cell death [PCD]) (Bayles 2014), the cellular level (e.g., morphological changes, and bacterial size regulation) (Westfall and Levin 2017) and the molecular level (e.g., gene expression, and protein production) (Abee and Wouters 1999).
These stress responses render bacterial cells the ability to detoxify the lethal agents or repair the damages to achieve final survival. The occurrence of stress response in foodborne pathogen could result in bacterial persistence and incomplete decontamination, posing potential risk to food safety and public health.

Plasma, partially or wholly ionized gas, is regarded as the fourth state of matter, which is distinct from the solid, liquid, and gas states (Bourke et al. 2018). Plasma is consisted of abundant species, including various radicals, charged particles (electrons and ions), neutral particles (ground/excited atoms and molecules), and quantum photons (visible and ultraviolet light) (Scholtz et al. 2015). The generation of plasma can be achieved under low pressure (vacuum), atmospheric pressure, and high pressure. Based on the electron temperature ($T_e$), plasma can be divided into thermal plasma ($T_e: 10^6–10^8$ K) and cold/nonthermal plasma ($T_e: 10^4–10^5$ K). The temperature of the whole plasma is mainly determined by the temperature of heavy particles (Niemira 2012). When the applied electromagnetic field is enough for the ionization, free electrons are subsequently produced, followed by the occurrence of collisions between electrons and gas molecules. Regarding cold plasma, the free electrons transfer only a small amount of energy to the surrounding particles, resulting in the electron temperature is much higher than the temperature of other particles and rendering the plasma in non-equilibrium thermodynamics. Generally, the temperature of the whole cold plasma remains in the range of 30–60 °C (Bourke et al. 2018). Thermal plasma remains in an equilibrium thermodynamics, in which all the particles have almost the same temperature. Therefore, the temperature of thermal plasma could reach over thousand degree Celsius. Due to the low working temperature and high bactericidal performance, cold plasma holds great promise for food decontamination application and has attracted a lot of interest in the last two decades. Common sources for the generation of cold plasma include dielectric barrier discharge (DBD), microwave discharge, corona discharge, gliding arc discharge, glow discharge, and atmospheric pressure plasma jet (APPJ). DBD and APPJ are the most widely used configurations for cold plasma generation due to the advantages of simple and easy to modification (Misra et al. 2011; Scholtz et al. 2015) (Fig. 10.1). Due to the high bactericidal efficiency, less damages in food organoleptic quality and environmentally friendly, cold plasma have been reported to be applied for the decontamination of various foods, including fruits and vegetables, nuts, cereals, meat, dairy products, juices, etc. (Ekezie et al. 2017; Liao et al. 2017). A lot of studies have investigated the efficacy of cold plasma on microbial inactivation in foods; however, there are limited studies concerning the potential risk of bacterial stress response against cold plasma exposure.

In this chapter, the bactericidal efficacy of cold plasma, the influencing factors and the mathematical models for inactivation kinetics are introduced. Additionally, the mechanisms underlying cold plasma-induced microbial inactivation are discussed as well. Then, the following part includes a comprehensive review of the bacterial stress toward cold plasma exposure from the aspect of phenotypic and genetic levels.
10.2 Microbial Inactivation by Cold Plasma

10.2.1 The Bactericidal Efficacy of Cold Plasma

As early as 1960s, Menashi (1968) reported the application of an argon plasma for decontamination of glass bottle surface. In the last decades, cold plasma technology has been applied for the microbial removal from foods or food packages. Lis et al. (2018) employed cold plasma to inactivate *L. monocytogenes* and *S. Typhimurium* on ready-to-eat ham rolls for 20 min. The reduction levels of *L. monocytogenes* and *S. Typhimurium* could achieve 1.02 and 1.14 log$_{10}$ CFU/cm$^2$, respectively. Pasquali et al. (2016) made use of an atmospheric DBD plasma to treat fresh radicchio, decreasing *E. coli* by 1.35 log$_{10}$ MPN/cm$^2$ while no significant reduction for *L. monocytogenes*. There are many factors affecting the bactericidal performance of cold plasma. The influencing factors can be divided into the terms of plasma generation sources, treatment modes (direct or indirect), working gas types and gas flow rates, bacterial characteristics (e.g., Gram-negative and-positive, initial inoculum, and growth phases), environment conditions (e.g., humidity, temperature), and food properties (e.g., constituents, viscosity, acidity, water activity, surface properties). Lu et al. (2014) reported that when the voltage and the oxygen content of working gas increased, a higher reduction of bacteria by an atmospheric DBD plasma was achieved. It is speculated that the greater oxygen resulted in the production of higher level of hydroxyl radicals and ozone in plasma, which exhibit higher reactive characteristics than other radicals. Mai-Prochnow et al. (2016) compared the resistance of Gram-positive and Gram-negative bacteria toward an argon plasma jet treatment. It is found that a 10-min cold plasma treatment resulted in 0.5–2 log reduction of Gram-positive bacteria (*B. subtilis, Staphylococcus epidermidis, Kocuria carniphila*), while 3.5 log reduction was achieved for Gram-
negative bacteria (*Pseudomonas libanensis*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*). The results were attributed to the differences in the cell wall thickness. The most resistant Gram-positive bacteria *B. subtilis* possesses the cell wall with a thickness of 55.4 nm, and the cell wall thickness of Gram-negative *P. aeruginosa* was only 2.4 nm. Han et al. (2016a) demonstrated that the damaged targets by cold plasma were different between Gram-negative bacteria (*E. coli*) and Gram-positive bacteria (*Staphylococcus aureus*). It is reported that the inactivation of *E. coli* was mainly resulted from the damages on the cell envelopes, while the intracellular components (e.g., DNA) of *S. aureus* were the major target affected by cold plasma. In addition to the microbial properties, Song and Fan (2020) reported that cold plasma brought out over 5 logs inactivation of *S. Typhimurium* inoculated on tomatoes and apples, while only 2.8–3.6 log reduction of *Salmonella* on tomato stem scars and cantaloupe rinds. The rough surface of tomato stem scars and cantaloupe rinds could shield the bacteria from the attack of cold plasma, resulting in low bactericidal efficacy. The complex structure of food surface, such as porosity (e.g., eggs), roughness (e.g., strawberries), and pit (e.g., cooked chicken breast), bring out the difficulties in the contact between bacterial cells and reactive plasma species and result in the incomplete inactivation.

### 10.2.2 The Kinetic Models for the Plasma-Induced Deactivation on Microbes

Predictive mathematical models are usually used as the tools to describe the microbial inactivation kinetics induced by a decontamination technology (Perez-Rodriguez and Valero 2013). Generally, predictive models can be sorted into primary, secondary, and tertiary models (Fakruddin et al. 2011). The primary models aim to describe the kinetics with the use of few parameters. The secondary models present the relationship between the environmental factors or other external influencing factors and the parameters obtained from the primary models. Regarding tertiary models, they are achieved through software, with which the users can simply and quickly obtain the model outputs after entering the inputs. The most common primary inactivation models are exhibited in Table 10.1, including the Linear model, Weibull model (Mafart et al. 2002), Shoulder/Tail model (Geeraerd et al. 2000), modified Gompertz model (Linton et al. 1995), biphasic linear model (Lee et al. 2009), and log-logistic model (Chen and Hoover 2003). The goodness of models is generally evaluated with indexes, including the root mean square error (RMSE), and the coefficient of determination ($R^2$) (Den Besten et al. 2006). The smaller RMSE value is and the $R^2$ value is closer to 1, the better fitness the model has. In addition, the validation is an indispensable step to confirm the practicability of the established models. Generally, there are two types of model validation methods: internal and external validations (Zurera-Cosano et al. 2006; teGiffel and Zwietering 1999). When the observed data are obtained from the same experimental
conditions as those applied for the model establishment, the validation is called internal validation. Regarding external validation, the fitted data are observed and obtained under the distinct conditions from the ones used for the model establishment.

So far, some studies have reported the application of various predictive models for the description of inactivation kinetics with cold plasma exposure (Hertwig et al. 2015; Surowsky et al. 2014). Surowsky et al. (2014) found that the inactivation kinetics of *C. freundii* in apple juice treated by an argon/oxygen cold plasma jet were fitted well by Weibull models. Generally, the shape parameters of Weibull models are highly related to the stress resistance of the treated bacterial cells. In the above study, the shape parameters of the fitted Weibull models were more than 1, indicating that the *C. freundii* became susceptible toward cold plasma as the treatment time increased. However, Hertwig et al. (2015) found that the fitted Weibull model for an argon plasma jet induced inactivation of *Bacillus atrophaeus*, *B. subtilis*, and *S. enterica* had the shape parameters less than 1, indicating that the remaining bacterial population became increased resistance toward cold plasma exposure. Such differences in cold plasma resistance might be attributed to the variations in bacterial strains as well as the working gas compositions for plasma generation. In the study of Mendes-Oliveira et al. (2019), the change of the generated reactive

<table>
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<th>Predictive models</th>
<th>Mathematical equations</th>
<th>Fitting parameters</th>
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<tr>
<td>Linear model</td>
<td>( \log N_t = \log N_0 - k t )</td>
<td>( k ): The first-order rate constant</td>
</tr>
<tr>
<td>Weibull model</td>
<td>( \log \frac{N_t}{N_0} = -\frac{1}{\alpha} \left( \frac{t}{\beta} \right)^\beta )</td>
<td>( \alpha ): Scale parameter ( \beta ): Dimensionless shape parameter</td>
</tr>
<tr>
<td>Shoulder/ tail model</td>
<td>( N_t = [(N_0 - N_{\text{res}}) \exp(-k_{\text{max}}t)] \left[ \frac{\exp(-k_{\text{max}}tL)}{1 + \exp\left((-k_{\text{max}}tL) - 1\right) \exp(-k_{\text{max}}t)} + N_{\text{res}} \right] )</td>
<td>( k_{\text{max}} ): The maximum specific decay rate ( t_L ): The lag time before inactivation ( N_{\text{res}} ): The residual population density</td>
</tr>
<tr>
<td>Modified Gompertz model</td>
<td>( \log \frac{N_t}{N_0} = a \exp[-\exp(b + ct)] - a \exp[-\exp(b)] )</td>
<td>( a ), ( b ), and ( c )</td>
</tr>
<tr>
<td>Biphasic linear model</td>
<td>( \log \frac{N_t}{N_0} = \log_{10} \left[ (1 - f) \cdot 10^{-\frac{D_{\text{sens}}}{D_{\text{res}}}} + f \cdot 10^{-\frac{D_{\text{res}}}{D_{\text{sens}}}} \right] )</td>
<td>( f ): The fraction of resistant microbial population ( D_{\text{sens}} ): Decimal reduction times of microbial population that are sensitive to the treatment ( D_{\text{res}} ): Decimal reduction times of microbial population that are resistant to the treatment</td>
</tr>
<tr>
<td>Log-logistic model</td>
<td>( \log \frac{N_t}{N_0} = \frac{A}{1 + e^{\frac{\alpha}{\sigma} + \frac{\tau}{t}}} )</td>
<td>( A ): The upper asymptote-lower asymptote ( \sigma ): The maximum inactivation rate ( \tau ): The log time to the maximum inactivation rate</td>
</tr>
</tbody>
</table>

\( N_t \): the number of microbial populations at time \( t \); \( N_0 \): the initial number of microbial populations; \( t \): treatment time
plasma specie-ozone with time was taken into consideration for the establishment of inactivation Weibull models as the following equation:

$$\log \frac{N_t}{N_0} = -b(C) (t)^{n(C)},$$

where $C$ is the concentration of the reactive plasma species, $n(C)$ are the parameters associated with reactive plasma species concentration, and $b(C)$ is the inactivated rate, which can be presented in the following equation:

$$b(C) = \ln \left[1 + \exp \left(k(C - C_C)\right)\right],$$

where $C_C$ is a concentration above which the inactivation of that microbial cells becomes effective.

### 10.3 The Mechanisms of Cold Plasma on Bacterial Inactivation

So far, the exact inactivation mechanisms underlying cold plasma on microorganisms are still obscure (Von Woedtke et al. 2013). It is generally believed that the combined effects of plasma species (e.g., UV photons, electric field, charged particles, and reactive oxygen/nitrogen species [RONS]) are the major contributors to microbial inactivation induced by cold plasma.

1. **UV photons**

   It is well known that UV photons, especially the UVC light at around 260 nm, can induce the formation of thymine dimers in bacterial DNA, blocking the process of genetic replication and transcription. However, the exclusion of UV photons by MgF$_2$ layer could not protect the *E. coli* from the cold plasma-induced inactivation, indicating the minimal contribution of UV to the bactericidal effect of cold plasma (Dobrynin et al. 2009). Ehlbeck et al. (2010) also demonstrated that the UV irradiation intensity was extremely small due to the absorption by the surrounding air. Similarly, Lunov et al. (2015) made use of sapphire glass to only allow the UV light to penetrate into the microbial cells. The results indicated that the pure UV irradiation took hours to achieve efficient microbial inactivation, while few minutes were enough for the plasma without sapphire glass to kill bacteria. Therefore, the UV irradiation generated from plasma poses a negligible effect on bactericidal activity.

2. **Electric field**

   The applied electric field to generate the plasma could result in the deformation of cell morphology. However, the electric field intensity tends to be dramatically decreased when the distance from the electrode exceeds 300 µm (Zimmermann
et al. 2012). Therefore, the effect of electric field can be generally ignored in most conditions due to the relatively large distance between the electrode and treated foods.

3. RONS

In addition, the oxidative damages induced by RONS on multiple cellular targets are thought to cause bacterial death. Generally, the major ROS include superoxide anion (O$_2^-$), hydroxyl radical (•OH), hydroperoxyl (HO$_2$), carbonate radicals (CO$_3^-$), hydrogen peroxide (H$_2$O$_2$), ozone (O$_3$), singlet oxygen (^1O$_2$), and peroxynitrite (ONOO$^-\$). In addition to ROS, the common plasma RNS are nitric oxide (NO), nitrogen dioxide (NO$_2$), nitrate (NO$_3^-$), nitrous acid (HNO$_2$), nitrosyl cation (NO$^+$), nitrosyl anion (Pankaj et al. 2016), and dinitrogen trioxide (N$_2$O$_3$). The types and concentrations of the RONS are highly dependent on the plasma generation sources, voltage, working gas, temperature, and humidity (Graves 2012). Among the cellular targets, the cell walls/membranes, and the outermost structure of bacterial cells, are more likely to be virulent toward plasma species. Especially, the polyunsaturated fatty acids (PUFA) in cell membranes tend to be attacked by ROS. The presence of double bonds reduces the bond energy of the C–H bonds present on the adjacent carbon atoms. It has been reported that the hydroxyl radical (•OH) and hydroperoxyl (HO$_2$) could attack the allylic hydrogen and initiate the oxidation of PUFA (Halliwell and Gutteridge 2015). In the initial phase, PUFA is attracted to a hydrogen atom and changes into fatty acid radicals. Subsequently, it is further oxidized into lipid hydroperoxide (ROOH). Regarding the charged radicals, like superoxide anion (O$_2^-$), the charged property prevents them to pass easily through the lipid bilayer structure of the bacterial cell membranes, so it is not easy for them to abstract the hydrogen atom from the fatty acids. In addition to fatty acids, another major constituent in cell walls/membranes-proteins can be modified or inactivated by the reaction with plasma reactive species (Suwal et al. 2019). Among those radicals, hydroxyl radical (•OH) is the most reactive one that can directly attack the peptide bonds within the proteins. Hydrogen peroxide (H$_2$O$_2$) can oxidize the -SH group in proteins, and superoxide anion (O$_2^-$) is capable of damaging the Fe-S cluster in some enzymes. Proteins are consisted of various amino acids with more than 20 types. The chemistry of oxidative damages in proteins is complex and depends on the number and type of the amino acid. In addition to the fatty acids and proteins, some bacterial strains, like S. aureus, have the pigment molecule within the cell membrane, which is also vulnerable to the reactive plasma species (Zhang et al. 2013). In the study of Zhang et al. (2013), the color of the S. aureus suspension was observed to be changed from yellow to white after 20-min plasma-activated water (PAW) exposure (Fig. 10.2), which indicated the oxidization of staphyloxanthin.

Similarly, Yang et al. (2020) reported that the S. aureus defected in staphyloxanthin-associated genes (crtM and crtN) exhibited increasing sensitivity toward cold plasma exposure. Staphyloxanthin belongs to the carotenoid pigments and is synthesized by a serial of enzymes encoded within the crtOPQMN operon (Pelz et al. 2005). Some studies also reported the oxidative damages in DNA induced
by the reactive plasma species (Privat-Maldonado et al. 2016; Yost and Joshi 2015). The radicals are capable of resulting in the breakage of DNA or the modification in DNA bases or deoxyribose (Dizdaroglu and Jaruga 2012; von Sonntag 2006). Among the four types of DNA bases, guanine shows most susceptible to oxidative attack. For instance, hydroxyl radical ($\cdot$OH) adds to guanine at position C-8 within the purine ring, and the C-8 OH-adduct radical can be subsequently produced and further oxidized into 8-hydroxyguanine (8-OHdG), a common biomarker of DNA oxidative damage (Yost and Joshi 2015). Privat-Maldonado et al. (2016) conducted the DNA damage diffusion assay to confirm the breaks in the double-stranded DNA within single bacterial cells after a DBD cold plasma jet treatment (Fig. 10.3). With the use of SYBR Gold fluorescent probes, the intact DNA is presented as a bright, condensed center and a peripheral region of DNA loops (Fig. 10.3c, untreated bacterium). If the DNA strands are damaged, there are fragments that disperse around the core (Fig. 10.3c, gas-treated bacterium).

In addition, Alkawareek et al. (2014) confirmed that a plasma jet treatment with the use of a gas mixture of 0.5% oxygen and 99.5% helium could result in the single/double-strand breaks within the plasmid DNA in a supercoiled (SC) form with the use of gel electrophoresis analysis (Fig. 10.4).

In the study of Ranjbar et al. (2020), the computer simulation with the use of constraint-based reconstruction and analysis (COBRA) Toolbox was conducted to estimate the effect of plasma on the biomolecules involving in the glycolysis pathway of *E. coli* cells. It was found that the plasma-induced oxidation led to the reduction in biomass production, with the decrease in the rates of 46 reactions and the increase in the rates of 2 reactions (Fig. 10.5). The two reactions with enhanced rates include the conversion from fumarate to succinate with the fumarate reductase and the production of nicotinamide adenine dinucleotide reduced (NADH) from NAD$^+$, which could contribute to the defense of *E. coli* toward the oxidative stress.

4. **Ion bombardment**

Apart from UV photon and reactive species, the ions produced from the plasma could be accelerated by the electric field and produce a directed flux of energetic particles to attack on the target sample, known as ion bombardment (Liu et al. 1990).
It has been reported that the ion bombardment of plasma could result in the physical disruption of microorganisms in the study of Lunov et al. (2016), who made use of Stopping and Range of Ions in Matter (SRIM) simulations and revealed that a helium plasma ion-induced bombarding directly disrupt the bacterial outer structure (cell wall and membrane), resulting in the complete inactivation (Fig. 10.6). Additionally, the accumulation of ions on the bacterial surface could result in the electrostatic tension, and if the electrostatic pressure exceeds the tensile strength, the bacterial
shell will be unstable with subsequent loss in membrane integrity (LarouSSI et al. 2003; Lunov et al. 2016; Mendis et al. 2000).

10.4 The Bacterial Stress Response Toward Cold Plasma

When exposed to fluctuating environmental stresses, bacteria are able to initiate the complex stress response systems to deal with the harsh conditions. As mentioned in Sect. 10.3, cold plasma treatment poses various stressors (e.g., charged particles, RONS) on bacterial cells. In turn, bacterial cells also make use of various mechanisms in response to those plasma-induced stressors to achieve survival upon cold plasma exposure.
Fig. 10.5  The core maps of *Escherichia coli* under no plasma stress (a) and plasma stress (b), active reactions are presented in line with the color. The color bar in the bottom right exhibited the reaction rate. This figure is reprinted from Ranjbar et al. (2020), with the permission of Elsevier.
10.4.1 Morphological Changes

The intact cell morphology plays an important role in the normal growth and survival of bacteria. Under some harsh environments, the changes in bacterial morphology could render the bacteria with the ability to deal with the stressors. In the study of Jahid et al. (2015), the colony morphology of S. Typhimurium changed from smooth to rugose under an oxygen cold plasma treatment (Fig. 10.7). The ruggedness in morphology has been reported as a survival strategy when exposed to nutrient depletion, high temperature, and high salinity (Ali et al. 2002; Martinez-Urtaza et al. 2004; Wai et al. 1998). It is reported that the rugose colonies tend to

Fig. 10.6 Stopping and Range of Ions in Matter (SRIM) simulation of plasma helium (He) ion penetration. SRIM simulation results of a low voltage plasma produced He ion penetration through a (a) Gram-positive and (b) Gram-negative cell outer structure. SRIM simulation results of a high-voltage plasma produced He ion penetration through a (c) Gram-positive and (d) Gram-negative cell outer structure. This figure is reprinted from Lunov et al. (2016), with permission of Elsevier

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produce more extracellular polymeric substances (EPS) and cause more formation of biofilms. Biofilms are the bacteria community attached to the contact surface and surrounded by EPS (O’Toole et al. 2000). It has been demonstrated that biofilms can efficiently protect bacterial cells from the penetration of some bactericidal agents (e.g., antibiotics). The formation of biofilms is believed to be regulated by quorum sensing (QS), which is a common communication phenomenon among bacterial cells through the signaling molecules (e.g., acyl-homoserine lactones and autoinducing peptide) (Whitehead et al. 2001; Ng and Bassler 2009; Saenz et al. 2000). When the concentration of signaling molecules reaches the threshold value, these molecules react with the receptors, and the information transduction will be subsequently initiated. In addition, the bacterial cells show the tendency to decrease the cell size under cold plasma exposure (Ziuzina et al. 2013). Ziuzina et al. (2013) employed scanning electron microscopy (SEM) and observed that a 3 s-DBD plasma exposure resulted in obvious shrinkage and dehydration of E. coli cells. Similarly, in the study of Joshi et al. (2011), the morphology of plasma-treated E. coli cells was observed to be changed into a coccoid shape with a reduced surface area. The

Fig. 10.7 The Salmonella Typhimurium colony formation after an oxygen cold plasma treatment. (a) Smooth colonies on a plate; (b) rugose colonies on a plate; (c) smooth colonies (40× magnification); (d) rugose colonies (40× magnification). This is reprinted from Jahid et al. (2015) with the permission of Elsevier
regulation of bacterial cell size and shape in response to the fluctuating conditions has been regarded as a strategy for survival (Chien et al. 2012). It has been found that the bacteria (e.g., *Salmonella*, *B. subtilis*, *S. aureus*) tend to have a larger cell volume under a suitable environment (e.g., rich nutrients) than the same cells in a harsh condition (e.g., starvation) (Kjeldgaard et al. 1958; Sargent 1975; Watson et al. 1998). The decreased cell size could minimize the energy consumption to retain the bacterial survival when encountering various stressors, such as acid and oxidation (Crompton et al. 2014). The nucleotide second messenger, alarmone guanosine tetraphosphate-(p)ppGpp, has been reported to be one of the major contributors to reduce the bacterial cell size and render bacteria with higher resistance toward external stressors (Westfall and Levin 2017; Chatnaparat et al. 2015). In addition to (p)ppGpp, c-di-AMP, another kind of nucleotide second messenger, has also been reported to be associated with the cell size reduction of *S. aureus* under extreme cell membrane/wall stresses (Corrigan et al. 2011). Furthermore, Kwandou et al. (2018) reported the drying effect of an atmospheric air dielectric barrier discharge could result in the aggregation of extracellular polysaccharides and change the biofilm structure, which rendered the increased resistance of *E. coli* biofilm toward cold plasma exposure.

### 10.4.2 Sigma Factors

In 1969, it was first identified that the initiation of transcription in bacteria requires the regulation of a subunit of RNA polymerase, called sigma factors (Burgess et al. 1969). Sigma factors contribute to directing RNA polymerase (RNAP) to bind to the specific promoter site of the gene and help the expression of the associated genes (Fig. 10.8) (Mooney et al. 2005; Paget 2015). The primary sigma factor is responsible for the transcription during bacterial growth. And the alternative sigma factors contribute to the regulation of the specific gene expression under stressful conditions (Helmann and Chamberlin 1988).

Based on the differences in the sequences, bacterial sigma factors can be classified into two kinds of families: $\sigma^{54}$ and $\sigma^{70}$. $\sigma^{54}$ factors generally take part in the regulation of nitrogen metabolism, carbohydrate metabolism, biofilm formation, bacterial movement, and quorum sensing RopN protein in *B. cereus* and SigL protein in *B. thuringiensis* are the examples of $\sigma^{54}$ factors (Hayrapetyan et al. 2015; Peng et al. 2015). Most of the sigma factors belong to the $\sigma^{70}$ family, which can be further divided into four groups (Vassylyev et al. 2002). Group I sigma factors are primary sigma factors, which are responsible for the transcription of diverse genes within bacterial cells (e.g., RopD protein in *E. coli*) (Davis et al. 2017). Groups II sigma factors are non-essential for bacterial growth and might contribute to the recognition of similar promoter sequences, for example, the RpoS protein in *Salmonella*. Group III sigma factors can selectively regulate the gene expression (e.g., SigI protein in *B. subtilis*) under specific conditions, such as high temperature, extreme osmotic pressure, etc. Group IV sigma factors (also called extracytoplasmic
function [ECF]), the most abundant group in $\sigma^{70}$ family, contribute to the response toward the signal from the external environment, for instance, AlgU protein in *Pseudomonas*. It has been reported that bacterial sigma factors played an important role in general stress response through regulation a serial of gene expression to deal with diverse stressors (Wiedmann et al. 1998). For instance, *L. monocytogenes* sigma factors are responsible for the regulation of over 140 genes, including stress response, virulent properties, transcription, metabolism, and transportation (Fig. 10.9) (Raengpradub et al. 2008).

The sigma factors have been attributed to the resistance of pathogens toward plasma exposure (Cui et al. 2021; Patange et al. 2019; Prieto-Calvo et al. 2016; Han et al. 2016a, b; Xu et al. 2015). In the study of Xu et al. (2015), the expression of sigma factor-sigB gene in *S. aureus* was found to be significantly upregulated by 3.84 and 2.77 folds after an air plasma jet treatment for 10 and 30 min, respectively. Similarly, Patange et al. (2019) reported that 1-min DBD cold plasma exposure induced *L. monocytogenes* EGD-e to overexpress the sigB gene by over 15 times and the sigB depletion resulted in significantly enhanced sensitivity of *L. monocytogenes* toward the cold plasma exposure. Similarly, Cui et al. (2021) also demonstrated the increase in the production of sigB factors in *L. monocytogenes* biofilms by 3.43 folds was induced by a cold nitrogen plasma for 220 s. It is found that it was easier for an air plasma jet to inactivate the verocytotoxigenic *E. coli* (VTEC) with mutation in the sigma factor gene rpoS compared with the wild-type ones (Prieto-Calvo et al. 2016). Apart from sigma factors, the anti-sigma and anti-anti-sigma factors have been found to be the regulators of bacterial cells (Brown and Hughes 1995; Gerardo Trevino-

![Fig. 10.8](https://example.com/fig10.8.png) A cycle of sigma factors. Sigma factors incorporate to core RNA polymerase (RNAP) to produce a holoenzyme, which further binds to the promoter of DNA to form an open complex (OC). $\sigma$ is released when an elongation complex (EC) is completed with the assistance of elongation factors (NusA). When the transcription is finished, DNA and RNA are released from the RNAP. Then, a new cycle of sigma factors is initiated. This figure is reprinted from Mooney et al. (2005), with the permission of Elsevier.
Anti-sigma factors can inhibit the transcription of genes that are upregulated by sigma factors. And the function of anti-anti-sigma factors (e.g., RsbV protein in *Listeria monocytogenes*) is to regulate and control the anti-sigma factor-mediated gene expression or metabolic activities to maintain the physiological

**Fig. 10.9** Overview of the sigma B factor-mediated regulation in *Listeria monocytogenes*. Operon and model of the $\sigma^B$ regulatory mechanism. (a) The sigma B factor operon of each gene; (b) Upon the stress, the phosphorylation of RsbR and RsbS occurs with the kinase, RsbT, which is subsequently released from the stressosome complex. With the incorporation with RsbT, RsbU turns into a phosphatase for the dephosphorylation of the anti-anti-sigma factor RsbV. The anti-sigma factor, RsbW, exhibits a stronger affinity for unphosphorylated RsbV, which results in the release of sigma B factors in a stressed cell. Sigma B factors then bind to RNA polymerase and initiate transcription of the sigma B factor gene regulon. This figure is reprinted from Dorey et al. (2018), with permission of Elsevier

Quintanilla et al. 2013). Anti-sigma factors can inhibit the transcription of genes that are upregulated by sigma factors. And the function of anti-anti-sigma factors (e.g., RsbV protein in *L. monocytogenes*) is to regulate and control the anti-sigma factor-mediated gene expression or metabolic activities to maintain the physiological
homeostasis within the bacterial cells (Utratna et al. 2014). To our best knowledge, the roles of anti-sigma and anti-anti-sigma factors in the bacterial response to cold plasma treatment remain unknown and more investigations are required in the future.

10.4.3 Metal Homeostasis

Intracellular metal ions (e.g., Fe, Cu, Mn, Zn) generally act as the cofactors of enzymes and are involved in diverse physiological activities (Papp-Wallace and Maguire 2006). In addition, ion homeostasis has been associated with bacterial resistance toward stresses, including oxidation, osmosis (Argandona et al. 2010; Brenot et al. 2007; Cornelis et al. 2011). Irons are widely acted as the important cofactors of numerous enzymes, including ribonucleotide reductase, RNA polymerase III, amino acid hydroxylases, dioxygenases, catalase, superoxide dismutase, and peroxidase (Wooldridge and Williams 1993). It is difficult for bacteria to use the ferric irons directly. Some bacteria acquire the irons by reducing the ferric irons into ferrous irons at the cytoplasmic membranes, followed by the transportation inside the bacterial cells. Also, siderophores are another strategy for bacteria to acquire irons (Fig. 10.10) (Bradley et al. 2020). Siderophores have a strong affinity to ferric irons, so they can supply the irons to bacterial cells (Neilands 1981). After the iron acquisition, it will be stored in the iron chelator proteins, like ferritin. In the study of Yau et al. (2018), the bacterioferritin B (BfrB) in P. aeruginosa was found to be upregulated by over 3.3 folds after an argon cold plasma exposure, and it was also found that the ΔbfrB mutant strains are more susceptible to plasma-mediated inactivation with the completed reduction, which was significantly higher than the inactivation level of their wild-type counterparts (0.74 logs). Bacterioferritin, an iron-storage protein, contains a ferroxidase center, which oxidizes Fe$^{2+}$ into Fe$^{3+}$ to regulate the iron homeostasis and contributes to the bacterial oxidative defense response (Carrondo 2003). In addition, bacterioferritin can also sequester the iron to protect bacteria from damages induced by ROS (Cornelis et al. 2011). Sharma et al. (2009) demonstrated that an atmospheric pressure argon plasma plume could induce the over expression of iron starvation genes fhuA and fhuF. The fhuA gene is responsible for the production of ferrichrome outer membrane transporters to facilitate the uptake of irons, and the fhuF gene encodes the ferric iron reductase protein, which contributes to reduce the ferric irons in cytoplasmic ferrioxamine B (Andrews et al. 2003).

Manganese is another important cofactor of bacterial arginase. In addition, manganese also plays an important role in the detoxification of ROS within the bacterial cells. For instance, the superoxide dismutase requires Mn$^{2+}$ to retain the enzyme activity involved in the dismutation reaction of superoxide anion radicals (Clements et al. 1999). There are two types of transporters (MntABC and Nramp) for the supply of manganese into the bacterial cells. Yost and Joshi (2015) reported that cold plasma exposure made E. coli upregulate the gene of mntH, which is responsible for the production of MntABC-type manganese transporter. Apart from iron...
and manganese, zinc (Zn) also acts as the cofactors for some enzymes, including DNA polymerase and dehydrogenase. The regulation of Zn is associated with oxidative stress regulators, such as Fur and PerR (Lee and Helmann 2007), which might also contribute to the stress response against cold plasma. In addition, copper is an essential trace metal, which involves a range of bacterial physiological activities, including respiration, metabolisms, and oxidative stress (Puig and Thiele 2002). So far, bacterial metal homeostasis has not been associated with the resistance to plasma exposure. The plasma-induced upregulation of proteins (e.g., superoxide dismutase [SOD], iron-binding ferritin-like antioxidant protein [Dps, MgrA], bacterial non-heme Ferritin-like protein [FnB]) that chelated with metals have been reported in the previous studies (Joshi et al. 2015; Mols et al. 2013; Yost and Joshi 2015), indicating the important role of metal homeostasis in the microbial resistance to plasma treatment.
**10.4.4 Oxidant Detoxification Enzymes**

Cold plasma produces abundant RONS, which cause oxidative stress on bacterial cells (Han et al. 2016a). The oxidant detoxification enzymes within bacteria can sufficiently help the alleviation of the oxidative attack caused by cold plasma exposure. SOD, a metalloenzyme, plays an important role in neutralizing the intracellular superoxide anion radical through the dismutation reaction into hydrogen peroxide ($O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$). There are four major types of SOD in bacteria, including copper and zinc (Cu/Zn)-SOD, manganese (Mn)-SOD, iron (Fe)-SOD, and nickel (Ni)-SOD (Fridovich 1995). Ritter et al. (2018) made use of a proteomic analysis and demonstrated the overexpression of (Cu/Zn)-SOD in S. Enteritidis after a DBD plasma (~170 W) exposure for 90 min. Regarding H$_2$O$_2$, the catalase within bacterial cells could catalyze the conversion of H$_2$O$_2$ into water and oxygen. Based on the structure and sequence, catalases can be classified into three groups: monofunctional catalase/typical catalase, catalase-peroxidase, and Mn-catalase (Zhang et al. 2010). The previous studies have indicated the contribution of catalase to the bacterial response against the plasma exposure (Alshraiedeh et al. 2016; Joshi et al. 2011; Xu et al. 2015; Yost and Joshi 2015). In the study of Joshi et al. (2011), the addition of catalase in the medium was found to protect E. coli cells from the floating-electrode (FE)-DBD exposure. Alshraiedeh et al. (2016) demonstrated the production of catalase was in a proportional relation to the cold plasma resistance of Burkholderia cenocepacia. The research conducted by Yost and Joshi (2015) found that the E. coli defective in the SOD and/or catalase-associated genes of katG/E and/or sodA/B tended to be more sensitive to cold plasma exposure. Apart from the direct cold plasma exposure, plasma-activated solution (e.g., plasma-activated water [PAW]) can also induce the expression of antioxidant enzymes in bacteria (Qian et al. 2020). In addition to SOD and catalase, the alkyl hydroperoxide reductase (AhpCD) is also one of the antioxidant enzymes within bacteria (Panmanee and Hassett 2009). AhpCD, belonging to the peroxiredoxin family, contributes to the detoxification of various oxidant agents, such as H$_2$O$_2$, organic peroxides, and peroxynitrite (Cosgrove et al. 2007). Winter et al. (2011) employed the two-dimensional (2-D) gel-associated proteomic technique and reported the overexpression of AhpC proteins by over three times in B. subtilis after an exposure to cold plasma treatment. Similarly, the proteomic analysis revealed the 3.96-fold upregulation of the AhpCD in cold plasma-treated S. Enteritidis from the study of Ritter et al. (2018). Similarly, Xu et al. (2015) found that the expression of ahpC gene in S. aureus was significantly enhanced by 3.40 and 3.21 folds after 10- and 30-min plasma exposure, respectively. Most of the aforementioned oxidant detoxification enzymes are activated by OxyS, which is a small noncoding RNA (Altuvia et al. 1997). Sharma et al. (2009) found that oxyS gene in cold plasma-treated E. coli increased by 43-fold times compared with that in untreated ones. In addition to the aforementioned detoxifying enzymes, flavohemoglobin (FlavoHb), with a ferredoxin-NADP$^+$ oxidoreductase-like domain, is also associated with the relief of oxidative agents within bacterial cells (Bonamore and Boffi 2008).
10.4.5 DNA Protection and Repair Systems

The oxidative stress predominantly causes the strand breakage of DNA within bacteria cells. Such damages can be repaired by the recombination-associated systems. The Gram-positive and Gram-negative bacteria might conduct distinct pathways for the recombinational DNA repair. For Gram-negative cells, RecBCD exonuclease/helicase complex is mainly used for the DNA break repair, and Gram-positive bacteria, like staphylococci, tends to make use of the AddAB nuclease/helicase complex (Yeeles and Dillingham 2010). In the study of Yost and Joshi (2015), the significant upregulation of recB and recD was found in the E. coli cells after cold plasma treatment. The genes of recB and recD are responsible for the production of RecB and RecD proteins, respectively, which are the subunits of RecBCD complex. Initially, RecBCD complex binds to the blunt end of a double-stranded DNA break and subsequently, RecB and RecD, acting as the helicase, unwinds the double-stranded DNA until it encounters a Chi sequence (5'-GCTGGTGG-3') from the 3' direction. RecC recognizes and binds to Chi site. Subsequently, RecA binds to 3' tails, and RecBCD complex disassembles. RecA facilitates the strand invasion for the performance of homologous DNA recombination and repair. The nucleotide excision repair (NER) is another pathway for bacterial cells to repair DNA damages induced by oxidative agents. NER pathway is constitutive of a series of Uvr proteins. The UvrAB complex is responsible for the detection of DNA damages (Fig. 10.11) (Ghodke et al. 2020). Specifically, UvrB binds to the injured DNA sequence, and UvrA is replaced by UvrC. UvrB proteins excise four nucleotides upstream of the damaged site, and UvrC proteins cleave the seven nucleotides downstream of the damaged site. Then, UvrD proteins, the helicase, dissociate the base pairs and allow the nucleotide segment at the damaged site to be released. The DNA polymerase is responsible for the synthesis of a new strand based on the complementary DNA strand (Dale and Park 2013). The significant upregulation of Uvr protein-coding genes (uvrA, uvrB) and Rec protein-coding genes (recA, recX, recN) was found in E. coli after an argon plasma exposure for 120 s in the study of Sharma et al. (2009). In addition, Xu et al. (2015) reported the expression of recG and recN was significantly upregulated in S. aureus by over two times after the plasma exposure of 10 and 20 min.

In addition to the NER and recombinational repair mechanisms, DNA-encoding protein from staved cells (Dps) and metalloregulation DND-binding stress protein (MrgA) are considered to be involved in the protection of DNA from oxidative damages (Nair and Finkel 2004). Dps and MrgA, ferritin super family proteins, non-specific bind to DNA under oxidative stress, and they can induce the nucleoid condensation and protect DNA damages (Chen and Helmann 1995; Martinez and Kolter 1997). It has been found that the gene dps was overexpressed by over 2 folds when exposed to a nitrogen plasma jet for 1 to 2 min in the study of Mols et al. (2013). Winter et al. (2005) reported the increased expression of MrgA proteins in B. subtilis by more than twofolds with the exposure of both air and argon plasma. In addition, SOS response, the global DNA damage repair system, also contributes to...
the bacterial resistance toward cold plasma exposure (Joshi et al. 2015; Sharma et al. 2009; Yost and Joshi 2015). Generally, SOS response is responsible for the regulation of more than 50 DNA damage repair-associated genes, such as endonuclease of nucleotide excision repair, DNA polymerase IV, DNA-damage-inducible protein, DinF MATE transporter, ATP-dependent helicase, cell division protein, DNA polymerase II, etc. Under the suitable condition, LexA proteins, as the repressors, bind to the promoter of SOS genes to prevent their expression (Maslowska et al. 2019). The activator of SOS responses is the accumulation of single-stranded (ss) DNA, which have been confirmed to be induced by various stressors, including the nutrient starvation, UV irradiation, high pressure, and antibiotics (e.g., fluoroquinolones). RecA protein binds tightly to the ss DNA and is activated in the presence of ATP. The activated RecA proteins further result in the proteolytic self-cleavage of LexA proteins from SOS box and initiate the expression of SOS genes for the DNA repair (Maslowska et al. 2019).
10.4.6 Protein Damage Repair Systems

The bacterial thioredoxin system contributes to the maintenance of the intracellular proteins in the reduced state (Scharf et al. 1998). Thioredoxin reductase and thioredoxin are the two major components of a thioredoxin system (Fig. 10.12) (Peng et al. 2018). Thioredoxin reductase is mainly responsible for maintaining the thioredoxin in a reduced form through using the electrons from nicotinamide adenine dinucleotide phosphate (NADPH) (Holmgren 1985). The expression of the thioredoxin reductase and thioredoxin was significantly upregulated by 6.21 and 11.55 folds, respectively, in S. Enteritidis after a cold plasma exposure for 60 min, which indicated that the activation of the thioredoxin system was a bacterial response against the plasma-induced oxidative damages in proteins (Ritter et al. 2018). Joshi et al. (2015) reported the overexpression of trxC gene, which encoded the production of thioredoxin in E. coli after the exposure of DBD plasma-activated PBS solution. Methionine of proteins is found to be highly sensitive to oxidative agents (Dean et al. 1997). The oxidation of methionine might result in the structural changes in enzymes and inactivate the enzymatic activity. The outcome of methionine oxidation is the

![Fig. 10.12](image-url)
diastereomeric methionine sulfoxide (Brot et al. 1981). The methionine sulfoxide reductases are generally employed by bacteria to repair this kind of protein damage (Moskovitz et al. 1996). The enzymes MrSA and MrSB contribute to the reduction of methionine sulfoxide into methionine (Russel and Model 1986). In addition to methionine, the iron-sulfur clusters within the proteins are also susceptible to oxidization. The repairing of iron-sulfur clusters within bacteria is reported to be associated with the iron-sulfur cluster repair proteins, which is called YtfE in E. coli (Djaman et al. 2004), and ScdA in S. aureus (Brunskill et al. 1997).

In addition to the protective mechanisms, the removal of damaged proteins is also a vital strategy for bacteria to survive after the cold plasma attack. Vaze et al. (2017) reported that the expression of ClpX protein-coding gene clpX was required for E. coli cells against cold plasma exposure. It is found that the E. coli cells defected in gene clpX could be completely inactivated by a DBD plasma while there were around 4-log survivals of wild-type E. coli cells. ClpX, an ATP-dependent protease, mainly contributes to the degradation and removal of the damaged proteins to retain the cell homeostasis (Alexopoulos et al. 2013). Also, the heat shock proteins (Hsp), as the molecular chaperones, contribute to the inhibition of protein aggregations through the binding to the unfolded proteins with the hydrolysis of ATP (Winter et al. 2005). In the study of Krewing et al. (2019), the survival rate of E. coli defected in the hslO, an Hsp33-encoding gene, was reduced to 35% after a DBD plasma treatment, while it was 70% for the strains complemented with gene hslO. The authors conducted the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and found that the addition of Hsp33 before plasma exposure could decrease the level of insoluble proteins with a high molecular weight, indicating the delay of protein aggregation by Hsp33.

10.4.7 Mutations

Mutations are the major drivers of the evolution of all the organisms through the genetic variability in the world (Rosenberg 2001). Generally, the mutations are likely to occur at a constant rate under the unstressed environment, which is called spontaneous mutagenesis (Lea and Coulson 1949). Once the external stress was applied, the rates of mutations tend to be increased, rendering bacteria with the resistance toward the stressors, which is called stress-induced mutagenesis (Foster 1999). So far, several mechanisms underlying the stress-induced mutagenesis have been proposed in the previous works. The accumulation of ROS and damaged DNA within bacterial cells is thought to be the direct contributor to the induction of stress-induced mutagenesis under a harsh environment (Kohanski et al. 2010). In addition, the downregulation of mismatch repair (MMR) system has also been reported to contribute to stress-induced mutagenesis. MMR system mainly consists of three key proteins, including MutS, MutL, and MutH. MutS is responsible for the recognition of the mismatched bases in the DNA strands and binds to MutL. Subsequently, the MutS-MutL complex activates the MutH, an endonuclease. The error-containing
DNA strand without methylation is further repaired by DNA polymerase and ligase. Therefore, the loss of MMR system in bacteria could render bacteria with high frequency in mutations. In the study of Zhu et al. (2014), *E. coli* defected in *mutL* gene exhibited higher resistance to butanol, osmotic pressure (NaCl), and heat (50 °C). Bacterial SOS response is also regarded as one of the mechanisms for the induction of stress-induced mutagenesis (McKenzie et al. 2000). The Pol IV and PolV DNA polymerases are activated for the replication of the damaged DNA during an SOS response. Those polymerases, however, tend to lack processivity and exhibit low fidelity, which easily results in errors when copying the DNA and could lead to the occurrence of mutations (Foster 2007). Apart from SOS response, the *E. coli* Lac system is also a well-studied mechanism for adaptive mutations (Ponde et al. 2005). Lac-mediated mutation is believed to be induced by the breaks in the DNA double strands and the associated error-prone repair (Hersh et al. 2004). Both MMR system and SOS response (the production of error-prone DNA polymerases) have been demonstrated to be regulated by the sigma factors (Gibson et al. 2010; Layton and Foster 2003), the regulators of the global gene expressions, which has been reviewed in Sect. 10.4.2. It has been reported that bacterial mutations could render the resistance toward various stresses, such as antibiotics, starvation, and heat (Cebula and LeClerc 1997; MacLean et al. 2013; Sung and Yasbin 2002). In the study of Mai-Prochnow et al. (2015), the whole genome DNA sequencing combined with the single nucleotide polymorphism (SNP) analysis was used for identifying the mutations within the plasma-resistant *P. aeruginosa* compared with the control ones. The results indicated four changes in the genes that were associated with the phenazine biosynthesis pathways. The further transposon mutant analysis suggested that the production of phenazine was involved in the stress response of *P. aeruginosa* toward the exposure of a plasma jet.

### 10.5 The Elimination of Microbial Stress Response Toward Cold Plasma Exposure

The development of microbial stress response should be efficiently avoided during the plasma exposure. The optimization of processing parameters (e.g., voltage, frequency, gas composition) should be carefully conducted to achieve efficient inactivation of microorganisms. Vaze et al. (2017) discovered that *E. coli* recovered their respiratory activity and regrew with the incubation for 8 h after a low dose exposure of the air DBD plasma (27.3 J/mL), while the completed inactivation was achieved by the lethal dose plasma treatment (41.6 J/mL). In addition, the hurdle technology, combing the cold plasma with other bactericidal treatments, could be an efficient strategy for the inhibition of the stress response within foodborne pathogens (Helgadóttir et al. 2017; Liao et al. 2018; Pan et al. 2020). In the study of Liao et al. (2018), the pretreatment of acidity (pH 5.5), osmosis (0.3 M NaCl) and heat (45 °C) for 24 h could enhance the sensitivity of *E. coli* toward an air DBD
plasma (50 W) exposure for 30 s. Pan et al. (2020) reported that the pretreatment of ultrasound (500 W, 40 kHz) significantly compromised the resistance of *L. monocytogenes* toward the following the DBD plasma exposure (1000 W) thorough the damages on the outer structure of bacterial cells. In addition to the ultrasound preexposure, the inactivation level of *L. monocytogenes* with the preincubation temperature at 10, 25, 37, and 42 °C were 2.50, 3.16, 3.29, and 3.78 logs by the cold plasma exposure, respectively. The endogenous ROS under the temperature of 42 °C were highest, which contributed to the maximum reduction by the plasma treatment.

### 10.6 Conclusions

In this chapter, the efficacy of cold plasma on bacteria, the associated affecting factors (e.g., processing parameters, environmental conditions, food characteristics, microbial properties), and the inactivation kinetics models are exhibited in detail. Though the microbial inactivation mechanisms caused by cold plasma is still obscure, several hypotheses have been proposed in the previous studies and reviewed in this chapter, including UV irradiation-induced DNA damages, electroporation caused by the electric field, as well as the oxidative damages of RONS. In addition, under cold plasma exposure, the bacterial cells tend to conduct the associated response in phenotypic and genetic levels to deal with the hazardous plasma agents and achieve survival. The development of stress response of microbial cells, especially the pathogens, could lead to the incomplete inactivation by cold plasma as well as the retention or even enhancement of the virulence, which potentially threatens food safety and human health. Therefore, it is of importance to take the microbial stress response into consideration during the optimization and implementation of cold plasma sterilization in order to achieve complete inactivation.

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Chapter 11
Response of Foodborne Pathogens to Ultraviolet Light

Xuan Li and Hongshun Yang

Abstract Ultraviolet (UV) light is a widely used non-thermal technology. Thereinto, UV-C is considered to be the most effective type for the inactivation of pathogens and other microorganisms. The cellular targets affected by UV light are mainly concentrated in genetic materials and cell membranes (protective layer). The production of photo-dimerizations and reactive oxygen species (ROS) is the main contributor to the microbial inactivation by UV light. Meanwhile, photooxidation caused by UV destroys proteins and lipids, thereby affecting both the structure and metabolism of pathogens, ultimately leading to microbial death. Bacterial strains, food properties, and treatment parameters will significantly affect the resistance development in foodborne pathogens to UV light exposure. Importantly, microbes have developed two mechanisms (photoreactivation and dark repair) to repair damage caused by ultraviolet light. In addition to the traditional UV treatment, the combinations of UV with other methods are also being continuously developed, which make up for the shortcomings of a single method and enhances safety. The combined methods can cause additional damage based on the damage from UV, resulting in a synergistic lethal effect. This chapter provided a systematical review of various aspects of UV light on foodborne pathogens, including inactivation mechanisms, the affecting factors, the associated microbial UV light resistance, and the development of the UV light-based hurdle.

Keywords Application · Dark repair · Foodborne pathogen · Molecular mechanism · Photoreactivation · Response · UV
11.1 Introduction of Ultraviolet (UV) Light Technology in Food Industry

11.1.1 A Brief Introduction and Advantages of UV Light Used in Food Industry

Among the series of new technologies studied so far, light-based technologies have shown great potential for antimicrobial purposes. It is noteworthy that UV light is a novel technology that has been successfully used in the food industry to inactivate microorganisms, especially pathogens to ensure food safety (Gautam et al. 2017). Compared to thermal processing, which is a traditional method used in food processing and preservation, this emerging tool shows the advantages as a non-thermal technology for improving food quality and extending the food storage period. When heating processing may destroy heat-sensitive nutrients in foods, such as vitamins, color, flavors, etc., resulting in a decline in product quality and sensory properties (Iqbal et al. 2019; Morales-de la Peña et al. 2019), non-thermal processing can not only ensure the safety of products, but also maintain or improve the quality, and has the advantage of saving energy to a certain extent (Jambrak 2019; Ojha et al. 2016).

UV light is a portion of the electromagnetic spectrum located in the electromagnetic spectrum between visible light and X-rays, with a range of wavelengths from 100 to 400 nm, corresponding to photon energies from 124 to 3 eV (Koutchma et al. 2021). Compared to ionizing radiation, UV has lower penetration due to its low inherent energy of photons. Generally, the UV light can be divided into the following categories due to the wavelengths: vacuum UV (100–200 nm), UV-A (315–400 nm), UV-B (280–315 nm), and UV-C (200–280 nm) (Koutchma 2014b). Thereinto, UV-C is considered the bactericidal range since it effectively inactivates a wide range of microorganisms (Singh et al. 2020). Additionally, UV-C light is a physical treatment method that does not produce chemical residues in products (Hamamoto et al. 2007, Ochoa-Velasco et al. 2018). Some studies indicated that ultraviolet processing could also help improve the shelf-life, nutritional quality, such as increasing vitamin content, total carotenoid content, antioxidant capacity, and physical properties in various food materials (Augusto et al. 2015; Baykuş et al. 2021; Braga et al. 2019; Fenoglio et al. 2020; Ganan et al. 2013).

UV technology is the effective method that has strong potential within the food industry via meeting the current and developing consumers’ demands, increasing the acceptability of products, as well as having the advantages and possibilities for a wide variety of treatment situations. Thus, it plays an important role in controlling and killing foodborne microorganisms for esculent fields.
11.1.2 UV Light Types and Sources

11.1.2.1 UV-A, UV-B, and UV-C

Typically, the UV spectrum is divided into three bands, UV-A (320–400 nm), UV-B (280–320 nm), and UV-C (200–280 nm). However, based on the different pieces of literature, the range of UV-A and UV-B can be 315–400 nm and 280–315 nm, respectively (Keklik et al. 2012; Liao et al. 2019). Limited research has been conducted on the disinfectant effectiveness of UV-A (315–400 nm) and UV-B (280–315 nm) due to their relatively weak bactericidal capacity compared to UV-C (Koutchma and Popović 2019). Usually, UV-B and UV-A are applied in the agriculture field, which affect plant morphology and physiology, and the plant–insect interaction (Belén et al. 2021; Brazaitytė et al. 2019; Chen et al. 2021; Neugart and Schreiner 2018; Ortega-Hernández et al. 2020; Zhang et al. 2020).

UV-A is not efficiently absorbed by DNA and therefore does not cause serious damage through dimer formation (Hamamoto et al. 2007). But UV-A can indirectly inactivate microorganisms by destroying proteins and producing hydroxyl and oxygen free radicals, thereby damaging cell membranes and other cellular components (Brem et al. 2017; Hirakawa et al. 2003; Sinha and Häder 2002). Bactericidal effects of UV-B have not been widely explored. Theoretically, UV-B exhibits the intermediate effectiveness between UV-A and UV-C. And the oxidization of lipids and the damage of proteins are crucial to antimicrobial activity under UV-B (Kim et al. 2017; Kovács et al. 2007; Santos et al. 2013). The formation of DNA photoproducts induced by UV-C, especially cyclobutane pyrimidine dimers (CPDs) and pyrimidine 6–4 pyrimidone photoproducts (6–4 PPs), will significantly inhibit the DNA replication and decrease metabolic activities (Hinds et al. 2019). The summary is shown in Table 11.1.

Table 11.1 Summary of bactericidal mechanisms for UV-A, UV-B, and UV-C. Adapted from Koutchma and Popović (2019)

<table>
<thead>
<tr>
<th>UV Type</th>
<th>Wavelength (nm)</th>
<th>Photon energy (eV, aJ)</th>
<th>Induced compound</th>
<th>Main target</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-A</td>
<td>320–400</td>
<td>3.10–3.94 (0.497–0.631)</td>
<td>Reactive oxygen species (ROS)</td>
<td>Membrane and genetic material</td>
<td>Oxidize lipids and damage proteins, and damage DNA indirectly</td>
</tr>
<tr>
<td>UV-B</td>
<td>280–320</td>
<td>3.94–4.43 (0.631–0.710)</td>
<td>Photoproducts</td>
<td>Genetic material</td>
<td>Oxidize of lipids and damage proteins, and damage DNA directly (Block DNA replication and RNA transcription)</td>
</tr>
<tr>
<td>UV-C</td>
<td>100–280</td>
<td>4.43–12.4 (0.710–1.987)</td>
<td>Photoproducts</td>
<td>Genetic material</td>
<td>Damage DNA directly (Block DNA replication and RNA transcription)</td>
</tr>
</tbody>
</table>
11.1.2.2 UV Light Sources

There are certain types of continuous UV light sources on the market, including low-pressure mercury lamps (LPM) and medium pressure mercury lamps (MPM) lamps, low-pressure amalgam (LPA), and emitting lights (Els) (Koutchma 2014d). Nowadays, LPM (monochromatic light) and MPM (polychromatic light) pressure are mainly utilized for UV light sources. However, its drawbacks limit the applications; large size, high energy consumption, high heat emission, and the presence of mercury (Hinds et al. 2019). In recent years, UV light-emitting diodes (UV-LEDs), ranging from 255 to 405 nm, with the advantages of adjustable and wider treatment parameters, has been employed for food decontamination (Koutchma et al. 2021).

Pulsed UV light is a novel technology with high-intensity light pulses, which is different from continuous UV light (Mandal and Pratap-Singh 2021). It is non-chemical and non-ionizing, and nearly non-thermal at short processing time, like traditional UV light (Keklik et al. 2012). Inert gas flashlamps (e.g., xenon) can produce intense light pulses of electromagnetic radiation (100–1100 nm), including UV, visible and infrared light (Mahendran et al. 2019).

Both continuous light and pulsed light can have a wide range of applications in the field of inhibiting pathogens, and these technologies are in continuous development.

11.1.3 Recent Applications for Inhibition of Foodborne Pathogens

11.1.3.1 Brief Introduction of Foodborne Pathogens

In the food industry, inappropriate or insufficient decontamination processing can cause foodborne diseases, leading to gastrointestinal symptoms, serious organ damages, disability, or mortality (Fonseca and Cané 2020). The bacteria, yeasts, molds, and viruses predominantly can cause enteric diseases by intoxicating products with microbial enzymes and toxins (Majumdar et al. 2018). To date, it has been most reported that UV light can inactivate the following food pathogens: Campylobacter, Escherichia coli O157:H7, Listeria monocytogenes, Salmonella, Staphylococcus aureus, and parasites (Garvey and Rowan 2019; Jeon and Ha 2018; Singh et al. 2020). Under UV-C exposure, pathogens can have three types of subpopulation: undamaged or repaired cells (normal metabolic activity), nonculturable-state cells (low metabolic activity), and dead cells (Fan et al. 2017).
11.1.3.2 Applications of UV with Anti-pathogens Effect in Food Industry

The utilization of UV for disinfection purposes has been applied since the 1900s (Keklik et al. 2012; Miller et al. 1999). Two decades earlier, the UV light sources consist of low-pressure mercury lamps with a wavelength of 253.7 nm have been approved for microbial inactivation in potable water, food and food products, and juice products (FDA 2000). Also, FDA has allowed pulsed light (200–1100 nm) to process food with the maximum total cumulative 12.0 J/cm² (FDA 1996). For its wide applications, UV can be applied in water, food products, the environment, and equipment for microbial inactivation purposes (Hinds et al. 2019; Keklik et al. 2012; Ochoa-Velasco et al. 2018).

It has been confirmed that UV can inactivate viruses, bacteria, bacterial spores, bacteriophages, and protozoa in drinking water (Hijnen et al. 2006; Wang et al. 2020). For solid products, UV can effectively inactivate the pathogens on the surface without adverse sensory evaluation (Allende and Artés 2003; Ganan et al. 2013; Izmirlioglu et al. 2020). Then, UV-C light can be an extremely useful alternative to traditional thermal processing for liquid food, such as fresh juices, soft drinks, and other derived beverages. And different processing means lead to various results of physicochemical characteristics, sensory properties, and antimicrobial effectiveness. (Antonio-Gutiérrez et al. 2019). In addition, there are some applications during food transport and processing conditions. UV-C could be used to reduce the population of Campylobacter jejuni and Enterobacteriaceae for crates that transported chickens (Moazzami et al. 2021).

Since the significant synergistic effects enhance microbial safety, the combination of UV with other processing methods is in continuous development. The combination of slightly acidic electrolyzed water (SAEW) with available chlorine concentration (ACC) of 20 mg/L and UV light (10.2 ± 0.3 W/cm²) showed higher bactericidal effectiveness against Salmonella Enteritidis after 4 min of treatment (Bing et al. 2019). Similarly, the simultaneous treatments with organic acids (acetic acid, benzoic acid, citric acid, fumaric acid, gallic acid, lactic acid) can also enhance the reduction of pathogens (Escherichia coli O157:H7, Salmonella Typhimurium, and Listeria monocytogenes, mainly by cell membrane disruption (de Oliveira et al. 2017; Ding et al. 2018; Jeon and Ha 2020; Rosario et al. 2021; Seok and Ha 2021). To achieve the desired purpose, the assistance of heat and other compounds, such as aqueous ozone, or the formulation of hydrogen peroxide, EDTA (Ethylenediaminetetraacetic acid), and nisin are also helpful (Gayán et al. 2013b; Gouma et al. 2020; Mukhopadhyay et al. 2015; Pagal and Gabriel 2020). More information shows in the following Tables 11.2 and 11.3.
11.2 The Cellular Targets Affected by UV Light

11.2.1 Genetic Materials

Alterations in genetic materials trigger signals that induce apoptosis (Pantic et al. 2012). Thus, UV light can achieve a germicidal effect against pathogens by destroying their genetic materials (DNA and RNA) structures to prevent further replication or microbial growth (Koutchma et al. 2021; López-Malo 2005; Turtoi and Borda 2013). The first is the photo-dimerizations. Ultraviolet can induce mutagenic DNA lesions by generating the two main photoproducts: cyclobutane

<table>
<thead>
<tr>
<th>Target pathogens</th>
<th>Treatment conditions</th>
<th>Matrix</th>
<th>Microbial reduction (log CFU/mL)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campylobacter jejuni (CCUG 43594)</td>
<td>265 nm UV-C 20.4 mJ/cm² (1 min) 61.2 mJ/cm² (3 min)</td>
<td>Transport crates for chickens</td>
<td>2.0 ± 0.5 3.1 ± 1.0</td>
<td>Moazzami et al. (2021)</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td></td>
<td></td>
<td>1.5 ± 0.3 1.8 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>254 nm UV-C 0.6 J/cm²</td>
<td>Raw salmon fillet muscle surface</td>
<td>0.9</td>
<td>Holck et al. (2018)</td>
</tr>
<tr>
<td>L. monocytogenes (CECT 4032, CECT 7467, and Scott A)</td>
<td>Pulsed light (PL) 11.9 J/cm²</td>
<td>Surface of ready-to-eat dry cured meat products</td>
<td>1.5 ± 0.3</td>
<td>Ganan et al. (2013)</td>
</tr>
<tr>
<td>Salmonella Typhimurium (CECT 7159 &amp; CECT 4371)</td>
<td></td>
<td></td>
<td>1.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>S. Enteritidis (PT8)</td>
<td>Pulsed light (PL) 5.6 J/cm² (45 s.)</td>
<td>Shelled walnuts</td>
<td>3.18 ± 0.3</td>
<td>Izmirlioglu et al. (2020)</td>
</tr>
<tr>
<td>S. Typhimurium (ATCC 14028)</td>
<td>254 nm UV-C 26.4 J/cm² (10 min)</td>
<td>Coconut water</td>
<td>5.96–7.32</td>
<td>Ochoa-Velasco et al. (2018)</td>
</tr>
<tr>
<td>Staphylococcus aureus (ATCC 25923)</td>
<td>254 nm UV-C 216.9 J/mL</td>
<td>Bovine milk</td>
<td>2.74</td>
<td>Atik and Gumus (2021)</td>
</tr>
<tr>
<td>Escherichia coli (ATCC 35218)</td>
<td>254 nm UV-C 98.4–216.9 J/mL</td>
<td></td>
<td>&gt;4</td>
<td></td>
</tr>
</tbody>
</table>

Table 11.2 Applications of UV light in killing foodborne pathogens
<table>
<thead>
<tr>
<th>Target pathogens</th>
<th>Treatment conditions</th>
<th>Matrix</th>
<th>Microbial reduction (log CFU/mL)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em> Enteritidis (CVCC 2184)</td>
<td>254 nm UV-C 10.2 ± 0.3 W/cm²</td>
<td>SAEW ACC: 20 mg/L</td>
<td>6.54 ± 0.11</td>
<td>Bing et al. (2019)</td>
</tr>
<tr>
<td></td>
<td>4 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. Typhimurium</em> (ATCC 14028)</td>
<td>254 nm UV-C 0.36 J/cm²</td>
<td>Lactic acid 7.7% (v/v)</td>
<td>1.3</td>
<td>Rosario et al. (2021)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> O157:H7</td>
<td>356 nm UV-A 0.16 J/cm²</td>
<td>Citric acid 0.5% (w/v)</td>
<td>3.63</td>
<td>Seok and Ha (2021)</td>
</tr>
<tr>
<td><em>S. Typhimurium</em></td>
<td>30 min</td>
<td>Sliced cheese</td>
<td>3.21</td>
<td></td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>90 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7 (ATCC 35150, ATCC 43889, &amp; ATCC 43890)</td>
<td>356 nm UV-A 0.03 mW/cm²</td>
<td>Fumaric acid 0.1% (w/v)</td>
<td>6.65 ± 0.11</td>
<td>Jeon and Ha (2020)</td>
</tr>
<tr>
<td><em>S. Typhimurium</em> (ATCC 19585, ATCC 43971, &amp; DT 104)</td>
<td>30 min</td>
<td>Apple juice</td>
<td>6.27 ± 0.12</td>
<td></td>
</tr>
<tr>
<td><em>L. monocytogenes</em> (ATCC 15313, ATCC 19111, &amp; ATCC 19115)</td>
<td>30 min</td>
<td></td>
<td>6.49 ± 0.21</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>365 nm UV-A light 3.63 J/cm²</td>
<td>Benzoic acid –15 mM BA</td>
<td>5.8 ± 0.3</td>
<td>Ding et al. (2018)</td>
</tr>
<tr>
<td><em>S. enterica</em> (Newport H1275, Stanley H0558, &amp; Montevideo G4639)</td>
<td>254 nm UV-C 0.6 kJ/m²</td>
<td>Aqueous ozone –1 ppm</td>
<td>3.13 ± 0.47</td>
<td>Mukhopadhyay et al. (2015)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixture (hydrogen peroxide, EDTA &amp; nisin)</td>
<td>4.71 ± 0.25</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> (STCC 4201)</td>
<td>254 nm UV-C 27.10 J/mL</td>
<td>Mild heat –54.2 °C</td>
<td>&gt;5</td>
<td>Gayán et al. (2016)</td>
</tr>
</tbody>
</table>
pyrimidine dimers (CPDs) and pyrimidine 6–4 pyrimidone photoproducts (6–4PPs) (Fig. 11.1), especially for UV-B and UV-C (Baysal 2018; Oguma et al. 2002; Pathak et al. 2019). The pyrimidine residues are covalently linked via the cyclobutane ring (CPD) or the bond between carbon 6 and carbon 4 (6–4 PP) (Beauchamp and Lacroix 2012). For DNA, the nitrogen bases of purines and pyrimidines are very sensitive to UV radiation and they are the main participant for absorbing a large amount of UV radiation (Rastogi et al. 2010). And in the normally used UV-C spectrum, the maximum absorption range of DNA is 260–265 nm (Gayán et al. 2014). Thus, when the pathogens are under UV exposure, the radiation travels through the cell wall to the nucleus, where it finds a region of DNA or RNA with neighboring pairs of thymine or cytosine pyrimidines and then induces one of the bases to absorb a photon and forms a high-energy dimer (Antonio-Gutiérrez et al. 2019; Koutchma and Popović 2019). Generally, during the processing of UV, CPDs is the most common lesion in the genome, and this kind of UV-induced DNA damage product accounts for three-quarters of the total (Matsunaga et al. 1993; Sinha and Häder 2002). The research indicated that the sensitivity of bacteria against the formation of CPDs was different, during the period of UV irradiation. At doses higher than 3 J/cm², the genome of L. monocytogenes showed higher natural resistance to UV light than that of the genome of E. coli. The amount of CPDs and 6–4 PPs produced by the DNA of L. monocytogenes was 35% and 10% less than that from E. coli, respectively (Beauchamp and Lacroix 2012).

Secondly, UV light indirectly plays an important role in the production of reactive oxygen species (ROS), which also react with DNA (Santos et al. 2012; Wu et al.
Thus, the details of ROS must be mentioned, they can induce oxidative stress, and finally lead the cellular damage and accelerate cell death. ROS includes mainly singlet oxygen ($^1\text{O}_2$), superoxide anion ($\text{O}_2^{-}$), hydrogen peroxide ($\text{H}_2\text{O}_2$), hydroxyl radical ($\text{OH}^\text{•}$), and perhydroxyl radical ($\text{HO}_2^\text{•}$) that cause damage to DNA (Haidera et al. 2020). However, the damage of genetic materials may not kill the pathogens but reduce the activity of them, which means the metabolism can still be undergoing. Because the repairing systems (photoreactivation and dark repair) will be initiated by the pathogens (Fitzhenry et al. 2021; Sanz et al. 2007). This will be discussed in more detail in the later section.

### 11.2.2 Cell Membrane (Protective Layers) and Other Constituents

Besides DNA damage, the damage of essential proteins and lipids, as well as the compromise in energy transfer are also the possible inactivation factors (Koutchma and Popović 2019; Luksiene 2010). Theoretically, due to the energy of photons, UV-C may affect these bonds (O-H, C-C, C-H, C-N, H-N, S-S) that commonly existed in cells, if pathogens absorbed the irradiation (Koutchma 2009, 2014b).

Cell membranes can be regarded as the protective layers of pathogens, which are the structures that maintain normal morphology, regulate the transportation of compounds in and out, and balance the pressures between internal and external (Bouyahya et al. 2019). When the destroy happened, the integrity of the cell is lost, and the normal metabolisms, such as the absorption of nutrition and elimination of the waste will be disrupted (Huang et al. 2014; Torres and Velazquez 2005). The utilization of fluorescent probes has been widely used to explore the germicidal mechanisms via catching and observation of the changes of physiology properties, including the membrane potential and integrity, permeability, enzyme activity, metabolic activity, and DNA damage (Díaz et al. 2010; Ha and Kang 2014). For example, the membrane-impermeable dye propidium iodide (PI) can be used to indicate the damage of membrane integrity, because it can only bind with nucleic acids through the damaged cell membrane (Pietkiewicz et al. 2015). Kim et al. (2017) have reported Gram-positive bacteria had a higher ratio of PI absorption than gram-negative bacteria did, which deduced the damage of membrane is twice as great in gram-positive bacteria as in gram-negative bacteria after 1 mJ/cm$^2$ UVC-LED irradiation. The cell walls of gram-positive bacteria are composed of only peptidoglycan and phospholipid, while gram-negative bacteria have the outer protective layer with lipopolysaccharide and glycoproteins that can help against more outside forces (Kohler et al. 2010; Zhang et al. 2021).

Photooxidation is the most common type of photosensitizing reaction. In microorganisms, the photo-degradation of organic compounds will generate ROS, including hydrogen peroxide and superoxide anion (Garg et al. 2011; Wang et al. 2019b). Some constituents from cells, like amino acids, unsaturated fatty acids, and
phospholipids are light sensitive (Taze et al. 2021). ROS can react with DNA to inhibit the further replication or growth of pathogens. Additionally, ROS can also bring about a cytotoxic effect by interacting with the lipids and proteins in cells (Luksiene 2010). The generation of ROS is highest under UV-A treatment, while the amount is lowest under UV-C light (Seok and Ha 2021). UV light-induced damages in proteins have been considered as one of the major causes to achieve the antimicrobial purpose. The damage can inhibit the reactivation of bacteria (Li et al. 2017). And the damage of membrane proteins can be regarded as the first step to disintegrate the protective layer. Since the existing of aromatic amino acids (phenylalanine, tryptophan, and tyrosine) in proteins, which are the main participants to absorb the 280 nm UV light, the proteins can catch the energy, and then transform to an excitation state, leading to the structural destruction and higher vulnerability of the proteins in the protective layer (Kim et al. 2017). As for viruses, the research about human adenovirus type 2 (HAdV2) which is one of the most resistant pathogens to UV irradiation illustrated that the protein damage contributed to the antimicrobial effect. When exposed to 280 nm UV-LED, it was observed that the minimal damage of proteins occurred at the dose lower than 186 mJ/cm², while accelerated damage degree showed at a higher dose around 300 mJ/cm² (Beck et al. 2014; Beck et al. 2017). And the damages of protein may be explained by UV light structural changes because of breakdown (Eischeid and Linden 2011).

Photochemical damage occurs in the double bonds of unsaturated fatty acids, and the absorption of high energy results in the generation of free radicals, which increase lipid autoxidation (Rosario et al. 2021). It was reported that UV processing with 265–305 nm stimulated the oxidation of lipids (Spikes 1981). Lipids play important roles in maintaining the normal metabolism of cells. They can be the structural components of cellular membranes and for signal communications, as well as the regulators that participate in some processes, like cell division, proliferation, and death (Flores-Romero et al. 2020). Thus, the disruption will affect the normal metabolism of organelles and even the whole cells, finally inhibiting the activity of pathogens or causing death. Moreover, based on the report, ROS can follow this damage sequence in microorganisms: phospholipids > peptidoglycan, lipopolysaccharides > phospholipids > peptidoglycan, glycoproteins > glucans > chitin (Bogdan et al. 2015).

The lethal effects of UV light can be enhanced by the combination with other techniques. The UV light combined with organic acids has been developed as a hurdle for microbial decontamination (Ding et al. 2018; Jeon and Ha 2020; Jeong and Ha 2019). The pH value of an organic acid solution may be a major determinant of its effectiveness against bacteria, as it affects the concentration of undissociated acids formed in the solution (Davidson et al. 2012). The internal pH reduction caused by organic acids in pathogens will disrupt the transport of substances by altering the permeability of cell membranes. With the use of field emission scanning electron microscopy (FESEM), higher shrinkages or membrane disruptions of L. monocytogenes were observed after applying UV-A (0.16 J/cm²) and 0.5% citric acid processing (Seok and Ha 2021). Similarly, the morphological shrinkage and
membrane damage of pathogens were also observed after the combination treatment of UV-A (0.16 J/cm²) and 0.5% acetic acid (Jeong and Ha 2019).

Another widely studied combination is UV and heat processing. Gayán et al. (2012) concluded that the bactericidal activity on E. coli under UV light was higher while the temperature increased from 20 to 40 °C. And even the temperature reached 50 °C, the UV resistance of E. coli barely changed. Furthermore, the synergistic method can observe the discontinuity of the plasma membrane, and the leakage of the contents, the separation of the plasma membrane from the cell wall, the destruction of organelles, and the general rupture of the membrane (Carrillo et al. 2018). To analyze the mechanism, one of the reasons, heating will increase the fluidity of cell membranes, making the affected cells more sensitive to UV irradiation, resulting in inhibition of DNA excision repair. Then, the existed damage leads the death (Gayán et al. 2013a). In addition, UV and heat will associatively interfere with the activity of some protective enzymes, such as catalase (CAT) or superoxide dismutase (SOD) that reduce or inhibit the action of ROS to prevent oxidative damages (Castro-Alférez et al. 2017; Imlay 2008).

It is worth mentioning that pulsed UV light not only induces photo-dimerizations and ROS to inactivate the microorganisms but also associates with photothermal and photophysical effects caused by high-energy pulses to damage the cell structure (Keklik et al. 2012). Because the localized heating of bacteria will happen during the pulsed processing, the vaporized water in the cell will cause the rupture (Krishnamurthy et al. 2010). The damaged cell wall, shrunken cytoplasmic membrane and collapsed internal cellular structure of Staphylococcus aureus after treatment of 4.95 J/cm² pulsed UV light were observed by the analysis of Fourier-transformed infrared spectroscopy (FTIR) and transmission electron microscopy (TEM). Finally, leakage of cellular contents from the cytoplasm led to cells’ death (Krishnamurthy et al. 2010).

11.2.3 Biofilms

Biofilm is a complex community of microorganisms attached to biotic or abiotic surfaces. Its presence will increase the risk of the spread of pathogens and strengthen their resistance to disinfectants and other sanitizing methods (Bridier et al. 2011; Chen et al. 2020a). E. coli, Salmonella, and Vibrio cholerae can form biofilms that cling tightly to the inner surfaces of water pipes (Fu et al. 2020). Not only bacteria but protozoan parasites and viruses have also been shown to attach to biofilms, such as Cryptosporidium and Poliovirus (Nocker et al. 2014).

The reduction of biofilm formation was observed under UV-C treatment. After a UV-C treatment for 30 min (26.2 kJ/m²) on the surface of stainless steel and rubber, Alicyclobacillus acidocaldarius biofilm showed an approximately 2 log CFU/mL reduction (do Prado et al. 2019). Similarly, the reduction in metabolic activity of the biofilm was observed when treated with the combination of UV-A light (2646 ± 212 µW/cm²) and 10 mmol/L gallic acid against E. coli O157:H7 for
30 min (Cossu et al. 2016). However, UV treatment may not be the effective way. The bacteria may have been blocked by biofilm to prevent radiation, allowing it to survive (Pozos et al. 2004). It also pointed out that unless some physical cleaning is performed, continuous UV irradiation on the surface may not be able to completely prevent the formation of biofilms for a long time (Torkzadeh et al. 2021). The specific mechanism of UV damage to biofilm is not clear yet, but it might be related to the damaged genetic materials and other metabolic pathways (Kim et al. 2016; Zhang et al. 2019).

11.3 The Main Factors Affecting Resistance Development in Foodborne Pathogens to UV Light Exposure

11.3.1 Bacterial Strains

The unique response of each microorganism to various UV wavelengths is due to the different protein and nucleic acid compositions of specific microorganisms. This concept is called the action spectrum of microorganisms (Sholtes and Linden 2019; Sinha and Häder 2002). According to the structure, the thickness of the protective layer, the composition of the cell, and the amount of UV absorbance by nucleic acids, the antimicrobial efficiency of UV light is varied (Koutchma 2014c). Generally, Gram-negative bacteria are more sensitive to UV-C light than Gram-positive bacteria, then followed by yeast < bacterial spores < molds < viruses (Antonio-Gutiérrez et al. 2019; Gayán et al. 2016; Sastry et al. 2000). Viruses are generally more UV-resistant than Cryptosporidium, Giardia, and bacteria (Hijnen et al. 2006). In addition, pathogens that produce mature biofilms could form a thicker matrix, which is expected to be difficultly penetrated by light, leading to higher resistance to UV processing (Argyraki et al. 2016).

Compared with laboratory-grown strains, the isolated strains from environment exhibit higher resistance to UV, which means that higher UV fluences are required to obtain sufficient inactivation (Hijnen et al. 2006).

11.3.2 Food Properties

Several physicochemical characteristics (pH, total acidity, total soluble solid content, water activity, viscosity, density), optical properties (absorption coefficient, turbidity, color), and an initial microbial load of food are also considered as the important factors affecting the effectiveness of UV processing (Atilgan et al. 2020). Because food can provide foodborne pathogens the shelters to resist the irradiation. The ability of UV light to penetrate food depends on the color and transparency of the target product (Delorme et al. 2020). The presence of colored compounds will lead to
poor UV-C transmittance, resulting in low efficiency of microbial inactivation (Char et al. 2010). Therefore, the limited permeability must be considered when using this technology (Keklik et al. 2012).

For solid food, the shadow effect due to the roughness and irregular shape of the products limits the applications and efficacy of UV rays. For example, in fruits, bacteria may stay in pores, trichomes, or cracks and then escape from a UV-C light. (Liu et al. 2015; Ortiz-Solà et al. 2021). As for liquid food, the type, suspended matter, and soluble solids significantly affect the penetration of UV-C irradiation. The suspended solids in the liquid can protect the microbes since UV-C radiation rarely penetrates the opaque materials (Antonio-Gutiérrez et al. 2019; Ochoa-Velasco and Beltrán 2013). At the same time, the pH of the liquid also affects the UV light-mediated sterilization effect. In general, juice with a low pH can achieve inactivation under lower temperature treatment conditions than those liquids with a high pH (Gayán et al. 2016). Another vital parameter for liquid is flow rate. In this research of milk, it showed a progressive decrease in the count of pathogens (S. Typhimurium, L. monocytogenes, S. aureus, E. coli) when the flow rate from 18 to 5 mL/min, which may be related to the more homogeneous exposure under higher flow rate (Atik and Gumus 2021).

11.3.3 Treatment Parameters

11.3.3.1 Dose

In order to achieve effective disinfection, it is important to determine the UV dose required to appropriately reduce the microbial loads. The dose, also called fluence of UV light, is one of the vital intrinsic process parameters for treatment. Dose (J/m² or mJ/cm²) is defined as the amount of radiant energy delivered or received per unit area (Koutchma 2014a), which can be calculated by intensity $I$ (the amount of irradiation energy per unit area, W/m²) times the residence time $t$ (the time exposed to irradiation, s) (Antonio-Gutiérrez et al. 2019), showing in Eq. 12.1:

$$D = I \times t \quad (12.1)$$

$D$: Dose or fluence (J/m²)
$I$: Intensity (W/m²)
$t$: Residence time (s)

For fluids, the unit of dose can be expressed in J/L or J/mL (Gautam et al. 2017; Miller et al. 1999; Saucedá-Gálvez et al. 2020).

Some researches illustrated that higher UV intensity with lower exposure time had more desirable inactivated results than that applied low intensity with longer illumination duration because prolonged exposure time could cause microbial accumulation (Chen et al. 2020c; Zhou et al. 2017). In the attention of the COVID-19
epidemic diseases, one research reported the 245 nm UV-C could inactivate the virus SARS-CoV-2 effectively in vitro, and the lethal doses, LD$_{90}$ and LD$_{99.999}$ in this research were 0.016 mJ/cm$^2$ (0.01 s), and 108.714 mJ/cm$^2$ (49.42 s), respectively (Sabino et al. 2020). Tables 12.1 and 12.2 in the application section shows more examples, but other parameters are required for further research on the UV resistance of a variety of microorganisms.

11.3.3.2 Temperature

Due to the different requirements of the food and processing conditions, the temperature of treatments will be diverse. Normally, higher heat can assist the sanitizing processing.

UV-C with mild heat treatment led to a synergistic inactivation effect, and the degree of this effect depends on the treatment temperature and microbial heat resistance (Fenoglio et al. 2020; Gayán et al. 2011, 2013b, 2016). However, if the temperature is not high enough, the effect will not be obvious. It was indicated that upon the same UV-C treatment, the inactivation of $S$. Typhimurium, $L$. monocytogenes, $S$. aureus, and $E$. coli from 4 °C to 25 °C in milk did not have significant change, analyzing by three-dimensional surface response graphs (Atik and Gumus 2021). For the treatment of $Listeria$, lower temperature (4 °C) could increase irradiance from 0.138 to 0.149 mW/cm$^2$ under the 268 nm UV-LEDs with the dose of 7 mJ/cm$^2$, resulting in 6–7 s reduction of processing time, compared to room temperature (25 °C). This example validates the utilization of UV light under cold conditions (Green et al. 2018).

11.3.3.3 Wavelength

As for UV light, the longer wavelength corresponds to the lower energy of the photon and consequently the penetration depth (Koutchma and Popović 2019). It is reported that microbial cells exhibited highest survival rate under UV-A and the lowest under UV-C, indicating that the wavelength pose effects on the antimicrobial efficacy of UV light treatment (Santos et al. 2013). Importantly, the lethality of UV radiation to pathogenic bacteria was related to the DNA absorbance of bacteria. It is indicated that the microbial inactivation would be higher under the wavelengths between 250 and 270 nm, which is closer to the DNA absorption of bacteria (Lu et al. 2021; Wang et al. 2019a) In most cases, the treatments from UV-LEDs (258 ~ 268 nm) showed greater inactivation effectiveness than those from LPM lamps (253.7 nm) (Green et al. 2018). It is reported that $S$. enterica diarizonae and $E$. coli O157:H7 exhibited the highest overall UV sensitivities at 259 nm, while the maximum sensitivity of $L$. monocytogenes occurred at 268 nm (Green et al. 2018; Koutchma and Popović 2019). However, when the wavelengths increased beyond 268 nm, microbial UV sensitivity showed a decreasing trend (Green et al. 2018). Similarly, it was summarized that the lowest inactivation rate constant of all
microorganisms appears at the end of the bactericidal spectrum, near 285 nm (Sholtes and Linden 2019).

Because different UV wavelengths can target various key cell components, the application of multi-wavelength UV treatment seems to be more effective (Sholtes and Linden 2019). Green et al. (2018) reported that the UV light treatment (total dose 14 mJ/cm²) with multiple wavelengths of 259 and 289 nm showed a synergistic effect on antimicrobial efficacy against *Listeria seeligeri* and *E. coli*. However, some studies observed no synergic effect from the combination treatment (Beck et al. 2017; Li et al. 2017; Nyangaresi et al. 2018). Therefore, whether to use the superposition of wavelengths should be evaluated according to the actual situation.

11.4 The Molecular Mechanisms Underlying the Resistance of Foodborne Pathogens to UV Light

11.4.1 Photoreactivation

DNA can be repaired by protein factors, for example, the two non-covalent cofactors (FADH₂ and MTFH or 8-HDF) of photolyase enzymes (PLs) in microbial cells (Kavakli et al. 2019; Yajima et al. 1995). Specifically, the DNA PLs contain two chromophores: 5,10-methenyltetrahydrofolate (MTHF) or 8-hydroxy-7,8-didemethyl-5-deazariboflavin (8-HDF), and FAD. As for the first chromophore, it can absorb the UV light effectively and then transfer the energy to the second chromophore FAD, helping to collect the light. And the FAD, which is a type of flavin found in most living species, has three states, classified by redox states: FAD₉ (fully oxidized), FADH⁻ or FAD⁻⁻ (semi-reduced), and FADH⁻⁻⁻ or FADH₂ (fully reduced). Only when FAD is at the fully reduced states, PLs exhibit catalytic activity (Wang et al. 2015). Then, PLs can separate the dimers of thymines and other pyrimidines to monomers, resulting in correcting/reversing modified nitrogenous bases to the normal state. But after the pathogens experienced a photoreactivation, the cells may be more resistant to second-applied UV light (Antonio-Gutiérrez et al. 2019; Guerrero-Beltrán and Barbosa-Cnovas 2004; Stevens et al. 1998). The generally accepted mechanism with three steps is summarized in Fig. 11.2 (Müller and Carell 2009; Saxena et al. 2006; Wang et al. 2015).

Light intensity, temperature, and wavelength affect the DNA repair rate catalyzed by the photolytic enzyme to respond to the damage from thymine dimer (Hu and Quek 2008). The research of Guo et al. (2009) found that after exposing *E. coli* to UV with the dose of 40 mJ/cm², the percentage of photoreactivation was less than 1% because the high dose induced more pyrimidine dimers that inhibit the system to repair effectively. Another research indicated that UV-A treatment with a dose of higher than 7.5 × 10⁵ J/cm² could inhibit the light repair process due to the mass-produced ROS (Xiao et al. 2018). Generally, photoreactivation has faster rates to repair than those of dark repair. Moreover, the degree of photoactivation and dark
repair varies among species and strains, as well as the various treatment conditions (Shang et al. 2009).

**11.4.2 Dark Repair**

Under the dark environment, photoreactivation is inhibited and another repair way (nucleotide and base excision) is conducted for developing the resistance toward UV treatment (Jungfer et al. 2007). This repair system can replace the damaged DNA with undamaged parts by enzymes, and it is light-independent, called dark repair (Rastogi et al. 2010).

UV irradiation can activate the SOS response in bacteria that adapt to the harsh environment and then increase survival, which is a conserved pathway (Foti et al. 2010). This response plays an important role in DNA repair and restart of stalled or collapsed replication forks via regulation by the repressor LexA and activator RecA (Butala et al. 2009; Cox 2007). It is usually composed of enzymes involved and essential in DNA repair (e.g., exonucleases, helicases, and recombinases) or enzymes involved in the translesion synthesis of DNA (e.g., translesion DNA polymerase) (Van der Veen and Abee 2011).

Additionally, bacteria can activate the dark repair system, which is regulated by the key gene called recA (Sinha and Häder 2002). Importantly, the vital point recA can coordinate the induction of more than 20 genes for SOS response. Moreover, the multifunctional protein RecA is the genic products that induce by recA, which coordinates many cellular processes, including homologous recombination, DNA repair, SOS response, and cell division (Stohl et al. 2003). Therefore, the amount of recA-specific mRNA and protein RecA might be recognized as damaged single-stranded DNA indicators. During the dark repair processing, the SOS response is
activated when bacteria detect the DNA damage induced by UV irradiation. Then the combination of protein RecA and single-strand DNA induced by UV will accelerate the cleavage of the general SOS LexA repressor, as well as increase the expression of recA in cells. The SOS genes are depressed (Jungfer et al. 2007).

Earlier studies reported the overproduction of RecA inhibits repair because it binds CPDs prematurely and blocks the dark repair pathway (Fridrichova et al. 1993; Slezáriková et al. 1996). For the research of E. coli, after applying the method of pre-radiation (UV-A) combined with second-radiation (UV-C), the amount of CPDs and RecA was higher than that after only UV-C treatment in 2018. It could be explained the pre-radiation (UV-A) inhibit dark repair by overproducing the protein RecA (Xiao et al. 2018). Jungfer et al. (2007) found that the activity of the recA gene can be detected in drinking water, indicating that the dark repair might be still working after a UV dose of higher than 400 J/m².

From a summary perspective, the two main approaches are base excision repair (BER) and nucleotide excision repair (NER) (Caldecott 2020; Raper et al. 2021; Souza and Koutchma 2021; Wang et al. 2018). The mechanisms have three main steps: recognition, excision, and resynthesis as shown in Tables 11.4 and 11.5.

11.5 Prospectives

Looking forward, it is not easy to study the reaction and metabolism of microorganisms. The results are changeable and unintuitive under different strains and experimental conditions. Advanced issues such as establishing kinetic and process models (Atilgan et al. 2020; Visuthiwan and Assatarakul 2020), visualized spectrum (Liu et al. 2019; Zhao et al. 2019), and NMR or MS-based metabolic profiles (Chen et al. 2020b; Li et al. 2020; Wu et al. 2021b), can also be further explored to better understand the response of foodborne pathogens or the food matrix to ultraviolet light.

11.6 Conclusions

UV light is a widely used disinfection technology that can improve food quality and prolong food shelf life. It can effectively extinguish a variety of pathogenic bacteria in water, food, the environment, and related equipment. UV rays directly or indirectly cause the inactivation of foodborne pathogens mainly through the formation of photodimers and photooxidation. Additionally, the combination of UV rays and organic acids or heat will cause additional damages on microbial cells, enhancing the inactivation efficacy. Bacterial strains, food properties, and treatment parameters are the main factors affecting resistance development under UV light exposure discussed in this chapter. The resistance of microorganisms to UV treatment is mainly developed by the ability to repair damaged genetic material. Microbes have
<table>
<thead>
<tr>
<th>Step</th>
<th>Enzymes</th>
<th>Reactive point</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recognition</td>
<td>DNA glycosylases</td>
<td>Damaged bases of DNA (Between base and the deoxyribose)</td>
<td>Breaking the N-glycosidic bond</td>
</tr>
<tr>
<td>Excision</td>
<td>AP endonuclease or AP lyase</td>
<td>Apurinic/apyrimidic (AP) site of DNA</td>
<td>Breaking the DNA strand</td>
</tr>
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<td></td>
<td>Phosphodiesterase</td>
<td>Remaining deoxyribose phosphate residue</td>
<td>Removing and producing a gap</td>
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<tr>
<td>Step</td>
<td>Description</td>
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<tr>
<td>Resynthesis</td>
<td>DNA polymerase and DNA ligase</td>
<td></td>
<td></td>
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<tr>
<td>DNA polymerase and DNA ligase</td>
<td>Cutting gap of DNA</td>
<td></td>
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<tr>
<td>Filling and sealing the strand</td>
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<tr>
<td>Step</td>
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<td>Reactive point</td>
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<tr>
<td>Recognition</td>
<td>Recognizing and triggering the repair</td>
<td>Damaged DNA (Dimer)</td>
<td>XP proteins</td>
</tr>
<tr>
<td>Excision</td>
<td>Forming an open complex, opening the DNA double helix</td>
<td>Damaged DNA (Dimer)</td>
<td>XPA, RPA and the general transcription factor TFIIH</td>
</tr>
<tr>
<td></td>
<td>Unwinding and cleaving DNA duplex at both sides</td>
<td>DNA around the damaged sites</td>
<td>DNA helicase (XBP and XPD in TFIIH)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nuclease enzymes (PG and XPF-ERCC1)</td>
</tr>
</tbody>
</table>

**Table 11.5** Mechanism of nucleotide excision repair

X. Li and H. Yang
Resynthesis

Cutting gap of DNA

DNA polymerase and DNA ligase

Filling (synthesis) and sealing the strand

Repaired DNA
developed two mechanisms to repair damage caused by UV light. These mechanisms are called light (photoreactivation) and dark repair. As for photoreactivation, the generally accepted mechanism with three steps: recognition, catalytic reaction, and separation. Base excision repair (BER) and nucleotide excision repair (NER) are the two main approaches to dark repair. Because of different experimental environments, the same pathogen may have different responses under UV treatment. The optimized processing parameters and antimicrobial effect should be verified according to actual needs. The overview is shown in Fig. 11.3.

References


Carrillo MG, Ferrario M, Guerrero S (2018) Effectiveness of UV-C light assisted by mild heat on Saccharomyces cerevisiae KE 162 inactivation in carrot-orange juice blend studied by flow


Response of Foodborne Pathogens to Ultraviolet Light


Chapter 12
Response of Foodborne Pathogens to Irradiation

Mohammad Shakhawat Hussain

Abstract Irradiation, an important food processing technique, has been used to ensure food safety and public health. In this process, ionizing radiation emitted from radioisotopes produced from radiation sources to food commodities is needed to kill and inhibit foodborne and pathogenic microorganisms. Irradiation, also called “cold pasteurization,” is safe because it does not leave any nuclear residues in food. Irradiation directly or indirectly interacts with bacterial structures, leading to cell injury and death. Cellular DNA damage due to the direct irradiation effect results in DNA lesions. However, ionization indirectly reacts with water or any other molecule in the cells to produce reactive oxygen species (ROS), which interact with DNA, resulting in damaged bond breaks or bases. Irradiation is useful to eliminate microorganisms that cause foodborne illness in different raw and produced foods. However, the effects of irradiation on foodborne pathogens may vary according to the growth and environmental conditions. Bacteria may develop resistance to irradiation exposure as a survival response. Induction of an efficient DNA double-strand (DBS) break repair system, formation of a mutant strain through an evaluation process, nucleoid structure, and protection of proteome oxidation play important roles in rice irradiation resistance.

Keywords Foodborne · Pathogens · Ionizing radiation · DNA lesions · Resistance · DNA repair system

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T. Ding et al. (eds.), Stress Responses of Foodborne Pathogens,
https://doi.org/10.1007/978-3-030-90578-1_12
12.1 Introduction of Irradiation Technology in the Food Industry

12.1.1 General Introduction

Radiation is energy emitted from a source that transmits through space and penetrates various materials in the form of waves or particles (Tonnessen and Pounds 2011). Radiation can be either ionizing or nonionizing depending on the energy level of the radiated particles or how it affects matter. Ionizing radiation, also called irradiation, is radiation with sufficient energy to remove bound electrons from an atom, causing the atom or molecule to become ionized (Berlman 1967).

Food irradiation technology is the application of ionizing radiation to food and has been widely used to eliminate microorganisms, extend the shelf life or delay sprouting and ripening to ensure food safety (Erkmen and Bozoglu 2016). Irradiation can damage DNA in the cells to inactivate living cells; therefore, microorganisms, insects, and plant meristems cannot reproduce. This mechanism represents the effective use of ionizing radiation in food (Thayer 1990). Food irradiation has already been applied for almost all food commodities, including grains, meats, poultry fish, crops, fruits, tubers and bulbs, and is very effective, with an additional advantage of being harmless to the environment (Farkas 2006).

Based on scientific evolution and research, food irradiation does not leave any “radioactive” residue in the food. Additionally, with specified low to medium doses, it has no and/or little negative effects on food nutrients, including vitamins that are usually supplied for human consumption (Crawford and Ruff 1996). The Food and Drug Administration (FDA) in the USA and European Food Safety Authority (EFSA) in the European Commission have evaluated food irradiation for different food commodities and have approved several food and food products, such as fruits and vegetables, fresh meat, sprouts, poultry, shell eggs, and molluscan shellfish (Pauli and Takeguchi 1986; Cef 2011a).

12.1.2 History of Food Irradiation Technology

Antoine-Henri Becquerel was the first to identify radioactivity (Grigorev 1996). However, Wilhelm Röntgen discovered X-rays at the end of 1895 (Röntgen 1896). After these two important discoveries, H. Minsch from Germany published a proposal that ionizing radiation can be used to preserve food by destroying spoilage microorganisms (Molins 2001). The British Patent (No. 1609, January 26, 1905) published to J. Appleby and A.J. Miller, analytical chemists to “bring about an improvement in the condition of foodstuffs” and in “their general keeping quality,” was the first documented use of ionizing radiation in food (Loaharanu and Thomas 2001). In 1960, Canada approved food irradiation for potatoes; in 1963–1964, the FDA approved the irradiation of bacon, wheat, flour, and potatoes (Molins 2001). In
1990, the FDA approved the irradiation of poultry to control *Salmonella* contamination (Sebranek et al. 2014). The World Health Organization (WHO) convened an expert committee to reexamine the safety of irradiated foods in 1990 and reconfirmed that irradiated foods are safe (Molins 2001). Currently, irradiation is used for many purposes in the food sector, including elimination of foodborne pathogens, preservation, control of insects, delay of sprouting and ripening, and sterilization.

### 12.1.3 Radiation Dose Measurement

The international system of units (SI) of irradiation dose measurement is the Gray (Gy) (Allisy-Roberts 2005). One gray represents one joule of energy absorbed per kilogram of irradiated product. In food irradiation and sterilization, the dose is also measured using the conventional unit “rad”. One “Gy” is equal to 100 rad. The kilo (1000) of Gy is commonly used in irradiation dose measurement with the unit “kGy.” In food, the desired irradiation dose is achieved by calculating the time of exposure and location of the product from the irradiation source (Eichholz 2003). The absorbed dose depends on the application purpose and mass, bulk density, and thickness of the food (Table 12.1).

<table>
<thead>
<tr>
<th>Dose level</th>
<th>Purpose</th>
<th>Product examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low dose</strong> Disinfestation/delay in ripening (up to 1 kGy)</td>
<td>– Inhibits the growth of sprouts&lt;br&gt;– Kills insects and larvae found after harvesting&lt;br&gt;– Slows the ripening process&lt;br&gt;– Kills certain harmful parasites&lt;br&gt;– Reduces or eliminates different foodborne pathogens</td>
<td>Potatoes, onions, garlic, root ginger, bananas, mangoes and certain other noncitrus fruits, cereals and pulses, dehydrated vegetables, dried fish and meat, fresh pork</td>
</tr>
<tr>
<td><strong>Medium dose</strong> pasteurization (1–10 kGy)</td>
<td>– Reduces the number of or eliminates certain microbes and parasites</td>
<td>Fresh fish, strawberries, grapes, dehydrated vegetables, fresh or frozen seafood, raw or frozen poultry, and meat</td>
</tr>
<tr>
<td><strong>High-dose sterilization</strong> (10–50 kGy)</td>
<td>– Sterilizes food for various uses, such as meals for hospital patients who have immune disorders and can eat only bacteria-free foods&lt;br&gt;– Eliminates some disease-causing viruses and spores of foodborne pathogens&lt;br&gt;– Decontaminates certain food additives and ingredients</td>
<td>Meat, poultry, seafood, and other food prepared for sterilized hospital diets, spices</td>
</tr>
</tbody>
</table>
12.1.4 Food Irradiation Sources

Food irradiation technology involves high-energy irradiators capable of producing wavelengths < 200 nanometers (gamma rays) or 2000 angstroms (X-rays) (McKeen 2012). Additionally, high-speed electron beams are used to irradiate food.

Gamma irradiators are the most widely used type of source in the food sector because of their ability to continuously penetrate and emit in all directions (Bisht et al. 2021). Gamma rays are emitted from radioactive forms of the element cobalt-60 (\(^{60}\text{Cobalt}\)) or cesium-137 (\(^{137}\text{Cesium}\)) (Kevin 2012). The \(^{60}\text{Co}\) irradiator produces gamma (\(\gamma\)) rays with energies of 1.173 and 1.332 MeV and has a half-life of 5.27 years, whereas \(^{137}\text{Cs}\) produces \(\gamma\) rays with an energy of 0.662 MeV and has a longer half-life of 30.1 years.

X-rays are produced during high-energy collisions of gamma rays and heavy elements (e.g., Tungsten) into food (Marshall 2012). X-rays are also widely used in medicine and industry to produce images of internal structures. They have limited practical application because of the low conversion efficiency of gamma to X-rays.

Electron beams, also called E-beams, are similar to X-rays and are a stream of high-energy electrons propelled from an electron accelerator into food (Miller 2006). They are produced by linear accelerators and are coherent and directional beams of high-energy electrons. The source is portable—i.e., no reactor is required. E-beams are not inherently radioactive. The establishment of the E-beam requires less shielding than gamma radiation. Because E-beam involves a flip of switch technology with less penetration depth but more exposure time. Critically, E-beams are not inherently radioactive.

12.1.5 Commercialization of Food Irradiation

The increasing number of studies on the safety and applications as well as the availability of more ionizing sources and nuclear reactors make food irradiation a technically and commercially feasible process. Worldwide, 60 countries have passed regulatory approvals for the use of irradiation in food processing and include China, the USA, the UK, Mexico, Thailand, the Netherlands, Belgium, Brazil, Bangladesh, and Australia (Roberts 2016). Therefore, in recent years, the commercialization of food irradiation has increased. Irradiation technology has been used to decontaminate vegetables, fruits, meats, poultry, fish, and seafood to improve food safety and extend shelf life.

Vegetables, ground beef, poultry, eggs, seafood, and dairy products are common sources of foodborne-related illness. The most serious contaminants in beef and beef products are \textit{Escherichia coli} and \textit{Listeria monocytogenes}. In Minnesota, the first irradiated frozen ground beef in the USA was introduced commercially on the market to reduce \textit{E. coli} O157:H7 contamination and subsequent disease outbreaks (Eustice 2018). Presently, approximately 515 irradiated plants are being used in...
22 countries, including the UK, France, Germany, and Japan. Commercial food irradiation is expanding for several purposes, including eliminating microorganisms, extending the shelf life, controlling insects, delaying sprouting and ripening, sterilizing, and improving food safety. Irradiation can be used to eliminate microorganisms that cause foodborne illnesses, such as *Salmonella*, *E. coli*, *Bacillus cereus*, and *L. monocytogenes*. Additionally, this technique can be used to preserve and extend the shelf life of different foods, such as seafood (Arvanitoyannis et al. 2009), meat (Verde et al. 2013), fish (Cef 2011b), juice (Hakguder Taze et al. 2015), pork meat (García-Márquez et al. 2012), and chicken meat (Hassanzadeh et al. 2017). Commercial irradiation technology can also be used to sterilize foods, including porridge (Park et al. 2020), bibimbap (Park et al. 2012), ready-to-eat chicken breast (Feliciano et al. 2014), hamburger steak (Lee et al. 2005) and meat (Galán et al. 2011), ham (Concepción Cabeza et al. 2007), and smoked mullet (Robertson et al. 2006), which have been used for a long time even without refrigeration. Irradiated sterilized foods are useful in space for astronauts, hospitals for immunosuppressed patients, and patients with AIDS or undergoing chemotherapy. In this chapter, we will mainly focus on the use of irradiation technology on foodborne pathogens.

Foodborne pathogen-related illnesses affect millions of people and are a high concern in global public health, economy, and food safety issues. Estimates of the global burden of foodborne diseases show that approximately 1 in 10 people become sick from eating contaminated food, and approximately 420,000 die every year (WHO 2019). *E. coli*, *Salmonella*, *Campylobacter*, and *L. monocytogenes* are among the most common bacteria that cause foodborne illness with severe life-threatening outcomes (de Oliveira Vieira et al. 2021). Foodborne pathogens also cause substantial economic loss due to high losses from food infestation, contamination, and spoilage. Food irradiation can be an effective tool to eliminate these microbes to improve food safety, secure the public and reduce economic losses. The Centers for Disease Control and Prevention (CDC) in the USA, WHO, and other health organizations approved irradiation technology against foodborne pathogens. In addition to the single use of irradiation technology, hurdle applications using two or three different disinfectants may enhance the effects of this technology against foodborne pathogens (Berrios-Rodriguez et al. 2020).

*E. coli* O157 is responsible for >300 million illnesses and nearly 200,000 deaths caused by diarrheagenic syndromes globally and 100,000 cases in the USA per year (CDC, 2020). *E. coli*-related infections lead to severe life-threatening complications, including hemolytic uremic syndrome, which can cause kidney failure and death. Ground beef is the most commonly identified source of *E. coli* infection. Several studies have shown that the irradiation of ground beef is effective against *E. coli*. For example, Schilling et al. (2009) found that E-beam and X-ray irradiation (2 kGy) eliminated inactivated *E. coli* O157:H7 from ground beef below the limit of detection and that irradiation did not affect consumer acceptability compared with untreated samples. Complete elimination or the radiation dose needed to inactivate 90% of *E. coli* (D10) in food also largely depends on the type of food. For example, Song et al. (2019) revealed that a radiation dose of 5 kGy decreased the pathogen levels in pistachios (*Pistacia vera* L.) to under the detection limit without affecting
the color. However, Thayer et al. (2003) demonstrated that a 2-kGy dose of radiation decreased *E. coli* O157:H7 levels up to 3.3 logs on alfalfa seeds used in food sprout production. In Jeong and Kang (2017), total elimination of *E. coli* O157:H7 was achieved at a 3-kGy radiation dose in ready-to-bake cookie dough. This study also showed that, at this irradiation dose, the color of this product was unaffected compared with the untreated sample. However, Fallah et al. (2010) found that *E. coli* O157:H7 was reduced to undetectable levels in barbecued chicken samples at a 4.5-kGy irradiated dose. The advantages of irradiation, such as the minimum or no increase in product temperature, make it a convenient technology to disinfect many products, including ice cream. For example, Jo et al. (2007) reported that low-dose irradiation (1 kGy) decreased the levels of *E. coli* by >5.5 logs in ice cream stored at −20 °C.

*Campylobacter jejuni* is commonly found in poultry and has been associated with human gastroenteritis and an acute neurologic disorder called Guillain–Barre´ syndrome (GBS) (Wakerley and Yuki 2013). Disinfection treatment, including heating or irradiation, effectively eliminates *Campylobacter* from contaminated foods. In a recent study, Gunther et al. (2019) demonstrated that a 3.8-log reduction in *C. jejuni* was achieved at 0.8 kGy of radiation followed by 1 week of storage at −20 °C.

*Salmonella* is commonly found in foods of origin, including meat, poultry, raw milk, and eggs. According to the CDC, USA, *Salmonella* is responsible for approximately 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the States every year. Song et al. (2019) found that a 5-kGy radiation dose completely inactivated *Salmonella* Typhimurium from pistachios (*Pistacia vera* L.). Irradiation at medium doses (less than 3.0 kGy) may eliminate or decrease vegetative cells of foodborne pathogens, including *Salmonella*, in different food products. For example, Thayer et al. (2003) revealed that a 2-kGy radiation dose was effective in reducing *Salmonella* up to 2 logs on alfalfa seeds. Total removal of *S. Typhimurium* was observed at a 3-kGy radiation dose in cookie dough.

The opportunistic pathogen *L. monocytogenes* causes estimated cases of severe invasive illness per year worldwide (Zhang et al. 2021). Song et al. (2019) showed a complete reduction to under the detection limit of *L. monocytogenes* at a 5-kGy radiation dose from pistachios (*Pistacia vera* L.). Jeong and Kang (2017) reported complete elimination of *L. monocytogenes* at 3 kGy in ready-to-bake cookie dough without affecting quality (Table 12.2).

Irradiation technology can be useful to eliminate foodborne viruses for different food products. Gamma irradiation may disrupt virion structure and degrade viral proteins as well as genomic RNA, resulting in virus injury and death (Feng et al. 2011). However, viruses are much more resistant to irradiation than foodborne bacteria (Feng et al. 2011). Norovirus contamination and outbreaks are serious food safety and public health concerns worldwide (Hall et al. 2014). Therefore, irradiation treatment against foodborne viruses may play an important role in ensuring food safety and hygiene. For example, Feng et al. (2011) reported that a reduced dose of 5.6 kGy inactivated human norovirus surrogates (murine norovirus 1) up to 1.7- to 2.4-log in fresh produce (strawberries, romaine lettuce, and spinach). This study also found that a 3.3-log vesicular stomatitis virus (VSV) was reduced
Table 12.2 List of microorganisms and foods that have been widely studied to prevent foodborne illness using irradiation

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Food</th>
<th>Dose</th>
<th>Reduction level</th>
<th>$D_{10}$ value</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> O157:H7</td>
<td>Ground beef</td>
<td>2 kGy</td>
<td>Elimination</td>
<td>–</td>
<td>Schilling et al. (2009)</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>Pistachios</td>
<td>5 kGy</td>
<td>Elimination</td>
<td>0.85 kGy</td>
<td>Song et al. (2019)</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>Alfalfa seed</td>
<td>2 kGy</td>
<td>3.3 logs</td>
<td>0.55 kGy</td>
<td>Thayer et al. (2003)</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>Cookie dough</td>
<td>3 kGy</td>
<td>Elimination</td>
<td>0.53 kGy</td>
<td>Jeong and Kang (2017)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Broth</td>
<td>3.6 kGy</td>
<td>Elimination</td>
<td>0.35 kGy</td>
<td>Trampuz et al. (2006)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Raw beef liver</td>
<td>5.3–5.5 kGy</td>
<td>$10^5$ CFU/g</td>
<td>–</td>
<td>Kawasaki et al. (2019)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Lettuce</td>
<td>1.0 kGy</td>
<td>3–4 logs</td>
<td>0.02–0.35 kGy</td>
<td>Gomes et al. (2009)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Ice cream</td>
<td>1 kGy</td>
<td>&gt;6.5 logs</td>
<td>0.28–0.38 kGy</td>
<td>Jo et al. (2007)</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>Barbecued chicken</td>
<td>3 kGy</td>
<td>Undetected</td>
<td>0.397 kGy</td>
<td>Fallah et al. (2010)</td>
</tr>
<tr>
<td><em>Salmonellae</em></td>
<td>Alfalfa seed</td>
<td>2 kGy</td>
<td>2 logs</td>
<td>0.97 kGy</td>
<td>[86]</td>
</tr>
<tr>
<td><em>Salmonellae</em></td>
<td>Spinach leave</td>
<td>–</td>
<td>–</td>
<td>0.190 kGy</td>
<td>Gomes et al. (2011)</td>
</tr>
<tr>
<td><em>S. Typhimurium</em></td>
<td>Pistachios</td>
<td>5 kGy</td>
<td>–</td>
<td>0.86 kGy</td>
<td>Song et al. (2019)</td>
</tr>
<tr>
<td><em>S. Typhimurium</em></td>
<td>Cookie dough</td>
<td>3 kGy</td>
<td>–</td>
<td>0.51 kGy</td>
<td>Jeong and Kang (2017)</td>
</tr>
<tr>
<td><em>S. Typhimurium</em></td>
<td>Baby food</td>
<td>8 kGy</td>
<td>–</td>
<td>0.98 kGy</td>
<td>Hong et al. (2008)</td>
</tr>
<tr>
<td><em>S. Typhimurium</em></td>
<td>Barbecued chicken</td>
<td>4.5 kGy</td>
<td>Undetected</td>
<td>0.397 kGy</td>
<td>Fallah et al. (2010)</td>
</tr>
<tr>
<td><em>S. Enteritidis</em></td>
<td>Raw beef liver</td>
<td>8.2–8.5 kGy</td>
<td>$10^5$ CFU/g</td>
<td>–</td>
<td>Kawasaki et al. (2019)</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>Chicken liver</td>
<td>1.8 kGy</td>
<td>&gt; 5 logs</td>
<td>0.361–0.748 kGy</td>
<td>Gunther et al. (2019)</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>Carrot</td>
<td>0.5 kGy</td>
<td>3.24 log CFU/g</td>
<td>–</td>
<td>Berrios-Rodriguez et al. (2020)</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>Tomato</td>
<td>0.5 kGy</td>
<td>(1.65 log CFU/g)</td>
<td>–</td>
<td>Berrios-Rodriguez et al. (2020)</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>Pistachios</td>
<td>5 kGy</td>
<td>1.02 kGy</td>
<td>–</td>
<td>Song et al. (2019)</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>Cookie dough</td>
<td>3 kGy</td>
<td>–</td>
<td>0.51 kGy</td>
<td>Jeong and Kang (2017)</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>Smoked salmon</td>
<td>3 kGy</td>
<td>6.59 logs</td>
<td>–</td>
<td>Badr (2012)</td>
</tr>
<tr>
<td><em>Listeria spp.</em></td>
<td>Spinach leave</td>
<td>–</td>
<td>–</td>
<td>0.213 kGy</td>
<td>Gomes et al. (2011)</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>Barbecued chicken</td>
<td>4.5 kGy</td>
<td>Undetected</td>
<td>0.680 kGy</td>
<td>Fallah et al. (2010)</td>
</tr>
</tbody>
</table>

(continued)
under the same treatment, indicating higher susceptibility to this virus under irradiation treatment than to human norovirus surrogates.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Food</th>
<th>Dose</th>
<th>Reduction levela</th>
<th>D10 value</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. monocytogenes</td>
<td>Smoked catfish</td>
<td>2.0 kGy</td>
<td>Undetected</td>
<td></td>
<td>Mahmoud et al. (2012)</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>Baby food</td>
<td>8 kGy</td>
<td></td>
<td>1.22 kGy</td>
<td>Hong et al. (2008)</td>
</tr>
<tr>
<td>B. cereus spore</td>
<td>Mesquite pod flour</td>
<td>6 kGy</td>
<td>Lower than 1 log</td>
<td>1.31 kGy</td>
<td>Fan et al. (2015)</td>
</tr>
<tr>
<td>Enterobacter sakazakii</td>
<td>Baby food</td>
<td>16 kGy</td>
<td>Elimination</td>
<td>4.83 kGy</td>
<td>Hong et al. (2008)</td>
</tr>
<tr>
<td>Murine norovirus</td>
<td>Fresh produce</td>
<td>5.6 kGy</td>
<td>1.7- to 2.4-log</td>
<td>–</td>
<td>Feng et al. (2011)</td>
</tr>
<tr>
<td>Vesicular stomatitis virus</td>
<td>Fresh produce</td>
<td>5.6 kGy</td>
<td>3.3-log</td>
<td>–</td>
<td>Feng et al. (2011)</td>
</tr>
</tbody>
</table>

*aUndetected—lower than detection limit*

12.2 Inactivation Mechanisms of Irradiation on Foodborne Pathogens

Irradiation can directly or indirectly interact with bacterial structures, causing “ionization,” thereby initiating a chain of events leading to cell injury and death (Lehnert 2007). The critical effect of irradiation on bacterial cells is DNA damage; therefore, DNA is considered a “radiation sensitive” portion (Potera 2007). High-energy linear transfer (LET), such as neutrons or alpha (α) particles of irradiation, may directly damage the DNA of bacteria, such as cross-links that prevent microbes from growing or reproducing and cause death (Azzam et al. 2012). Ionization can occur in any molecule in bacterial cells, resulting in the creation of a cation radical and an electron. The produced electron can react with another molecule or can become solvated before further reactions. The radical site can be transferred to another nearby molecule. Cation radicals can react and become neutralized by losing a proton; for example, irradiation-induced cations react with water. H2O+ reacts with neighboring water molecules to produce OH. Consequently, cellular DNA can be damaged in several ways, such as direct ionization of DNA and reactions between the DNA and electrons, including OH, H2O+ or other radicals. Irradiation produces several DNA lesions, including damage to purine or pyrimidine bases, DNA single bonds, and double bond breaks. Single strand DNA base damage occurs because of abstraction of any of the deoxyribose hydrogens (Biswas and Chakraborty 2019). Irradiation results in DNA damage, and other related direct and indirect effects have been studied in foodborne pathogens, including E. coli. For example, in a study by Krisko and Radmana (Krisko and Radman 2010), irradiation caused E. coli death by oxidative damage with the loss of maintenance activities, including the DNA repair
system. In another study, Krisko and Radman (Krisko and Radman 2010) demonstrated that proteome oxidation (i.e., protein carbonylation) in *E. coli* is correlated with cell killing following irradiation exposure. The decreased biosynthetic efficacy of the cellular proteome during irradiation suggests that oxidative damage with consequential loss of the DNA repair system is the primary cause of cell killing.

Direct and indirect irradiation can alter bacterial membrane permeability and other cellular functions, which can also cause cell injury or cell death. For example, Ayari et al. (2009) revealed that irradiation causes membrane cellular damage in *B. cereus* and *S. Typhimurium*. Figure 12.1 presents a generalized schematic diagram of microbial inactivation mechanism of irradiation.

12.3 Factors Affecting Resistance Development in Foodborne Pathogens

The effects of irradiation in foodborne pathogens may vary based on the environment of the treatment process. Bacteria may develop resistance to irradiation exposure as a survival response. Resistant pathogens may exhibit higher irradiation
D$_{10}$ values; the dose requires achieving a 90% (1 log) reduction compared with susceptible cells. Bacterial resistance to irradiation depends on several factors, such as specific species, strains, the growth phase, oxygen availability, temperature, water activity, and other physical agents.

Resistance development in food pathogens may vary depending on their structural differences, including the structure of DNA and presence or absence of efficient mechanisms for the DNA repair system. Pathogenic bacteria are commonly eliminated at irradiation doses such as 3 to 10 kGy. At this high dose, microbes usually cannot develop resistance. However, in the case of low-dose irradiation exposure to foodborne pathogens, damaged DNA can be repaired using specialized enzymes in cells. For example, Levanduski and Jaczynski (2008) found that *E. coli* O157:H7 increased resistance to irradiation after exposure to repetitive irradiation at sublethal doses. In the present study, the D$_{10}$ values of *E. coli* increased during sublethal repetitive irradiation (E-beam) starting at 0.63+/−0.02 kGy. After four cycles of irradiation treatment, isolate L3 survived even at a 3.0-kGy dose, indicating that *E. coli* can develop irradiation resistance following repetitive sublethal irradiation exposure.

Bacterial cells in the exponential or log phase are less resistant to irradiation exposure than cells in stationary phase (Sukhi et al. (2009)). Sukhi et al. (2009) demonstrated that *D. radiodurans* R1 is four times more sensitive in the latent phase to irradiation (2–20 kGy at ice temperature) than cells in the exponential or early stationary phase. This study concluded that the shortage of nutrients at the latent growth phase may reduce the metabolic capability of the cells to repair DNA damage in irradiation exposure. By contrast, Mendonca et al. (2004) found that nutrient starvation at the exponential phase caused *L. monocytogenes* Scott A to be more resistant to irradiation exposure than cells grown in the stationary phase.

Aerobic bacteria exhibit lower survival capacity in irradiation treatments than their anaerobic counterparts (Obodovskiy 2019). Bacteria are more readily damaged in the presence of oxygen during irradiation. The irradiation resistance of bacteria can be different among isolates of the same species. According to Xu et al. (2019), irradiation resistance differed between clinical and food isolates of *E. coli* in ground chicken meat. In the present study, the D$_{10}$ values for the clinical isolates and nonclinical isolates were 0.36 kGy and 0.27 kGy, respectively, indicating more radiation resistance of clinical isolates than food isolates. Temperature is also an important factor affecting irradiation resistance development in bacteria (Kawasaki et al. 2019). For example, the temperature at which a product is irradiated may influence the resistance development of a pathogen (Thayer and Boyd 1995). Foodborne pathogens show higher resistance at low temperatures (Palumbo et al. 1986; Thayer and Boyd 1995). Thayer and Boyd (1995) investigated the effect of temperature (from −60 °C to +15 °C) of *L. monocytogenes* on the beef irradiation dose (2.0 kGy). In this study, no change was observed in the resistance of *L. monocytogenes* to irradiation from 15 °C and 0 °C; however, between 0 °C and −5 °C, a marked increase in resistance occurred. Palumbo et al. (1986) found that decreasing the temperature during irradiation increased the radiation resistance (higher D10 values) of *Aeromonas hydrophila* in ground bluefish.
Bacterial spores are more resistant to irradiation than their vegetative counterparts. Therefore, conditions suitable for spore formation facilitate the development of resistance to irradiation. For example, Wang et al. (2006) found an up to 7-log reduction in E. coli at 1 kGy in cantaloupe juice, whereas B. subtilis spores were reduced by only 1 log, demonstrating higher resistance of spores. In the present study, the D$_{10}$ values of E. coli and B. subtilis spores were 0.9908 and 1.192 kGy, respectively, indicating that spores were highly resistant to irradiation compared with E. coli. Spores of common foodborne pathogens, including Clostridium perfringens and B. cereus, are particularly important and show higher resistance to irradiation exposure. Therefore, the Joint IAEA and WHO study group recommends a very high dose of 45 kGy to inactivate bacterial spores in meat, poultry and fish products (Joint and Organization 1999).

The cell wall structure of bacteria also plays an important role in resistance development against irradiation. For example, Gram-negative bacteria, including E. coli O157:H7 and S. Typhimurium, are more sensitive to irradiation than Gram-positive bacteria, such as L. monocytogenes (Jeong and Kang 2017).

Microbes may develop resistance against irradiation during adaptation to stress conditions, including nutrient starvation and antibiotic exposure (Gaougaou et al. 2018). Gaougaou et al. (2018) observed that E. coli promotes resistance to irradiation during antibiotic exposure by inducing the expression of different genes. In that study, E. coli was exposed to kanamycin or carbenicillin (25, 15, or 7 μg/mL) and irradiated at 0.4 kGy or lethal 1.5 kGy doses. Antibiotic-adapted E. coli was resistant to 0.4 kGy and could survive after a lethal 1.5 kGy irradiation dose. The study concluded that the ampC and ampG genes regulated the resistance of E. coli to irradiation after exposure to antibiotics. Black and Jaczynski (2007) revealed that the increased salt content (NaCl) in meat products induced higher resistance of E. coli O157:H7 to irradiation. Skowron et al. (2018) demonstrated that antibiotic-resistant strains of L. monocytogenes exhibited higher resistance to irradiation than susceptible strains. That study found faster induction of the irradiation-induced DNA damage repair system in antibiotic-resistant strains than in antibiotic-susceptible L. monocytogenes strains. The presence of acid in the growth medium or food assists the development of resistance to irradiation in microbes. For example, Buchanan et al. (2004) found that the presence of different organic acids increased D-values by 1.2–3.3-fold in an enterohemorrhagic E. coli strain (E. coli O157:HEnt-C9490) following irradiation treatment. However, that study revealed that pH values ranging from 4.0 to 5.5 do not affect the irradiation resistance of E. coli. Starvation and heat were also shown to have no effect on the irradiation resistance of Salmonella (Osaili et al. 2016).

The composition and type of food and food matrix play important roles in irradiation resistance. Hong et al. (2008) described that the formulation of baby food products affects the irradiation sensitivity of E. sakazakii. For example, Lee et al. (2007) investigated the effect of irradiation treatment of E. sakazakii on dehydrated infant formula, and a D$_{10}$ value of 0.76 kGy was observed. However, Osaili et al. (2007) observed a D$_{10}$ value of 0.37 kGy for E. sakazakii on rehydrated infant formula, whereas a D$_{10}$ value of 4.83 kGy was observed in
dehydrated weaning food rich in rice flour (Hong et al. 2008). These studies clearly demonstrated the strong difference in irradiation among different food compositions.

12.4 Mechanisms Underlying the Resistance of Foodborne Pathogens to Irradiation

Bacteria use defense systems or responses against stress, including irradiation, to protect against cell damage and death. The best-studied irradiation-resistant bacterium is *Deinococcus radiodurans* (Cox and Battista 2005). The mechanism of extreme resistance of *D. radiodurans* to irradiation remains unclarified. The irradiation resistance of this bacterium is hypothesized to be attributed to Mn$^{2+}$-mediated protein oxidation (Webb and DiRuggiero 2012), DNA repair system processes (Minton 1994), and evolution capacity to survive extreme environments (Wassmann et al. 2010). However, the resistance development of foodborne pathogens has not yet been extensively studied because most of these species are sensitive to high doses of irradiation. A few foodborne bacteria, including *E. coli*, *Micrococcus radiodurans*, and *Acinetobacter* sp. (formerly *Moraxella* sp.), may survive a very high irradiation dose as *D. radiodurans* (Sommers 2012).

12.4.1 ROS-Mediate Oxidation Damages

The first line of resistance against irradiation is the adaptation of oxidative stress generated from irradiation. The presence of antioxidant enzymes, including superoxide dismutases (SODs) and catalases, may contribute to protecting cells from ROS-mediated damage. SOD catalyzes the conversion of oxygen superoxide (O$_2^-$) to hydrogen peroxide (H$_2$O$_2$), which is subsequently converted to H$_2$O by catalases or peroxidases. For example, Misra et al. (2004) found that overproduction of the ROS scavenger pyrroloquinoline-quinone (PQQ) protects *E. coli* against irradiation. PQQ reacts with reactive oxygen species to produce nonreactive molecular products, and the decreased ROS support *E. coli* cells showing higher resistance to irradiation. Additionally, PQQ promotes the protection of plasmid DNA and proteins from oxidative damage caused by irradiation exposure. In general, PQQ is considered a bacterial glucose dehydrogenase redox cofactor found in several biological fluids, such as plants, bacteria, and animals (Mitchell et al. 1999).
12.4.2 **Bacterial Evolutionary Processes**

Some foodborne pathogens may develop resistance attributed to evolutionary processes. For example, irradiation resistance developed in the irradiation-sensitive foodborne pathogens *E. coli* (Erdman et al. 1961), *Clostridium botulinum* (Erdman et al. 1961), and *Staphylococcus aureus* (Erdman et al. 1961) through evolution (Harris et al. 2009). Repeated irradiation of survivors of foodborne pathogens may develop more resistant strains than the initial cultures. Harris et al. (2009) demonstrated that *E. coli* adapted to repeated exposure to high doses of irradiation and developed higher resistance than *D. radiodurans*. The study revealed that a single mechanism did not cause this resistance against irradiation, but multiple mechanisms were involved in this evolutionary acquisition, including multiple genes, alleles of some genes and several evolutionary pathways. In the *E. coli* K-12 strain, the *recA* gene and e14 prophage play an important role in evolutionary acquisition against irradiation exposure (Harris et al. 2009). Similarly, repeated exposures of 84 cycles to gamma irradiation resulted in the development of an irradiation-resistant culture of *S. Typhimurium* showing a D10 value 200-fold higher than that of the initial strain (Davies and Sinskey 1973). Recently, Gaougaou et al. (2020) revealed that gamma irradiation triggers a global stress response in *E. coli* O157:H7. Irradiation exposure of this microbe at a nonlethal dose (0.4 kGy) promoted changes in various metabolic pathways, including base excision repair and nucleotide excision repair pathways, sulfur and histidine metabolisms, and virulence mechanisms. Additionally, irradiation treatment upregulated the *sulA* gene encoding a cell division repressor together with other genes involved in the SOS response and repair mechanism, such as *recA*, *recN*, *recI*, *recQ*, *mutM*, and *uvrB*. The study also revealed that as an early response to irradiation stress, *dnaK*, *groEL*, *ibpA*, sulfur metabolism genes, and genes related to oxidative stress are upregulated, while histidine biosynthesis genes are downregulated.

12.4.3 **DNA Double-Strand (DBS) Break Repair System**

The efficient induction of the DNA double-strand (DBS) break repair system is particularly important in irradiation resistance development in microbial cells. Some microbes have the exceptional ability to repair hundreds of DNA double-strand breaks (DSBs) generated by high doses of irradiation (Minton 1994). For example, Gram-positive bacteria show higher resistance to irradiation than Gram-negative bacteria using effective metabolic systems, including sulfur metabolism pathways that can repair irradiation-induced DNA damage (Ray and Bhunia 2008). Previous studies have demonstrated that irradiation resistance is associated with high efficacy of the DNA repair system (Zhang et al. 2020). Zhang et al. (2020) observed in *Bacillus subtilis* that genes associated with DNA repair-deficient mutants such as Δ*recA*, Δ*Ku ΔligD*, Δ*exo Δnfo*, Δ*uvrAB*, and Δ*sbcDC* show reduced resistance,
indicating that DNA damage is induced during irradiation treatments. The recA gene is crucial for spore resistance under treatment conditions. Several pathways are involved in the DSB repair system, such as homologous recombination (HR) and nonhomologous DNA end joining (NHEJ) (Dillingham and Kowalczykowski 2008). DSB repair requires the formation of single-stranded DNA tails, which can be obtained from the activities of multiple helicases and nucleases (Yeeles and Dillingham 2010).

12.4.4 Structure Modifications

The association of bacterial nucleoids with proteins and chromosomes may correlate with irradiation resistance development. For example, the presence of high histone-like protein HU in E. coli nucleoids is associated with cell survival after irradiation exposure (Boubrik and Rouviere-Yaniv 1995).

The bacterial membrane may be modified in a cellular response to survive through irradiation stress. For example, Ayari et al. (2009) showed the fatty acid (FA) and murein (peptidoglycan) composition of B. cereus and Salmonella Typhimurium in response to a sublethal radiation dose of 1 kGy. The study demonstrated that the fatty acid composition is modified with an increase in the unsaturated fatty acid percentage and that the muropeptide profile was changed under this low-dose irradiation stress. ROS produced by irradiation treatment induce changes in protein expression, altering enzymatic activity and peptidoglycan biosynthesis (Bonura et al. 1975) (Fig. 12.2).

12.5 Conclusions

Irradiation technology has been used to reduce or eliminate spoilage and/or pathogenic microorganisms; therefore, irradiation plays a critical role in ensuring food safety. The FDA in the USA, EFSA in the European Commission, and WHO along with 41 countries evaluated food irradiation for different food commodities and allowed disinfection of foodborne pathogens. Commercial food irradiation technology involves high-energy irradiators such as 60Co and 137Cs, X-rays, and high-speed E-beams. Irradiation directly causes bacterial DNA damage, resulting in cell death, or indirectly interacts with bacterial biomolecules, including water, producing ROS. The produced ROS may destroy or change the structure of cellular membranes or cause irreversible changes to nucleic acids, leading to cell inhibition or death. The commercialization of food irradiation is increasing because of recent advantages in irradiation facilities. This technology is now used to decontaminate foodborne pathogens such as E. coli, Salmonella, L. monocytogenes, and B. cereus in many products, including vegetables, ground beef, poultry, eggs, seafood, and dairy products. Most foodborne pathogens are sensitive to irradiation treatment, except
for some spores and viruses. However, the effects of irradiation in foodborne pathogens may vary depending on the growth and environmental conditions, including species, strains of each species, growth phase, and temperature. A few pathogens, including \textit{E. coli}, may develop higher resistance to irradiation. The molecular mechanism of the development of this resistance to irradiation in foodborne pathogens is not yet completely understood. The induction of an efficient DSB repair system, formation of a mutant strain using an evaluation process, nucleoid structure, and protection of proteome oxidation may contribute to the development of irradiation resistance. The development of resistance of foodborne pathogens against irradiation is the result of complex cell networks, and further extensive research is required to understand molecular pathways that are involved in irradiation resistance.

\textbf{Fig. 12.2} Schematic mechanism of the development of resistance of foodborne pathogens to irradiation exposure. The effect of ROS during irradiation may be neutralized because of the presence of superoxide dismutases (SODs), pyrroloquinoline-quinone (PQP), and catalases, which may contribute to protecting cells from ROS-mediated damage. Induction of the DNA double-strand (DBS) break repair system contributes to irradiation resistance development. Protein carbonylation or protein oxidation contributes to protecting and repairing damaged proteins. Increased levels of fatty acids (FAs) and mureins (peptidoglycan) may be associated with bacterial survival after irradiation.

\textbf{References}


Pauli GH, Takeguchi CA (1986) Irradiation of foods – an FDA perspective. 2:79–107


Chapter 13
Response of Foodborne Pathogens to Phytochemicals

Mahmoud Ela’fy and Chao Shi

Abstract Many organisms of public health concern have the potential to contami-
inate foods and are therefore of great concern to food safety researchers. Because
most foodborne pathogens, especially those that exhibit multidrug resistance, pose a
potential threat to human health, the food industry urgently needs effective control
strategies. Phytochemicals are a promising method for inhibiting the growth of
pathogenic bacteria. Many plant extracts can effectively control bacteria, yeasts,
and molds and are therefore considered good candidates for use in food preservation.
The objective of this chapter is to provide a systematic summary of how different
types of phytochemicals can be applied to foods. The antimicrobial efficacy of
phytochemicals and the major factors affecting phytochemical stability are also
discussed in detail.

Keywords Phytochemicals · Natural additives · Foodborne pathogens ·
Mechanism · High-quality foods

13.1 Definition of Phytochemicals

Phytochemicals refer to various chemical substances produced in plants, which
generally provide a defense against microorganisms (Huang et al. 2016). Phyto-
chemicals can be divided into six main categories based on their characteristics and
chemical structure: lipids (monounsaturated, polyunsaturated and saturated fats,
fatty acid); carbohydrates (monosaccharides, disaccharides, polysaccharides, and
oligosaccharides); phenolic acids (flavonoids, phenolic acids, tannins, coumarins,
benzofurans, and phenylpropanoids); terpenoids (carotenoids, monoterpenoids, diterpenoids, triterpenes, sesquiterpenoids, and polyterpenoids); and alkaloids and other nitrogen-containing metabolites (glucosinolates, amaryllidaceae, betalain, diterpenoids, indole, isoquinoline, piperidine, quinolone, steroids, tropane, amino acids, amines, and purines) (Campos-Vega and Oomah 2013). It has been reported that phytochemicals can contribute to reducing the risks of chronic diseases, such as heart disease and diabetes in humans (Liu 2013).

### 13.2 Extraction and Identification of Phytochemicals in Foods

Polyphenols, carotenoids, phenolic acids, and glucosinolates are the most widely used phytochemicals of plant origin (Table 13.1). Phytochemicals can be extracted by several methods such as supercritical fluid extraction, microwave-assisted extraction, sonication extraction, Soxhlet extraction, hydro-distillation extraction, steam distillation extraction, and hot water extraction (Doughari 2012; Ingle et al. 2017). The advantages and disadvantages of these methods have been compared by Azmin et al. (2016) (Table 13.2).

### 13.3 Antimicrobial Activity of Phytochemicals Against Foodborne Pathogens

Tables 13.3, 13.4, and 13.5 show that phytochemicals have been reported to exhibit antimicrobial activity against several pathogens (Khameneh et al. 2016).

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### Table 13.1 Classification of the main bioactive phytochemicals found in fruits and vegetables, and their sources (Fig. 13.1)

<table>
<thead>
<tr>
<th>Category</th>
<th>Subcategory</th>
<th>Chemical constituents</th>
<th>Food sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic compounds</td>
<td>Triterpene</td>
<td>Phytosterols</td>
<td>Almonds, nuts, avocados</td>
</tr>
<tr>
<td></td>
<td>Phenolic acids</td>
<td>Ellagic acid</td>
<td>Cranberries, walnuts, coffee</td>
</tr>
<tr>
<td></td>
<td>Polyphenols</td>
<td>Hydroxycinnamic acids</td>
<td>Muscadine grape, berries</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anthocyanins</td>
<td>Peanuts, soy, red clover</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isoflavones</td>
<td>Citrus fruit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flavonones</td>
<td>Apple, almond, tea</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flavonols</td>
<td></td>
</tr>
<tr>
<td>Terpenoids</td>
<td>Carotenoids</td>
<td>Lutein</td>
<td>Spinach, turnip greens</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lycopene</td>
<td>Tomatoes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B-carotene</td>
<td>Tomatoes, carrots, pumpkin</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Glucosinolates</td>
<td>Isothiocyanate</td>
<td>Cabbage, broccoli</td>
</tr>
</tbody>
</table>
### 13.3.1 Alkaloids

Alkaloids are defined as organic compounds in living organisms that contain more than one nitrogen atom (Kurek 2019). In plants, the most common types of alkaloid are piperine, pyrrolidine, piperidine, quinazoline, isoquinoline, glyoxaline, ungeremine, tomatidine, imidazoline, resperine, and ellagic acid (Fig. 13.1) (Kurek 2019).

There are several types of natural alkaloids: for example, berberine, an isoquinoline alkaloid extracted from a Chinese herb, is used for treating gastrointestinal tract disorders (Qiu et al. 2014). The antimicrobial activity of berberine arises from its interaction with DNA, RNA, and DNA gyrase (Iwasa et al. 2001; Domadia et al. 2008). Berberine inhibits DNA gyrase, which is responsible for supercoiling and uncoiling bacterial DNA, thus inhibiting bacterial replication (Khameneh et al. 2019). The inhibition of the cell division protein, FtsZ (Filamenting temperature-sensitive mutant Z), has been suggested as contributing to the antimicrobial activity.

<table>
<thead>
<tr>
<th>Extraction methods</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supercritical fluid extraction</td>
<td>CO₂ is used as a solvent and it is not expensive. The obtained extractions are pure and clear. Solvent recycling can be done during the extraction process.</td>
<td>Loss of desirable phytochemical components during extraction.</td>
</tr>
<tr>
<td>Microwave-assisted extraction</td>
<td>Used for both laboratory and industrial scales. Less time consuming.</td>
<td>Low efficiency for nonpolar target compounds or solvent or highly viscous solvent.</td>
</tr>
<tr>
<td>Sonication extraction</td>
<td>Less time consuming. Used in the large-scale commercial application. The obtained extracts characterize by a high quality.</td>
<td>The active part of ultrasound is only the vicinity of the ultrasonic emitter. Has a limited effect on oil extraction.</td>
</tr>
<tr>
<td>Soxhlet extraction</td>
<td>Inexpensive and simple method. Temperature during extraction process can be managed.</td>
<td>Long time consuming. Use a large amount of solvent Thermal decomposition of heat-labile compounds.</td>
</tr>
<tr>
<td>Hydro-distillation extraction</td>
<td>Inexpensive method.</td>
<td>Long time consuming to separate water from the product. Thermal decomposition of heat-labile compounds. Energy consuming.</td>
</tr>
<tr>
<td>Steam-distillation extraction</td>
<td>Water insoluble compound can be extracted.</td>
<td>No acceleration of extraction process even by agitation.</td>
</tr>
<tr>
<td>Hot water extraction</td>
<td>Inexpensive method. Saving for both energy and investment costs.</td>
<td>Thermal decomposition of heat-labile compounds.</td>
</tr>
</tbody>
</table>

#### Table 13.2 Advantages and disadvantages of different methods for extracting phytochemicals
Table 13.3 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of some alkaloid phytochemicals against foodborne pathogens

<table>
<thead>
<tr>
<th>Plant extracts</th>
<th>The types of phytochemicals</th>
<th>Target bacteria</th>
<th>MIC (mg/ml)</th>
<th>MBC (mg/ml)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mangrove leaf extract</td>
<td>Alkaloid</td>
<td>Enterococcus faecium</td>
<td>4</td>
<td>4</td>
<td>Behbahani et al. (2018)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Klebsiella pneumoniae</td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Ethanol leaf extracts of P. nigrum</td>
<td>Alkaloid</td>
<td>Staphylococcus aureus</td>
<td>12.5</td>
<td>25</td>
<td>Akthar et al. (2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Escherichia coli</td>
<td>12.5</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S. Typhimurium</td>
<td>12.5</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pseudomonas aeruginosa</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Garlic extract</td>
<td>Alkaloid</td>
<td>Escherichia coli</td>
<td>100</td>
<td>200</td>
<td>Garba et al. (2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Staphylococcus aureus</td>
<td>200</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pseudomonas aeruginosa</td>
<td>200</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Coix lacryma-jobi oil (Pyridine)</td>
<td>Alkaloid</td>
<td>Escherichia coli</td>
<td>31</td>
<td>125</td>
<td>Diningrat et al. (2020)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Staphylococcus aureus</td>
<td>31</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacillus subtilis</td>
<td>31</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>Alternanthera brasiliana leaf extract</td>
<td>Alkaloid</td>
<td>Escherichia coli</td>
<td>0.625</td>
<td>1.25</td>
<td>Kannan et al. (2014)</td>
</tr>
<tr>
<td>Methanolic extract of Piper (P.) sarmentosum</td>
<td>Alkaloid</td>
<td>MRSA</td>
<td>50</td>
<td>100</td>
<td>Fernandez et al. (2012)</td>
</tr>
<tr>
<td>Callistemon citrinus</td>
<td>Alkaloid</td>
<td>Staphylococcus aureus</td>
<td>0.0025</td>
<td>0.835</td>
<td>Mabhiza et al. (2016)</td>
</tr>
<tr>
<td>Swietenia macrophylla</td>
<td>Alkaloid</td>
<td>Staphylococcus aureus</td>
<td>12.5</td>
<td>50</td>
<td>Mohammed et al. (2014)</td>
</tr>
<tr>
<td>Brown Algae (Sargassum hornschuchii)</td>
<td>Alkaloid</td>
<td>Staphylococcus aureus E. coli (EHEC) O157</td>
<td>125 125 500</td>
<td>500</td>
<td>Alghazer et al. (2021)</td>
</tr>
</tbody>
</table>

of berberine (Boberek et al. 2010). Berberine has also been reported to damage the bacterial cell membranes, proteins, and DNA thus resulting in bacterial death (Feng et al. 2016).

Piperine, a piperidine-type alkaloid obtained from black pepper, has been reported to exhibit antimicrobial activity against foodborne pathogens such as Escherichia coli, Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa, and Klebsiella pneumoniae, with MIC values of 6.25, 50, 12.5, 100, and 25 mg/mL, respectively (Aldaly 2010). Piperine also exhibited an antimicrobial effect against Candida albicans ATCC10231 with an inhibition zone of 1.90 mm (Moraru et al. 2019). It has also shown a synergistic effect against foodborne pathogens, especially when
Table 13.4 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of some flavonoid phytochemicals against foodborne pathogens

<table>
<thead>
<tr>
<th>Plant extracts</th>
<th>The types of phytochemicals</th>
<th>Target bacteria</th>
<th>MIC (mg/ml)</th>
<th>MBC (mg/ml)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous extract of <em>R. communis</em></td>
<td>Flavonoid</td>
<td><em>Staphylococcus aureus</em></td>
<td>3.1</td>
<td>300</td>
<td>Suurbaar et al. (2017)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>3.1</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Klebsiella pneumonia</em></td>
<td>12.5</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td>Methanolic extract of <em>S. litwinowii</em></td>
<td>Flavonoid (quercetin)</td>
<td><em>Escherichia coli</em></td>
<td>25</td>
<td>50</td>
<td>Bazzaz et al. 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Bacillus cereus</em></td>
<td>25</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Staphylococcus aureus</em></td>
<td>6.25</td>
<td>6.25</td>
<td></td>
</tr>
<tr>
<td>Ethanol leaf extracts of <em>P. nigrum</em></td>
<td>Flavonoid</td>
<td><em>Escherichia coli</em></td>
<td>12.5</td>
<td>25</td>
<td>Akthar et al. (2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>S. Typhimurium</em></td>
<td>12.5</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Satureja Montana essential oils</td>
<td>Flavonoid (carvacrol)</td>
<td><em>Bacillus subtilis</em></td>
<td>0.25</td>
<td>0.25</td>
<td>CibuSiÇ and BeziÇ (2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Enterococcus faecalis</em></td>
<td>0.25</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Staphylococcus aureus</em></td>
<td>0.12</td>
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<td>0.06</td>
<td>0.06</td>
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<td></td>
<td></td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>&gt;6</td>
<td>&gt;6</td>
<td></td>
</tr>
<tr>
<td>Adiantum philippense crude extract</td>
<td>Flavonoids (quercitrin, quercetin, lagochilin, orientin)</td>
<td><em>Escherichia coli</em></td>
<td>0.062</td>
<td>0.2</td>
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</tr>
<tr>
<td></td>
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<td><em>Staphylococcus aureus</em></td>
<td>0.031</td>
<td>0.125</td>
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<tr>
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<td><em>Shigella flexneri</em></td>
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<td></td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>0.2</td>
<td>0.5</td>
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</tr>
<tr>
<td>Garlic extract</td>
<td>Flavonoid</td>
<td><em>Escherichia coli</em></td>
<td>100</td>
<td>200</td>
<td>Garba et al. (2013)</td>
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<tr>
<td></td>
<td></td>
<td><em>Staphylococcus aureus</em></td>
<td>200</td>
<td>300</td>
<td></td>
</tr>
<tr>
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<td></td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>200</td>
<td>300</td>
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</tr>
<tr>
<td>Alternanthera brasiliana</td>
<td>Flavonoid</td>
<td><em>Escherichia coli</em></td>
<td>0.625</td>
<td>1.25</td>
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<tr>
<td>Methanolic extract of <em>Piper (P.) sarmentosum</em></td>
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<td>MRSA</td>
<td>50</td>
<td>100</td>
<td>Fernandez et al. (2012)</td>
</tr>
<tr>
<td>Lemon oil (100%)</td>
<td>Flavonoid (catechin)</td>
<td><em>Staphylococcus aureus</em></td>
<td>3.125</td>
<td>&gt;25</td>
<td>Yazgana et al. (2019)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Klebsiella pneumonia</em></td>
<td>6.25</td>
<td>&gt;25</td>
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</tr>
<tr>
<td></td>
<td></td>
<td><em>Vibrio Vulnificus</em></td>
<td>25</td>
<td>&gt;25</td>
<td></td>
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</table>

(continued)
Table 13.4  (continued)

<table>
<thead>
<tr>
<th>Plant extracts</th>
<th>The types of phytochemicals</th>
<th>Target bacteria</th>
<th>MIC (mg/ml)</th>
<th>MBC (mg/ml)</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td>Proteus mirabilis</td>
<td>6.25</td>
<td>25</td>
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</tr>
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<td>Serratia</td>
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<td></td>
<td></td>
<td>Pseudomonas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oregano oil</td>
<td>Flavonoid (catechin)</td>
<td>Staphylococcus</td>
<td>50</td>
<td>100</td>
<td>Moghrovyan et al. (2020)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aureus</td>
<td>50</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacillus subtilis</td>
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<tr>
<td>Methanolic extr-</td>
<td>Flavonoid</td>
<td>Salmonella</td>
<td></td>
<td></td>
<td>Mahboubi et al. (2015)</td>
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<td>action of flowers of</td>
<td></td>
<td>Typhimurium</td>
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<td>Punica granatum</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>L. (Punicaceae)</td>
<td></td>
<td></td>
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</table>

Table 13.5  Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of some terpenoid phytochemicals against foodborne pathogens

<table>
<thead>
<tr>
<th>Plant extracts</th>
<th>The types of phytochemicals</th>
<th>Target bacteria</th>
<th>MIC (mg/ml)</th>
<th>MBC (mg/ml)</th>
<th>References</th>
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<tbody>
<tr>
<td>Aqueous extract of</td>
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<td>Staphylococcus</td>
<td>3.1</td>
<td>300</td>
<td>Suurbaar et al. (2017)</td>
</tr>
<tr>
<td>R. communis</td>
<td></td>
<td>aureus</td>
<td>3.1</td>
<td>200</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Pseudomonas</td>
<td>12.5</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>aeruginosa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Klebsiella</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pneumonia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol leaf</td>
<td>Terpenoid</td>
<td>Staphylococcus</td>
<td>12.5</td>
<td>25</td>
<td>Akthar et al. (2014)</td>
</tr>
<tr>
<td>extracts of</td>
<td></td>
<td>aureus</td>
<td>12.5</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>P. nigrum</td>
<td></td>
<td>Escherichia</td>
<td>12.5</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>coli O157</td>
<td></td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Bunium persicum</td>
<td>Terpenoid (Terpinene)</td>
<td>Staphylococcus</td>
<td>0.75</td>
<td>0.75</td>
<td>Oroojalian et al. (2010)</td>
</tr>
<tr>
<td>essential oils</td>
<td></td>
<td>aureus</td>
<td>0.18</td>
<td>0.18</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Bacillus cereus</td>
<td>0.75</td>
<td>0.75</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Listeria</td>
<td>1.50</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>monocytogenes</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Escherichia</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>coli O157</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cuminum Cyminum</td>
<td>Terpenoid (Terpinene)</td>
<td>Staphylococcus</td>
<td>0.75</td>
<td>1.50</td>
<td>Oroojalian et al. (2010)</td>
</tr>
<tr>
<td>essential oils</td>
<td></td>
<td>aureus</td>
<td>0.37</td>
<td>0.37</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Bacillus cereus</td>
<td>1.50</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Listeria</td>
<td>3.0</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>monocytogenes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Escherichia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>coli O157</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
administered with antibiotics. For example, a treatment combining piperine 1 with ciprofloxacin had a more potent inhibitory effect against *S. aureus* (Khan et al. 2006) and with gentamycin, a significant inhibitory effect against methicillin-resistant *S. aureus* (MRSA) (Khameneh et al. 2015).

Quinolone alkaloid has been reported to have the ability to inactivate microbial cells (Heeb et al. 2011). Dictamine (C₁₂H₉NO₂) and maculine (C₁₃H₉NO₄), quinolone alkaloids obtained from the stem of *Teclea azelii*, have been reported to exhibit antimicrobial activity with an MIC value of 19.53 μg/mL on *E. coli*, *Bacillus subtilis* and *Microsporum audorium* (Kuete et al. 2008). Alkyl methyl quinolones are also a quinoline alkaloid, which provides antimicrobial activity by reducing the oxygen consumption of bacterial cells so they act as a respiratory inhibitor (Tominaga et al. 2002).

### Table 13.5 (continued)

<table>
<thead>
<tr>
<th>Plant extracts</th>
<th>The types of phytochemicals</th>
<th>Target bacteria</th>
<th>MIC (mg/ml)</th>
<th>MBC (mg/ml)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Satureja Montana</td>
<td>Terpenoid (Terpinene)</td>
<td><em>Bacillus subtilis</em></td>
<td>0.25</td>
<td>0.25</td>
<td>CibuSiÇ and BeziÇ (2004)</td>
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<tr>
<td>essential oils</td>
<td></td>
<td><em>Enterococcus faecalis</em></td>
<td>0.25</td>
<td>0.25</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td><em>Staphylococcus aureus</em></td>
<td>0.12</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Escherichia coli</em></td>
<td>0.06</td>
<td>&gt;6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>0.25</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>&gt;6</td>
<td>&gt;6</td>
<td></td>
</tr>
<tr>
<td>Adiantum Philippense</td>
<td>Terpenoid compounds (ursolic acid, betulin, polygodial, and carvone)</td>
<td><em>Escherichia coli</em></td>
<td>0.062</td>
<td>0.031</td>
<td>Adnan et al. (2020)</td>
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<td>crude extract.</td>
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<td>0.2</td>
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<tr>
<td></td>
<td></td>
<td><em>Shigella flexneri</em></td>
<td>2</td>
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<td>0.5</td>
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<td>Garlic extract</td>
<td>Terpenoid</td>
<td><em>Escherichia coli</em></td>
<td>100</td>
<td>200</td>
<td>Garba et al. (2013)</td>
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<tr>
<td></td>
<td></td>
<td><em>Staphylococcus aureus</em></td>
<td>200</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>200</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Lemon oils (100%)</td>
<td>Terpenoid (β-pinene, Cymen)</td>
<td><em>Staphylococcus aureus</em></td>
<td>3.125</td>
<td>&gt;25</td>
<td>Yazgana et al. (2019)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Klebsiella pneumoniae</em></td>
<td>6.25</td>
<td>&gt;25</td>
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<tr>
<td></td>
<td></td>
<td><em>Vibrio vulnificus</em></td>
<td>25</td>
<td>&gt;25</td>
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<td><em>Serratia liquefaciens</em></td>
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<td><em>Pseudomonas</em></td>
<td>6.25</td>
<td>25</td>
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<tr>
<td>Sea weeds extract</td>
<td>Terpenoid</td>
<td><em>Escherichia coli</em></td>
<td>1.25</td>
<td>2.5</td>
<td>Sumayya et al. (2020)</td>
</tr>
</tbody>
</table>
Ungeremine is an iso-quinoline alkaloid, obtained by methanolic extraction from bulbs of *Pancratium illyricum* L. The antimicrobial action observed with ungeremine might be caused by damage to bacterial DNA by inhibiting the topoisomerase IA that is responsible for DNA supercoiling (Casu et al. 2011; Schrader et al. 2013).

Reserpine is an indole alkaloid which has a potent antimicrobial activity. Reserpine alone exhibited antimicrobial activity against *P. aeruginosa* with an MIC of 800 μg/mL (Parai et al. 2018). Combining reserpine with antibiotics has shown a synergistic inactivation effect on several bacterial species such as *Acinetobacter baumannii*, *Staphylococcus* spp., *Streptococcus* spp., and *Micrococcus* spp. (Jia et al. 2015; Sridevi et al. 2017). Brenwald et al. (1998) reported that 273 strains of *Streptococcus pneumoniae* were less susceptible to norfl oxacin or ciprofloxacin (MIC of norfl oxacin, > 8 μg/mL; MIC of ciprofloxacin, > 1 μg/mL), but in the presence of reserpine (10 μg/mL), 124 out of 273 isolates became more susceptible to norfl oxacin or four times or more to ciprofloxacin.

Sanguinarine, an alkaloid obtained from plants such as *Macleaya cordata* and *Chelidonium majus* has been reported to possess antimicrobial activity. Sanguinarine has been found to inhibit the growth of *S. aureus* and *E. coli* O157: H7 with an MIC of 1 and 4 μg/mL and an MBC of 64 and 16 μg/mL, respectively (Hamoud et al. 2016). The antimicrobial action of sanguinarine is caused by the release of autolytic enzymes which damage the bacterial cell walls (Obiang-Obounou et al. 2011).

Holarrhena antidysenterica is a steroidal alkaloid which can inhibit microbial growth (Siriyong et al. 2017). It exhibits a synergistic effect in combination with

![Fig. 13.1 Chemical structures of some selected alkaloids](image-url)

Ungeremine is an iso-quinoline alkaloid, obtained by methanolic extraction from bulbs of *Pancratium illyricum* L. The antimicrobial action observed with ungeremine might be caused by damage to bacterial DNA by inhibiting the topoisomerase IA that is responsible for DNA supercoiling (Casu et al. 2011; Schrader et al. 2013).

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Holarrhena antidysenterica is a steroidal alkaloid which can inhibit microbial growth (Siriyong et al. 2017). It exhibits a synergistic effect in combination with
antibiotics against A. baumannii (Zhou et al. 2017) and has exhibited efflux pump inhibitory activity against AdeIJK EP which has a vital role in pumping several antibiotics thus providing A. baumannii with antibiotic resistance (Siriyong et al. 2016).

Tomatidine is a steroidal alkaloid obtained from plants such as the tomato and potato (Friedman 2002). It has been reported that it exhibited antimicrobial activity against S. aureus ATCC 29213, Bacillus cereus ATCC 11778, and Listeria monocytogenes ATCC 13932 with an MIC >64 μg/mL (Guay et al. 2018). It also has a potent antimicrobial activity both alone or combined with aminoglycosides against foodborne pathogens, especially S. aureus (Mitchell et al. 2012).

13.3.2 Organosulfur Compounds

An organosulfur compound, an organic compound containing carbon–sulfur bonds, include examples such as sulforaphane, allicin, ajoene, s-allyl-mercapto cysteine (Fig. 13.2) (Sobolewska et al. 2015).

Allicin, a sulfur-containing compound obtained from garlic, exhibits antibacterial activity against MRSA, Streptococcus agalactiae and P. aeruginosa (Reiter et al. 2017). It also enhances the bactericidal effect of several antibiotics such as ciprofloxacin and tobramycin (Cai et al. 2008). Allicin inhibits sulfhydryl-dependent enzymes or the synthesis of DNA and protein, thus leading to microbial cell death (Davis 2005; Lanzotti et al. 2014).

Ajoene, another sulfur-containing compound, is extracted from garlic (Nakamoto et al. 2020). It has a greater antibacterial activity than allicin against Gram-negative and Gram-positive bacteria, fungi, and protozoa. The antimicrobial action of ajoene

![Chemical structures of some selected organosulfur compounds](image-url)
is similar to that of allicin as they can both inhibit the formation of biofilms and quorum sensing (QS) but ajoene also suppresses the thiol-dependent enzymes necessary for protecting cells against irreversible and toxic damage (Rehman and Mairaj 2013). Garlic ajoene extract exhibited antimicrobial activity against some foodborne pathogens such as Salmonella and S. aureus with MIC values of 0.125 to 1 μg/mL (El-Azzouny et al. 2018).

Sulforaphane, a natural compound originating from plants such as Diplotaxis harra, acts as a potent antimicrobial agent against Gram-positive pathogens such as S. aureus and L. monocytogenes (Benzekri et al. 2016). It has also exhibited a strong inhibitory effect against Salmonella Typhimurium with an inhibition zone of 25 mm (Abukhabta et al. 2021).

Allyl ITCs (AITCs) are sulfur-containing compounds of plant origin exhibiting antimicrobial activity against several pathogens (Lu et al. 2016), including a bactericidal effect against Gram-negative bacteria such as E. coli and Gram-positive bacteria such as S. aureus. They also enhanced the effect of erythromycin against Streptococcus pyogenes when combined (Palaniappan and Holley 2010). The antimicrobial activity of AITCs is caused by damage to the bacterial cell wall and internal structure (Luciano and Holley 2009; Nedorostova et al. 2009).

Phenethyl isothiocyanate, another sulfur-containing compound, is found in brassica vegetables (Jang et al. 2010). It exhibits antibacterial activity against both Gram-negative and Gram-positive bacteria (Aires et al. 2009). Phenethyl isothiocyanate also has potent antifungal activity against Alternaria brassicicola (Drobnica et al. 1968). Exposure of microbial cells to phenethyl isothiocyanate has been reported to decrease the oxygen consumption rate, intracellular accumulation of reactive oxygen species (ROS) and depolarization of the mitochondrial membrane (Calmes et al. 2015).

Isothiocyanates are organosulfur compounds obtained from the reaction between myrosinase enzyme and plant glucosinolates (Park et al. 2013). Isothiocyanates exhibited bactericidal activity against Helicobacter pylori by decreasing the urease activity necessary for adapting to undesirable conditions (Fahey et al. 2013) and a potent antimicrobial effect against Clostridia with an MIC of 1.25 to 5 μg/mL (Dufour et al. 2012).

### 13.3.3 Phenolic Compounds

Phenolic compounds, a group of phytochemicals containing hydroxyl groups bonded directly to an aromatic hydrocarbon group, include phytosterols, hydroxyphenylacetic acids, flavonones, anthocyanins, isoflavones, resveratrol, baicalein, kaempferol, quercetin, and epigallocatechin gallate (EGCG) (Fig. 13.3) (Górniai et al. 2019). Several studies have investigated the effect of phenolic compounds incorporated into food packaging materials (Table 13.6).

Resveratrol is a natural phenolic compound that provides antimicrobial activity by enhancing EPI against Campylobacter jejuni and Mycobacterium smegmatis (Lechner et al. 2008; Klancnik et al. 2017). It has been reported to exhibit...
Table 13.6 Examples of phenolic compounds for potential use in food packaging materials

<table>
<thead>
<tr>
<th>Sources of phenolic compounds</th>
<th>Type of food</th>
<th>Major results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley husks</td>
<td>Salmon</td>
<td>Increase the oxidative stability</td>
<td>De Abreus et al. (2010)</td>
</tr>
<tr>
<td>Rosemary extract</td>
<td>Beef</td>
<td>Increase the oxidative stability of beef</td>
<td>Barbosa-Pereira et al. (2014)</td>
</tr>
<tr>
<td>Green tea</td>
<td>Pork sausage</td>
<td>Decrease bacterial and mold counts by 4.1 and 2.5 log CFU/mL during 12 days of storage, respectively</td>
<td>Siripatrawan and Noipha (2012)</td>
</tr>
<tr>
<td>Lemongrass oil, clove oil, ginger oil</td>
<td>Minced meat</td>
<td>Antimicrobial activity (1.3 log CFU/mL reductions in total bacterial count)</td>
<td>Barbosa et al. (2009)</td>
</tr>
<tr>
<td>Oregano</td>
<td>Lamb steaks</td>
<td>Extend aroma from 8 to 13 days</td>
<td>Camo et al. (2011)</td>
</tr>
<tr>
<td>Grapefruit seed extract</td>
<td>Salmon</td>
<td>Decrease <em>L. monocytogenes</em> counts by 0.5 logCFU/g within 15 days of storage</td>
<td>Hye et al. (2012)</td>
</tr>
</tbody>
</table>

Fig. 13.3 Chemical structures of some selected phenolic compounds
antimicrobial activity against *B. cereus* (MIC = 50 μg/mL), *H. pylori* (MIC = 25 to 50 μg/mL), and *Enterococcus faecalis* (MIC = 100 to 200 μg/mL) (Abedini et al. 2021).

Baicalein, another phenolic compound, is obtained from the roots of plants such as *Thymus vulgaris* and *Scutellaria lateriflora* (Chen et al. 2013). Bazzaz et al. (2011) reported that the antimicrobial activity of baicalein against *E. coli*, *S. aureus*, and *B. cereus* led to MIC values of 25, 6.25, and 25 and MBC values of 50, 6.25, and 25 (mg/mL), respectively. Baicalein has been reported to inhibit NorA, which allows microorganisms to regulate their internal environment by removing toxic substances, including antimicrobial agents (Chan et al. 2011), and also to enhance the effectiveness of tetracycline against *E. coli* by inhibiting EP (Fujita et al. 2005).

Kaempferol is a phenolic compound recommended for use against resistant pathogens such as fluconazole-resistant *C. albicans* and MRSA (Randhawa et al. 2016; Shao et al. 2016). The antimicrobial activity of kaempferol against MRSA might be due to it inactivating the NorA pumps with the combined effect of kaempferol and ciprofloxacin increasing the inactivation rate of MRSA by eightfold compared with ciprofloxacin treatment alone (Holler et al. 2012). Kaempferol has also been reported to exhibit antimicrobial activity against *S. aureus*, *P. aeruginosa*, and *S. Typhimurium* with MIC values of 256, 512, and 64 μg/mL and MBC values of 256, 512, and 128 μg/mL, respectively (Tatsimo et al. 2012).

A methanolic extract of grape (*Vitis vinifera* L.) pomace showed a significant inhibitory effect against MRSA with an MIC between 0.3 and 3 mg/mL (Sanhueza et al. 2017). Ethanolic extracts of blueberry pomace (*Vaccinium corymbosum* L.) and blackberry (*Rubus fruticosus* L.) exhibited a high level of inactivation against *C. jejuni* with an MIC of 0.4 mg/mL (Lima et al. 2019). A methanolic extract of pomegranate (*Punica granatum* L.) peel (10–100 mg/mL) was found to inhibit the growth of the pathogens, *Listeria innocua*, and *S. aureus*, with MIC values of 20 and 30 mg/mL and MBC values of 50 and 60 mg/mL, respectively (Tatsimo et al. 2012).

Clove and thyme can inhibit the growth of *L. monocytogenes* in cheese by 99% (Yousef et al. 2020). Menon and Garg (2001) found that a 1% clove oil could reduce the count of *Listeria* by 3 log10 CFU/g cycles compared with the control samples. Rosemary essential oils at a concentration of 0.5% completely inhibited the growth of *Arcobacter butzleri* in cooked minced beef stored at 4 °C (Irkin et al. 2011). Tea tree essential oil from *Melaleuca alternifolia* at a concentration of 1.5% exhibited an antilisterial effect on artificially contaminated minced beef (Silva et al. 2019).

EGCG is another phenolic compound that has a potent antibacterial effect against multidrug-resistant bacteria and also has efflux inhibitory activity (Gibbons et al. 2004). It has also been found to inhibit the growth of *P. aeruginosa* and *E. coli* with MIC levels of 200 and 400 μg/mL, respectively (Jeon et al. 2014).

Curcumin, a polyphenolic compound obtained from turmeric, has been reported to exhibit bacteriostatic and bactericidal effects against *E. coli* and *S. aureus* (Tyagi et al. 2015). The MIC for curcumin against *E. coli*, *Yersinia enterocolitica*, *S. aureus*, and *B. subtilis* has been reported as 250, 250, 62.5, and 12.5 μg/mL, respectively (Wang et al. 2009). Quercetin and apigenin are two flavonoid
compounds, which can inhibit the d-alanine:d-alanine ligase of *E. coli*, essential enzymes for synthesizing the cell walls of bacteria, leading to bacterial death (Wu et al. 2008).

The mechanisms of the antimicrobial action of phenolic compounds are not only based on inhibiting the efflux pump but also on inactivating the DNA gyrase (Anderle et al. 2008; Patel et al. 2015). Several natural phenolic compounds such as tannins, chebulinic acid, and anthraquinones have been reported to inhibit DNA gyrase (Gradisar et al. 2007; Duan et al. 2014; Patel et al. 2015). Phenolic compounds can also interact with some vital enzymes that are responsible for the integrity of bacterial cell membrane such as KAS II and III (beta-ketoacyl acyl carrier protein synthase) or enzymes that are responsible for fatty acid elongation such as FabG, FabI, and FabZ (Donadio et al. 2021). For example, one study has demonstrated the effect of flavanones such as eriodictyol and taxifolin on *E. faecalis*, revealing a significant inhibitory effect through the inactivation of KAS III (Jeong et al. 2009).

Another microbial inactivation mechanism provided by phenolic compounds is the inhibition of dihydrofolate reductase, sortase, and urease. For example, dihydrofolate reductase was inactivated thus inhibiting the growth of *Stenotrophomonas maltophilia* when exposed to EGCG at concentrations between 4 and 256 μg/mL for 24 h (Navarro-Martínez et al. 2005), with a concentration of 128 μg/mL causing a reduction in the count of almost 6 log₁₀ CFU/mL cycles. Turmeric, *Curcuma longa*, had a potent inhibitory effect against sortase A and also exhibited a bactericidal effect against *S. aureus* (Park et al. 2005). Trihydroxyl-isoflavene has been reported to inactivate urease thus reducing the viability of *H. pylori* (Xiao et al. 2013).

### 13.3.4 Terpenes and Terpenoids

Terpenes belong to the largest class of secondary metabolites with a basic structure consisting of five carbon isoprene units connected together, while terpenoids are a modified class of terpenes with different functional groups and an oxidized methyl group moved or removed at different positions (Perveen 2018). Terpenes, the main constituents of essential oils, are derived from the isoprenoid pathway, and produced from plant tissues (Perveen 2018). They are composed of isoprene units (C5), the basis for their classification, i.e., two isoprene units form monoterpenes (C10), three units form sesquiterpenes (C15), four units form diterpenes (C20), six units form triterpenes (C30), and eight units form carotenoids (C40) (IUPAC 2019).

Terpene standards containing borneol, camphor, carvacrol, cymene, citral, and eugenol have been examined to determine their antimicrobial activity, showing MIC values against *B. cereus* of 0.12, 0.25, 0.03, 0.25, 0.06, and 0.07 mg/mL and against *S. Typhimurium* of 0.12, 0.25, 0.015, 0.25, 0.07, and 0.17 mg/mL, respectively (Guimarães et al. 2019). Terpenoids extracted from *Annona muricata* (soursop) seeds exhibited antimicrobial activity against *S. aureus*, *P. aeruginosa*, and *E. coli*
with inhibition zones of 11, 13, and 14 mm, respectively (Abdel-Rahman et al. 2019) (Fig. 13.4).

### 13.3.5 Saponins

Saponins are compounds with a rigid skeleton of at least 4 hydrocarbon rings to which sugars are attached and can be subdivided into triterpenoid and steroid glycosides (Sparg et al. 2004). These high molecular weight glycosides consist of saccharide chain units (1 to 8 residues) linked to a triterpene (triterpene saponins) or to a steroid (steroid saponins) (Hostettmann and Marston 1995). The chemical structure of saponins may change during storage or processing because the links between the sugar chain and the aglycones as well as those between the individual sugars can be hydrolyzed during acid or heat treatment (Abed El Aziz et al. 2019).

Saponins have been reported to be present in more than 100 plant families, being found in dicotyledonous plants such as the seeds of hippocastani, the roots and flowers of primulae, the leaves of hedrae, and the roots of ginseng. Legumes such as soybeans, beans, and peas are very rich in triterpenoid saponins but cereals and grasses have a limited saponin content, except for oats (Moses et al. 2014). Water, methanol, and ethanol can be used as aqueous and alcoholic surfactants for extracting saponins as well as the Soxhlet method. Other methods, such as pressure, microwave, and ultrasound, can also be used to increase the efficiency of extraction (Doughari 2012).

Guerrero et al. (2016) found that essential oils from the peel of mango (Mangifera indica) containing saponin exhibited an inhibitory effect against *B. subtilis, S. aureus, E. coli*, and *P. aeruginosa*. A saponin fraction from the stem of the Mojave yucca (*Y. schidigera*) has also been reported to exhibit a potent inhibitory effect against food damaging yeasts with MIC values ranging from 31.3 to 125 μg/mL (Miyakoshi et al. 2000).

In general, the inactivation mechanism of saponins occurs because of their interaction with the lipid A part of lipopolysaccharides which increases the permeability of the bacterial cell wall. These lipid A-saponin complexes might then increase the uptake of antibiotics such as colistin and ampicillin into the cells of inherently resistant bacteria (Arabski et al. 2012) (Fig. 13.5).
13.3.6 Anthranoids

Anthranoid compounds are widely distributed in many plants, especially Aloe, Cassia, Rheum, and Frangula (Paneitz and Westendorf 1999). Anthranoids have often been reported to exhibit antimicrobial activity. Wu et al. (2006) reported that the inhibitory effect of three anthraquinones, rhein, emodin, and 1,8-dihydroxyanthraquinone, on S. aureus ranged, in order, from high to low. Wang et al. (2010) determined that the sequence of antimicrobial activity against Bifidobacterium adolescentis of five hydroxyanthraquinones extracted from rhubarb from high to low was rhein, emodin, aloe-emodin, chrysophanol, and physcion. Laure et al. (2007) examined the antimicrobial activity of the bark of cyclicodiscus gabunensis, which contains anthranoids, against the foodborne pathogens P. aeruginosa, S. aureus, E. coli, and Shigella flexneri, reporting MIC values of 0.10, 0.00078, 0.0031, and 3.2 mg/mL and MBC values of 3.2, 0.0031, 0.25, and 6.4 mg/mL, respectively (Fig. 13.6).

Fig. 13.5 General chemical structures of saponins

Fig. 13.6 General chemical structures of anthraquinone
13.4 Major Factors Affecting the Activity of Phytochemicals

13.4.1 pH

One important factor associated with the antimicrobial activity of phytochemicals is pH. Salama and Marraiki (2010) examined the antimicrobial activity of Polygonum aviculare L. (Polygonaceae), which grows naturally in Egypt, against E. coli, S. Typhimurium, and S. aureus at pH values ranging from 2.5 to 10. The diameter of the inhibition zone of a chloroform extract (100 mg/mL) for E. coli, S. Typhimurium, and S. aureus was 15 mm or less at a pH greater than 7 but more than 25 mm at an acidic pH between 2 and 6. Gupta et al. (2014) evaluated the antimicrobial activity of Solanum sisymbriifolium against B. subtilis, reporting inhibition zones for the hexane and chloroform extracts at a pH of 3, 6, and 9 as 9.08, 9.12, and 8 mm, respectively.

Arora and Sood (2017) investigated the antimicrobial activity of Gymnema sylvestre leaves against several foodborne pathogens, reporting inhibition zones at a pH of 3, 5, and 9 of between 14 and 23 mm with the best antimicrobial effect of 23 mm at pH 5 because of the stability of phytochemicals at a slightly acidic pH, while at the alkaline pH of 9, the inhibition zones for S. Typhimurium and E. coli were 14.3 and 19.6 mm, respectively.

Abd El-Monem et al. (2018) examined the effect of pH on the antimicrobial activity of Spirulina platensis, a planktonic filamentous cyanobacterium, against S. aureus NCTC-7447, reporting a maximum inhibition zone of 20 mm at a pH of 8.0 and 10, while at pH 8.0 it exhibited the maximum inhibition zone of 17 mm against E. coli NCTC-10418. This was attributed to the greatest content of total phenolic compounds and flavonoids of spirulina platensis being obtained at a pH of 8.0 and 10.0. In contrast, Chakraborty et al. (2015) showed that the maximum inhibition zone from a water extract of Spirulina platensis was 19 and 18 mm for S. aureus and E. coli, respectively.

13.4.2 Characteristics of Pathogens

Phytochemicals have a slightly stronger inactivation effect on Gram-positive than on Gram-negative bacteria (Kotzekidou et al. 2008; Klančnik et al. 2011). This is caused by a structural difference in the cell walls of these bacteria. Gram-negative bacteria possess an outer membrane which acts as a barrier for many antimicrobial substances including some natural agents (Briers and Lavigne 2015). This explanation is also supported by Silva et al. (2016) who evaluated the antimicrobial activity of Cleome spinosa Jacq. (Cleomaceae) against 17 species of bacteria and molds. The results showed that the MIC and MBC values of Gram-positive bacteria were lower than those of Gram-negative bacteria. For example, the MBC values using the
ethanolic extract of the leaves and roots of *Cleome spinosa* Jacq. against *B. subtilis* and *E. coli* were 6.25 and 12.5 mg/mL, respectively.

Unlu et al. (2010) reported that cinnamon oils caused a higher reduction in Gram-positive than in Gram-negative bacteria, with inhibition zones for *S. aureus* and *Salmonella* of >40 and 25 mm, respectively.

Verma et al. (2018) found that an ethanolic extract of *Jasminum mesnyi* had a greater antimicrobial effect on various Gram-positive bacteria than on Gram-negative bacteria, with an MIC against *S. aureus* ranging from 10.5 to 15 mm while there was no inhibitory effect on *E. coli*.

Stobnicka and Gniewosz (2018) evaluated the antimicrobial activity of swamp cranberry (*Vaccinium oxyccos* L.) fruit and pomace extracts on minced pork meat artificially contaminated with Gram-positive *L. monocytogenes* and Gram-negative bacteria such as *Salmonella* Enteritidis and *E. coli*. Both types of extract had a stronger inhibitory effect against Gram-positive bacteria (5.5 log_{10} CFU/g reductions) than Gram-negative *S. Enteritidis* (4 log_{10} CFU/g reductions) after 48 h of exposure.

### 13.4.3 Concentrations of Phytochemicals

Several studies have reported the effects of the concentrations of phytochemicals on their antimicrobial activity against foodborne pathogens. Selim (2011) evaluated the inhibitory effects of natural oils containing thyme at different concentrations (0.5 and 1%) on beef and cheese artificially inoculated with *E. coli* O157:H7 and bancomycin-resistant enterococci. The thyme oils at a concentration of 0.5% significantly reduced the bacterial counts (4.5 log_{10} CFU/mL cycles) in both cheese and beef, while thyme oils at a concentration of 1% completely inactivated these pathogenic bacteria within 7 d of storage. Stobnicka and Gniewosz (2018) evaluated the antimicrobial activity of swamp cranberry (*Vaccinium oxyccos* L.) fruit and pomace extracts at a concentration of 2.5% in minced pork meat artificially contaminated with *L. monocytogenes*, *S. Enteritidis*, and *E. coli*, showing 4 log_{10} CFU/g reductions in pathogenic bacteria after 4 d of storage at 4 °C. Raeisi et al. (2019) examined the effect of *Mentha piperita* essential oil at various concentrations (0.3, 0.5, 1, and 2% v/w) on artificially contaminated beef meat stored at refrigeration temperatures for one week. The results showed that the *Mentha piperita* essential oil at the highest concentration (2%) caused a 2 and 3 log_{10} CFU/g reduction in the counts of *L. monocytogenes* and *S. Typhimurium*, respectively, with concentrations of 0.3, 0.5, and 1% leading to 1.1, 1.4, 1.8, and 0.8, 1.3, 2 log_{10} CFU/g reductions in the counts of *L. monocytogenes* and *S. Typhimurium*, respectively. Márquez-Rodríguez et al. (2020) investigated the antibacterial activity of a phenolic extract of *Hibiscus sabdariffa* L. at various concentrations (250, 500, 750, 1000, and 1250 mg/L) against contaminated beef. The counts of psychrotrophic bacteria decreased by 1, 2, and 4 log_{10} CFU/mL at extract concentrations of 250, 500, and 750 mg/mL, respectively. Mahros et al. (2021) reported on the inhibitory effect of black seed oils (BSO) on *E. coli* O157:H7 in ground mutton stored at refrigeration
temperature (4 °C) for 21 d, with concentrations of 1, 2, and 3% BSO causing reductions of 0.1, 0.5, and 0.9 log10 CFU/g in the counts of *E. coli* O157:H7, respectively.

### 13.5 Mechanisms Underlying the Antimicrobial Actions of Phytochemicals

#### 13.5.1 Membrane Disruption

The bacterial cell membrane is a vital structure for osmoregulation, the transportation process and respiration, so any disruption in membrane integrity leads to bacterial death (Hartmann et al. 2010). Phytochemicals have been shown to exhibit antimicrobial activity because of the interaction of phytochemicals with lipid bilayers. This interaction involves the association of the non-polar partition of compounds in the hydrophobic interior of the cell membrane with the formation of hydrogen bonds between the polar head groups of lipids and the hydrophilic flavonoids at the membrane interface (Tsuchiya 2015). The interaction of phytochemicals with phospholipids can induce structural changes and disturbances in the bacterial cell membranes (Arora et al. 2000). Catechin has been reported to exhibit an antibacterial effect by rupturing the bacterial cell wall through binding to the lipid bilayers and inhibiting intracellular enzymes (Reygaert 2014). Ollila et al. (2002) found that the main antimicrobial activity of the flavones, acacetin and apigenin, and the flavonols, morin, and rhamnetin, could be attributed to the destabilization of the bacterial membrane structure, leading to undesirable changes in the membrane fatty acids. Sanver et al. (2016) reported that the flavonols, quercetin rutin (quercetin-3-O-rhamnoglucoside), and tiliroside, reduced the thickness of lipid bilayers so disrupting the lipid monolayer structures. Fathima and Rao (2016) have reported that bacterial death caused by some antimicrobial agents such as catechin and EGCG was due to the generation of reactive oxygen species (ROS) altering the membrane permeability.

#### 13.5.2 Efflux Pump Inhibition

Efflux pumps (EPs) are important for pathogens to develop resistance against antimicrobial agents. Pinostrobin has been reported to inhibit pathogenic bacteria such as *E. coli* and *S. aureus* by damaging EPs and disrupting the formation of biofilm (Christena et al. 2015). EGCG has been found to inhibit bacterial growth and the expression of specific virulence genes and also to inhibit biofilm formations (Lee and Tan 2015).
Nanmeni et al. (2021) reported that an ethanolic extract of *Plectranthus glandulosus* exhibited no antimicrobial activity against pathogens such as *E. coli* and *P. aeruginosa* but when combined with the efflux pump inhibitor, phenylalanine-arginine-β-naphthylamide (PAβN), it exhibited an inhibitory effect against *E. coli* and *P. aeruginosa* with MIC values of <8 and 265 μg/mL, respectively. This inhibitory effect could be attributed to PAβN which blocked the efflux pumps thus increasing the intracellular concentration of the active principle of *P. glandulosus*.

### 13.5.3 Inhibition of Cell Envelope Synthesis

Several phytochemicals can inhibit the synthesis of the cell envelope. Quercetin has been found to exhibit an inhibitory activity against the synthesis of the bacterial-type II fatty acid necessary for generating the fatty acid components of phospholipids in *H. pylori* (Zhang et al. 2008). The hydrogen bonds between the flavonoid hydroxy groups at positions C-4 and C-5 of the B ring and the KAS III enzyme (B-ketoacyl acyl carrier protein synthase) amino acid residues Arg38 and Phe308 have been reported as the key to the antimicrobial activity of flavonoids (Jeong et al. 2009). Elmasri et al. (2017) have reported that pentahydroxy flavone and dimethoxyflavone inactivated the enzymes responsible for type II fatty acid synthesis (FAS-II). Zhang and Rock (2004) found that EGCG from green tea inhibited the specific reductase enzymes responsible for FAS-II.

Peptidoglycan is a vital component of the bacterial cell wall so inhibiting its synthesis has also been reported as a target for the action of phytochemicals (Eumkeb et al. 2012). The flavonol, galangin, has been reported to exhibit antimicrobial activity against *E. coli* by inhibiting the synthesis of peptidoglycan and ribosomes (Eumkeb et al. 2012). Catechins can also interfere with bacterial growth by binding with the peptidoglycan layer (Zhao et al. 2001).

### 13.5.4 Inhibition of Nucleic Acid Synthesis

DNA gyrase is a vital enzyme for replicating DNA so is a target for most phytochemicals (Plaper et al. 2003). Ohemeng et al. (1993) have reported that the DNA gyrase of *E. coli* can be inhibited by apigenin. Plaper et al. (2003) also showed that quercetin linked to the B subunit of gyrase, thus hindering DNA replication in *E. coli* and preventing its growth and multiplication. Fang et al. (2016) used molecular docking simulation and reported that flavonoids inhibited nucleic acid synthesis by interacting with the ATP (adenosine triphosphate) binding site of the DNA gyrase B subunit.
Helicases are responsible for separating and/or rearranging nucleic acid duplexes in reactions fueled by ATP hydrolysis (Shadrick et al. 2013). One flavone, luteolin, has been reported to inhibit the replication of helicases in *E. coli* (Xu et al. 2001).

Another mechanism underlying the antimicrobial activity of phytochemicals related to nucleic acid is through phytochemical-DNA intercalation. Mori et al. (1987) showed that EGCG inhibited the synthesis of RNA and DNA in *S. aureus* through the direct interaction of phytochemicals with nucleic acids.

### 13.5.5 Inhibition of Electron Transport Chain and ATP Synthesis

Phytochemicals have been reported to be efficient antimicrobial agents against *E. coli* by inhibiting ATPase (Chinnam et al. 2010). ATP synthase is a highly conserved enzyme with two sectors, F1 and FO. ATP hydrolysis and synthesis have been reported to occur in the F1 sector, and proton movement in the FO sector (Senior et al. 2002). A wide range of phytochemicals can bind with F1 and inhibit ATP synthesis (Gledhill et al. 2007). Baicalein and morin have shown inhibitory activity against *E. coli* ATPase (Chinnam et al. 2010). Ulrey et al. (2014) reported that A-type proanthocyanidins exhibited inactivation activity against *P. aeruginosa* through inhibiting ATP synthesis (Fig. 13.7).

Phytochemicals can kill or inhibit bacterial cells by several ways, such as causing membrane disruption (1) and inhibition of nucleic acid synthesis (2a— inhibition of dihydrofolate reductase (DHFR), 2b— helicase inhibition, 2c— gyrase/topoisomerase inhibition), as well as inhibit bacterial virulence as quorum sensing, which impairs their ability to form biofilms (4). Antimicrobial action can be also executed through inhibition of cell envelope synthesis, which involves inhibition fatty acid synthase (FAS—5) and peptidoglycan synthesis (7a— inhibition of Ala-Ala dipeptide synthesis, 7b— inhibition of peptidoglycan cross-linking). Inhibit efflux pumps as well, which can lead to reversing antimicrobial resistance (6). Moreover inhibition of NADH-cytochrome c reductase activity in the bacterial respiratory chain (8) and inhibition of ATP synthase (9). This figure is reprinted from Górniak et al. (2019), licensed under CC BY.

### 13.6 The Regulatory Response of Microbial Cells to Phytochemicals

Several studies have investigated the microbial response to phytochemical treatment. For example, Ren et al. (2019) studied the effect of pterostilbene derived from the Xinjiang wine grape on the growth of *E. coli* and *S. aureus*. Confocal laser scanning microscopy was used to study the effect of pterostilbene on the total DNA content of
Fig. 13.7 Mechanisms of antimicrobial effect of flavonoids against pathogens growth
the bacterial cells using the fluorescent dye, DRAQ5, with the total DNA decreasing after pterostilbene treatment. The expression of genes associated with antimicrobial activity (tocL, dinF, pal, mtgA, and nagA) was also analyzed using RT-qPCR. The results showed that pterostilbene upregulated dinF, an oxidative stress gene, due to exposing the bacterial cells to oxidative stress and downregulated pal, MtgA, and NagA, genes involved in cell wall synthesis. The tocL gene, associated with bacterial virulence, was revealed by this study to be upregulated by pterostilbene treatment.

Muthaiyan et al. (2011) studied the effect of terpeneless cold-pressed Valencia orange essential oil (CPV) on the expression of genes related to cell lysis in MRSA. The results showed that in S. aureus treated with 0.1% CPV 431 genes were upregulated and 551 downregulated. The transcriptional profiling analysis showed that about 62 and 36 genes related to the cell envelope were under- and overexpressed, respectively. Some cell wall stress genes such as penicillin-binding protein PBP1, PBPbp2 (mecA), and peptidoglycan biosynthesis-related genes such as murB, murD, murG, murC, and murE were also downregulated. The two-component response regulators encoding genes vraS, vraR, arlS, and arlR and the transcriptional regulator, tcaR, and the staphylococcal accessory regulator encoding genes sarA, sarV, sarY were upregulated by between two- and fivefold. Genes associated with cell division and stress resistance in S. aureus were downregulated by CPV treatment which indicated that CPV had acted on the membrane of S. aureus.

Visvalingam et al. (2013) conducted a whole-genome transcriptomic profiling of E. coli O157:H7 after exposure to cinnamaldehyde. The results showed that cinnamaldehyde significantly reduced the expression of genes responsible for DNA replication, protein synthesis (including RNA synthesis and 50S and 30S ribosomal protein synthesis), o-antigen synthesis and fimbrial synthesis. At 2 h, the highest expression (6.1-fold) was observed after 2 h in the gene encoding alcohol dehydrogenase, yqhD, which was followed by a fivefold increase in the gene encoding 2,5-diketo-D-gluconate reductase A, dkgA. However, after 4 h, there was no difference in expression between these two genes. The genes encoding the cold shock proteins, cspH and cspG, were downregulated by more than 2.4-fold at 2 h then upregulated by more than sixfold at 4 h. The expression of genes involved in glutamate-dependent acid resistance and acid-resistant proteins were downregulated by more than 3.5-fold at 2 h but there was no difference in expression at 4 h.

Chueca et al. (2017) investigated the transcriptional response of E. coli MG1655 exposed to the oxygenated monoterpenes, citral, and carvacrol. This showed that carvacrol treatment (100 ppm for 10 min) resulted in 76 genes being significantly expressed (p ≤ 0.05). A total of 61 and 15 genes were upregulated and downregulated after carvacrol treatment, respectively. While the E. coli cells treated with citral exhibited 27 upregulated genes, the genes coding for efflux pumps (acrA and mdtM), the biosynthesis of arginine (argC, argG, and artJ), and those related to the phage shock response (pspA, pspB, pspC, pspD, pspF, and pspG) were upregulated in E. coli cells treated with carvacrol alone.
13.7 Conclusions

Foodborne pathogens have an adverse impact on human health and the economy, especially in low-income countries with poor healthcare and sanitation facilities. The widespread presence of antibiotic-resistant bacteria is causing a global health crisis so controlling such bacteria is a matter of urgency. The interaction between the use of chemical food preservatives for controlling the growth of pathogens and extending the shelf-life of foods and consumers has been controversial because of their mistrust of the use of food additives. Finding natural, effective and safe substances as a method of controlling the growth of pathogens is therefore essential. Phytochemicals such as epigallocatechin, kaempferol, apigenin, and quercetin have been shown to effectively inhibit the growth of foodborne pathogens. Some phytochemicals have also exert a synergistic and maximized effect when combined with antibiotics for controlling the growth of pathogens where the effectiveness of antibiotics is limited. For practical application in foods, making use of the inhibitory effect of phytochemicals against pathogens is a promising method for providing safe foods for human consumption and also effective barriers against economic problems caused by the poor management of food safety.

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Part IV
Stress Response Mechanisms of Foodborne Pathogens
Abstract  Viable but nonculturable (VBN C) refers to a dormant state that bacteria tend to enter under unfavorable environments. VBN C bacteria still maintain metabolic activity but cannot grow or proliferate in medium. VBN C bacteria are different from regular cells in morphological, physiological, and genetic properties. Additionally, VBN C bacteria can be resuscitated and regain culturability under suitable conditions. The presence of VBN C bacteria in foods or food processing environments is difficult to detect by conventional culture-based methods and they could pose a huge potential threat to public health. This chapter systematically discusses cutting-edge research on VBN C bacteria, including the induction of VBN C bacteria in the food industry, the formation mechanism of VBN C bacteria, culture-independent detection techniques, the resuscitation of VBN C bacteria, and the corresponding mechanisms and pathogenic potential of bacteria in the VBN C state.

Keywords  Viable but nonculturable · Foodborne pathogens · Characteristics · Diagnostics · Mechanisms · Resuscitation · Virulence
14.1 Introduction

Microbial food contamination can occur in every phase of the food production chain, including raw food harvest, processing, distribution, and marketing, representing a major type of food safety risk (Begley and Hill 2015). Consumption of contaminated food is the primary cause of foodborne diseases worldwide (WHO 2015). Bacillus cereus, Enterohaemorrhagic Escherichia coli, Listeria monocytogenes, Salmonella, Campylobacter, Clostridium botulinum, and Staphylococcus aureus are recognized as common pathogenic bacteria associated with food contamination (Khaneghah et al. 2020). What makes them even more dangerous is that these pathogens can survive unfavorable environments, such as extreme temperature, high osmotic pressure, and antimicrobial treatment, by entering a nonculturable state, the viable but nonculturable (VBNC) state. VBNC bacteria are metabolically active and are believed to regain culturability and pathogenicity through resuscitation under suitable conditions. Conventional culture-based methods cannot detect VBNC cells, as they lose culturability (Gao et al. 2021). The presence of VBNC pathogens in the food production chain is a challenge to food safety and public health.

In the 1970s, Vibrio cholerae and other Vibrio strains were isolated from the ocean and estuary in the summer when the water temperature was high. However, these bacterial strains could not be detected in the ocean and estuary when the water temperature dropped below 10 °C in winter. Researchers could not give any reasonable explanation for this phenomenon. In 1982, Xu et al. (1982) found that E. coli and V. cholerae entered a dormant state in cold seawater and proposed the concept of viable but nonculturable (VBNC) for the first time. VBNC explains the well-known periodic occurrence of cholera. Currently, additional species have been reported to enter a VBNC state, including Campylobacter jejuni, S. aureus, L. monocytogenes, Enterococcus faecalis, and Salmonella (Oliver 2005).

To date, only limited outbreaks of foodborne diseases have been associated with VBNC pathogens. Makino et al. (2000) reported the first VBNC-associated outbreak due to the consumption of salted salmon roe in Japan in 1998. They found that enterohemorrhagic E. coli O157 was the cause of the outbreak; however, the bacterial loads in the salted salmon roe measured by the most probable number (MPN) were far less than the infectious dose. The authors hypothesized that EHEC O157 might enter a VBNC state during salting of salmon roe, which resulted in an underestimation of the degree of contamination. In 2011, an outbreak related to E. coli O104:H4 contamination on sprouts occurred in Germany, leading to more than 3000 people developing bloody diarrhea and over 700 cases of hemolytic uremic syndrome. Researchers confirmed that E. coli O104:H4 was the cause of the infection by positive cultures from the feces of the patients; however, the culture-based detection of the suspected sprouts was negative (Aurass et al. 2011; Scheutz et al. 2011). Polymerase chain reaction (PCR) confirmed the existence of E. coli O104:H4 in the sprouts. Hence, E. coli O104:H4 most likely remained in a VBNC state on the sprouts, regained culturability after consumption, and subsequently caused disease. We should pay more attention to the occurrence of VBNC bacteria,
especially pathogenic bacteria, due to their potential risk to food safety and public health.

In this chapter, the characteristics of VBNC bacteria, the formation of VBNC pathogens in the food industry, the mechanisms underlying the entrance of pathogens into the VBNC state, the diagnostics of VBNC bacteria and the resistance and virulence properties of VBNC pathogens are introduced in detail.

14.2 The Characteristics of Viable But Nonculturable Bacteria

Bacteria in the VBNC state are significantly different from their culturable counterparts. Morphological changes in VBNC cells are one of the most obvious signs. In most cases, the cell morphology is transformed from a rod shape into a spherical shape when the bacteria enter the VBNC state (Citterio et al. 2004; Gupte et al. 2003; Lázaro et al. 1999; Su et al. 2013; Vattakaven et al. 2006). For example, Gupte et al. (2003) used scanning electron microscopy (SEM) to characterize the morphological changes of VBNC S. Typhimurium cells transitioning from rods into spherical shapes. Citterio et al. (2004) observed the dynamic cell shape change of Helicobacter pylori from spiral to U-shaped within 5 to 8 days and then to V-shaped at 9 to 11 days during the state transition of VBNC. Su et al. (2013) showed that V. parahaemolyticus decomposed its original cell wall and formed a new thin layer cell wall when entering the VBNC state (Fig. 14.1). In addition, for some V. parahaemolyticus cells with a lower cytoplasmic density, the formation of cell buds with an empty cytoplasm (Fig. 14.1iv) and others with a cytoplasm (Fig. 14.1v) was observed, as indicated by the arrows. The authors hypothesized that the aberrant shapes of some V. parahaemolyticus were the result of the expansion of cell walls during the nascent cell wall synthesis process. However, not all bacteria shrink in the VBNC state. Signoretto et al. (2000) reported that Streptococcus faecalis slightly elongated itself when entering the VBNC state. C. jejuni induced by low temperature to enter VBNC was reported to maintain its normal cell morphology (Lázaro et al. 1999).

The cellular components of bacteria are affected during the VBNC transition. Linder and Oliver (1989) used gas chromatography to analyze the membrane fatty acids of VBNC V. vulnificus and E. coli. The authors found that the ratios of fatty acids with fewer than 16 carbons increased significantly when V. vulnificus and E. coli cells lost culturability, while the percentages of long-chain fatty acids, i.e., palmitic (C_{16}) and palmitoleic (C_{16:1}) acids in the VBNC V. vulnificus cell membrane and palmitic (C_{16}), palmitoleic (C_{16:1}), and oleic (C_{18:1}) acids in the VBNC E. coli cell membrane decreased dramatically. In the study of Signoretto et al. (2000), during the VBNC transition of Enterococcus faecalis, the percentages of trimers, tetramers, pentamers, and oligomers were increased in the peptidoglycan
composition. The cross-linking might contribute to a more rigid cell wall of VBNC E. faecalis (Signoretto et al. 2000).

With the development of the VBNC state, the nucleic acid density within VBNC bacteria was significantly reduced. Falcioni et al. (2008) found that the surface properties of V. parahaemolyticus were remodeled with decomposed structural antigen components, which remarkably impaired its cellular affinity to a polyclonal antibody (generated from BALB/c mice infected by V. parahaemolyticus Conero strain) compared to their culturable counterparts. However, this observation about VBNC cells remains controversial. Senoh et al. (2012) reported that VBNC V. cholerae induced by low temperature (4 °C) retained its surface structural antigens.

It is believed that VBNC bacteria have relatively low metabolic activity compared with culturable bacteria. Zhao et al. (2013) compared the enzymatic activities between culturable and VBNC E. coli O157:H7 and demonstrated that two enzymes

Fig. 14.1 Transmission electron microscopy images of VBNC Vibrio parahaemolyticus cells induced by incubation at 4 °C in starving medium: (i, ii, iii) represent the cell morphology after three days of incubation and (iv, v, vi) represent four days of incubation. This figure is reprinted from Su et al. (2013), with the permission from Elsevier
(leucine arylamidase and β-galactosidase) were inhibited in VBNC cells, but VBNC cells retained activity of five enzymes, including alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, leucine arylamidase, and β-galactosidase (Fig. 14.2).

Guo et al. (2019) investigated the metabolic activity of VBNC *E. coli* and *S. aureus* at the single-cell level by D$_2$O labeling coupled with a Raman spectroscopy. The hydrogen atoms of water were substituted by deuterium isotope atoms. In this way, newly synthesized molecules are labeled by forming C-D bonds. The accumulation of the isotope-labelled molecules can reflect the cellular metabolic activity from the perspective of biosynthesis. Subsequent Raman spectroscopic characterizations revealed that VBNC *Aeromonas* sp., *Pseudomonas* sp., *E. coli* and *S. aureus* induced by UV treatment demonstrated impaired synthetic activity as the C-D band intensities decreased (Fig. 14.3). Fu et al. (2020) had similar observations using a parallel setup of isotope labeling coupled to a confocal Raman microspectroscopic system. D/(H + D) was used as the indicator of metabolic activity, and it was calculated from the share of the integrated spectral intensity of the C-D (2040–2300 nm) signature region in the integrated spectral intensity of both the C-D (2040–2300 nm) and C-H (2800–3100 nm) signature regions. The D/(H + D) of the VBNC cells was 5.04%, while it was 13.42% for their culturable counterparts.
14.3 The Formation of VBNC Pathogens in the Food Industry

14.3.1 Common Food Production-Associated Stressors for the Induction of VBNC Pathogens

Foodborne pathogens encounter various stress factors throughout food production chains, such as extreme temperature, high osmotic pressure, and acid agents (low pH). These stress factors might induce foodborne pathogens into a VBNC state.

Low temperature is a well-known condition to retard bacterial proliferation and it has been used as a major strategy for food preservation (Kondratowicz and Matusiūnas 2002). As shown in Table 14.1, many studies have reported that low temperature, as a stressor, can induce bacteria into the VBNC state. As early as 1991, Brauns et al. (1991) found that the colony counts of *V. vulnificus* decreased to less than 10 CFU/mL after incubation at 5 °C for 18 days. However, the viability of these
cells was confirmed by PCR amplification using hemolysin gene as the target gene, which indicated the presence of VBNC *V. vulnificus* due to low temperature. The ability to enter the VBNC state varies among bacteria in different metabolic states. Magajna and Schraft (2015) demonstrated that *C. jejuni* biofilm cells entered the VBNC state at 4 °C more rapidly than their planktonic counterparts did.

As one of the most well-recognized methods, thermal processing can efficiently inactivate microbes in food by causing damage to bacterial macromolecules (Fellows 2009). Typical thermal processing in the food industry includes blanching, pasteurization, and sterilization. Blanching is carried out with hot water at up to 100 °C. Pasteurization refers to mild heating, and the treatment temperature can be adjusted according to the desired treatment time. For instance, a temperature of

<table>
<thead>
<tr>
<th>Low-temperature conditions</th>
<th>Bacterial strains</th>
<th>Matrix</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen storage at −20 °C for three months or three freeze-thaw cycles (being kept at −20 °C for 5 days + thawed at 25 °C for 30 min)</td>
<td><em>Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio cholerae</em></td>
<td>Cooked shrimps</td>
<td>Moon et al. (2020)</td>
</tr>
<tr>
<td>−20 °C in the dark for 9 days</td>
<td><em>Vibrio parahaemolyticus</em></td>
<td>3% (w/w) sterile NaCl solution</td>
<td>Cao et al. (2019)</td>
</tr>
<tr>
<td>4 °C for 150 d</td>
<td><em>Vibrio parahaemolyticus</em></td>
<td>Artificial seawater</td>
<td>Yoon et al. (2019)</td>
</tr>
<tr>
<td>4 °C for 120 d</td>
<td><em>Escherichia coli</em></td>
<td>LB broth</td>
<td>Vilhen et al. (2019)</td>
</tr>
<tr>
<td>4 °C for 35 d</td>
<td><em>Vibrio cholerae</em></td>
<td>Artificial seawater</td>
<td>Xu et al. (2018)</td>
</tr>
<tr>
<td>4 °C for 16 d</td>
<td><em>Vibrio cholerae</em> O1</td>
<td>Freshwater microcosm</td>
<td>Mishra et al. (2012)</td>
</tr>
<tr>
<td>4 °C for 42 d</td>
<td><em>Campylobacter jejuni</em></td>
<td>Bolton broth</td>
<td>Chaisowwong et al. (2011)</td>
</tr>
<tr>
<td>4 °C for 60 d</td>
<td><em>Vibrio cholerae</em></td>
<td>Artificial seawater</td>
<td>Asakura et al. (2007)</td>
</tr>
<tr>
<td>4 °C for 10 d</td>
<td><em>Vibrio vulnificus</em></td>
<td>Artificial seawater</td>
<td>Abe et al. (2007)</td>
</tr>
<tr>
<td>4 °C for 25 d</td>
<td><em>Campylobacter jejuni</em></td>
<td>Brucella broth</td>
<td>Tangwatcharin et al. (2006)</td>
</tr>
<tr>
<td>4 °C for 9 d</td>
<td><em>Vibrio cholerae</em></td>
<td>Artificial seawater</td>
<td>González-Escalona et al. (2006)</td>
</tr>
<tr>
<td>4 °C for 5 d</td>
<td><em>Vibrio vulnificus</em></td>
<td>Microcosms of half-strength artificial seawater</td>
<td>Smith and Oliver (2006)</td>
</tr>
<tr>
<td>4 °C for 10 d + 8 °C for 18 d</td>
<td><em>Listeria monocytogenes</em></td>
<td>Salmons</td>
<td>Neunlist et al. (2005)</td>
</tr>
<tr>
<td>5 °C for 3 d</td>
<td><em>Vibrio vulnificus</em></td>
<td>Artificial seawater</td>
<td>Kong et al. (2004)</td>
</tr>
</tbody>
</table>
63 °C requires 30 min, while 15 s treatment is sufficient when the temperature is 72 °C. The temperature used in sterilization is generally over 100 °C to inactivate bacteria and even spores. Previous studies suggested that bacteria can survive thermal processing by entering the VBNC state (Fu et al. 2020; Gunasekera et al. 2002; Cervero-Aragó et al. 2019). Cervero-Aragó et al. (2019) reported that *Legionella pneumophila* could enter the VBNC state in response to various thermal conditions: 3–8 h at 55 °C, 60 min at 60 °C, and < 2 min at 70 °C. Recently, Liu et al. (2020) and colleagues confirmed that the individual treatment of boiling (~97.8 °C) for 1–10 min or microwave heating (2450 MHz, 1200 W) for 1.5 min could induce *E. coli* O157:H7 to enter the VBNC state. The complete induction of VBNC *E. coli* O157:H7 was achieved at 50 °C for 2 h (Fu et al. 2020).

Osmotic pressure can inactivate bacterial cells by dehydration and thus it can be used as an important food preservation strategy (Gutierrez et al. 1995). Sugar and salt are commonly used osmotic additives in the production of foods, including jams, jelly, and fermented pickles. It has been reported that osmotic pressure can induce bacteria to enter the VBNC state (Table 14.2). Khezri et al. (2020) reported that the induction of VBNC *E. coli* O157:H7 could be achieved in water microcosms or salted silver carp fish when the concentration of NaCl reached 10 and 30%, respectively.

Food preservatives are mainly used to prevent food spoilage caused by microorganisms. Common food preservatives can be classified as organic acids (e.g., citric acid, sorbic acid, and lactic acid) or inorganic preservatives (e.g., nitrite) (Russell and Gould 2003). Bai et al. (2019) confirmed that citric acid (pH 4.0), a common preservative used in beverages and candies, could force *S. aureus* into a VBNC state after 20 days of incubation at 4 °C. In the study of Cunningham and Oliver (2009), it was reported that exposure to sorbate potassium (pH 4.0) for 7 h could trigger VBNC in *L. monocytogenes*. Li et al. (2020) reported that *S. aureus* entered the VBNC state when incubated in saline solution with the addition of 0.3, 0.7, or 1% acetic acid.

### Table 14.2: Summary of the formation of VBNC bacteria induced by osmotic conditions

<table>
<thead>
<tr>
<th>Osmotic conditions</th>
<th>Bacterial strains</th>
<th>Matrix</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 and 30% NaCl</td>
<td><em>Escherichia coli</em> O157:H7</td>
<td>Water microcosms and silver carps</td>
<td>Khezri et al. (2020)</td>
</tr>
<tr>
<td>7% (w/v) NaCl</td>
<td><em>Campylobacter jejuni</em></td>
<td>Water</td>
<td>Lv et al. (2019)</td>
</tr>
<tr>
<td>1.2 M NaCl at 4 °C</td>
<td><em>Salmonella enterica</em> serovar Typhimurium ATCC 14028, <em>Salmonella enterica</em> serovar Enteritidis phage type PT4 578</td>
<td>Water</td>
<td>Salive et al. (2020)</td>
</tr>
<tr>
<td>30% NaCl at 4 °C</td>
<td><em>Listeria monocytogenes</em></td>
<td>Water microcosm</td>
<td>Zolfaghari et al. (2020)</td>
</tr>
<tr>
<td>13% NaCl</td>
<td><em>Escherichia coli</em> O157:H7</td>
<td>Water</td>
<td>Makino et al. (2000)</td>
</tr>
</tbody>
</table>
(v/v); however, the induction of VBNC *S. aureus* was inhibited when the culturing medium was switched to 25% tryptic soy broth (TSB).

In food processing, bacteria can encounter more than one single stressor. The induction of VBNC bacteria by a combination of various stressors has also been investigated (Chen et al. 2009; Masmoudi et al. 2010; Zhong et al. 2018). Chen et al. (2009) applied the concomitant exposure to cold (15 °C) and carbon starvation (in a marine minimal medium) to induce the formation of VBNC *V. parahaemolyticus*. The combined effect of potassium sorbate (10 mM) and low temperature (4 °C) was also found to induce *V. parahaemolyticus* to enter the VBNC state (Zhong et al. 2018).

### 14.3.2 The Induction of VBNC Bacteria by Emerging Physics Nonthermal Techniques

With the improvement of living standards, customers’ demands for minimally processed food are increasing. Emerging physics nonthermal techniques can inactivate bacterial cells through physics-associated stressors (e.g., voltage, electric field, and pressure) instead of heating. Therefore, physics nonthermal techniques have resulted in less deconstructive effects on food nutrients, natural flavor, and sensory characteristics and have attracted increasing attention in recent decades (Li and Farid 2016). However, physics nonthermal processing may incompletely inactivate bacteria and induce the formation of VBNC bacteria, resulting in potential risks to food safety (Table 14.3). Before the application of physics nonthermal techniques for the decontamination of food, the occurrence of VBNC bacteria should be carefully evaluated.

High-pressure carbon dioxide (HPCD) treatment is an emerging physics nonthermal technology for microbial decontamination in foods. As early as 1951, Fraser (1951) discovered that a combination of high pressure and carbon dioxide could kill bacteria. During HPCD processing, the food is placed in a batch or continuous processor and then exposed to a certain temperature and pressure (<50 MPa), which can efficiently inactivate microorganisms. HPCD treatment can induce the formation of VBNC *E. coli O157:H7* (Zhao et al. 2013; 2016; Wang et al. 2019). Zhao et al. (2013) found that *E. coli O157:H7* cells gradually lost culturability and entered the VBNC state under various HPCD treatments (5 MPa, 25 °C; 5 MPa, 31 °C; 5 MPa, 34 °C; 5 MPa, 37 °C) (Fig. 14.4).

Pulsed electric fields (PEFs) have emerged as nonthermal technologies in recent years (Ho and Mittal 2000). During PEF treatment, a pulsed electric field and magnetic field are generated between two electrodes. The electric field and the magnetic field act alternately to increase the permeability of the cell membrane and this results in electroporation of the outer structure of bacterial cells, eventually leading to microbial inactivation. In addition, the anions and cations produced near the electrodes interact with the components on the membrane, subsequently blocking
the normal biochemical reactions and metabolic processes of the membrane. As early as 2001, it was found that VBNC *B. cereus* and *L. monocytogenes* formed during PEF processing (Rowan 2004). However, another study from the same research group reported that the survival levels of *L. monocytogenes*, *B. cereus*, and *E. coli* determined by the 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) dye and plate count method were insignificantly different (*p* < 0.05), which indicated that PEF failed to induce VBNC bacteria (Yaqub et al. 2004). Therefore, the induction of VBNC bacteria by PEF is still under debate, and more investigations are required to explore it.

Ultrasound, as a relatively low-cost, nonhazardous, and environmentally friendly nonthermal technology, is commonly used in food processing (Chandrapala et al. 2012). Ultrasound refers to a mechanical elastic wave with a frequency of over 20 kHz. In the propagation process of ultrasonic waves, the molecules of the propagation medium are squeezed or dispersed, forming bubbles or cavities. The cavities subsequently collapse, elevating both temperature and pressure. High-intensity ultrasound could directly cause physical damage to the bacterial cell wall, cell membrane and internal structure, which is an irreversible inactivation process. Declerck et al. (2010) reported that ultrasound treatment (36 kHz, 0.064 kW/L, 15 and 30 min) could cause approximately 7% of *Legionella* to enter the VBNC state. In the study of Li et al. (2017), flow cytometry combined with double staining

<table>
<thead>
<tr>
<th>Physics nonthermal technologies</th>
<th>Bacterial strains</th>
<th>Matrix</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>High pressure carbon dioxide</td>
<td><em>Escherichia coli</em> O157:H7</td>
<td>0.85% (w/v) sterile NaCl solution (pH 7.0)</td>
<td>Zhao et al. (2013), Zhao et al. (2016), Wang et al. (2019)</td>
</tr>
<tr>
<td>Ultrasound</td>
<td><em>Legionella pneumophila</em></td>
<td>Acanthamoeba castellanii host cells</td>
<td>Declerck et al. (2010)</td>
</tr>
<tr>
<td></td>
<td><em>Staphylococcus aureus</em></td>
<td>0.85% (w/v) sterile NaCl solution</td>
<td>Li et al. (2017)</td>
</tr>
<tr>
<td>Cold plasma/Nonthermal plasma</td>
<td><em>Escherichia coli</em></td>
<td>Physiological solution (0.85% NaCl in distilled water)</td>
<td>Dolezalova and Lukes (2015)</td>
</tr>
<tr>
<td></td>
<td><em>Bacillus stratosphericus</em></td>
<td>Distilled water</td>
<td>Cooper et al. (2010)</td>
</tr>
<tr>
<td></td>
<td><em>Staphylococcus aureus</em> biofilm</td>
<td>Microwell dishes surface</td>
<td>Xu et al. (2017)</td>
</tr>
<tr>
<td>Ultraviolet irradiation</td>
<td><em>Escherichia coli</em></td>
<td>Saline phosphate buffer (PBS)</td>
<td>Said et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>Sterile saline solution</td>
<td></td>
<td>Zhang et al. (2015), Zhang et al. (2018)</td>
</tr>
</tbody>
</table>
of carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) was applied to confirm that the proportion of VBNCS. aureus increased from 30.45% to 45.75% when the ultrasound treatment time (20 kHz, 13.3 W/mL) was increased from 1 to 10 min. In addition to individual ultrasonic treatment, hurdle technology coupled with ultrasound and other techniques has also been reported to induce the formation of VBNCS. aureus (Liao et al. 2017, 2018). Liao et al. (2017) applied ultrasound exposure (380 W) incorporated with mild heat (53 °C), known as thermosonication (TS), to treat S. Typhimurium. The Salmonella cells was found to enter into VBNC as the TS treatment time was extended to 30 min. Another study from the same research group demonstrated that the generation of free radicals (carbon-centered radicals, hydroxyl radicals, hydrogen proton) from TS exposure contributed to the formation of VBNCS. Typhimurium in carrot juice (Liao et al. 2018). The higher TS intensities resulted in a higher level of radicals, subsequently leading to a higher level of VBNCS. Typhimurium (Fig. 14.5).

Ultraviolet (UV) is a promising physics nonthermal processing technology with the application of light ranging from 100 to 400 nm (Keyser et al. 2008). The UV spectrum can be divided into four categories: UVA (400–315 nm), UVB
UVC, ranging from 240 to 280 nm, is generally used for the inactivation of bacterial cells (Shin et al. 2016; Zhang et al. 2015). UV treatment can cause damage to DNA and RNA, which inhibits the reproduction of pathogenic cells, subsequently causing cell death. Several studies have found that UV exposure could induce the formation of VBNC bacteria (Said et al. 2010). Shin et al. (2016) reported that UV exposure at doses of 60 mJ/cm² for over 60 s could render *E. coli* incapable of forming colonies on media. In the study of Zhang et al. (2015), the application of a UV energy of 100 mJ/cm² resulted in 6.49-log survival of *E. coli* determined by the plate count method; however, it was 8.41 logs measured by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). The differences between the results from the plate count method and RT-qPCR were attributed to the occurrence of VBNC *E. coli*.

Fig. 14.5  The changes in the intensity of 5,5-dimethyl-1-pyrroline N-oxide (DMPO)/free radical spin adducts (measured by electron spin resonance) in carrot juice inoculated with *Salmonella Typhimurium* under different thermosonication (TS) processing parameters: (a) processing powers, (b) temperatures, and (c) treatment times. (d) The relationship between the intensity of free radicals and the formation of VBNC bacteria level, fitted with a Boltzmann model (the solid line, $R^2 = 0.96$). This figure is reprinted from Liao et al. 2018, with the permission of Elsevier.
Cold plasma, also known as nonthermal plasma, is an emerging nonthermal treatment technology that shows the advantages of high efficiency, low temperature and no residue of chemical compounds (Scholtz et al. 2015). The effects of UV, pH, electric field, electrons, reactive oxygen species (ROS), and reactive nitrogen species (RNS) all contribute to the sterilization mechanism of cold plasma. To date, several studies have reported the induction of VBNC bacteria by cold plasma exposure (Dolezalova and Lukes 2015; Cooper et al. 2010; Liao et al. 2020). Dolezalova and Lukes (2015) reported that a nonthermal atmospheric pressure plasma jet (APPJ) treatment for 15 min could force \textit{E. coli} to enter the VBNC state. In the study of Cooper et al. (2010), no colony of \textit{Bacillus stratosphericus} was found on solid medium after the treatment of dielectric barrier discharge (DBD) cold plasma at a voltage of 30 kV for 120 s. However, over 40\% of the subpopulation remained viable, which was determined by double staining with SYTO 9 (for viable cells) and PI (for dead cells) dyes. Therefore, the authors hypothesized that DBD treatment could induce the formation of VBNC \textit{B. stratosphericus}. In addition to bacterial cells in vegetative form, Xu et al. (2017) reported that treatment with a He/O\textsubscript{2} atmospheric plasma jet (APPJ) induced the formation of 5\% VBNC \textit{S. aureus} biofilm along with a 95\% dead subpopulation.

14.4 The Mechanisms Underlying the Entrance of Pathogens into a VBNC State

To date, the mechanisms underlying the formation of VBNC bacteria are still not clear. Some hypotheses about the molecular regulation of VBNC formation have been proposed over the past decades (Fig. 14.6).

![Fig. 14.6](image) Summary of the molecular regulation of VBNC formation. RNAP: RNA polymerase
14.4.1 The Accumulation of Alarmone Guanosine Pentaphosphate

The stringent response of bacteria can slow down the biosynthesis of proteins under amino acid starvation conditions. This mechanism is regulated by the signaling molecules guanosine pentaphosphate or guanosine tetraphosphate [(p)ppGpp] (Potrykus and Cashel 2008). The level of (p)ppGpp within bacterial cells is regulated by RelA and SpoT. RelA, guanosine triphosphate (GTP) pyrophosphokinase, is a (p)ppGpp synthase. In *E. coli*, the RelA protein with a molecular weight of 84 kDa catalyzes the transfer of the pyrophosphate group from ATP to the 3’ hydroxyl group of guanosine diphosphate (GDP) or GTP ribose to synthesize pppGpp or ppGpp. SpoT, with a molecular weight of 79 kDa, contributes to the hydrolysis of (p)ppGpp into GTP or GDP. To survive in harsh environments, bacterial cells can change their (p)ppGpp concentration to regulate a series of important cellular processes, such as blocking DNA replication, inhibiting and degrading rRNA synthesis, differentially expressing genes, and activating or inhibiting metabolic enzymes (Toulokhonov et al. 2001; Srivatsan and Wang 2008). RNA polymerase (RNAP) is the major target of (p)ppGpp. (p)ppGpp directly binds to RNAP to regulate the process of transcription. Crystal structure analysis revealed that (p)ppGpp is located near the RNAP catalytic center, which allows the entry of nucleotide triphosphate substrates as well as their entrance for the separation of newly synthesized RNA and RNAP after transcriptional hindrance (Artismovitch et al. 2004). Transcriptional inhibition by (p)ppGpp might be achieved by the following mechanisms. The combination of (p)ppGpp and the RNA polymerase-binding transcription factor DksA protein reduces the stability of the open complex formed by RNAP and DNA, thereby inhibiting transcription from rRNA promoters (Paul et al. 2004, 2005). In addition, RNAP is trapped in a closed complex by (p)ppGpp and cannot initiate the transcription process (Potrykus et al. 2006). On the other hand, (p)ppGpp could also contribute to transcriptional activation by promoting the affinity of housekeeping Sigma factor with the RNAP core (Jishage et al. 2002). The regulation of RelA, the (p)ppGpp synthase, has been implicated in the induction of VBNC bacteria (Jayakumar et al. 2020; Gonzalez-Escalona et al. 2006; Mishra et al. 2012; Wan et al. 2014; Zhang et al. 2020). In the study of Mishra et al. (2012), a significant upregulation of *relA* by 60.76-fold was found in VBNC *V. cholerae* O1 induced by freshwater microcosms at 4 °C. Jayakumar et al. (2020) found that *V. cholerae* 0395 defective in the *relA* gene exhibited a slower rate in entering the VBNC state compared with the wild-type strain, indicating a role of *relA* in the VBNC formation. However, Asakura et al. (2007) reported downregulation of the *relA* gene in *V. cholerae* when entering VBNC induced by cold stress (4 °C). Nowakowska and Oliver (2013) found no significant difference in the expression of *relA* between VBNC and culturable C-genotype *V. vulnificus*, while a significant upregulation of *relA* was found in E-genotype *V. vulnificus*. 
14.4.2 Proteases

Intracellular accumulation of proteases has been associated with the formation of the VBNC state. It is speculated that Clp, an ATP-dependent protease, probably participates in the induction of bacteria into the VBNC state. Kusumoto et al. (2013) reported that it took a long time for *S. Typhimurium* defective in the *clpX* gene to enter the VBNC state. This might be related to the degradation of the RNA polymerase sigma factor RpoS by the ClpXP protease complex, which is formed from a combination of ClpX and ClpP. In the study of Alleron et al. (2013), proteomic analysis was conducted to reveal the accumulation of ClpP proteins in VBNC *L. pneumophila* cells compared with their culturable counterparts. In addition to the degradation of RpoS, it has been reported that antitoxins could also be degraded by ClpP protease, resulting in the release of toxins and contributing to the formation of VBNC bacteria (e.g., *E. coli* and *S. aureus*) (Donegan et al. 2010; Diago-Navarro et al. 2013).

14.4.3 Toxin-Antitoxin Systems

Toxin-antitoxin (TA) systems are ubiquitous in bacteria and play an important role in bacterial adaption to external stressors, virulence, and biofilm formation (Gerdes and Maisonneuve 2012; Yamaguchi et al. 2011). TA systems have been widely studied in antibiotic-induced persistent bacteria and are the main contributors to the formation of persisters (Maisonneuve and Gerdes 2014). TA systems have also been proposed to regulate the induction of VBNC bacteria (Ayrapetyan et al. 2015a, b; 2018). It has been reported that when toxin genes are overexpressed, bacteria can enter the VBNC state, while the overexpression of associated antitoxin genes can reverse the dormant state (Demidenok et al. 2014; Pedersen et al. 2002; Christensen-Dalsgaard and Gerdes 2006). Demidenok et al. (2014) demonstrated that the expression of VapC, the toxin of the type II toxin-antitoxin (TA) system, was found to be significantly increased when *Mycobacterium smegmatis* transited into the VBNC state, while the overexpression of VapB, the antitoxin of the type II toxin-antitoxin (TA) system, could prevent *M. smegmatis* from becoming dormant. To date, several TA systems have been shown to regulate the bacterial VBNC state, including *hipAB* (Korch and Hill 2006), *higBA* (Christensen-Dalsgaard and Gerdes 2006), *relEB* (Pedersen et al. 2002), *chpAKAI* (Pedersen et al. 2002), and *vapBC* (Demidenok et al. 2014).
14.4.4 ATP Depletion

In recent years, ATP depletion and a decrease in cellular energy have been associated with the formation of the VBNC state. In the study of Pu et al. (2019), it was demonstrated that ATP depletion contributed to the promotion of protein aggregation within *E. coli*, which resulted in the formation of dormancy, including the persister and VBNC states. To date, the mechanisms underlying ATP depletion inducing the VBNC state are limited and they require further investigation.

14.5 The Diagnostics of VBNC Pathogens

VBNC bacteria cannot be detected by conventional plate count methods, and it is easy to underestimate microbial contamination, creating a potential threat to food safety and public health. Therefore, various culture-independent techniques have been developed for the qualitative and quantitative detection of bacteria in the VBNC state.

Direct viable count (DVC) is a classical detection method for VBNC bacteria that was first proposed by Kogure et al. (1979). The principle of the DVC method to distinguish live and dead bacteria is based on the fact that live bacteria have the ability to absorb substrates. After the addition of nalidixic acid to the medium to inhibit cell division of live bacteria, the bacteria continue to absorb nutrients and their cells elongate after 6 hours of incubation. Acridine orange is used for staining followed by fluorescence microscopy to identify the survival state of the bacteria. Nalidixic acid used with the DVC method can inhibit DNA synthesis in most Gram-negative bacteria, but it tends to be ineffective for Gram-positive bacteria. Some studies have shown that the application of ciprofloxacin or ofloxacin instead of nalidixic acid could effectively inhibit DNA synthesis in both Gram-negative and Gram-positive bacteria (Besnard et al. 2000).

In addition to combining DNA inhibitors with the DVC method, the electron acceptor 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) can also be added for microscopic detection (Baffone et al. 2003). CTCs make use of the characteristics of active redox metabolism in live bacteria. CTCs can penetrate bacterial cells and participate in redox reactions and subsequently be reduced to red or purple fluorescent substances. Generally, CTCs are coupled with DAPI for the determination of total viable bacterial counts (Cools et al. 2005; Peneau et al. 2007).

Live/Dead® BacLight kits produced by Molecular Probes are widely used for the detection of live bacteria. This kit contains two nucleic acid dyes, namely, SYTO9 and propidium iodide (PI). The principle of LIVE/DEAD® BacLight kits for detection is based on the integrity of the bacterial cell membrane. SYTO9 dye has the ability to penetrate the intact membrane into the bacterial cell and bind to DNA strands. The nucleic acids of viable bacteria dyed by SYTO9 emit green fluorescence at wavelengths of 480/450 nm. PI dye fails to pass through an intact cell membrane,
but it can enter dead cells through a damaged membrane to bind to DNA, exhibiting red fluorescence at 490/635 nm. The percentage of viable bacteria could be determined with the use of a fluorescence microscope or flow cytometer.

The cell wall surface of bacteria in the VBNC state still has the same surface antigens as that of bacteria in the normal state, which can bind to specific antibodies. Therefore, immunological methods combined with fluorescent staining can be used to detect live cells, combined with plate counting methods to detect the number of culturable cells to achieve rapid detection of bacteria in the VBNC state. Commonly used immunological methods include enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IF) (Biosca et al. 1997). The ELISA method is easy to operate with high specificity and low LOD value. And the IF method has the advantages of high efficiency, low detection limit, and the direct observation of cell membrane integrity with a fluorescence microscope.

Propidium monoazide (PMA) and ethidium monoazide (EMA) bromides are high-affinity photolytic DNA nucleic acid dyes (Chaveerach et al. 2003; Nocker et al. 2006). The azide groups can easily interact with hydrocarbons of nucleic acids to generate stable and strong covalent nitrogen-carbon bonds. PMA and EMA cannot pass through the intact cell membrane of living cells but they can pass through the damaged cell membrane of dead cells to intercalate with intracellular DNA. DNA intercalated by PMA or EMA cannot be amplified (Elizaquível et al. 2012). Generally, PMA is less toxic to living cells than EMA (Nocker et al. 2006). PMA combined with PCR, real-time PCR (qPCR) and loop-mediated isothermal amplification (LAMP) can distinguish viable cells from dead cells (Dinu and Bach 2013; Zhong et al. 2017). Dinu and Bach (2013) used PMA-qPCR to detect VBNC *E. coli* O157:H7 in lettuce and spinach, achieving a limit of detection (LOD) of $10^3$ CFU/g. In terms of the PMA-LAMP method, Han et al. (2020) designed four specific primers (two outer primers and two inner primers) targeting the *wzy* gene of *E. coli* O157:H7 and the *agfA* gene of *S. enterica*, and PMA coupled with LAMP detection achieved the rapid detection of viable *E. coli* O157:H7 within 0.5 h. The LOD of PMA-LAMP were 9.0 CFU/reaction for *E. coli* O157:H7 and 4.6 CFU/reaction for *S. enterica*, respectively. Zhong et al. (2016) used PMA coupled with real-time fluorescent loop-mediated isothermal amplification (RF-LAMP) for the quantitative detection of VBNC *V. parahaemolyticus*. This method could detect as few as 14 copies/g of *V. parahaemolyticus* within 30 min from seafood products, including pomfret, shrimp, scallop, oyster, and salted fish.

Green fluorescent proteins (GFP) have been used for the discrimination of viable bacteria from the death subpopulation. GFP was first discovered in jellyfish in 1962 (Shimomura et al. 1962). GFP proteins emit green fluorescence when exposed to light ranging from blue to ultraviolet wavelengths. The advantages of GFP proteins include being nontoxic to cells, emitting stable fluorescence, and requiring no exogenous substrates or cofactors to express the GFP encoding gene in cells. GFP-labeled cells in the VBNC state exhibited bright green fluorescence, while dead cells with incomplete cell membranes emit no fluorescence (Lowder and Oliver 2001). Using the GFP encoding gene as a reporter can effectively distinguish dead bacteria from viable ones, so it can be used for the detection of VBNC cells.
(Lowder et al. 2000). However, it was found that *Pseudomonas putida* labeled with GFP rapidly lost fluorescence in the VBNC state under starvation conditions (Lowder and Oliver 2001). The loss of fluorescence in VBNC bacteria might be resulted from the inhibition of GFP synthesis due to the cessation of nutrient acquisition under starvation conditions.

It has been proposed that mRNA can be used as an indicator of bacterial viability because the half-life of mRNA is only a few minutes (Caldera et al. 2015; Conway and Schoolnik 2003; Fu et al. 2015; Khezri et al. 2019; Liu et al. 2008; Fischer-Le Saux et al. 2002). Hence, reverse transcription quantitative PCR (RT-qPCR) can be used as an approach for the detection of VBNC bacteria. A study conducted by Yaron and Matthews (2002) applied RT-PCR for the detection of VBNC *E. coli O157:H7* targeting the *rfbE*, *fliC*, *stx1*, *stx2*, *mobA*, *eaeA*, *hly*, and *16S rRNA* genes. The results showed that *rfbE* was the most suitable target due to its stability in viable bacterial cells. González-Escalona et al. (2006) targeted *16S rRNA* and *tuf* (encoding the elongation factor Tu), *rpoS* (encoding the RNA polymerase sigma factor), and *relA* (encoding the bifunctional [p]ppGpp synthase/hydrolase RelA) genes using RT-PCR to detect *V. cholerae* in the VBNC state. Jiang et al. (2013) applied RT-qPCR targeting various genes for the detection of various bacterial strains. The *tufA*, *invA*, and *ipaH* genes were selected for the detection of *E. coli*, *S. Typhimurium*, and *S. flexneri*, respectively. The LODs of the RT-qPCR method for the detection of *E. coli*, *S. Typhimurium*, and *S. flexneri* were $10^2$, $10^2$, and $10^4$ MPN per reaction, respectively.

The immunological method is based on the specific binding of antigens and antibodies. Only bacteria with a complete cell wall structure can feedback a positive immune response. Fluorescent antibody staining and indirect enzyme-linked immunosorbent technology are widely used for the detection of VBNC bacteria. Liu et al. (2006) established an immunomagnetic capture-fluorescent PCR (IMC-FPCR) approach, which could quickly and sensitively detect VBNC *C. jejuni*. In the study of Dinu and Bach (2013), a Ridascreen® verotoxin enzyme immunoassay kit was used for the qualitative detection of VBNC *E. coli* by estimating the production of Shiga toxin.

Some researchers have proposed a method for the detection of VBNC cells with specific phages (Awais et al. 2006; Fernandes et al. 2014; Said et al. 2010). The principle is based on the fact that bacterial phages show differential infectious abilities against dead cells and VBNC cells (Awais et al. 2006). Said et al. (2010) proposed a detection method using *E. coli*-specific phage (Q/β phage) to evaluate VBNC cells. Researchers used Q/β phage to infect *E. coli* irradiated by UV. The counts of released mature Q/β progeny phage were directly proportional to the counts of viable *E. coli* ATCC 13965. This study found that *E. coli* entered the VBNC state when the UV doses were 60 mJ/cm² and 90 mJ/cm². Another method is to use phage magnetoresistive biochips to assess bacterial cell viability. Fernandes et al. (2014) combined a magnetoresistive sensor and a phage probe to detect *S. Enteritidis* cells in the VBNC state (Fig. 14.7). This study confirmed the correlation between the immobilized phage detection signal on the gold surface and the concentration of the VBNC cells.
In addition to the aforementioned methods, there are other methods that have been applied for the detection of bacteria in the VBNC state, such as isotope labeling (Guo et al. 2019), fluorescence in situ hybridization technology (Griffitt et al. 2011), ATP monitoring methods (Robben et al. 2019), spectrum-associated techniques (Cialla-May et al. 2017), biosensor-based techniques (Bertelsen et al. 2020; Cheng et al. 2011; Kuznetsov et al. 2004), and resuscitation-associated detection (Huff et al. 2012).

### 14.6 The Resuscitation of VBNC Pathogens

VBNC is a reversible state in which VBNC bacteria can resuscitate to culturable state under suitable conditions. Roszak et al. (1984) first proposed the concept of resuscitation that described the restoration of the cultivability of VBNC S. Enteritidis. The resuscitated bacterial cells regain cultivability and possessed high metabolic activity. However, the mechanisms underlying VBNC resuscitation are not completely clear. To date, some factors have been proposed to achieve the
resuscitation of VBNC bacteria, including temperature upshifts, the removal of stressors, the addition of protective agents against stressors (e.g., osmotic protectant), the addition of nutrients and some chemical agents (e.g., Tween 20, vitamin B), and host cells (Table 14.4). In the study of Sun et al. (2008), VBNC V. harveyi induced by seawater at 4 °C achieved resuscitation on yeast extract medium supplemented with Tween 20 or vitamin B at 26 °C. VBNC S. Enteritidis induced by hydrogen peroxide treatment can recover in M9 medium supplemented with sodium pyruvate, but supplementation using bromoacetone or phenylpyruvate failed to resuscitate VBNC S. Enteritidis (Morishige et al. 2013). Chaisowwong et al. (2011) demonstrated that Caco-2 cells could simulate the resuscitation of VBNC C. jejuni induced by 4 °C. Ayrapetyan et al. (2014b) found that the addition of density-sensing self-inducing molecule (AI-2) could achieve the resuscitation of VBNC V. vulnificus. Pyruvate, a well-known scavenger of ROS (e.g., hydrogen peroxide and hydroxyl radicals) (Desagher et al. 1997; Woo et al. 2004), has been reported to promote the resuscitation of VBNC bacteria (Morishige et al. 2013; Reissbrodt et al. 2002; Vilhena et al. 2019). In the study of Vilhena et al. (2019), it was found that upregulation of the high-affinity transporter BtsT encoding gene btsT (regulated by the BtsS/BtsR two-component system) contributed to the rapid uptake of pyruvate by VBNC E. coli cells, followed by initiating the biosynthesis of DNA and proteins and proliferation to achieve resuscitation.

Resuscitation-promoting factor (Rpf) was originally found in Micrococcus luteus and it can promote the resuscitation and growth of dormant bacteria (Pinto et al. 2015). Rpf is widely present in Gram-positive Firmicutes. The Rpf protein belongs to the family of transglycosylases, showing similar functions to lysozyme. The Rpf protein can hydrolyze the glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine. Hydrolyzed intermediates may interact with other factors and act as a second messenger to stimulate the resuscitation and growth of VBNC cells (Keep et al. 2006). Proteins with similar functions to Rpf have also been found in Gram-negative bacteria. For instance, YeaZ proteins in Salmonella and Vibrio can promote the resuscitation of VBNC cells, but it is not a homolog of Rpf (Aydin et al. 2011; Panutdaporn et al. 2006). Some studies have also proposed that a subpopulation of scout bacterial cells, known as exploration factors, are the key to the resuscitation of VBNC bacteria (Epstein 2009, 2013). In some strains, scout cells can secrete a specific signaling molecule to induce the resuscitation and regrowth of the surrounding dormant cells. Quorum sensing has been described as a phenomenon in which bacteria can trigger their adaptions in response to density changes in the population. Bacteria synthesize and release signal molecules through diffusion or transport systems. Once the density of the bacteria reaches a certain level, they can be recognized by sensors on the cell membrane, causing changes in gene expression (Galloway et al. 2011). It is hypothesized that the exploration factors of nonculturable bacterial populations are correlated with the quorum-sensing system. More rpf-like genes have been discovered in multiple bacterial strains (Kell and Young 2000). When the environment becomes harsh, scout cells, as exploration factors, respond rapidly and secrete signaling molecules that act on the rest of the population. This process can lead to the induction of the VBNC transition in the
entire population to protect against adverse environments (Ayrapetyan et al. 2014a). When the stress is relieved, scout cells can secrete another signaling molecule, the Rpf protein, which promotes the resuscitation of VBNC cells (Table 14.4).
14.7 Resistance and Virulence Properties of VBNC Pathogens

The resistance and virulence properties of pathogenic bacteria in the VBNC state have attracted much attention. Compared with normal cells, VBNC cells are more resistant to environmental stress, such as physical, chemical, and antimicrobial agents. This may be due to the increase in the cross-linking level of peptidoglycan, which results in decreased metabolic activity and stronger cell walls (Lothigius et al. 2010). Nowakowska and Oliver (2013) found that *V. vulnificus* in the VBNC state could withstand different stressors, including high doses of antibiotics, heavy metals, high temperature, high salinity, ethanol, and acid. In terms of chemical stressors, a study showed that VBNC *V. parahaemolyticus* cells were resistant to hydrogen peroxide and low-concentration salinity, but they were still sensitive to bile salts (Wong and Wang 2004). Regarding antibiotic stress, VBNC foodborne pathogens, such as *E. coli* O157:H7, *S. aureus*, *V. vulnificus*, and *C. jejuni*, have been found to be resistant to several antibacterial drugs, such as piperacillin, gentamicin, and vancomycin (Ramamurthy et al. 2014). Similarly, Lin et al. (2017) reported that compared with culturable counterparts, the VBNC *E. coli* induced by chlorination exhibited higher resistance toward 9 kinds of antibiotics, including ampicillin, gentamycin, polymyxin, ciprofloxacin, rifampicin, clarithromycin, chloromycetin, tetracycline, and terramycin. The enhanced antibiotic resistance of VBNC *E. coli* was attributed to the upregulation of genes involved in multidrug efflux pumps (*tolC, acrD, acrF, emrA, macA, macB*) and porins (*ompC, ompF*).

Some studies have shown that pathogenic bacteria in the VBNC state still retain the capacity to express toxin genes (Alleron et al. 2013; Dinu and Bach 2011; Fu et al. 2020; Yaron and Matthews 2002). Yaron and Matthews (2002) performed reverse transcription PCR and found that some toxin genes were expressed in VBNC *E. coli*, including *mobA* (encoding molybdenum cofactor guanylyltransferase), *rfbE* (encoding GDP-perosamine synthase), *stx1* (encoding Shiga toxin 1), and *stx2* (encoding Shiga toxin 2). Dinu and Bach (2011) also observed the constant expression of toxin genes—*hly*, *stx1*, and *stx2* in VBNC *E. coli* O157:H7 and the production of enterotoxins, indicating the potential virulence of VBNC cells. In a study conducted by Fu et al. (2020), the eaeA gene, encoding the intimin toxin, was overexpressed in VBNC *E. coli* O157:H7 compared with culturable and resuscitated daughter cells, while the expression of the *hytA* gene for the production of enterohemolysin exhibited insignificant differences among bacterial cells in various states. Patrone et al. (2013) detected the expression of the fibronectin-binding protein CadF in VBNC *C. jejuni* and observed that the VBNC population still maintained the capacity to attach to the intestine. In the study of Chaisowwong et al. (2011), it was also found that *C. jejuni* in the VBNC state still had the ability to invade human intestinal epithelial Caco-2 cells. Highmore et al. (2018) used *Caenorhabditis elegans* as a model to evaluate the pathogenicity of VBNC *L. monocytogenes* and *Salmonella* Thompson induced by chlorination (50 and 100 ppm free chlorine). It
was found that VBNC cells could significantly reduce the lifespan of *C. elegans* compared with their culturable counterparts.

However, contrary results were reported in other studies. Cappelier et al. (2005) reported that VBNC *L. monocytogenes* lost pathogenicity in human adenocarcinoma HT-29 cells and a mouse model. The research conducted by Lothigius et al. (2010) indicated that the enterotoxigenic *E. coli* (ETEC) in the VBNC state maintained the expression of the virulence genes *estA* and *eltB*, which encode heat-labile enterotoxins A and B, respectively, but those enterotoxin proteins could not be detected in VBNC cells by the GM1 ganglioside enzyme-linked immunosorbent assay (GM1-ELISA) method.

### 14.8 Conclusions

In this chapter, the characteristics of VBNC pathogenic bacteria were introduced in terms of cell morphology, cell wall/membrane compositions, and physiological activities. During food processing, bacterial cells encounter multiple stressors (e.g., cold, heat, low pH, and osmosis), some of which have been confirmed to trigger the formation of VBNC bacteria. Emerging physical processing technologies, such as HPP, ultrasound, and cold plasma, have received much attention in the food industry due to less destruction of food quality as well as excellent antimicrobial performance. However, some previous studies have reported the association of these mild techniques with the formation of VBNC bacteria, which should be carefully considered during the optimization of the processing parameters. To date, the mechanisms underlying the induction and resuscitation of VBNC bacteria are still unclear, and additional investigations are required. The efficient and accurate detection of pathogenic bacteria in the VBNC state is a prerequisite for ensuring food safety. The current diagnostic techniques for VBNC bacteria each have their advantages and disadvantages. Future efforts should be made to develop reliable and convenient technologies for the efficient detection of VBNC bacteria in the food production chain.

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Chapter 15
Persistence Phenotype

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Abstract In the past few years, the persistence of foodborne pathogens in food processing units has drawn significant attention. Persistence involves those microorganisms which can endure for a long time in specific habitats, particularly under hostile conditions. Various factors are involved in the development of persistence in pathogens, i.e., certain physical–chemical conditions, microbial natural habitats, transmission routes, and antimicrobial exposure. A common belief in the food industry is that, these bacteria can tolerate environmental stresses, including cleaning and disinfection that causes persistence in pathogenic bacteria, which leads to the continuous cross-contamination of food products. The existence of bacterial persistence can also lead to a higher risk to the violation of food safety, thereby threatening the public health. From a clinical perspective, persistent pathogenic bacteria can permanently colonize their host and cause either chronic or frequent infections that the immune system and antimicrobial treatments cannot eliminate. These persistent bacteria are adaptive to surviving very high doses of antimicrobial treatment and are believed to be among the factors that make these infections challenging. When the antimicrobial concentration reduces, persister cells can convert back to a normal phenotype which can guarantee the survival of the bacterial population. The present chapter summarizes the development of persistent bacteria, the associated food-stress factors and the mechanism involved in persistent bacterial formation. We also discussed the persistent and host interaction and the state-of-art approaches to detect persistent bacteria.

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T. Ding et al. (eds.), Stress Responses of Foodborne Pathogens,
https://doi.org/10.1007/978-3-030-90578-1_15
15.1 Introduction

One of the leading challenges for foodborne diseases is the occurrence and persistence of bacterial pathogens along the food chain (NicAogín and O’Byrne 2016). Persistent bacteria are those microorganisms that can survive for prolonged periods of time in certain habitats. In the food industry, these bacteria often remain on the surfaces after regular cleaning and disinfection. Persistent bacteria can cause severe public health concerns and affect the well-being of people in both developed and developing countries (Dandage et al. 2017; Adams et al. 2019; Bisht et al. 2021). For the first time, Hobby et al. (1942) identified the persistent phenotype of Staphylococcus aureus in 1942, and the researchers found that penicillin could not kill 1% population of bacterial cells and this sub-population became persister cells. Persistence is associated with previously existing heterogeneity within the bacterial populations arising due to the phenotypic interchange from normal growing cells due to the reduced growth rates. Persister cells are general in dormancy, while they are usually resistant toward some growth-inhibiting antimicrobials and they are frequently associated with the existence in biofilm (Bigger 1944). Persister cells are responsible for the resistance of bacteria during antimicrobial treatment, leading to repeated administration of antimicrobials, which in turn give rise to antimicrobial-resistant bacteria. Such infections pose a major public health problem. Bacteria such as Mycobacterium tuberculosis, Salmonella, Chlamydia, Brucella, Borrelia, Pseudomonas, pathogenic Escherichia coli, Staphylococcus, and Streptococcus can cause persistent infections (Monack et al. 2004; Palmer et al. 2009; Yue 2016; Paudyal et al. 2020). The magnitude of persistence level or sub-population is generally low (0.001 ~ 1%), depending on the type of bacteria. When genetically identical bacterial cells are exposed to stress factors such as high heat, acidic conditions, other sanitizing agents, or a sufficiently strong dose of antimicrobials, most of the population are killed which takes place at an exponential rate. However, after a few hours, the initial exponential killing rate slows down. Therefore, when the exogenous stress is removed, a small population still survive. The mechanism by which these cells survive involves the reduction in their metabolic rate, i.e., the bacteria may survive the harsh conditions or antimicrobials treatment by being dormant (Kim and Wood 2016). These cells have not genetically acquired antibiotic resistance and they can give rise to a new population that is sensitive to the antimicrobial (Balaban 2004). Day (2016) proposed that the inheritance through non-genetic mode may be involved in the persister cell formation. Miyaue et al. (2018) proposed that a stationary-phase cellular dwarfing is the typical phenotype of antimicrobial-resistant isolates.

The current chapter aims to offer an overview of persister cells, including their development in response to food-associated stressors, their virulence and toxicity,
and host interaction. Additionally, we will discuss various approaches that are used for the detection of persister cells.

## 15.2 The Relationship Between Persister Formation and Food-Associated Stressors

The food processing techniques, such as thermal treatment, irradiation, cold storage, acid treatment, disinfection, alkali treatment, antimicrobial drugs, are generally used to reduce the microbial pathogenic burden (Davidson et al. 2013; Wang et al. 2016a; Esbelin et al. 2018). Nevertheless, in most of these cases, the processing techniques are lethal, and for those survivors, they may activate stress responses. The stress responses could assist the microorganisms to deal with the sub-lethal treatments (Fig. 15.1) or make them cross-adapt to other hostile stimulus. Moreover, the overall outcomes may lead some virulence genes to be upregulated under stressful conditions, giving rise to persister cells (Wesche et al. 2009; Dong and Schellhorn 2010; Alvarez-Ordóñez et al. 2015).

### 15.2.1 Biofilm Formation

Biofilm formation is one of the ecological phenotypes by which a bacteria persistently reside in food processing environments (Bridier et al. 2015). It was previously

![Fig. 15.1 Factors mediating the persister cell formation. Persistent bacterial cells can arise stochastically in unstressed bacterial populations. Bacterial persister can also arise due to various stress factors, such as nutrient depletion-related biofilm formation, acid, alkali, thermal stress, quorum sensing, and environmental stimuli. Additionally, bacteria–host interaction can also lead to persister cell formation. Adapted from Maisonneuve and Gerdes (2014)](image)
reported that the biofilm-forming capability of bacteria is positively correlated with its tolerance to common sanitizing techniques (Wang et al. 2016b). The variations of biofilm formation among different isolates have been described previously, indicating the progression of biofilm development linked to the variation in genetic backgrounds. One of the examples is the 1/2b and 1/2a serotype of L. monocytogenes isolates, which are commonly present in the environment of food processing industry. These isolates are found to be highly effective in forming biofilms at temperatures 20 °C, 30 °C, and 37 °C in nutrient-rich medium when compared to serotype 4b isolates which are more commonly associated with clinical cases (Kadam et al. 2013).

Many studies have investigated the biofilm-forming ability among food-associated microorganisms. Biofilm formation is triggered due to the presence of food constituents in the medium or because of numerous environmental situations of the food processing. Glucose is the biofilm formation factor of Aeromonas hydrophila. The level of glucose can influence the synthesis of acyl-homoserine lactone, a quorum-sensing molecule (Jahid et al. 2013). Another constituent milk lactose, which is suggested to increase the biofilm formation of Staphylococcus aureus by promoting the synthesis of intercellular polysaccharide adhesin protein (Xue et al. 2014). In Bacillus subtilis, LuxS-mediated quorum-sensing system plays an important role in the biofilm formation (Duanis-Assaf et al. 2018). Presence of certain minerals can influence the biofilm formation of bacteria. For example, Bacillus cereus tends to develop a biofilm on the surface of stainless steel rather than on polystyrene, this could be attributed to the higher iron availability (Hayrapetyan et al. 2015).

The hostile environmental conditions existing in certain processing units can lead to biofilm formation (Fig. 15.1). In order to reduce the microbial growth, organic acids, i.e., lactic, acetic, citric, and propionic acid, are used at a pH range of 4 to 6. Acid stress may inhibit microbial growth by enzyme inactivation, cell damage, and cell apoptosis (Cruz-Romero et al. 2013; Wang et al. 2015). Decrease in medium pH to 3.6 was found to enhance the biofilm formation of Alicyclobacillus acidoterrestris when incubated at 37 °C for up to 3 days. At a lower pH condition, the pathogen showed strong cell adhesion and confluent biofilm formation (Shemesh et al. 2013). The biofilm not only shields specific bacterial cells from various handling and sanitation agents, but also serve as a microorganism reservoir with higher resistance toward an extensive variety of antimicrobial agents or other hostile conditions.

### 15.2.2 Stress Response

Along the food processing units, the microorganisms are continuously exposed to various stress factors. These factors can be either intrinsic factors associated with food matrix or it can be the extrinsic factors that have been applied to preserve food (Ruiz et al. 2017). Bacteria may endure in the food processing unit for months or
years (Lundén et al. 2000). Acidifying agents and heat treatment are significant in the eradication of micro-organisms from food processing units (Lundén et al. 2000).

15.2.2.1 Persistence Formation in the Food Processing

It has been proposed that organisms which are capable of forming biofilm in response to food constituents or to environmental conditions during industrial processing are the ones that are most proficient of persisting in food processing conditions by colonizing surfaces and equipment. The common places in food processing unit where these pathogens are isolated and commonly forms biofilms in floor drains, metals, walls, packaging, conveyors, freezer, gloves, wood, food product, cooling pipes, etc. (Tompkin et al. 1999; González-Rivas et al. 2018).

The adherence of microorganisms depends on the physicochemical properties of the surface, such as texture, surface charge, pH, temperature, hydrophobicity, and nutrient composition (Srey et al. 2013). Bacterial attachment is an intricate communication between planktonic bacteria, surface-related bacteria, and the surface, which leads to colonization and persistence (Carter et al. 2016). Boyd et al. (2001) reported that with use, the contact surfaces deteriorate and their flaws can act as retention sites for microorganisms and organic matter. Previous reports suggest that biofilm formation of food-related microorganisms is affected by the presence of food components in the medium or as a function of different environmental conditions that prevail during food processing. Several simple carbohydrates such as glucose can influence the biofilm formation in bacteria, which can regulate acyl-homoserine lactone quorum-sensing molecules in *Aeromonas hydrophila* (Jahid et al. 2013), and milk lactose, enhances biofilm formation in *S. aureus* intracellular adhesion protein. Earlier findings suggest that *Salmonella* strains isolated from produce have a higher ability to survive on polystyrene, polycarbonate, and stainless steel surface and acquire specific abilities to colonize contact materials and foods in the produce supply chain (Patel et al. 2013). Likewise, thermophilic strains of *Geobacillus* spp. and *Anoxybacillus flavithermus*, initially isolated from milk powders, formed good biofilms on stainless steel only in the presence of skimmed milk, which suggests the high substrate dependence of these species for biofilm formation (Sadiq et al. 2017).

Damages of RNA or DNA, lead to changes in the protein synthesis, which can cause damage to the cell membrane (Begley and Hill 2015). The sigma factors are the common stress response genes in bacteria that are involved in dealing with various stresses, including high temperature, low temperature, acid, osmolarity, and oxidative stresses (Cebrián et al. 2009). The sigma factor σS (RpoS) and σB (SigB) are the stress responses in Gram-negative and Gram-positive bacteria, respectively (Boor 2006).

In acid-induced stress conditions for *Salmonella*, the activity of sigma E (σE) activity is high which in turn increases bacterial survivability in the acidic conditions in the host mammal and assists them to evade the host immune system (Crouch et al. 2005; Muller et al. 2009). Numerous reports indicate that *Salmonella* Typhimurium has a number of two-component virulence systems, such as EnvZ-OmpR and CpxR-
CpxA, which are activated during acid stress conditions. The EnvZ-OmpR system leads to upregulations of genes involved in type III secretion system and cell replication (Fass and Groisman 2009). The CpxR-CpxA system is an antimicrobial peptide regulon, which helps *Salmonella* in establishing itself in the gut which in turn leads to *Salmonella*-induced colitis and gut inflammation (Fujimoto et al. 2018). The *E. coli* on the other hand develops an acid resistance by activating a sigma factor known as RpoS (Barnett Foster 2013). The resistance to a low pH condition is mainly regulated by RpoS; arginine decarboxylase and its regulator, CysB; GAD and γ-aminobutyric acid antiporter regulatory system (Lim et al. 2010). Moreover, in acid-resistant *E. coli*, there is a change in adhesion and flagellar protein expression promoting their colonization in the gut (Barnett Foster 2013). Hence, the acid exposure during food storage, augment tolerance to low pH, thereby guaranteeing successful transit of bacteria through the stomach to establish a foodborne infection. Similarly, acid-tolerant bacteria from contaminated meat products lead to bacterial resistance to acids during food processing or preservation.

Hyperosmotic stress is extensively used in food processing to eliminate foodborne pathogenic bacteria. Mainly, NaCl, KCl, NaNO₃, or NaNO₂ are used for preserving food, since they exert osmotic stress on the food microbes. Osmosis is important for bacteria, which influences the growth of bacteria, turgor, and transport activities between the bacterial cells and the surrounding environment (Csonka 1989). Salt prevents bacterial growth by disturbing the balance of osmosis among the intracellular and cytoplasmic membrane (Wesche et al. 2009). However, some bacteria have developed strategies to survive under hyperosmotic stress. Some underlying mechanism includes exchange or accumulations of ions like potassium and glutamate, variations in the constituents of cell envelope and amassing of osmoprotectants such as trehalose (Csonka 1989). Osmotic stress may also improve the resistance of microbes to a variety of antimicrobial agents, prompt biofilm development, and lead to a persistent behavior (Capozzi et al. 2009; Poole 2012). For example, the survivability of *L. monocytogenes* strain Scott A inoculated in egg products containing 10% NaCl was augmented around six times, when compared to the same bacteria in egg products without NaCl (Bartlett and Hawke 1995). This could be attributed to the reduced water activity in the bacterial growth medium, which provides a protective effect (Shebuski et al. 2000). Acidity also influences the thermotolerance of bacteria, like *L. monocytogenes*, to adapt to acidic conditions in fruit juices, which was proven to be more resistant to ensuing heat treatment (Mazzotta 2001). Previous reports suggest that bacteria can resist high thermal treatment in stationery phase when compared to exponential state (Jørgensen et al. 1995).
15.3 The Molecular Regulation in Persister Cells

The targets of antimicrobials include proteins, enzymes, or nucleic acids in metabolically active bacteria, therefore antibiotics may not damage persister cells because they are in a metabolically inactive dormant state. This may lead to a higher drug tolerance and increase the amount of bacteria within a lag time (Vulin et al. 2018). Some studies have reported that the dormant phenotype is due to the downregulation of the factors associated with energy production, i.e., the decrease of ATP production. Once the cell division resumes in the persister cells, and their growth rate is similar to the bacteria in exponential stage. Higher ribosomal content usually leads to faster revival of persister cells (Lewis 2005). Consequently, coherent diversity occurs because of the variations in the physical association of parental DNA strands. One parental DNA strand can physically connect with proteins required for a survival strategy whereas the second DNA strand could be involved with proteins suitable for a growth tactic leading to coherent diversity (Gangwe Nana et al. 2018).

Various factors influence the persister formation, which include switching of phenotype of limited organisms to a state of dormancy, a sudden, and reversible change in gene transcription or protein expression of those bacterial cells (Kussell 2005). Various environmental factors are also reported to give rise to persister cells, including starvation, transition in carbon source, indole signalling, host macrophages, SOS (Save Our Souls) response, quorum sensing, and antibiotics (Dörr et al. 2010; Leung and Lévesque 2012; Bernier et al. 2013; Amato et al. 2013). Also, it has been reported that persister cells can occur independently in the presence of antibiotics and additional environmental signals, and antibiotics disclose the persister population by killing cells that are intolerant to drugs (Helaine and Kugelberg 2014).

15.3.1 Genomics of Persister Cells

In the initial stages of infection, pathogenic bacteria might be exposed to a number of environmental stresses, which involve nutrient depletion and oxidative stress. In order to endure these hostile conditions, few known persister-related genes and pathways are present in bacteria. During nutrient limitation bacteria usually switch their gene expression profile from fast growth to a survival state by alarmone, a linear nucleotide guanosine tetraphosphate and pentaphosphate [(p)ppGpp], to regulate gene expression from replication and growth state to establishment and survival state (Nazir and Harinarayanan 2016). The whole process is known as the response to stringent conditions (Dalebroux and Swanson 2012). The synthesis of (p)ppGpp is generally ruled by two canonical enzymes RelA/SpoT (Wendrich et al. 2002; Potrykus and Cashel 2008). The (p) ppGpp biosynthesis usually occurs because of RelA/SpoT homologue proteins (RSH). The (p) ppGpp, can directly bind to a small RNA polymerase binding protein DksA, and then destabilize its open
complex (Haugen et al. 2008; Sanchez-Vazquez et al. 2019). On the other hand, (p) ppGpp indirectly regulates the gene expression of sigma factor σ70, which leads to the interaction of free RNA polymerases with other sigma factors, such as σ54, to activate stress response genes (Jishage et al. 2002; Potrykus and Cashel 2008). Another mode of action of (p) ppGpp is through the direct down-regulation of stable RNA and genes involved in transcription and translation, whereas it upregulates the genes involved in amino acid biosynthesis (Chiaramello and Zyskind 1990; Hernandez and Bremer 1993; Jishage et al. 2002). The ppGpp regulated bacterial persistence in E. coli is provided in Fig. 15.2. In bacteria, the PhoU, the fifth gene in the pstSCAB-phoU operon is involved in phosphate metabolism (bacterial Pi or inorganic Phosphate). It is a global negative regulator, which is essential for the development of persister cells.

In E. coli, the adenine methylase (Δdam) or Dam plays a chief role in the formation of persister cells (Stephenson and Brown 2016). The Δdam controls gene transcription, cell motility, DNA repairment, and metabolite transport processes. Therefore, Δdam methylation is a potential therapeutic target (Xu et al. 2021). Bojer et al. (2010) reported that clpK gene was involved in the persistence of Klebsiella pneumoniae, an important opportunistic pathogen that could cause a number of serious infections in intensive care units. Klebsiella isolate is relatively tolerant to heat due to which, it is persistent in the hospital environment. This tolerance is mainly due to an ATPase gene known as ClpK, which is encoded by a conjugative plasmid. In bacteria like E. coli, the genes involved in SOS response have been found upregulated during persistence (e.g., uvrA and uvrB) (Rossi et al. 2018). Various heat shock proteins, chiefly proteases (lon) and chaperons (dnaK, dnaJ, and clpB), are associated with persistence; deletion of those genes decreases persistence (Sendi and Proctor 2009; Hardt 2016; Geddes-McAlister et al. 2019) (Table 15.1).

### 15.3.2 Proteomics of Persister Cells

The persistence is a phenotypic state rather than a genetic change, therefore the proteomics is the most appropriate method to study persister cells. The proteomics helps us to understand the posttranslational modifications that are not understood during genomics analysis. This may help in understanding the mechanism for bacterial persistence. The LC-MS/MS (liquid chromatography/mass spectrometry) combined with two dimension-differential gel electrophoresis are common proteomics approaches used for studying persister cells. The data-independent acquisition strategy called Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra (SWATH-MS) has been recently used for understanding persistent mechanisms (Albrethsen et al. 2013). Factors such as availability of nutrients, concentration of oxygen, density of cell, and antibiotic concentration control the expression of certain proteins in the biofilm. For example, the protein adhesin and
the type of biofilm decide the rate by which the bacteria in the biofilm enter the state of persistence, leading to the antibiotic tolerance of the biofilm (Waters et al. 2016).

In a study by Albrethsen et al. (2013), the changes of *M. tuberculosis*’s proteome were determined using LC-MS/MS and two-dimensional differential gel electrophoresis. Prior to the analysis, cells were starved for around six weeks in order to mimic the host environmental stress. The study demonstrated that the toxin-antitoxin

![Fig. 15.2](image-url)
system were produced to a high level when cells were in the non-replicating state, resulting in the inhibition of protein synthesis (Buts et al. 2005). In another study, Schubert et al. (2015) used a data-independent acquisition strategy called SWATH-MS to understand the proteomic change of *M. tuberculosis*. The study revealed that regulon protein DosR was necessary for the persistence of *M. tuberculosis*. In a study by Li et al. (2015), LC-MS/MS analysis of *Candida albicans* biofilms treated by amphotericin B for 24 h, showed that there was a downregulation of the protein involved in various metabolic enzymes. This also included the once taking part in ergosterol biosynthesis—which is an azole drug class target. The result showed that persister cells had a downregulation of proteins associated with glycolysis, protein synthesis, and tricarboxylic acid cycle.

The phosphorylation of serine/threonine protein kinases is considered to play a critical role in the shift of *M. tuberculosis* lifestyle to persister cells. Prisic et al. (2010) compared the phosphoproteins among mycobacteria which were cultured using diverse carbon sources, at various phases of growth, and exposed it to host-related stressors, such as oxygen depletion, NO, and H$_2$O$_2$. They identified nearly 301 phosphoproteins in *M. tuberculosis* H37Rv which had a minimum of 516 phosphosites (Prisic et al. 2010). The phosphorylation happened on around 60% of threonine and 40% of serine. These phosphoproteins were mostly associated with cell wall and cell processes, and also highly enriched in the cellular metabolism pathway. In another study, two replicates of H37Ra and H37Rv were recovered at logarithmic and stationary phases. Out of 2709 proteins, around 257 proteins were found to be phosphorylated (Verma et al. 2017). Within these 257 proteins, 94 proteins were differentially expressed in stationary and log phases that were responsible for the persistent phenotype of bacteria. The expression of PhoU protein is mainly controlled by the changes in the availability of nutrients and age of culture, and it regulates the metabolic pathways of bacteria leading to persister formation (Li and Zhang 2007).

In low oxygen and nutrient-limited conditions, *M. tuberculosis* shifts from replicating state to non-replicating persistent state. The triacylglycerols and wax

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**Table 15.1** Major genes/proteins involved in persistence of bacteria

<table>
<thead>
<tr>
<th>Gene/protein</th>
<th>Major function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>uvrA</em>, <em>uvrB</em></td>
<td>SOS response</td>
<td>Rossi et al. (2018)</td>
</tr>
<tr>
<td><em>phoU</em></td>
<td>Global regulator</td>
<td>Li and Zhang (2007)</td>
</tr>
<tr>
<td>Δ<em>dam</em></td>
<td>Gene transcription, cell motility and DNA repair</td>
<td>Stephenson and Brown (2016)</td>
</tr>
<tr>
<td>ClpK</td>
<td>ATPase</td>
<td>Bojer et al. (2010)</td>
</tr>
<tr>
<td>RelA</td>
<td>Stringent response</td>
<td>Wendrich et al. (2002), Potrykus and Cashel (2008)</td>
</tr>
</tbody>
</table>
esters are the storage molecules for *M. tuberculosis*, through non-replicating persistence, reactivation and the use of triacylglycerols produces more energy when compared to other storage compounds like glycogen (Alvarez 2016). In oxidative stress, nutrient depletion, and NO treatment, various triacylglycerol synthase-like Tgs1 are activated in *M. tuberculosis* whereas the transition happens to a non-replicating state (Daniel et al. 2004). When exposed to high oxygen stress, the growth rate of *M. tuberculosis* is limited by shifting the carbon usage away from the tricarboxylic acid cycle (Baek et al. 2011). The amassing of wax esters also reduces the growth of pathogens by preventing nutrient uptake in the stress conditions. These triacylglycerol and wax esters tend to collect in the envelope of cells and they are exported by mycobacterial membrane protein large, MmpL11 (Wright et al. 2017).

15.4 Detection Methods of Persistent Foodborne Pathogen Cells

Culture-based and culture-independent methods, mainly nucleic acid- and phage-based approaches, have been used to identify the persistent behavior of bacteria in food.

15.4.1 Culture-Based Methods

Culture-based techniques are the “gold standard” for detecting bacteria in food. This method is extremely simple, selective, sensitive, very useful, and easily adaptable (Taskila et al. 2012). Conventional culturing technique mainly involves the capability of bacteria to grow and multiply on the laboratory media. However, this method is a very slow process and the bacteria can be damaged during handling procedures or in the processing like freezing, irradiation, refrigeration, drying, heat processing, low water activity, acidity, and starvation (Gracias and McKillip 2004; Shintani 2006). In order to overcome this, a non-specific pre-enrichment step is needed as the preliminary analysis. The following steps are required which include plating on selective media, and confirmation using in vitro biochemical methods (Splittstoesser and Vanderzant 1992; Bhunia 2014). The whole culture-based approaches require around 2–3 days from initial isolation to the final validation of typical easy-handled bacteria (Zhao et al. 2014).

The accuracy of culturing techniques varies due to the nonuniform distribution and presence of relatively lower amount of pathogens in the food sample, the heterogeneity of food matrices, and the presence of native bacteria which then obstruct the recovery of certain pathogens (Mandal et al. 2011). Injuries to bacteria or bacteria in viable but non-culturable (VBNC) state may also lead to inaccurate detection. Hence, the plate culturing technique is not fully reliable because it does not give the actual idea of the exact microbial population in the natural ecosystems.
15.4.2 Culture-Independent Methods

Nucleic acid and bacteriophage-based recognition techniques are the two major culture-independent methods used for detecting viable foodborne pathogens.

15.4.2.1 Nucleic Acid-Based Methods

To reduce the risk associated with foodborne outbreaks, accurate detection approach for viable bacteria is essential. The nucleic acid-based technique recognizes DNA or RNA sequences of target pathogenic microorganisms. Techniques, such as polymerase chain reaction (PCR) assays, are fast, and sensitive. The drawbacks of these methods include the overestimation of the level of viable cells due to false-positive amplification of DNA derived from dead cells or from environment (Rudi et al. 2005; Priyanka et al. 2016). Reverse-transcriptase PCR (RT-PCR) technique is another approach to detect viable foodborne pathogens. The principle of RT-PCR is that, mRNA of bacteria is susceptible to breakdown by intra- and extra-cellular RNases; and cell death can lead to a fast reduction of the level of mRNA indicating that mRNA can only be possessed by viable cells. Nevertheless, RT-PCR based method has disadvantages, such as lack of internal housekeeping genes as the expression profiles might vary in stress conditions. In addition, due to the greater susceptibility of RNA to contamination, the handling of RNA can be tiresome. Hence, RT-PCR is not an ideal approach for the detection of foodborne pathogenic bacteria (Barbau-Piednoir et al. 2015).

To overcome these drawbacks, cell viability dyes combined with viability PCR are used (Rudi et al. 2005). These detection assays are usually conducted by using ethidium monoazide (EMA) or propidium monoazide (PMA) dyes. These dyes have positive charge and they cannot enter a negatively charged bacterial cell wall, however, it can enter bacteria with damaged cell wall/membranes (Nocker et al. 2009). Inside cell, the azide group of dyes can intercalate with the nucleic acids after exposure to strong visible light. The light exposure will generate nitrene radical, which in turn binds to components in the near proximity such as DNA, which inhibits the amplification of DNA (Nocker et al. 2009). The two dyes act as intercalating stains, but their permeation properties are different. Compared to EMA, PMA is more effective in differentiating between live and dead cells. The type and concentration of dye, the type of organism, food matrices, ratio of dead and viable cells, the length of PCR amplicon, and light exposure conditions, all together affect the effectiveness of the viability detection.

15.4.2.2 Bacteriophage-Based Methods

In the past few years, bacteriophage has gained a lot of interest because of its specificity and sensitivity toward the targeted microbes (Koskella and Meaden 2015).
Bacteriophage or phage has the natural capability to precisely identify a certain strain of bacteria via its tail attachment. Among the 6300 phages discovered so far, 6196 belong to the group in recognition of bacteria (Ackermann and Prangishvili 2012). Within a bacterial cell, the bacteriophages display two different life cycles, one is lytic cycle and another is lysogenic cycle (Jamal et al. 2019). In the lytic cycle, they initially bind to the host bacterial cell and then insert their nucleic acid. They then gain control of the host machinery and start to replicate their genome, and initiate the production of their progenies (Garretto et al. 2019). In the lysogenic cycle, the genome of phage is divided as a part of the host genome and is vertically transmitted until the lytic cycle is prompted. The nutrient environment around the host, type of phage and state of bacteria decides the released viral particles and burst size (Ofir and Sorek 2018). Peng et al. (2014) developed an in vitro method involving a bioluminescent system, luciferase-flavin mononucleotide, nicotinamide adenine dinucleotide oxidoreductase combined with lysis of bacteriophages within bacteria for the detection of Vibrio parahaemolyticus. This system detected more than $10^7$ CFU/mL bacteria in pure culture and more than $10^8$ CFU/mL bacteria in oyster samples without pre-enrichment. After 4 h of enrichment, more than 100 CFU/mL bacteria in oyster samples could be detected.

Bacterial lysis will release various cellular components such as ATP and β-galactosidase that can be detected through bioluminescent assays using luciferase/luciferin enzyme (Wilson et al. 2017). The overall quantity of ATP will be far lower compared to the total metabolized ATP, which is released and this can be measured by total number of viable cells (Li et al. 2013). In a study, a T4 bacteriophage and recombinant T4 bacteriophages having biotin-binding peptide and cellulose-binding module were immobilized on nano-aluminum fiber-based filter (Disruptor™), streptavidin magnetic beads, and microcrystalline cellulose, respectively. This reduced the infectivity of bioluminescent *E. coli* and cell lysis confirmed by a test involving bioluminescent ATP. Bacterial cell counts, as low as $6 \times 10^3$ CFU/mL of *E. coli*, were detected in the sample within a short period (2 h) with high accuracy (CV = 1–5% in log scale). Tilton et al. (2019) developed a biosensor-based nanophotonic equipment using T7 bacteriophage to sense the release of β-galactosidase from *E. coli*. A nanophotonic device consisting of fluorescent enzyme substrate resorufin β-D-galactopyranoside was used for the detection. The device could detect *E. coli* BL21 as low as 10 CFU/mL from simulated spinach wash water within 8 h (Minikh et al. 2010).

Bacterial lysis triggered by bacteriophage will also result in the discharge of progeny, which can be served as an approach to identify bacteria (Haaber et al. 2016). The qPCR method is used to directly augment and identify the progeny nucleic acids of bacteriophage after proliferation. The qPCR and bacteriophages were combined for detecting *E. coli* O157:H7, *Mycobacterium avium*, and *Acinetobacter baumannii* (Botsaris et al. 2013; Luo et al. 2018). A detection method to sense viable *Salmonella* Enteritidis in chicken samples centered on the amplification of *Salmonella* bacteriophage vB_SenS_PVP-SE2, coupled with real-time PCR (qPCR) was established by Garrido-Maestu et al. (2019). This method could
detect around $10^3$ pfu/mL of viral particles and 0.22 fg/μL of pure virus DNA within 10 h.

Engineered phage-based biosensors can detect pathogens that are quantitative, rapid, and sensitive. The effectiveness of recognition technique depends on the type of biosensing elements that can specifically bind to the target pathogen. The usual sensing agents are antibodies, but they are relatively high in cost and are unstable in harsh conditions when used in biosensor platforms. In the past few years, bacteriophage has been used as a biosensing agent to detect the pathogen. The major advantages of bacteriophage as biosensing agent include robustness, high stability under high temperature and chemical treatment, high specificity, and easily being immobilized on the surface of transducer for various sensing platforms (Wang et al. 2017; Mack et al. 2017). An M13 bacteriophage-based electrode system for the detection of Salmonella spp. was developed by Niyomdecha et al. (2018). The system constituted of bacteriophage immobilized on a polytyramine/gold surface using a crosslinker glutaraldehyde. The biosensor can detect a range from $2 \times 10^2$ to $1.0 \times 10^7$ CFU/mL of bacteria within 40 min.

15.5 The Virulence of Persistent Foodborne Pathogens

Multiple mechanisms are involved in the emergence of persistent phenotype as an adaptive trait (Cabral et al. 2018). The modes of action of persister cells are conserved with respect to the function of genes and pathways involved among various bacterial species (Cui et al. 2016). Moreover, the mechanism of persister formation between Gram-negative and Gram-positive bacteria is different. Kim et al. (2018) reported that, the antibiotic corrupting proteins in persister cells are relatively low in concentration and that their restoration is heterogeneous which was demonstrated by (i) cell division that occurs immediately, (ii) followed by cell elongation, (iii) immediate cell elongation without division, (iv) delayed cell elongation/division, and (v) existence in viable but non-culturable forms where there is no growth of bacterial cells. After the removal of antibiotics, for example, ofloxacin, persister cells elongate and attain a maximum SOS induction. Ultimately, the cell division is resumed which gives rise to daughter cells (Goormaghtigh and Van Melderen 2019).

15.5.1 Toxin-Antitoxin System

Two genes in an operon encode for stable toxin-antitoxin systems. The toxin system that generally involves a mechanism that interrupts an important cellular process such as growth inhibition via mRNA degradation. Antitoxin can be a RNA or protein that prevents toxicity (Van Melder and Saavedra De Bast 2009). There are around six different toxin-antitoxin types, among which the type II system is well recognized as the one leading to the formation of persister cells. Compared to the
non-persister cells, the type II toxins, chiefly the ones that show RNase activity are upregulated extremely in the persister cells indicating their role in leading to dormancy (Keren et al. 2004; Shah et al. 2006).

The HipB-HipA is an important type II toxin-antitoxin system associated with the persister formation in *E. coli*. The mode of action of this system involves the growth inhibition of *E. coli* cells by phosphorylation of GltX toxin component by HipA in hipBA locus leading to inactivation of translational factor EF-Tu (Schumacher et al. 2009). This will result in the replacement of G22S and D291A amino acids in HipA toxin, leading to higher toxicity because of poor contact with the antitoxin, during the development of persistence. Moreover, there was an increase of persister cell fraction by about 1000-folds due to the overexpression of HipA (Korch and Hill 2006). It was also observed that the toxin-antitoxin-based persistence elevated the amount of (p)ppGpp in some cells as a result of the antitoxin proteolysis (Hansen et al. 2012).

Few studies have reported that the toxin-antitoxin systems can improve the lethality of antibiotics against bacteria, such as *Bacillus subtilis* and *Streptococcus pneumoniae* (Chan et al. 2012; Durand et al. 2012). In a study conducted with *Salmonella*, deletion of twelve diverse single toxin-antitoxin systems caused a reduction of macrophage-induced non-replicating bacteria and persister (Helaine et al. 2014). *Salmonella* toxin TacT, work by acetylating aminoacyl tRNAs, while hydrolysis of corrupted tRNA by peptidyl-tRNA hydrolase Pth was suggested to revive persister cells (Cheverton et al. 2016). A study of *Burkholderia pseudomallei* proposed that single TA deletions caused no effect in the examined tube-grown bacterial cultures, but reduced their viability in macrophages when treated with antibiotics (Ross et al. 2019). This variation in test outcome could be due to the differences in experimental conditions. The use of precise trial methods could overcome these drawbacks (Kaldalu et al. 2016; Levin-Reisman and Balaban 2016; Harms et al. 2017). Type I toxin-antitoxin system has also been associated with persistence. The type I toxin systems are, mainly, small proteins that caused cell membrane damage and the reduction of ATP. The antisense sRNA antitoxins inhibit the translation of these toxins (Berghoff and Wagner 2017). In *E. coli*, TisB/IstR-1 is one of the Type I toxin-antitoxin systems, that integrate into the SOS regulon and is triggered to augment persistence by fluoroquinolone (Dörr et al. 2009, 2010; Berghoff et al. 2017). DNA damage induces the promoter of *tisB* toxin gene which is suppressed by the regulator LexA (Dörr et al. 2010). This gives rise to polarized and depolarized subpopulation of bacterial cells (Berghoff et al. 2017). The deletion of *tisB* may reduce the persistence to fluoroquinolone (Dörr et al. 2010), whereas the regulatory RNA sequences deletion lead to an extremely persistent (hip) strain (Berghoff et al. 2017). During the higher production of GTPase Obg in *E. coli*, a toxin, HokB (belonging to the *hokB-sokB* type I system), the bacteria forms pores, which improve the survival against antibiotic treatment (Verstraeten et al. 2015). However, in a reverse scenario, the phenotypic reversal is achieved by HokB monomerization and membrane repolarization (Wilmaerts et al. 2019). The role of TisB/IstR-1-like systems for the persistence of pathogenic bacteria during infection is still unclear.
In *Salmonella* Typhimurium, the Phd/Doc and Tac toxin-antitoxin system produced multidrug-resistant *Salmonella* Typhimurium persist after being activated by nutrient-deprived environment in the vacuoles (Cheverton et al. 2016). Another study suggested type II toxin-antitoxin system involving toxin (Hha) and antitoxin (TomB), which plays a crucial role in the formation of persister cells by preventing the apoptosis of *S*. Typhimurium (Jaiswal et al. 2016). The toxin-antitoxin also has a crucial role in facilitating persistence of oral pathogens, i.e., *Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans*. *S*. *mutans* is a common pathogen in oral cavity which forms biofilm and causes major destruction to the hard tissue of teeth (Krzyściak et al. 2014). Within the *S. mutans* UA159 genome, a gene coding MazEF-like toxin-antitoxin was recognized, which may exemplify as cell growth modulator permitting the *S. mutans* persistence under hostile conditions (Syed et al. 2011). A recent study established that 11 types of operons coding the type II toxin-antitoxin system out of which two RelBE-like systems were known to be triggered in low pH situations and which suppress the translation of ribosome-associated mRNA leading to persistence phenotype for bacteria in the dental biofilm (Schneider et al. 2018).

### 15.5.2 Bacterial Persistence and Host Interactions

Various pathogenic microorganisms, i.e., *E. coli*, *Salmonella enterica*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, and, *Staphylococcus aureus*, are involved in persistent infections (Grant and Hung 2013). In most cases, the reasons are due to unsuccessful clearance of the pathogen from host due to deficiency of host immune system, or bacterial subversion or evasion (Monack et al. 2004; Fisher et al. 2017). Even after the treatments with antimicrobials, a considerable subpopulation of these pathogens endure in the tissue. Nevertheless, whether those survived bacteria are actual persister or not remains unclear.

Antimicrobial treatment to patients suffering from *M. tuberculosis* infection will lead to population with distinctive resistant characteristics that progress throughout the entire infection period (Meacci et al. 2005; Horsburgh et al. 2015). The higher persistence during antimicrobial treatment could be due to the stress-induced noise in RNA expression depending on the nutrient depletion and metabolically active bacterial subpopulations (Wakamoto et al. 2013; Manina et al. 2015). *S*. Typhimurium cells that were involved in persistent infection were suggested to reside in the granulomas of M2-like macrophages of spleen. The interaction between *S*. Typhimurium and macrophage generates considerable heterogeneity in both bacterial and host cells that will help the survival of pathogens throughout the antimicrobial therapy (Helaine et al. 2014; Stapels et al. 2018). Meanwhile, *S*. Typhi can form gallstone-accompanied biofilms that act as a carrier of persistent bacteria leading to amplified transmission over long periods of time. The persistence of *Pseudomonas aeruginosa* is mostly involved with the biofilms formation during cystic fibrosis (Moreau-Marquis et al. 2008). This is possibly related to a variety of
signalling molecules and stress signals produced within and exchanged between bacterial cells within a biofilm, such as (p)ppGpp in the stringent response and mediators of the SOS response (Spoering and Lewis 2001; Harms et al. 2016). Persistence also exists in *S. aureus* which leads to recurrent infections, especially osteomyelitis which are hard to eliminate using antimicrobials (Sendi and Proctor 2009; Olson and Horswill 2013). The reasons for the formation of persistent cells are usually due to poor penetration into biofilm and formation of small colony variants (Kahl et al. 2016). These colony variants arise, due to long lag time, are antimicrobial resistant and can resist the host immune system, leading to *S. aureus* persistence in clinics (Sendi and Proctor 2009; Olson and Horswill 2013).

### 15.5.3 Persistence in Intestinal Microflora

Gastro intestine has harsh environmental conditions, such as existence of pancreatic enzymes, acidic conditions, temperature conditions, anaerobic conditions, and competition among bacteria (Bernardeau et al. 2017). The virulence factors of bacteria play a chief role during persistence infection and can produce tolerant subpopulations (Bakkeren et al. 2020).

The pathogen–host interaction may produce stress and can cause selection for persistence (to evade the immune response). For example, in *S. Typhimurium*, the evolution of virulence has caused selection for better survival during antibiotic therapy. The virulence factors of *S. Typhimurium* *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2 islands affects the pathogen–host interaction in two different processes (Fookes et al. 2011; Desai et al. 2013; Diard and Hardt 2017). First, the SPI-1 and SPI-2 help the pathogen to stimulate mucosal inflammation to enhance gut-luminal blooms and speed up transmission (Diard et al. 2014). Second, these virulence factors enable *Salmonella* spp. in intracellular growth, which incidentally enables the persistence formation (Helaine et al. 2010). Moreover, the host factor, SLC11a1, that limits systemic growth of pathogens like *M. tuberculosis* and *S. Typhimurium* by reducing local Mg$^{2+}$ availability, produces heterogeneous bacterial growth rates linked with heterogeneous gene expression patterns.

Additionally, *S. Typhimurium* uses defensive virulence factors, like superoxide dismutase (SOD) to tackle the host cellular responses and thereby battle these defenses (Chakravortty et al. 2005; Yu et al. 2010). The antioxidant defense enzymes such as superoxide dismutase (SOD) and catalase or compounds such as glutathione and vitamin C, can eradicate ROS (Reactive oxygen species). This ROS includes hydrogen peroxide [H$_2$O$_2$], superoxide [O$_2^-_{}$], and hydroxyl radical [OH$^-_{}$]. Under various environmental stresses, such as UV, thermal or antimicrobial treatment, the level of ROS can increase. The rise of ROS can in turn lead to destruction of DNA, lipids, and proteins that will ultimately lead to apoptosis (Liu and Imlay 2013). The SOD and catalase are found to exhibit a defensive activity in *E. coli* (Van den Bergh et al. 2017).
Methicillin-resistant *S. aureus* (MRSA) the persister formation is associated with metabolic modification of tricarboxylic acid (TCA) cycle during ROS formation (Rosato et al. 2014). In *Vibrio cholerae*, two catalase enzymes KatB and KatG and the transcriptional regulator OxyR have been reported to contribute to ROS homeostasis (Goulart et al. 2016). Also, the cholix toxin in mediating ROS response also acts as a virulence factor which leads to ADP-ribosyltransferase action on the eukaryotic elongation factor 2 (EF-II) of host, resulting in eukaryotic cell death (Ogura et al. 2017).

### 15.6 Conclusions

Since the discovery of bacterial persister cells, the persister formation has been the topic of intense investigation for about 70 years. Persistent bacterial infections have led to increased morbidity and mortality worldwide. Persister cells are bacterial cells that are phenotypically tolerant to antimicrobials. This phenotype is always associated with the re-occurrence of bacterial infections that are persistent, and result in dissemination of antimicrobial resistance. Persistent foodborne pathogens occurring at the primary and secondary food processing units are a significant risk to public health. In the worst scenario of increasing numbers of immunocompromised patients and global aging population, these recurrent infections may become highly dominant in the coming future. An improved understanding of the intrinsic factors that regulate the development of persistent phenotypes and their re-occurrence may guide novel interventions and control strategies against bacterial infections.

### References


Chapter 16
Biofilm Formation of Food-Borne Pathogens

Junyan Liu, Yue Gu, and Zhenbo Xu

Abstract The pathogenic microbial biofilm is gaining increasing concern in the food safety field. Biofilms may exist in food raw materials, food processing equipment as well as food products and packages during transportation and storage. Since the microbial biofilms have strong survivability in various pressure conditions (pH, temperature, ultraviolet, antibiotics, disinfectants, etc.) in food processing, they become a nonnegligible source of foodborne diseases, thus have potential threat to human health. Here, we focus on discussing the general phenomenon of food-associated pathogen biofilms, the survival mechanism of biofilms under various stimuli in food processing, commonly used detection methods, and the characteristic of virulence of biofilm.

Keywords Biofilm · Food · Microorganism · Stimulus · Virulence

16.1 Introduction

In the seventeenth century, biofilm was observed for the first time by a Dutch scientist Anthony van Leeuwenhoek, who scraped plaque from his teeth and named the accumulation of moving tiny bodies noticed by his homemade microscope with “animalcules” (Donlan and Costerton 2002). In the 1970s, Costerton et al. (1978) first proposed the conception of “biofilm.” It is now acknowledged that biofilm is a self-protection state when microorganisms grow on the surface of an object (Yin et al. 2019). It is not a simple accumulation of bacteria or fungus, but a complex membrane-like substance formed by the interaction of microorganism cells and the secreted extracellular polymeric substances (EPS), that contribute to retain the moisture of the biofilm and protect the cells in the biofilm from the toxic substances, such as antibiotics (Cortés et al. 2011; Donlan 2002; Flemming and
Wingender 2010; Hurlow et al. 2015; Sutherland 2001). In fact, biofilms are ubiquitous in nature, and over 90% microorganisms exist in the form of biofilm (Costerton et al. 1999; Donlan and Costerton 2002; Shirtliff et al. 2002).

Biofilms have been considered as one of the potential problems to food safety and public health (Jahid and Ha 2012). In the food industry, the processing equipment and pipelines that contact with food for a long time can accumulate nutrients such as carbohydrates and proteins, which are required for the bacterial growth. Bacteria can adhere to raw food materials, factory floors, pipe bends, rubber seals, conveyor belts, stainless steel, and other surface to form biofilms, which are difficult to be completely removed or inhibited even using high-pressure water guns, disinfectants, or some other physical and chemical methods to clean the factory environment and equipment regularly (Chmielewski and Frank 2003, 2006; Mattila-Sandholm and Wirtanen 1992; Saitou et al. 1992; Simões et al. 2010). Biofilms not only corrode food processing equipment and increase the difficulty of equipment cleaning, but also have the potential to cause food spoilage and foodborne diseases (Jahid and Ha 2012). In the drinking water systems, bacterial biofilm attaching to distribution pipes diffuse into the tap water distribution system under the force of the water flow, which poses potential risks for human health (Ashbolt 2004; Flemming et al. 2018; Helmi et al. 2008). In the medical field, biofilm is a key cause of bacterial and chronic infection with about 65% to 80% of bacterial infections caused by biofilms (Jamal et al. 2018; Van Acker et al. 2014). They are associated with cystic fibrosis pneumonia, chronic otitis media, periodontitis, chronic sinusitis, osteomyelitis, recurrent urinary tract infections and other chronic diseases (Foreman et al. 2012; Fallah et al. 2017; Brady et al. 2008; Costerton 2001). Some biofilm infections are attributed from the contaminated medical equipment, including central venous catheters, artificial heart valves, and joint prostheses (Stewart and Costerton 2001; Hall-Stoodley and Stoodley 2009). Antibiotic resistance is one of the important characteristics of biofilm (Gebreyohannes et al. 2019). In vitro experiments have shown that Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus biofilm can still survive at hundreds or even a thousand times the minimum inhibitory concentration (MIC) of planktonic cells (Ceri et al. 1999). Besides, immune defense is another important feature of biofilm (Vuong et al. 2004). For example, white blood cells cannot penetrate the bacteria encapsulated in EPS and thus do not induce immune activity (Leid et al. 2002). Due to the above characteristics, infectious biofilm may persist in the human body for months or even years, causing multiple recurrences of inflammation (Marrie et al. 1982).

Compared with planktonic bacteria, the morphological structure and physiological characteristics of biofilms render them with stronger survival ability (Lewis 2008). The vitality is the prerequisite for bacteria to cause disease and food spoilage. The formation of biofilms and the associated stress response in the food system is a complicated process. Even if there is still no uniform standard and system for the detection of biofilms (Percival et al. 2015). Researchers can still characterize the biofilm from various aspects, such as morphology, matrix, multiple omics, and so on. This chapter provides a systematically review of the biofilm formation, biofilms
in response to stimuli and underlying molecular mechanism, biofilm detection methods, and the virulence of biofilm in food-associated systems.

16.2 Biofilm Formation under Food-Associated Stressors

16.2.1 Staging of Biofilm Formation

The formation of biofilm is a dynamic process, which mainly includes three stages (Fig. 16.1): the initial adhesion, maturation, and the late detachment stages (Bjarnsholt et al. 2013). It is mainly affected by the bacterial characteristics, the host surface properties, and the environmental factors.

At the initial stage, the planktonic bacteria adhere to the surface of an object. The adhesion strength between cell and the object depends on the chemical compositions and structural characteristics of the surface. Cells loosely adhere to the surface of the object. At this time, they are mainly affected by non-specific forces such as hydrophobic force, surface charge, and critical tension, which is a reversible adhesion process (Hall-Stoodley et al. 2004; Emily et al. 2003). For some species with flagella, such as *Bacillus subtilis, Listeria monocytogenes,* and *Pseudomonas aeruginosa,* the flagella can effectively promote the movement of planktonic bacteria to the surface, thereby contributing to the initial adhesion of cells (Kobayashi 2007; Lemon et al. 2007; O’Toole and Kolter 1998). For Gram-negative bacteria, the fimbriae assist the bacteria to slowly twitch on the surface, leading to the bacterial aggregation and position adjustment on the surface (Bradley 1980; O’Toole et al.)

![Fig. 16.1 Schematic illustration of the biofilm formation stages and contributory factors](image)
When bacteria begin to secrete specific adhesins, which usually occurs on living tissues, the adherence becomes irreversible (Ryu and Beuchat 2005). Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) is one kind of the adhesins that can help to specifically adhere to tissue surface proteins (Vazquez et al. 2011; Paterson and Orihuela 2010). For example, in S. aureus, Cna (collagen binding protein) can bind to collagen, and FnAB can bind to fibronectin (Foster et al. 2014; Kang et al. 2013). As more bacterial cells replicate and adhere to each other, large colonies are subsequently formed under the effect of intercellular adhesion (Armbruster and Parsek 2018).

The second step of biofilm formation is known as the maturation stage (Bjarnsholt et al. 2013). Bacterial cells continue to proliferate and adhere to each other. Specific genes are stimulated to express and the secretion of a large amount of EPS by the bacterial cells plays a key role for Staphylococcus in this stage (Kot et al. 2018). Multiple microcolonies contact and fuse with each other and grow upwards, gradually forming a mushroom-like structure (Klausen et al. 2003). Generally, it is recognized that the polysaccharide intercellular adhesion (PIA) or polymeric N-acetyl-glucosamine (PNAG) regulated by the icaABD operon contributing to form the polysaccharide-type biofilm membrane protein, which can be activated by the icaD regulatory product to produce N-acetylglucosamine oligomers. The expression of icaC can extend the oligomer chain and participate in the transportation of polysaccharides to the cell surface. The product of icaB gene is a protein attached to the cell surface, which can deacetylate poly N-acetylglucosamine molecules (O’Gara 2007). Studies have shown that the stimulation of the external environment by NaCl or ethanol can activate the sigB factor, which increases the PIA synthesis and biofilm formation of Staphylococcus epidermidis 1457 (Knobloch et al. 2001). However, more and more studies have shown that Staphylococcus can form protein-type biofilms depending on the surface proteins such as accumulation-associated protein (Aap) and Staphylococcal protein A(Spa) rather than polysaccharides produced by the ica regulatory system (Figueiredo et al. 2017; Pozzi et al. 2012; Rohde et al. 2007; Kogan et al. 2006). So we can point out that the same species has alternative biofilm formation regulation mechanisms. In addition, the regulation mechanisms for different species are different (Seneviratne et al. 2008; Sharma et al. 2016). For E. coli, the autotransporter Antigen 43 (Ag43) encoded by the flu gene helps to promote cell adhesion. The composition of its extracellular polysaccharide is different from that of Staphylococcus, mainly including b-1,6-N-acetyl-D-glucosamine polymer (PGA), cellulose and colanic acid (Sharma et al. 2016). Among them, PGA is regulated by the pgaABCD system (Itoh et al. 2008). In Candida parapsilosis biofilm, high concentration of glucose can induce the up-regulation of FKS1 and promote the secretion of b-1,3-glucan which is an essential component of extracellular matrix (Araujo et al. 2017). In addition, cell wall proteins Als1, Als3, and Hwp1 also play an important role in promoting the adhesion between Candida cells and the maturation of the biofilm (Henriques et al. 2006; Nobile et al. 2006). The mature biofilm is an uneven three-dimensional
network structure. There are transport channels in the biofilm, which can transport nutrients and oxygen and output metabolic waste (Sauer et al. 2004).

The third step is known as the post-mature stage. Theoretically, biofilm can continue to grow under a suitable condition. In the actual situation, biofilm is not always conducive to the survival of cells. The growth of biofilm enlarges the gradient of nutrients, oxygen, osmotic pressure, and pH inside (Williamson et al. 2012; Serra and Hengge 2014). Once the population of bacteria reach a threshold, or the acquisition of nutrients or oxygen below requirements, the bacterial cells launch the regulation through secretion of signal molecules, including enzymes that shed cells (Kaplan et al. 2003). For example, the glycoside hydrolase dispersin B produced by *A. actinomycetemcomitans* (Kaplan et al. 2003) and Spl (serine proteases) produced by *S. aureus* can decompose extracellular matrix to promote biofilm detachment (Boles and Horswill 2008). The specific mechanism of signal molecules regulating the dispersion of the biofilm is not clear. When studying *P. aeruginosa* biofilm, it was found that the decrease of c-di-GMP level may contribute to the cell movement and dispersion (An et al. 2010). Thermal nuclease can degrade the eDNA of *S. aureus* biofilm which may also be a method of active diffusion of the cells (Mann et al. 2009). The dispersion mechanism of enteropathogenic *E. coli* (EPEC) biofilm is related to the structure of type IV bundle-forming pili (BFP) (Cleary et al. 2004). When the structure of BFP changes from a thin conformation to a thick one, the adhesion of the fimbriae is adjusted to dispersion function (Knutton et al. 1999). The division of cells on the surface of the biofilm may also cause the dispersion (Kaplan 2010). In addition, the shear force of the fluid also promotes the shedding of the biofilms. When the shedding speed of the biofilm exceeds its adhesion, the biofilm volume decreases (Costerton et al. 1999). Biofilms conduct different ways of detachment. In a relatively stable environment, a small amount of biofilm cells are slowly released. When the external environment changes or as the volume of the matrix increases, the biofilm cells may fall off in large chunks (Rumbaugh and Sauer 2020). Biofilm cells are converted into a planktonic state after falling off and dispersing. Dispersed cells present a transitional state from biofilm to planktonic state in a short period of time. The dispersed cells usually have better mobility than biofilm cells, and their metabolic process, toxicity, and drug resistance have their own characteristics (Rumbaugh and Sauer 2020; Chua et al. 2014; Uppuluri et al. 2018; Liu et al. 2013; Vaysse et al. 2011). The spread of more detached virulent cells from biofilm may lead to more serious infections in vivo (Rumbaugh and Sauer 2020).

### 16.2.2 The Phenomenon of Biofilm Formation in Various Food Processing

Bacteria can adhere to the surface of food or processing equipment and further form biofilms (Chmielewski and Frank 2003, 2006; Saitou et al. 1992; Simões et al. 2010;
Mattila-Sandholm and Wirtanen 1992). Food or food residues on the surface of the processing equipment provide efficient nutrients for bacterial growth. However, an extreme temperature, pressure, acid and alkali substances, or disinfectants pose stress on bacterial survival.

In the dairy industry, the microbial biofilm on the milking equipment may be the source of milk contamination. Latorre et al. (2010) reported that the milk meters and rubber liners were contaminated by L. monocytogenes biofilms. The surface of the dairy equipment has also been found to be contaminated by Acinetobacter baumannii, Listeria, Enterococcus faecalis, Lactobacillus, Streptococcus, Corynebacterium callunae, Acinetobacter junii, and Stenotrophomonas maltophilia biofilms (Wang et al. 2019a, b). These biofilms may fall off with various production and processing processes and eventually infect dairy products. The production of milk generally undergoes processes, such as filtration, centrifugation, cooling, homogenization, sterilization, and packaging. Sterilization is the key step in the milk production process (Chen et al. 2012). Pasteurization and ultra-high temperature instantaneous sterilization (UHT) are commonly used for milk sterilization. However, the sterilized milk might be contaminated in the later packaging process (Kontominas 2010). The filling machine is the main source of contamination, and the biofilms may exist in the nozzle of the filling machine (Rysstad and Kolstad 2006).

In the milk powder production process, the milk needs to be dehydrated (Baldwin and Truong 2007). Flavobacterium and Geobacillus, with strong heat resistance, have been reported to form biofilm on the evaporator, which could cause contamination of the final product (Palmer et al. 2010; Hinton et al. 2002). The nonstarter lactic acid bacteria biofilm may contaminate the cheese, making the cheese surface turbid or producing unpleasant flavor (Somers et al. 2001). Somers et al. (2001) found that the Lactobacillus curvatus biofilm, one kind of nonstarter lactic acid bacteria, survived through the cleaning process and cause contamination of the cheese.

The beer/wine production process consists of four stages: malting, saccharification, fermentation, and canning (Kobayashi et al. 2008; Ndife et al. 2019; Morata 2021). Though the beer has been pasteurized and the antibacterial components (e.g., alcohol, organic acids) are produced during beer fermentation, beer might still subject to the microbial contamination. Timke et al. (2008) isolated wild-type yeast from the surface of the brewery’s processing equipment of two breweries and identified the biofilm-forming ability. The authors identified 6 C. pelliculosa that can form biofilms. Although the isolated Saccharomyces cerevisiae strains cannot form biofilms, they can grow in beer. So they concluded that Candida pelliculosa may attach to beer production equipment and form biofilms, while Saccharomyces cerevisiae may attach to later, thereby increasing the potential of beer deterioration. Pannella et al. (2020) simulated the environment of wine and found that the Lactobacillus plantarum Lpls22 biofilm exhibited a significantly higher survival rate under ethanol and acid conditions than the planktonic counterparts.

Common meat products include sausages, ham, bacon, sauced meat, and jerky, which are made from fresh meat supplemented with spices and different processing procedures (Sperber 2009). E. coli O157:H7 is ubiquitous found in meat, and this
bacterium is more difficult to form biofilms under the fluid shear (Moreira et al. 2013). However, a study has shown that E. coli O157:H7 existed in the biofilm of Acinetobacter calcoaceticus. It might happen when Acinetobacter calcineum secretes extracellular substances and wraps them together (Habimana et al. 2010). Yang et al. (2015) pointed out that E. coli may originate from the conveyor belt in the beef packaging plant. They survived through the daily cleaning of the plant and further contaminated the meat products. Iniguez-Moreno et al. (2019) found that the pathogenic or spoilage microorganisms, such as P. aeruginosa, B. cereus, and Salmonella, formed mixed-biofilm on meat extract, and P. aeruginosa and Salmonella have a higher density compared with B. cereus and have a stronger survival advantage in the mixed biofilm.

The naturally formed biofilm in the ocean may also make seafood contaminated (Mizan et al. 2015). Vibrio, Aeromonas spp., Salmonella spp., and L. monocytogenes are the common sources of seafood biofilm contamination (Mizan et al. 2015). During processing, seafood may also be contaminated by the biofilms when contacting with the processing facilities that have not been completely sterilized. Eating raw seafood also poses a higher challenge to food safety. For example, V. parahaemolyticus has been to form a biofilm on the chitin of diatoms and oysters and other marine organisms (Frischkorn et al. 2013; Aagesen et al. 2013). Even under high salt and temperature condition, Salmonella may contaminate shrimps, crabs, and other seafood (Lin et al. 2013; Bakr et al. 2011). Salmonella can also survive in the acidic environments, such as marinated shrimp and seafood salads (Wan Norhana et al. 2010). Even if the water and equipment are cleaned regularly, seafood products are still contaminated by bacteria. Seawater treated with the chlorination and ultraviolet (UV) radiation may also be contaminated when passing through the pipeline distribution system (Shikongo-Nambabi and Kachigunda 2010). Pseudomonas spp. and Enterobacteriaceae have been reported to be isolated from fish processing plants, and these bacteria that have adhered to the processing line can easily form a biofilm after obtaining nutrients from fish or cause repeated pollution (Shi and Zhu 2009).

Fruit and vegetable-associated products include dried fruits and vegetables, canned food, fruit and vegetable juices, pickled products, and preserved fruits (Siucińska and Konopacka 2014; Verma and Singh 2015; Ding et al. 2018; Blunden and Wallace 2003). The processes of drying, high temperature, or high osmosis are employed to extend the shelf life and retain the flavor of fruits and vegetables. Salad is one of the simplest and most common dishes. L. monocytogenes has been observed to attach to cabbages, especially on cut leaves, and develop into biofilms (Ells and Hansen 2006). Under low temperature conditions of 4 °C, E. coli O157:H7 was found to survive on lettuce leaves and form biofilm (Keskinen et al. 2009). Ng et al. (2017) found that Helicobacter pylori, which has a higher biofilm-forming ability, survives longer in vegetables compared with strains with low biofilm-formation ability. Various methods have been developed to remove the bacteria contaminated in fruit and vegetable. Amrutha et al. (2017) found that the organic acids inhibited the biofilm formation of E. coli and Salmonella membranes on the cucumbers, but they still cannot completely inhibit their biofilm formation. Cui et al.
successively used the cold nitrogen plasma (CNP) and phages to process the vegetables, such as lettuce, cucumber, and carrot. For example, \textit{E. coli} in biofilm was reduced by 5.71 log CFU/cm² after exposing to CNP for 2 min and 5% phage for 30 min successively.

16.2.3 Influence of Food-Associated Factors on Biofilm Formation

During food production chain, various physical, chemical, and biological factors have been associated with the biofilm formation (Table 16.1).

The physical factors include the surface material, temperature, pH, osmotic pressure, and ultraviolet radiation (Myriam 2011; Song et al. 2015; Schultze et al. 2021; Silva Meira et al. 2012). The bacterial adhesion to the surface is closely related to the surface characteristics. Bonaventura et al. (2008) found that at 37 °C, the level of biofilm produced by \textit{L. monocytogenes} on glass and stainless steel was higher than that of polystyrene, and the hydrophobicity at 37 °C was higher than lower temperatures. Song et al. (2015) concluded that the surface charge, hydrophobicity, roughness, and morphology were related to the adhesion and subsequent biofilm formation. Due to the negatively charged properties of cell surfaces, bacteria are more likely to adhere to the positively charged surfaces (Song et al. 2015). In addition, the increase in surface roughness might also contribute to the adhesion (Ammar et al. 2015). The hydrophobic force between the cell and the surface of the material also changes with temperature. Giao and Keevil (2014) cultured \textit{L. monocytogenes} isolated from the tap water at different temperatures and found that the bacteria cultured required only 4 h to form the biofilm at 37 °C, while it took 4–24 h to form biofilm at 4 °C. The result showed that the cultivability of \textit{L. monocytogenes} biofilm decreased with the increase of culture time and temperature. The effect of pH on biofilm formation is also obvious. Hoštacká et al. (2010) studied the biofilm formation ability of \textit{P. aeruginosa} at various pH values at 37 °C and found that the biomass of the biofilm more than doubled under alkaline condition (pH 8.5) than that of acidic condition (pH 5.5). Wang et al. (2020b) found that \textit{E. coli} O157:H7 J29 biofilm was highly resistant to lactic acid (pH 2.0), more than 4 log CFU/cm² cells remained after 12 h of lactic acid treatment. Nostro et al. (2012) found that when the pH value is 8.5, it has a stronger inhibitory effect on the \textit{S. aureus} biofilm than when the pH value is 7.2. From the above studies, we can infer that different microbial biofilms have their own suitable pH ranges for growth, and the ability of microorganisms to form biofilm at different pHs is related to their own tolerance to acid and alkali. The addition of NaCl or some other ingredients during processing does not directly chemically react with the biofilms but affects the osmotic pressure in the food system, which would furthermore affect the balance of osmotic pressure inside and outside the cells. When the NaCl concentration is lower than 6%, the biofilm biomass of \textit{S. aureus} isolated from the ham increases with the increase of the NaCl concentration (Nostro et al. 2012).
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<td>At 37 °C, the level of biofilm produced on glass and stainless steel was higher than that of polystyrene</td>
<td>Bonaventura et al. (2008)</td>
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<td>Temperature</td>
<td><em>Listeria monocytogenes</em></td>
<td>The cultivability of biofilm decreased with the increase of culture time and temperature.</td>
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<td>pH</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>The biomass of the biofilm more than doubled under alkaline condition (pH 8.5) than that of acidic condition (pH 5.5)</td>
<td>Wang et al. (2020b)</td>
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<td><em>Escherichia coli</em></td>
<td><em>E. coli</em> O157: H7 J29 biofilm is highly resistant to lactic acid (pH 2.0), more than 4 log CFU/cm² cells remained after 12 h of lactic acid treatment</td>
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<td>Pasquaroli et al. (2013)</td>
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(continued)
However, the opposite is true for *Salmonella enterica*. That is, the increase in NaCl content inhibits the growth of the biofilm (Iliadis et al. 2018). Ultraviolet (UV) irradiation is often used for disinfection in food factories. But for the mature biofilm, UV can only penetrate the outer extracellular matrix (Carvalho 2017). The microbial biofilms may be subjected to flow system, which might affect the biofilm formation. Aanterkker et al. (2003) established a growth model of microorganisms in fluid. The authors found that the growth of cells accelerated with the increase of flow rate which may due to the fact that the fluid is beneficial to the exchange of nutrients.

Chemical factors include antibiotics, disinfectants, metal ions, and other natural antibacterial substances, etc. (Penesyan et al. 2019; Wang et al. 2018). In addition to inhibit the formation of the biofilm, these antimicrobial compounds may also induce the biofilm cells into a viable but non-culturable (VBNC) state, in which cells exhibited a high resistance towards the environmental stressors (Trevors 2011).
After treating poultry and fish with over doses of antibiotics, these antibiotics might remain in meat or dairy products, and the antibiotic resistant bacteria (ARB) appear. Pavlickova et al. (2017) investigated the resistance of 105 strains of *E. coli* from wild animals to 13 antibiotics. The results showed that 46 of the 66 drug-resistant strains (70%) were able to form biofilms. The authors pointed out that there is a statistically significant correlation between the antibiotic resistance and the ability to form biofilms. Antibiotic resistance may be related to the formation of VBNC cells in the biofilm (Pasquaroli et al. 2018). Tobramycin, ciprofloxacin, daptomycin, vancomycin, quinupristin, and other antibiotics have been observed to induce bacteria to enter the VBNC state in biofilm (Mangiaterra et al. 2020; Pasquaroli et al. 2013, 2014). Exposed *P. aeruginosa* PAO1-N biofilm to tobramycin and ciprofloxacin with subinhibitory antibiotic concentrations (0.062 mg/L and 0.25 mg/L, respectively) for 60–170 days, a large number of VBNC bacteria was found, which may lead to the recurrence of infection in cystic fibrosis patients (Mangiaterra et al. 2020). Daptomycin-exposed *S. aureus* biofilms entered the non-cultivable state after 40 d, which may be attributed from the gene expression induced by low concentrations (2 μg/mL) of antibiotics (Pasquaroli et al. 2014). Besides, vancomycin and quinupristin have also been reported to introduce *S. aureus* biofilms to VBNC state (Pasquaroli et al. 2013). Disinfectants containing substances such as chlorine, ammonium, and hydrogen peroxide are also related to the induction of VBNC state bacteria in biofilm (Leriche and Carpentier 1995; Alleron et al. 2008; Ye et al. 2020; Brauge et al. 2020). *Listeria monocytogenes* biofilms, which may form in smoked salmon processing environments, can enter into the VBNC state after exposed to 2% (v/v) quaternary ammonium and 12.5 ml hydrogen peroxide based disinfectants with 20 min (Brauge et al. 2020). The chlorine, commonly used as water disinfectant, has been found to induce *Legionella pneumophila* to lose cultivability with 0.36 mg/L Cl₂ for several days (Giao et al. 2009). Chlorine or monochloramine can also induce the formation of *S. Typhimurium*, *E. coli*, and *L. pneumophila* biofilm (Leriche and Carpentier 1995; Alleron et al. 2008; Ye et al. 2020). Other factors such as copper ions, silver compounds, and oxygen all have been found to induce bacterial biofilm into the VBNC state (Moritz et al. 2010; Lleo et al. 2007; Zhong et al. 2020). In copper tap water pipes, it is reported that the copper ions induced the formation of VBNC in *P. aeruginosa* and *L. pneumophila* biofilms (Moritz et al. 2010). Silver nanoparticles and silver nitrate are also related to VBNC *P. aeruginosa* in biofilm (Lleo et al. 2007). The VBNC levels of *Campylobacter jejuni* biofilm under aerobic conditions were 95.5%, much higher than 13.9% of VBNC cells under micro-aerobic conditions (Zhong et al. 2020). Polyethylene glycol PEG enhanced the formation of biofilm of *Astragalus cicer microsymbionts* while sucrose showed the opposite effect (Wdowiak-Wróbel et al. 2016). After cultivated in a low nutrient medium containing 1/10 tryptic soy broth for 25 d, *Cronobacter sakazakii pgfpuv* entered into the VBNC state during the biofilm formation process (Sinaga and Dewanti-Hariyadi 2016).

The interaction between bacteria is the main biological factor that affects the biofilm formation. Mixed biofilm of multiple bacterial strains are commonly formed in the food system (Chmielewski and Frank 2003). The biovolume of *E. coli* O157:
H7 isolated in a meat processing plant when cultivated together with *Acinetobacter calcoaceticus* was 400 times that was in a monoculture (Habimana et al. 2010). This may be related to the fact that the biofilm structure of *A. calcoaceticus* is more likely to be colonized by *E. coli*. It was found that the single-species biofilms of pathogenic and spoilage microorganisms have a higher cell density than the multi-species biofilms (Iniguez-Moreno et al. 2019). Although EPS can indiscriminately protect the multi-microbial populations against the external stressors, there is competition or cooperation relationships among various bacterial species within the biofilm (Yang et al. 2011). On the one hand, the interaction between microorganisms can promote the formation of the biofilm. For example, the biovolume of *E. coli* O157:H7 isolated in a meat processing plant when cultivated together with *Acinetobacter calcoaceticus* was 400 times that was in a monoculture. This may be related to the fact that the biofilm structure of *A. calcoaceticus* is more likely to be colonized by *E. coli*. On the other hand, microorganisms can also inhibit the formation of biofilms by competing for nutrients or secreting antibacterial substances. Chopra et al. (2015) found that the bacteriocin *sonorensin* isolated from the marine microorganism *Bacillus sonorensis* MT93 can significantly inhibit the growth of spoilage bacteria *S. aureus* and *L. monocytogenes* and the biofilm formation in food. Therefore, we can speculate that in the mixed biofilm of *B. sonorensis* and spoilage bacteria, the secretion of bacteriocins will reduce the ability of spoilage microorganisms to form biofilms.

### 16.3 Biofilms in Response to Stimuli

#### 16.3.1 Characteristics of Biofilm

Microbial biofilm consists of one or more species and the extracellular matrix secreted by the microbial cells (Flemming and Wingender 2010). The volume of microorganisms accounts for about 15–20%, and the extracellular matrix occupies about 80–85% (Kokare et al. 2009). The microbes and extracellular substances are unevenly distributed in a gradient external environment, resulting in the subpopulations with different metabolic activities and different states within the biofilm (Stewart and Franklin 2008).

According to the cells position, the microorganisms in the biofilm can be divided into three types of planktonic, superficial, and inner microorganisms (Inglis et al. 1995). Planktonic and superficial cells are exposed to more nutrients and oxygen. These cells are relatively more active with larger sizes (Ramey et al. 2004). The inner layer cells can only acquire the nutrients, oxygen, and other materials through the transport channels in biofilm matrix (Wilking et al. 2013). These cells, with small size, have a relatively slow metabolic activity or even stop growing (Ramey et al. 2004). For multi-species microbial biofilm, the aerobic microorganisms prefer to live on the superficial layer, while anaerobic microorganisms located in the inner layer (Dufour et al. 2012). Due to the different microenvironments, the phenotypes of the cells might be various even for one species. From the perspective of the state of
microorganisms, the biofilms contain both live and dead cells (Àlvarez et al. 2013). In addition, there may also be dormant-like VBNC cells that do not produce spores (Trevors 2011). After entering this state, the composition and structure of the cell membrane/wall and the gene expression may change (Signoretto et al. 2000; Nowakowska and Oliver 2013). The respiration rate, nutrient transport, and macromolecule synthesis might be decreased. However, the adenosine triphosphate (ATP) can still maintain a high level (Trevors 2011; Oliver 2005; Lindbäck et al. 2010). In the biofilm, the dormant P. aeruginosa cells exhibited higher survival rate than the active cells when exposed to the tobramycin (10 mg/L, 23 mM, 30 min) and silver nitrate (10 mg/L, 169 mM, 30 min). Interestingly, the chlorine (0.2 and 0.5 mg/L, 3 and 7 mM, 30 min) has stronger inhibitory effect on dormant P. aeruginosa rather than on the active cells in biofilm. Kim et al. (2009) explained that metabolically active cells can produce more components combined with chlorine to reduce the antibacterial effect of chlorine. When the external environment changes, the biofilm cells respond quickly to improve their own survival ability. The respiration rate of Pseudomonas sp. biofilms was reduced 90% in one hour after being transferred to a medium without added carbon source (Bester et al. 2011). After the P. fluorescens biofilm is exposed to toxic copper ions for 4 h, its stress response induces the increased protein synthesis related to the extracellular matrix instead of the metabolic changes related to the TCA cycle and the pyruvate cycle (Booth et al. 2011).

In addition to microbial cells, another important part of the biofilm is EPS, which provides the main living environment for the cells (Flemming and Wingender 2010). Microorganisms regulate the secretion of EPS through enzymes. It has been reported that the combination of glucose pyrophosphorylase (GalU) and phosphoglucomutase (PGM) increased the amount of EPS secreted by the lactic acid bacteria (Levander et al. 2002). The main component of EPS is polysaccharides, but it also contains the proteins, fat, nucleic acids, etc. These components may be resulted from the secretion by the active cells, the cell lysis, the external environment, and the degradation of extracellular macromolecules (Salama et al. 2015a, b). EPS mainly accumulates around the biofilm cells. EPS is tightly arranged to the cells in the inner layer, but loosely arranged to the cells in the outer layer (Salama et al. 2015a, b; Sheng and Yu 2006). EPS possesses three major functions of the biofilm: structural function, nutritional function, and protection function. EPS is the basis of the three-dimensional structure of the biofilm. The network skeleton formed by the cross-linking of exopolysaccharides contributes to the mechanical strength and stability of EPS. Some proteins have been reported to be combined with polysaccharides for the formation and stabilization of matrix networks (Sutherland 2001). Fatty acids are combined with polysaccharides to form lipopolysaccharides, which act as the surfactants and assist the cell attachment (Matsuyama and Nakagawa 1996). Extracellular DNA (eDNA) is also found to be involved in the cell-to-cell adhesion (Yang et al. 2007). Under the starvation conditions, EPS can also be used as a source of nutrition for microorganisms. EPS can be degraded by the extracellular enzymes into small molecular components, which microbial cells can use for survival (Costa et al. 2018). EPS is also a contributor in the antibiotic resistance, immune escape, and
stress resistance of microbial cells within the biofilms (Stewart and Franklin 2008; Arciola et al. 2008).

### 16.3.2 Relevant Molecular Mechanisms

Microbial biofilms are usually more resistant to external stimuli, and the mechanisms of its tolerance can be divided into cell-related mechanisms and EPS-related mechanisms.

#### 16.3.2.1 Cell-Related Mechanisms

Formation of Highly Tolerant Cell Subpopulations

Persisters are the subpopulation with antibiotic resistance, and the existence of persisters within the biofilm might lead to the intractability of biofilms (Roberts and Stewart 2005). Most antibiotic resistant bacteria have the toxin-antitoxin systems (Yang and Walsh 2017). *hipA* is the first toxin gene which has been verified to exist in *E. coli* (Hansen et al. 2012). In the presence of antibiotic stress, Lon protease can degrade its antitoxin counterpart HipB, thereby activating HipA (Conlon et al. 2015). HipA inhibits glutamyl-tRNA synthetase (GltX) through the phosphorylation, thereby promoting the accumulation of uncharged tRNA and the synthesis of ppGpp (Germain et al. 2013). ppGpp inhibits the cell transcription and translation, inducing the formation of persisters in biofilms. Persistent cells cease the certain activities associated with the antibiotic binding targets, which renders them with antibiotic resistance (Lewis 2005). ppGpp not only plays a key role in the formation of planktonic persisters, but also contributes to the formation of persister in *E. coli* biofilms, but its impact on persister may be less related to HipA (Amato and Brynildsen 2014). Another toxin, TisB, has also been found to specifically induce the formation of persister (Durfee et al. 2008). When the sos repair (SOS) system responds to the DNA damage, it can induce TisB to act on the cell membrane to inhibit the proton transportation, and ultimately compromise the energy source required for cell metabolic activities to induce the generation of persister (Unoson and Wagner 2008). Some studies have been explored the genes (e.g., *dnaK*, *clpB*, *rpoS*, and *pspF* in *E. coli*) that involve in the induction of persister (Liu et al. 2017). Compared with the planktonic cells, the biofilm cells seem to be easier to become persisters (Conlon et al. 2013). This may be related to the persister-induced activity initiated by the stimulation of local nutrient deficiency in the biofilm.

Viable but non-culturable (VBNC) state is similar to dormancy in the biofilms (Giao and Keevil 2014). VBNC state is a kind of state that bacteria cannot grow on the conventional medium but still has the cell viability as a strategy for long-term survival (Li et al. 2014; Ramamurthy et al. 2014). Unlike dormant cells, VBNC cells still maintain a certain degree of metabolic activity (Trevors 2011; Oliver 2005;
After entering into the VBNC state, most of the pathogenic bacteria might shrink in size and still maintain a certain degree of toxicity, and can be revived under a suitable environment (Oliver 2010; Su et al. 2013). Quorum sensing (QS) refers to a group behavior in which microorganisms respond to a high population density by activating specific gene expression (e.g. AI-2 system) (Yu et al. 2012). Microorganisms employ the QS to regulate biofilm formation, virulence factor expression and motility, bioluminescence, nitrogen fixation, and sporulation (Pan and Ren 2009). It is found that QS also contributes to the entry of VBNC state in biofilms where bacterial density is higher than planktonic ones (Krzyzek and Gosciniak 2018). Diffusible signal factors (DSFs) are the fatty acid molecules that can promote the communication among the microorganisms and may stimulate *Helicobacter pylori* to enter into the VBNC state within biofilms (Krzyzek and Gosciniak 2018). Lauric acid and 7-(Z)-tetradecenoic acid are identified as the self-growth-inhibiting (SGI) compounds, which can cause *Helicobacter pylori* to lose cultivability (Yamashita et al. 2015). Correia et al. (2012) found that docosahexaenoic acid (DHA) also shrunk *Helicobacter pylori* from spiral to spherical VBNC cells. Similar to persister, it is easier for the cells within biofilms to enter into the VBNC state than the planktonic ones do. Wang et al. (2020a) subcultured the spoilage bacteria-*Lactobacillus* in beer at 26 °C, and found that *Lactobacillus* biofilm only needed 63 days to enter the VBNC state, while it took 119–217 days for their planktonic counterparts. This phenomenon may be due to the fact that the QS signal molecules gathered in the biofilm might be more effective in stimulating formation of VBNC cells.

Microorganisms such as *Bacillus* and *Clostridium* can produce dormant spores (Pendyala et al. 2019). It is easier to form spores under the stimulation of nutrient deficiency, dry moisture, and other stressed conditions (Setlow and Johnson 2019). Spores have very low water content and show strong survival capacity under various stressors along the food processing. Khot et al. (2006) found that the spores formed in *C. albicans* biofilms were ten times more resistant to amphotericin B than the planktonic cells did, and it may be related to the regulatory effects of ergosterol and β-1,6-glucan. *Bacillus cereus* biofilm formation in the first three days was found to be positively correlated with the spore content (Hussain and Oh 2018). Huang et al. (2020a) proposed that the formation of *Bacillus cereus* and *Bacillus subtilis* spores may be regulated by a common pathway. The protein-Spo0A promotes the secretion of EPS by *B. subtilis*, and it also stimulates the spore formation (Aguilar et al. 2010). The relationship between spores and the biofilm formation remains to be further studied. The spores can fall off along with the biofilm and germinate when the environment become suitable, leading to the food contamination. *B. cereus* spores are more hydrophobic than the vegetative cells, so they exhibit stronger adhesive capacity, which helps them avoid being washed away by fluids and to re-form the biofilms (Shaheen et al. 2010; Tauveron et al. 2006).
Stimuli Response of Biofilm Cells

The initiation of oxidative stress defense system has been considered as one of the proposed mechanisms associated with the antibiotic resistance of biofilms (Gambino and Cappitelli 2016). Under environmental stimuli, biofilm cells accumulate intracellular reactive oxygen species (ROS) (Das and Roychoudhury 2014). When ROS accumulates to a certain extent, cells might be killed (Petrov et al. 2015). Proteus mirabilis biofilm cells remove ROS by producing the antioxidant proteins, such as superoxide dismutase (SOD) and glutathione (Aiassa et al. 2010). P. aeruginosa biofilm can increase the antioxidant capacity by up-regulating \textit{katA} to produce catalase (Khakimova et al. 2013). It was reported that \textit{P. aeruginosa} and \textit{E. coli} enveloped cells launched the stringent response (SR) to initiate the activity of catalase to remove the excess ROS (Khakimova et al. 2013; Nguyen et al. 2011). The inhibition of ROS by the biofilm cells may be associated with the formation of persisters mentioned above (Van Acker et al. 2013). Persisters down-regulate the TCA pathway to reduce the intracellular content of ROS (Van Acker et al. 2013). The oxidative stress response-associated proteins may also contribute to the formation of biofilms to protect themselves. The adhesion protein encoded by \textit{agn43} renders the resistance of \textit{E. coli} to H$_2$O$_2$ and also stimulates the formation of biofilm (Schembri et al. 2003; Danese et al. 2003). It was reported that the YjcC protein regulates c-di-GMP to respond against ROS and promotes the production of \textit{Klebsiella pneumoniae} biofilms (Huang et al. 2013).

Efflux pumps are another important regulation mechanism employed by the microorganisms against the metal ions, disinfectants, antibiotics, metabolites, and other toxic substances (Sun et al. 2000). Efflux pumps are the membrane proteins that can excrete the antibiotics from the cell with the hydrolysis of ATP (Van Bambeke et al. 2000). More and more studies have shown that efflux pumps also contributed to the formation of microbial biofilms. The \textit{lsrA} gene, encoding one ABC superfamilies multidrug resistance efflux pump, was found to mediate the transport of the signal molecule AI-2, which might promote the maturation of \textit{E. coli} biofilms. For the gram-negative bacteria \textit{P. aeruginosa}, the MexAB-OprM pump contributes to the efflux of N-3-oxododecanoyl-L-homoserine lacacyl-homoserine lactones (3OC12-HSL), which contributes to the QS and biofilm formation (Gillis et al. 2005). Therefore, the efflux pumps may involve in the formation of the biofilm by the efflux of signal molecules. In addition, the efflux pumps may also directly expel the components of extracellular matrix. It has been found that the \textit{E. coli} efflux pump (\textit{bcr}, \textit{norE}, \textit{ydeE}, and \textit{yeeO} multidrug-efflux transporter genes) can transport short peptides L-Alanyl-L-glutamine (Ala-Gln), which are part of the EPS, to the outside of the cells (Hayashi et al. 2010). Additionally, the efflux pumps can also protect the biofilms. The genes \textit{cdr1} and \textit{mdr1}, encoding the ATP-binding cassette (ABC) and major facilitator superfamily (MFS) pumps, respectively, can enhance the tolerance of \textit{C. albicans} to fluconazole within 2 h, the rapid response can well protect the biofilm cells in the adhesion stage (Mateus et al. 2004). It is worth noting that the efflux pump system of biofilm cells against the external stimuli may be distinct from that of planktonic cells (Pamp et al. 2008). The researchers found that
mexAB-oprM can specifically regulate the tolerance of *P. aeruginosa* biofilm to colistin, but posed insignificant effect on their planktonic counterparts (Pamp et al. 2008).

### 16.3.2.2 EPS-Related Mechanisms

The EPS in biofilm, which mostly account for over 90% of the dry matter, also acts as the contributor for protection of microorganisms against a variety of stimuli (Booth et al. 2011). EPS protects or maintains the microbial vitality by supplying nutrition and water, alleviates the stimuli environment, and promotes the signal exchange among cells (Flemming and Wingender 2010). EPS maintains the nutrients and moisture in the community (Myszka and Czaczyk 2011). When it comes to the nutritional deficiency condition, EPS provides microorganisms with nutrients to maintain their viability (Flemming et al. 2007). Various enzymes including protease, α-glucosidase, β-xylosidase, and lipase are potentially employed to degrade macromolecular substances into nutrients for their use by microorganisms (Wimpenny and Colasanti 1997; An and Friedman 2000; Wolfaardt et al. 1999). Under a drying pressure, the hydration state of biofilm also exhibits the capacity to keep moisture (Fröslser et al. 2017).

The extracellular substances act as a protective barrier of biofilms against some external stimuli, especially from toxic substances (Henriques and Love 2007; Bridier et al. 2011; Wang et al. 2019a, b). Acids, alkalis, disinfectants, antibacterial agents, and other toxic substances may form a concentration gradient during the diffusion from the outside into the deep layer of the biofilm, which makes the various concentration of toxic substances around cells in different locations (Koechler et al. 2015; Najera et al. 2005). Therefore, the toxic substances may not reach the deep layer of the biofilm. Jang et al. (2006) found that the chlorine at 25 mg/mL penetrated up to 100 mm of the biofilm in the dairy processing pipeline. In addition, EPS may neutralize the toxic substances. Ampicillin penetrates slowly in the biofilm of the wild-type *Klebsiella pneumoniae*, which may be caused from the neutralization of positively charged antibiotics by the negatively charged substances in EPS (Anderl et al. 2000; Shigeta et al. 1997). Additionally, the heterogeneity of EPS also contributes to a microenvironment for withstanding the toxic substances (Machineni 2020). The oxygen content is low in the depth of the biofilms. Some toxic substances (e.g., aminoglycoside) might fail to launch the inactivation effect on the deep biofilm cells under a hypoxic environment (Pamp et al. 2008). Biofilm cells are also adaptive to the toxic substances at the sublethal doses, which might be attributed from the strong cell viability at the bottom of the biofilms. When *Salmonella* biofilm was continuously exposed to the sublethal concentrations of benzalkonium chloride, it proved to be adaptable (Mangalappalli-Illathu et al. 2008).

Biofilm contributes to the gene information exchange among the cells, which may increase resistance against the environmental stressors through the regulation of gene expression (Frederick et al. 2011). EPS may provide cells with a suitable environment for the gene mutation and genetic sequence exchange and to adapt to
the external stressful conditions. Driffield et al. (2008) found that the cell mutation rates in the biofilm of *P. aeruginosa* reached 105 times over that of the planktonic ones. A higher mutation rate contributes to a high probability to form the mutants that can adapt to a stressful environment. The mutation of *P. aeruginosa* biofilm cells was found to produce a wrinkled colony, which makes the biofilms more adaptable to H\textsubscript{2}O\textsubscript{2}. In addition, the microbial community with a high cell density within the biofilm releases a large amount of DNA, which may facilitate the transfer of resistance genes such as *qac* among the cells, thereby improving the survival of the biofilms through stressful conditions (Bridier et al. 2011).

16.4 Detection Methods of Biofilm Cells

The techniques for the detection of biofilm have been developed. In this section, the biofilm detection techniques are introduced.

16.4.1 Cell-Based Methods of Biofilm Detection

Cell-based research includes research on cell quantitative analysis, vitality, and metabolism properties, and so on.

Major cellular characteristics include cultivability and viability, and thus most of cell-based methods of biofilm detection have based on these 2 characteristics. Cultivability refers to the fact that individual cells of a microorganism could form colonies when streaked on solid medium plate. Since only living cells can form colonies, the purpose of quantifying living cells can be achieved by counting the colonies. After the microbial biofilm is washed and homogenized, it is diluted on the agar to the extent that the cells can be separated and is evenly spread on a suitable nutrient medium to form colonies. The colony forming units (CFU) value can be calculated according to the number of colonies and the dilution factor. This method is easy to operate with relatively low cost, but there are many factors that affect the cultivability of biofilm samples. The elements that affect the cultivability of live microorganisms can be divided into two categories: internal factors and external factors (Barer and Harwood 1999; Kumar and Ghosh 2019). Internal factors include: (1) cell aging or damage; (2) microorganisms enter dormancy or VBNC state; (3) slow cell growth or replication. External factors include: (1) unsuitable nutritional conditions; (2) environmental conditions, including pH, oxygen, osmotic pressure, toxic substances and other pressures on the growth of microorganisms; (3) interaction between microorganisms, including symbiosis and competition relationship. Among them, external factors could trigger internal factors. In fact, most microorganisms in nature are not cultivable under laboratory conditions. There are many explanations for the specific reasons, and they have not been fully understood at present. Flow cytometer is an instrument that can perform automatic optical
detection and sorting of cells. It is often used for quantitative analysis of biofilm cells. Usually, the biofilm cells are washed out, dispersed in phosphate buffer or saline, and homogenized to obtain a pretreated sample. The cells flow through the narrow sample pipe one by one and then are emitted. At the same time, they are irradiated by the laser and scatter light. The detected information mainly comes from non-fluorescent scattering signals or specific fluorescent signals. The scattered light signal is related to the cell volume (forward scatter, FSC) and internal structure (side scatter, SSC); the strength of the fluorescence signal can distinguish the source of fluorescence (Nunez 2001). For example, the specific fluorescence emitted by fluorescein is much stronger than the fluorescence emitted by itself. According to the characteristic parameters of the cells, the targets can be classified, screened, and quantitatively analyzed. After the biofilm cells are pretreated into a uniform suspension, the total amount of biofilm cells can be quantified by flow cytometry. And the biofilm cells can be stained with Live/Dead double dyes and then cell viability can be measured by flow cytometry. SYTO 9 and propidium iodide (PI) stain the microbial biofilm. SYT 9 can penetrate the wall of live and dead cells and bind to DNA, while PI cannot penetrate the wall of living cells. Because the integrity of the cell wall of dead cells is destroyed, PI can penetrate the wall of dead cells and replace SYT 9 to bind to DNA. Therefore, the DNA of living cells finally binds to SYT09, and the DNA of dead cells finally binds to PI (Macià et al. 2018). The fluorescent dye can be selected from the aforementioned SYTO 9/PI, or SYTO 9 can be replaced with SYBR (Manoil et al. 2014; Cerca et al. 2011). Moreover, we can also try to choose a more cost-effective single-dye method for vitality determination. Monique Kerstens et al. (2013) selected TO-PRO®-3 iodide (TP3) combined with flow cytometry to quantitatively determine the viability of Candida albicans. Since TP3 can only penetrate through damaged cell membranes, live cells that emit low fluorescence intensity and dead cells that emit strong fluorescence signals after staining can be distinguished. This method is fast and accurate to detect the viability of microorganisms. But the cost is high and the test results may be affected if the biofilm cells are not completely dispersed.

Molecular methods can also measure biofilm cells. Quantitative polymerase chain reaction (q-PCR) can quantitatively analyze biofilm cells by measuring target genes. One of the major limitations of qPCR is that it cannot distinguish between living and dead cells, but combining it with DNA intercalating dyes can overcome this defect. The most commonly used DNA intercalating dye is propidium monoazide (PMA), which is a derivative of PI and can only penetrate through damaged cell membranes. The azide group of PMA is converted into a nitroso group under light and then bind to DNA, causing its structure to change and cannot be amplified (Taylor et al. 2014). And their binding products are insoluble in water and easily removed as impurities in the following DNA extraction step (Nam et al. 2011). In addition to dead cells, those planktonic cells that adhere to the surface of the biofilm can also be removed by soaking in saline after the pretreatment process. Therefore, the samples treated with PMA can be used to quantitatively measure the number of living cells by qPCR. Ethidium monoazide (EMA) is used as DNA intercalating dye, too. However, EMA
can also penetrate some living cells, resulting in lower viability results (Rueckert et al. 2005). So now researchers are more inclined to choose PMA instead of EMA.

The tetrazolium salt can be used to detect the respiration metabolism of biofilm cells. 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) is one of the most commonly used tetrazolium salt stains (Alonso et al. 2017). XTT is converted into orange-yellow formazan by the dehydrogenase in the respiratory chain of the viable cells (Xu et al. 2016). In addition, 2,3,5-triphenyltetrazolium chloride (TTC), cyanoditolyl tetrazolium chloride salt (CTC), and 3-(4,5-dimethyl-2-thiazole)-2,5-diphenyl tetrazolium bromide (MTT) can also be used for detecting the viability of biofilms. Living cells in the biofilm can also convert the colorless, non-fluorescent fluorescein diacetate (FDA) into yellow, highly fluorescent fluorescein, by the esterase produced, so FDA can also be used to detect the viability of biofilm cells. The fluorescence of FDA is very stable, and the calculation of biofilm activity is accurate and sensitive (Peeters et al. 2008).

### 16.4.2 Other Biofilm Detection Methods

Morphology-based method is the most direct method to observe microbial biofilm (Kim et al. 2010). The confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM) and atomic force microscopy (AFM), and other instruments have been used to observe the morphology of the biofilms (Huang et al. 2020b). CLSM is based on the fluorescence microscope with a laser scanning device (Teng et al. 2020). Laser as the scanning light source quickly scans the object point by point, line by line, and side by side (Michels and Gorb 2012). Fluorescence microscope can observe the three-dimensional structure of the cell without damage the samples (Turillazzi et al. 2008). It can also be used to quantitatively calculate the volume, thickness, and roughness of the biofilm. In addition to the morphological observation and structure measurement of the biofilm, CLSM can also be combined with fluorescence probes (e.g., SYTO 9/PI) to distinguish live and dead cells. After dyeing, the alive and dead cells emit green and red fluorescence, respectively, under observation by a CLSM. The intensity ratio of green fluorescence to green and red fluorescence volume could represent the bacterial viability (Shen et al. 2010; Seneviratne et al. 2009). Although CLSM has powerful functions, the cost is relatively high and it is difficult to realize daily inspection (Kumar et al. 2016; Ceresa et al. 2015). SEM scans the samples with a focused, narrow high-energy electron beam, to generate the signals between light and matter, for the morphological analysis (Kuo et al. 2011). Compared with CLSM, the resolution of SEM can reach a nanometer level, and it can be used to observe the adhesion of individual cells. However, it cannot provide the information about the three-dimensional structure of the biofilm (Boguslavsky et al. 2018; Norton et al. 1998). AFM is used to analyze the properties of substance surface by detecting the weak interatomic interaction force between the object and a miniature force-sensitive element (Okada
et al. 2019). It can achieve atomic resolution detection and 3-D scanning (Arman et al. 2015). When using an AFM, the sample does not need to be specially processed, so it is suitable to detect the living tissues (Li et al. 2013). In addition to calculate the thickness of the biofilm, AFM is also used to measure the interaction force between the microbial biofilm and the surface, including the strength, elasticity, and toughness (Chatterjee et al. 2014; Phang et al. 2010). In addition to the aforementioned instruments, transmission electron microscopy (TEM), scanning transmission X-ray microscopy (STXM), and nuclear magnetic resonance (NMR) are also applied for the morphological observation of biofilms. Researchers can choose the corresponding instrument or combination for morphological research according to the purposes (Achinias et al. 2020).

The total biofilm biomass and the components of EPS have also been determined (Ommen et al. 2017; Flemming 2016). Dry or wet weight can represent the total amount of the biofilm, and the weight per unit area or volume is the biofilm density (Khatooon et al. 2014). The slide with the biofilm grown is placed in the incubator to a constant weight. The difference between the weight of the dry biofilm and the slide and the weight of the dry slide is calculated as the biofilm dry weight (Guzzon et al. 2008). This method is simple and easy to operate and can be used to estimate the biofilm biomass and growth rate as a whole (Wilson et al. 2017). Crystal violet (CV) is a common stain used to measure the biofilm biomass (Ommen et al. 2017). Before staining, the biofilms are usually washed with saline to remove planktonic cells attached to the surface, which could lead to the loss of biofilm cells. Xu et al. (2016) classified the biofilm-forming ability of S. aureus according to the ratio of the optical density (OD) of biofilm sample to the control OD (ODc), namely the value of resource unit (RU, RU=OD/ODc), into strong (4 < RU), medium (2 < RU < 4), and weak (0 < RU < 2) levels. In addition, for extracellular polysaccharides, 1,9-dimethylmethylene blue (DMMB) staining combined with spectrophotometry is used to characterize the extracellular matrix. DMMB can combine with sulfated polysaccharides in the biofilm matrix to form insoluble complex products. By detecting the absorbance value of the dye released by adding the decomplexing solution, the amount of sulfated polysaccharide present in the biofilm matrix can be measured (Lin et al. 2017; Marshall et al. 2012). Regarding the specific components of the extracellular matrix, the physically and chemically destructive methods were used to destroy the biofilm and extract the target substances (polysaccharides, proteins, lipids, etc.), which are further detected (Azeredo et al. 2017).

Multi-omics (genomics, transcriptomics, proteomics, or metabolomics, etc.) are the novel technologies for the biofilm detection. Transcriptomics is used to determine the expression of all mRNA. Proteomics is employed for the quantification of the expression pattern of all the proteins. Metabolomics is to measure the metabolites of small molecules. Specific to microbial biofilms, currently, multi-omics is mainly applied to the research about biofilm formation, drug resistance, quorum sensing, biofilm inhibition, the interaction, between the biofilm and the host, or the interaction between the microorganisms, etc. Researchers can make preliminary predictions of the phenotypes that may occur about the biofilms according to genome. They can
also infer the genetic lead to the difference in biofilm phenotype by comparing the differences in genome sequences. Król et al. (2019) employed proteomics and found that *E. coli* C strain lacks genes associated with the biofilm formation, such as the *flu* gene encoding antigen 43. When compared the genome of *E. coli* C with other *E. coli* strains, the authors found that the presence of IS3-like insertion sequence in front of the *csrA* gene and the presence of IS5/IS1182 in front of the *csgD* gene might contribute to the weaker ability of other *E. coli* to form a biofilm. Tan et al. (2015) found that methicillin-sensitive *S. aureus* (MSSA) strain ATCC25923 exhibited different inhibitory effects from methicillin-resistant *S. aureus* (MRSA), and the RNA-seq technology was used to reveal that it may be due to the deletion of the *agr* gene of the MSSA strain. Rajendran et al. (2016) compared the transcriptomics data between *Candida albicans* with strong biofilm formation ability and those with weak biofilm formation ability and found that the aspartate aminotransferase gene *aat1* might contribute to the ebiofilm formation. Liu et al. (2018) used RNA-seq technique to explore the antibiotic resistance of *S. aureus* biofilm cells. The authors found that the biofilm cells up-regulated genes encoding the penicillin binding protein PBP1, PBP1a/2, and PBP3 and multidrug resistance efflux pump AbcA. Resch et al. (2006) made use of 2-D PAGE and ESI-MS/MS to separate and identify the proteins expressed by the *S. aureus* biofilms. The results confirmed that the regulation of SarA on the intercellular adhesion operon promotes the biofilm formation. Valliammai et al. (2020) revealed the inhibitory mechanism of citral on the biofilm formation and virulence of *S. aureus* with the assistance of proteomics. The authors demonstrated that citral down-regulated the expression of cell wall homeostasis (IsaA), regulation of exotoxin secretion (SaeS), and the proteins related to the cell adhesion and pathogenesis by *S. aureus* biofilm. Bao et al. (2017) used LC-MS/MS to investigate the interaction of microorganisms in the mixed species biofilm. The authors found that the newly introduced *Anaeroglobus geminatus* caused a significant increase in the number of *P. intermedia* in the biofilm and affected the expression of related proteins from carbon metabolism and iron transport pathway. According to proteomic analysis, Kugadas et al. (2019) reported that the abundance of α-mannosidase produced by neutrophils of the host increased during biofilm infection, indicating that the host increases resistance by degrading the extracellular polysaccharide component of the biofilm. Metabolites can be detected by a mass spectrometry (MS), but some metabolites cannot be ionized. The use of MS combined with nuclear magnetic resonance (NMR) can provide more systematic information of the metabolic pathways in biofilms. Sadiq et al. compared the difference in the metabolome between the biofilm and planktonic bifidobacteria. The results showed that 16 metabolites in biofilm cells were up-regulated, 48 metabolites in planktonic cells were up-regulated. The production of poly-N-acetylglucosamine, a component of the extracellular matrix of biofilms, is over four times higher than the planktonic ones. Kart et al. (2020) compared the metabolic differences between *C. albicans* biofilm alone and co-cultured with *Proteus mirabilis*. It was found that the growth of *C. albicans* was inhibited during co-culture, its carbohydrate, amino acid and other metabolism changed, and the
metabolite putrescine was up-regulated by 230 times. So far, most of studies on the multi-omics analysis of the microbial biofilm employed two of the omics, especially the combination of transcriptomics and proteomics or proteomics and metabolomics. The studies combining three or even four omics methods are limited (Ellepola et al. 2019; Sun et al. 2020; Favre et al. 2019). A combination of proteomics, transcriptomics, and metabolomics had been employed to study the evolved Nontypeable Haemophilus influenzae biofilm formation mechanism. The quantitative analysis identified 29 proteins, 55 transcripts, and 31 metabolites. It was observed that pyruvate is a key point in the metabolic pathway related to changes in cAMP phosphodiesterase activity during the formation of biofilms (Harrison et al. 2019).

16.5 Virulence

Biofilms tend to induce chronic infections, which may be related to the sustained release of virulent factors (Phillips and Schultz 2012). On the one hand, the protective function of the biofilm creates suitable conditions for the continuous production of the virulence of pathogenic bacteria. On the other hand, the extracellular matrix of biofilm may play a role in buffering the release of virulent factors. For example, the half-lethal dose of Streptococcus suis HA9801 biofilm cells is more than 40 times that of the planktonic ones (Wang et al. 2011). The pathogenic mechanism of planktonic cells and biofilm cells may be different. Different patterns of gene expression may lead to a distinct response of the host. Research by Patrick R Secor et al. (2011) showed that S. aureus biofilm triggered the production of p inflammatory cytokines in the host, while the planktonic cells induce a significant increase in the level of cytokines. In addition, various studies have shown a connection between the virulence genes and biofilm (Fattahi et al. 2015; Ghazalibina et al. 2019; Baldry et al. 2016). The virulence genes fimA, papC, and hly of E. coli strains isolated from the urinary tract infection were reported to contribute to the biofilm formation, among which papC is the most highly related one (Fattahi et al. 2015). Mehran Ghazalibina et al. (2019) summarized 14 articles on the clinically isolated strains of P. aeruginosa from Iran, and discovered that their virulence genes including exoA, ppyR, algU, exoY, and pslA. They finally concluded that there is clearly relationship between these virulence genes and biofilm formation in most of the research articles. Similarly, the inhibition of virulence genes also affects the biofilm formation. Norlichexanthone is combined with AgrA to reduce the expression of the virulence gene hla, encoding the α-hemolysin, which further reduced the damages to neutrophil and inhibits the formation of S. aureus biofilms (Baldry et al. 2016). Ma et al. (2012) found two of antimicrobial agents CCG-203592 and CCG-205363, which can inhibit the expression of the virulence factor streptokinase (SK) in S. aureus and biofilm formation by more than 25%. The mixed bacterial biofilms are more complicated. It was found that the Streptococcus sanguinis or/and Lactobacillus casei posed opposite effects on the formation of Streptococcus mutans
biofilm, but their regulatory trends on selected virulence factors \textit{spaP} and \textit{gbpB} were roughly the same (Wen et al. 2010).

16.6 Conclusion

Microbial biofilms exist in various food production processes. Due to its extracellular matrix’s protection function for biofilm cells, the formation of cell subpopulation, and the molecular regulation of stress response system, they can survive under various food-related stimuli. Eventually, it can sustain releases toxins and lead to potential food safety hazards. In the past, researchers paid more attention to biofilm in the medical and environmental fields. Nowadays, the potential social health threats and economic losses brought by biofilm in the food field have attracted more and more attention. However, there are relatively few studies on biofilm in various food-related conditions such as food raw materials, processing, transportation, and storage. Due to the food-related environments (oxygen, pH value, nutrient composition, various additives and processing) are different from the human body and the natural environment, the applicability of models suitable for medical and environmental fields in the food is unknown. At present, there is no effective way to completely inhibit and remove the biofilm. Establishing models that simulate the food environment to study the structure, composition, characteristics, virulence, and mechanism of the biofilms can provide more targeted support for removal methods in the food field. In addition, although there have been various research methods for biofilm, there is still a lack of relevant gold standards. Due to the heterogeneity of the biofilm, the development of in-situ analysis methods for the biofilm would have good effect on further understanding their drug resistance and cell subgroups; At present, morphological methods are commonly used to distinguish the biofilm state from the planktonic one. The omics method will contribute to a more comprehensive and deep analysis of biofilm from the molecular level and has the potential for distinguishing the state of microorganisms and providing a novel technology for the research related to biofilm identification and removal in the future.

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Chapter 17
Bacterial Spores

Ruiling Lv and Donghong Liu

Abstract As a dormancy state of bacteria, spores exhibit resistance toward various stresses during food processing. However, once the environment becomes suitable, the bacterial spores will germinate and resume growth, which might pose potential risk to public health. To date, heat treatment is the most widely used method to inactivate the enzyme and microbe to assure food safety. In order to inactivate the spores, thermal treatment at a relatively high temperature (121 °C) is required for more than 20 min. This inevitably leads to the deterioration of food quality, such as nutrition, color, function, texture, and aroma. Currently, numerous studies focus on the sporicidal effect and the associated mechanism by various non-thermal technologies. This chapter provides a comprehensive introduction to bacterial spores, including the formation and germination of spores, the structure and resistance, the inactivation effect and mechanisms used by food processing technologies.

Keywords Spore · Dormancy · Sporulation · Resistance · Germination · Food safety

17.1 Introduction

Spores are the dormant state of bacteria with a multilayer structure, which is a remarkable example of survival strategies to deal with adverse environmental conditions (Paul et al. 2019). Spores have strong resistance to various processing techniques and bactericides, they are difficult to inactivate directly. Thus, spores are considered as one of the potential risks to public health and industry. The formation of spores is of great significance for bacteria to resist external stress. The control of spore is important in food industry. However, the resistance and dormancy are vital characteristics of spores in medicine, crop protection, and other fields.
The unpredictability of natural ecosystem environmental conditions is a continuous challenge to survival. Therefore, an organism needs to strive against the external factors, which are not conducive to growth and proliferation in its lifetime. This phenomenon is attributed to various reactions, one of which is decreased metabolic activity in dormant cells, reducing the adaptation cost of dormancy caused by environmental stresses for the population (Paul et al. 2019).

Bacterial species have been defined by the different strategies used to survive challenging environmental conditions. The above strategies may be involved in an array of response systems of the whole community, such as biofilm formation (Kreft 2004), or help single cells to survive under stresses. Bacterial dormancy has been as one of the most widely explored survival strategies, which contribute to the survival of communities and individuals.

17.2 Spore Formation Induced by Various Food-Related Stressors

17.2.1 The Formation of Bacterial Spores

When some Gram-positive bacteria (such as Bacillus and Clostridium) were subjected to environmental stresses such as nutrient deficiency, they formed a dormant structure: spores (Sunde et al. 2009). Sporogenesis is conducted by bacteria to resist adverse environment. Spores are not only a kind of resistant structure that can maintain dormancy for long periods of time, but also are carriers that can improve the transmission rate, avoiding the local adverse conditions hindering optimal growth (Nicholson 2002). The dormancy process of these bacteria can be summarized as three stages: initial stage, dormancy stage, and recovery stage (Lennon and Jones 2011).

17.2.2 Factors Affecting Spore Formation

The environmental factors that affect the growth of spore-forming bacteria also have a profound impact on the formation of spores. This section summarizes the factors that affect spore formation (Table 17.1).

17.2.2.1 Temperature

To date, no spores have been found to form outside the allowable temperature, water activity ($a_w$), or pH range. Bacterial cells more easily form spores under the optimum culture temperature, pH, and $a_w$. When the temperature, pH, or $a_w$ deviated from the
optimal conditions, sporulation decreased and the process was prolonged (Baril et al. 2012; Planchon et al. 2011). It is reported that the sporulation time was 3 days under optimal conditions for *Bacillus subtilis* ATCC 31324 (37 °C in nutrient broth). However, it took 10 and 14 days for *B. subtilis* to form spores at 45 °C and 19 °C, respectively (Minh et al. 2011).

It has been reported that the maximum specific growth and sporulation rate of *Bacillus weihenstephanensis* and *Bacillus licheniformis* were affected by pH and temperature (Baril et al. 2012). The sporulation yield of *Bacillus* remained high under various environments (Garcia et al. 2010; Minh et al. 2008).

### 17.2.2.2 Osmolality

Spore-forming medium containing 1.2 M of NaCl could not produce phase bright *B. subtilis* spores. The inhibition effect of high salinity (7% NaCl) on the sporulation of *Bacillus subtilis* occurred in the early stage because of the impairment of the activity of Spo0A and σ^H^ (Widderich et al. 2016).

### 17.2.2.3 Oxygen Concentrations

The production of *B. thuringiensis* and *B. cereus* spores was reported to be influenced by the O_2_ concentrations: the number of spores generated under O_2_ limitation was lower than that under aerobic conditions (Boniolo et al. 2012; Abbas et al. 2014).

**Table 17.1** Factors affecting spore formation

<table>
<thead>
<tr>
<th>Factors</th>
<th>Strains</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth temperature</td>
<td><em>Bacillus subtilis</em></td>
<td>The sporulation time was 3 days at 37 °C and 10 and 14 days at 45 °C and 19 °C, respectively.</td>
</tr>
<tr>
<td>Osmolality</td>
<td><em>Bacillus subtilis</em></td>
<td>Medium containing 1.2 M of NaCl failed to produce spores.</td>
</tr>
<tr>
<td>Oxygen concentrations</td>
<td><em>Bacillus thuringiensis</em> and <em>Bacillus cereus</em></td>
<td>The number of spores formed under oxygen limitation was lower than that under aerobic conditions.</td>
</tr>
<tr>
<td>Metal ions</td>
<td><em>Bacillus subtilis</em></td>
<td>Mn^{2+}, Mg^{2+}, Zn^{2+}, and Ca^{2+} increased the sporulation efficiency and improved the stability of spores. The sporulation time of <em>B. subtilis</em> in the absence of Ca^{2+} was five times longer than that in the presence of Ca^{2+}.</td>
</tr>
<tr>
<td>Saccharide</td>
<td><em>Bacillus subtilis</em></td>
<td>The concentration of glucose (1.04 g/L) increased the spore yield by a factor of 17.</td>
</tr>
</tbody>
</table>
17.2.2.4 Metal Ions

The addition of Mn$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, or Ca$^{2+}$ had been shown to increase the sporulation efficiency and improve the stability of spores, causing the spores to no longer germinate spontaneously (Atrih and Foster 2001). The complete sporulation time of *B. subtilis* in the absence of Ca$^{2+}$ was nearly five times longer than that in the presence of Ca$^{2+}$ (Minh et al. 2011).

17.2.2.5 Amino Acids and Saccharide

The sporulation time and yield were closely related to the concentration of amino acids and carbohydrates in the growth conditions (Schaeffer et al. 1965). Glucose and ribose have been found to increase the sporulation of *B. cereus* and *B. subtilis* (Monteiro et al. 2005; de Vries et al. 2005). In the study of Posada-Uribe et al. (2015), the concentration of MgSO$_4$·7H$_2$O (0.59 g/L) and glucose (1.04 g/L) at 30 °C and 150 rpm was further optimized to increase the sporulation yield of *B. subtilis* strain by a factor of 17.

17.2.3 Factors Affecting the Structure and Composition of Spores

Changes in sporulation conditions also affected the structure and composition of spores. In the process of sporulation, spores accumulate minerals (mainly Ca$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$) in the core (Bressuire-Isoard et al. 2018). The concentration of core minerals varied greatly and was highly dependent on the spore-forming conditions (Bressuire-Isoard et al. 2018). The core had a high content of dipicolinic acid (DPA), which might form an inorganic polymer to maintain the glassy or gel-like state of the core (Setlow and Li 2015). The CaDPA polymer might contribute to spore resistance by fixing proteins and molecules within the core (Cowan et al. 2003, 2004). *B. subtilis* spores could not synthesize DPA themselves. If *B. subtilis* spores sporulated in the growth medium without DPA, the water content in spore core was much higher than those formed in the medium with DPA (Paidhungat et al. 2000). The spores of several *Bacillus* strains formed on nutrient agar containing metal ions (e.g., Fe$^{2+}$, Ca$^{2+}$, Mg$^{2+}$, K$^+$, Mn$^{2+}$) exhibited lower water content than those generated on the medium containing only Mn$^{2+}$ or no Mn$^{2+}$ (Cazemier et al. 2001; Minh et al. 2011). The temperature for spore formation was also one of the most important factors affecting the DPA synthesis and core water content. Nevertheless, the exact effect of temperature remains unclear (Kaieda et al. 2013; Melly et al. 2002). The DPA concentrations of *B. anthracis* and *B. cereus* spores generated under 30 and 45 °C were higher than those under 10 and 25 °C (Planchon et al. 2011; Baweja et al. 2008). The water containing in the *B. subtilis* spores generated under relatively
higher temperatures was lower than those formed at lower temperatures (Melly et al. 2002; Minh et al. 2011). In addition, a higher temperature for spore formation was also associated with a higher mineralization level of core spores (Igura et al. 2003). It is found that the temperature slightly contributes to the changes in structure of cortex peptidoglycan, and the percentage of cross-linked teichoic acid in spores generated at higher temperatures increased slightly (Melly et al. 2002). Compared with the enriched medium, the structure of cortical peptidoglycan in Bacillus spores generated in an oligotrophic medium lacking carbon source also changed substantially (Atrih and Foster 2001).

Changes in pH, sporulation temperature, or inorganic salt led to significant changes in the volume of spores. The size of B. subtilis spores generated in a medium lacking Ca$^{2+}$ was observed to be around half of those formed in a medium with Ca$^{2+}$ (Minh et al. 2011). B. cereus spores generated in a liquid medium were significantly smaller than those in an agar medium or biofilm (van der Voort and Abee, 2013). It was reported that the surface roughness of spores was affected by the medium water activity or growth temperature (Minh et al. 2011). The spore expansion of B. thuringiensis and B. subtilis under different relative humidities and core hydration/dehydration may indicate the relationship between spore size and spore hydration/dehydration (Sunde et al. 2009; Westphal et al. 2003). Besides, the spore formation conditions led to variations in spore volume (Zhou et al. 2017). Other modifications in structure were also related to the sporulation conditions, which were observed using electron microscopes. For example, the exospores of B. cereus were destroyed and isolated at higher temperatures (Faille et al. 2007). B. subtilis spore coat was thicker in propane sulfonic acid buffered 2x Schaeffer’s-glucose liquid medium (broth) than on solid 2x Schaeffer’s-glucose agar plates (Abhyankar et al. 2016).

The profiles of B. subtilis spore coat proteins were distinct under various temperatures or in broth/agar medium (Abhyankar et al. 2016; Rose et al. 2007; Melly et al. 2002). However, there were no differences between α/β-type small acid-soluble proteins (SASPs) under the same environment for sporulation (Rose et al. 2007; Melly et al. 2002). The electrophoretic bands of CotA, CotG, CotB, and CotS prepared at sporulation temperatures of 22 or 30 °C were lower than those at 48 °C (Rose et al. 2007). The electrophoretic bands of CotA, CotB, CotG, and CotS were different in Bacillus cereus spores generated under 20 or 37 °C (Bressuire-Isoard et al. 2016). The proportion of CotE proteins in spore extracts produced at 20 °C was higher than in spore extracts produced at 37 °C (Rose et al. 2007). These differences might be due to differences in protein quantity and/or extractability. For example, the total amount of proteins extracted from B. subtilis spores generated on agar was higher than for spores formed in broth (Rose et al. 2007). However, the coat protein contents of spores generated in broth or agar had no significant difference (Driks 1999).

The fatty acid composition of B. subtilis and B. cereus spores showed significant differences under various temperatures for sporulation. The total anteiso fatty acid content of the spores increased when formed at lower temperatures (Planchon et al. 2011). With decreasing temperature, the ratio of anteiso/iso branched-chain fatty
acid and the content of unsaturated fatty acid in the inner membrane of *B. subtilis* spores increased (Cortezzo and Setlow 2005). Additionally, higher anteiso/iso fatty acid ratios were observed in the inner membrane of spores formed in a Petri dish compared to those formed in a broth (Rose et al. 2007).

### 17.3 The Multilayer Structure and Resistance of Bacterial Spores

Resistance to extreme temperatures was the difference between vegetative cells and spores, since the inactivation temperature of spores was about 45 °C higher than that of vegetative cells (Checinska et al. 2015; Setlow 2014). The wet-heat inactivation of spores was mainly achieved through the denaturation of metabolism enzymes and damages in core proteins (Setlow 2014; Coleman et al. 2007; Wells-Bennik et al. 2016). Spores were saturated with DNA through α/β-type SASPs, high DPA, as well as low water content in spore core and low mineral content, which prevented molecular fluidity in the core and protected proteins from undergoing irreversible aggregation and denaturation (Setlow 2014; Sunde et al. 2009). Through cross-linking peptidoglycan, the cortex also contributes to keeping the dehydrated state of *B. subtilis* spore core (Driks 1999; Atrih and Foster 1999). Nevertheless, it is still unknown how this change of cortical components could maintain the dehydration of the spore core. In the process of *Bacillus subtilis* spore maturation, as the cross-linking of coat proteins increased, the heat and moisture tolerance of the spores was found to be enhanced (Abhyankar et al. 2015; Sanchez-Salas et al. 2011). Resistance to dry heat was associated with DNA saturation by α/β-type SASP in the core and activation of systems (such as RecA protein) that repaired dry heat induced DNA damages (Setlow and Setlow 1996; Nicholson et al. 2000; Setlow 2014). Furthermore, spores can also tolerate multiple instances of physical damages, including drying, freezing–thawing cycles, high-pressure processing, ultraviolet (UV) and gamma radiation, and chemical damages (Setlow 2014; Checinska et al. 2015). Spores were 10–50 times more resistant to UV radiation than the cells in the vegetative form (Nicholson et al. 2000). The UV resistance of spores involved two main factors: the binding of α/β-type SASPs to DNA and the DNA repair system (Checinska et al. 2015). In addition, other factors affected the UV resistance of spores, such as high DPA level in the spore core and low water content (Setlow 2014).

Strong acid treatment, high-temperature organic solvents, and hydrogen peroxide could cause serious damage to the inner membrane (Setlow 2006). The oxidation of membrane proteins might lead to cell rupture (Cortezzo and Setlow 2005; Cortezzo et al. 2004). Strong alkali treatment mainly damaged the spore coat and hydrolyzed the cortex (Setlow et al. 2002). Spore coat was considered as the first barrier against the macromolecules targeting the spore cortex and it has been reported to contribute to the protection of spores from chlorine dioxide, hypochlorite, peroxynitrite, and
other oxidants (Young and Setlow 2003). Contributing factors for the resistance of spores to chemicals include detoxification enzymes (e.g., catalase and superoxide dismutase) in the cell wall of spores, the low permeability of the spore endomembrane, α/β-type SASPs for DNA protection, and the ability to reduce the capability of the DNA repair system (Setlow 2006, 2014).

17.4 The Inactivation of Spores by Food Processing Technologies

17.4.1 Thermal Treatment

Although spores were resistant to high temperatures (about 45 °C higher than vegetative cells), the most common and effective inactivation method for spores was thermal treatment (Setlow 2006). Thermal treatment at 88 °C for up to 90 min inactivated 99.9% of *B. cereus* spores (Coleman et al. 2010). Alex et al. (2013) investigated the effects of thermal treatment on the surface structure of spores via an atomic force microscopy. The results indicated that thermal treatment damaged the surface structure of spores. During the thermal treatment of spores, the release of DPA appeared to be an all-or-nothing phenomenon, since there were no spores with partial DPA release (Zhang et al. 2010). Besides, it seems that the release of DPA mainly occurred after spore death, and the spores that retained DPA still died when recovering in the rich medium (Fan et al. 2019b). For example, the release of DPA was unsuccessful in nonviable *B. subtilis* spores with the vitality of DPA (Coleman and Setlow 2009). During the process of thermal treatment, the structure of the sporophyte changed after the release of DPA. More interestingly, thermally treated spores that retained DPA could still germinate (Coleman and Setlow 2009). However, some DPA-filled spores killed by thermal treatment could not germinate, which indicated that these non-germinating spores had accumulated more damage (Coleman and Setlow 2009). Spores with high levels of denatured protein caused by thermal treatment were hardly able to germinate (Coleman et al. 2007). Thermal treatment inactivated *B. subtilis* spores by destroying one or several essential proteins. Thermal treatment more easily inactivates spores with compromised ATP production, indicating that thermal treatment inactivated spores by damaging metabolic enzymes (Coleman et al. 2010). Thus, the characteristics of the enzymes targeted by thermal treatment should be further investigated (Coleman et al. 2010).

17.4.2 Ultrasound

As a green non-thermal physical sterilization technology, ultrasound treatment can destroy microorganisms in a short period time (Shankar and Pagel 2011). At the
same time, it maintained food quality and reduced the damage to functional components in food, which had a broad application prospect in the food industry (Tremarin et al. 2017; Yu et al. 2020). The main mechanism of ultrasonic action was cavitation, involving both a thermal and mechanical effect (Tremarin et al. 2017). During the ultrasonic process, once the cavitation bubbles reached a critical size, they would collapse and locally produce intensively mechanical and chemical energy (Jose et al. 2014).

Lv et al. (2019b) found that a short duration of ultrasound treatment (200 W, 1 min) could make the outer-most layer unattached, which reduced the hydrophobicity of B. cereus spores. Additionally, individual ultrasound contributed to insignificant effect on the elimination of B. cereus spores due to the high resistance of spores (Lv et al. 2019a). However, ultrasound could enhance the inactivated efficacy of spores in combination with other thermal and non-thermal treatments including cold plasma, UV radiation, and electrolyzed water (Evelyn and Silva 2015). Simultaneous ultrasound and high-pressure treatment caused the release of the majority of DPA in the spore core by triggering the germinant receptors and releasing ions, proteins, and CaDPA (Lv et al. 2019a). Acidic electrolyzed water inactivated 1 log CFU/mL of B. cereus spores, while the effect of ultrasound was negligible. However, the inactivation effect of ultrasound combined with electrolyzed water was synergistic (2.29 log CFU/mL) (Lv et al. 2020). The synergistic mechanisms were that electrolyzed water disrupted the inner membrane, and ultrasound mainly hydrolyzed the cortex and damaged the structure (Lv et al. 2020). Fan et al. (2019a) investigated the inactivation effect of the thermosonication of B. subtilis spores and found that thermosonication wreaked havoc on the spore structure (cortex, coat, and inner membrane) and metabolic enzymes. It is worth noting that the inner membrane of spores acts as a barrier against chemicals. Damage to the inner membrane induced by thermosonication treatment caused the subsequent leakage of intracellular substances, which might be the main reason for the inactivation of B. subtilis spores (Fan et al. 2019b). Also, it was reported that there were 167 differentially expressed proteins with the exposure of thermosonication; these proteins were mainly involved in the metabolic processes including amino acid and energy metabolism (Fan et al. 2020). Several chemical compounds, including chitosan, nanoparticles, and L-alanine combined with ultrasound were reported to cause a synergistic inactivation effect on spores (Guerrero et al. 2005; Sagong et al. 2013). Based on previous studies, Fig. 17.1 shows the mechanisms underlying sporicidal effect of ultrasound combined treatment.

17.4.3 Non-Thermal Plasma

Plasma is the fourth state of matter, composed of partially or completely ionized gas (Liao et al. 2019) and exists as two types: thermal and non-thermal plasma (Murugesan et al. 2020). In non-thermal plasma, also called cold plasma, the electrons exhibit much higher temperatures than other reactive species, leading to
an overall temperature of non-thermal plasma below 60 °C (Liao et al. 2019). Cold plasma can be generated through multiple methods, including gliding arc discharge, dielectric barrier discharge, glow discharge, and corona discharge (Liao et al. 2019).

Cold plasma treatment (power density: 35 mW/cm²) for 3 min reduced *B. subtilis* spores by more than 5 log CFU/mL, while 2-log reduction of spores could be achieved by 30-min exposure (Wang et al. 2016). In the study of Hertwig et al. (2017), the plasma-induced *B. subtilis* and *B. atrophaeus* spore inactivation curves were fitted with the Weibullian-power law model, which fully described the plasma-induced inactivation behavior, and curve shapes are various for different generated plasma. The sterilizing effect of cold plasma included physical effect, chemical effect, and electrical effects (Lopes et al. 2018). Boudam et al. (2006) emphasized the use of reactive species produced by plasma to induce damage to plasma membrane, proteins, and DNA of spore. The destruction of spore proteins, especially inner membrane proteins, by reactive oxygen species (ROS) could be the main cause for the microbiocidal effect by cold plasma (Klämpfl et al. 2012). After cold plasma treatment, the damage and rupture of spore structure were observed, followed by the leakage of cytoplasmic components (Klämpfl et al. 2012). Besides, some studies showed that DNA damage was the primary mechanism of the inactivation caused by the UV radiation of plasma (Roth et al. 2010; Li et al. 2018; Taylor et al. 2020). Generally, cold plasma results in direct inactivation on spores, rather than the induction of germination. Liao et al. (2019) reviewed the detailed targets of plasma-induced spore inactivation and summarized the inactivation mechanism (Fig. 17.2).

Fiebrandt et al. (2018) investigated the spore inactivation by cold plasma with various wavelengths ranging from 100 to 400 nm and found that the sporicidal rates of broadband plasma emissions were similar to those of monochromatic light. In addition, the effects of ions, free radicals, and metastable state on the plasma-induced
sporicidal effect were slight, and radiation was considered as the major contributor to spore inactivation.

Cold plasma is environmentally friendly and possesses the advantages of high antimicrobial efficacy and low working temperature. However, direct plasma exposure might result in compromised food quality (Lopes et al. 2018). Thus, plasma-activated water (PAW) and its combination with heat treatment have been proposed for microbial inactivation (Lopes et al. 2018). Bai et al. (2020) reported that a lower initial *B. cereus* spore concentration, higher temperature, and lower volume of water improved inactivation efficiency.

17.4.4 Ultraviolet

Ultraviolet (UV) radiation has been utilized as a green and safe sterilization technology for over 140 years (Delorme et al. 2020). The primary mechanism of UV for sterilization is the UV-induced formation of dimers in the pyrimidine base of DNA, leading to the inhibition of DNA replication and microbial inactivation (Delorme et al. 2020). UV with wavelengths ranging from 200 to 280 nm (especially 250–260 nm), also known as UV-C, is widely used for food decontamination (Ochoa-Velasco et al. 2020). Taylor et al. (2020) reported that 222-nm UV radiation could be a substitute for 254-nm UV radiation commonly used for disinfection. SASPs, DNA repair proteins, and a high concentration of CaDPA were found to enhance the UV tolerance of spores (Taylor et al. 2020). Do Prado et al. (2019) found that 20-min UV-C at an intensity of 16.8 kJ/m² decreased *Alicyclobacillus* spp. spores by 4 log CFU/mL, and the formation of biofilm was also reduced. Setlow (2006) indicated that *B. cereus* spores exhibited higher resistance to UV than *B. subtilis*.

Begyn et al. (2020) established a directed evolution experiment to carry out sporulation, germination, and outgrowth after the UV treatment of *Bacillus cereus* spores. This study indicated that the resistance of spores to UV-C was easily
improved but at the cost of a compromise between thermal tolerance and germination efficiency. Additionally, the tolerance of spores to UV could not be retained once they became vegetative cells (Begyn et al. 2020). Besides, part of the sub-lethal spores could repair the UV-induced damage (Begyn et al. 2020). Photolyases could attach to dimers and reverse dimer formation with the application of visible light-generated energy (Setlow and Li 2015). In order to achieve complete inactivation, novel UV-based hurdle technologies were developed to overcome spore resistance to UV radiation and the photolytic enzyme repair. Sauceda-Galvez et al. (2020) combined UV radiation with high-pressure homogenization technology to eliminate Alicyclobacillus acidoterrestris spores in apple juice, indicating that UV radiation and high-pressure homogenization had an additive synergistic inactivation effect. Tremarin et al. (2017) applied the combination of UV radiation with ultrasound technology for spore inactivation, and the inactivation rate of the combined treatment was significantly higher than that of the individual methods. It was reported that UV light-emitting diodes have a synergistic effect with chlorine for the elimination of spore, and reactive free radicals generated from UV/chlorine might be a major contributor to the synergistic inactivation (Li et al. 2018).

17.4.5 High-pressure Processing

Spores can resist the harsh factors including heat, high osmotic pressure, and low nutrients and moisture. Nevertheless, as spores germinate into vegetative form, their stress tolerance decreases keenly (Reineke et al. 2013b). Thus, the induction of spore germination might be a strategy to achieve spore inactivation (Modugno et al. 2020). High-pressure processing (HPP) was considered as a promising disinfection technology, with a pressure in the range of 100–600 MPa usually employed for commercial use (Serment-Moreno et al. 2014).

The nutrient germinant receptors (nGRs) were receptors for germination induced by HPP since spores lacked nGRs genes could not germinate under HPP (Paidhungat et al. 2002). So far, nGR triggered spore germination was the most thoroughly studied non-nutrient germination pathway (Zhang et al. 2020). Spore strains that lacked nGRs could not germinate under proper pressure, which indicates that nGRs were receptors for pressure-induced germination (Paidhungat et al. 2002). In addition, HPP was found to activate the cortex-lytic enzymes for the degradation of the cortex, leading to the subsequent rapid release of CaDPA and the reduced resistance of spores (Reineke et al. 2013a, b, c). Kong et al. (2014) detected the spore germination rate under different pressures, and showed that more than 95% of Bacillus spores achieved germination with 40 min exposure under 150 MPa, however, the kinetics of spore germination was heterogeneous.

It is found that the HPP at 400–700 MPa and room temperature can induce a reduction of B. subtilis spores by about 4 log CFU/mL (Black et al. 2007). Changes in the internal structure of the spores induced by HPP were analyzed by flow cytometry and electron microscopy (Black et al. 2007). The results showed that
150-MPa HPP at 37 °C could induce the release of DPA and the degradation of DNA-bound SASPs (Reineke et al. 2013a, b, c).

The inactivation rate of *B. subtilis* spores was over 3 log CFU/mL, and the germination rate was over 5 log CFU/mL after pressure treatment between 0.2 and 0.6 MPa (65 °C) for 10 min (Hauck-Tiburski et al. 2019). The germination and inactivation rate of spores increased as the temperature increased (Hauck-Tiburski et al. 2019). Wang et al. (2017a, b) applied the combination of slightly acidic electrolyzed water (SAEW) with HPP for eliminating *B. cereus* spores. They found that spores in SAEW were sensitive to HPP, which meant that SAEW inactivated spores synergistically with HPP. SAEW combined with HPP treatment destroyed spore structure, especially the exosporium. Interestingly, more than 90% DPA of spore released after combined treatment which decreased the resistance of spore. In addition, the results showed that the combination of HPP and SAEW could inactivate spores without germination (Wang et al. 2017a, b).

In conclusion, HPP can be used as an effective tool for both spore germination and inactivation. Spores have a strong resistance to environmental stresses and cannot be inactivated by individual pressure treatment. The combined application of HPP technology with heating, nisin, ethanol, and other technologies has a synergistic effect on spore inactivation, which can be widely applied in the food industry.

### 17.5 Spore Germination

Physiological germinates, such as specific amino acids and sugars, are recognized by germinant receptor (GR) protein complexes in the inner membrane, which can initiate a cascade of spore germination reactions (Setlow et al. 2017; Xing and Harper 2020). Spores had various GRs with distinct and/or overlapping germinant specificities, and various GRs usually cooperatively triggered spore germination (Setlow et al. 2017; Borch-Pedersen et al. 2016).

Except for amino acid and sugar germinants, spore germination can be induced by other specific agents (Setlow et al. 2017). High hydrostatic pressure (HHP) was used to induce spore to be germinated by GRs under a pressure of 150–400 MPa and 37 °C or by the SpoVA channel for CaDPA under a pressure of over 500 MPa and 50 °C (Borch-Pedersen et al. 2016). HHP treatment under higher pressure of 600–700 MPa at an initial temperature of around 90 °C has been used for producing shelf-stable foods and maintained the future prospects of sterile foods. However, it is still not clear if HHP directly affects GRs/SpoVA or indirectly causes the spore inner membrane to change proteins embedded in the inner membrane (Setlow et al. 2017).
17.6 Biotechnological Applications of Spore-Forming Bacteria

The understandings of spore diversity and ecology can be directly applied in various fields of biotechnology (Zhang et al. 2020). For example, since endospore formation under adverse environmental conditions may promote the persistence of *Bacillus* in soil, it may contribute to the development of efficient inoculants (Kumar et al. 2011). Spores are also used as the potential delivery systems for drugs, nucleic acids, or antigens (Farjadian et al. 2018).

17.7 Conclusion

Spores with high resistance are a major concern in the food industry. To date, most studies focused mainly on the inactivation and germination effects and the underlying mechanisms of thermal treatment and HPP. However, the sporicidal effects of novel non-thermal technologies were found to be limited. Therefore, the hurdles faced by technology in efficiently inactivating spores need to be further investigated.

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Chapter 18
Sublethal Injury Adaptation in Foodborne Pathogens

Imran Khan, Shehla Sammi, Bashir Ahmad, Inam Ullah, Sumaira Miskeen, Muhammad Liaquat, and Muhammad Jahangir

Abstract Various environmental stresses and food processing methods (physical and chemical) can cause sublethal damage to microorganisms. The presence of sublethally injured microbial cells in foods poses a great threat to food safety, as these cells have the ability to repair their damaged components when they encounter suitable conditions. Sublethally injured cells can go undetected during food processing, increasing the risk of spoilage, and toxin production. Therefore, sublethally injured foodborne pathogens should not be ignored. In this chapter, the adaptive responses of sublethally injured microorganisms and their associated molecular mechanisms, virulence and pathogenicity are systematically discussed.

Keywords Sublethal injury · Foodborne pathogens · Food safety · Resuscitate

18.1 Introduction of a Sublethally Injured State in Foodborne Pathogens Under Stresses

Foodborne pathogens subjected to physical or chemical treatments could undergo injury that could be repaired through the resuscitation process under suitable conditions (Fig. 18.1). Such sublethal injury of foodborne pathogens poses a potential threat to food safety (Horn and Bhunia 2018; Lan et al. 2019). Sublethal injury has been observed for numerous foodborne pathogens when subjected to sublethal stressors, such as high-pressure carbon dioxide; organic acids; electrolyzed water; the process of freezing, air-drying, or freeze-drying; heat; dyes; sodium azide; irradiation; heavy metals; high hydrostatic pressure; aerosolization; chemical or natural antimicrobial compounds; antibiotics; sanitizing compounds; and essential oils (Table 18.1) (Bi et al. 2015; Han et al. 2018; Lan et al. 2019; Wu 2008). The
Fig. 18.1 Effect of stressors on foodborne pathogens in foods

- Refrigeration, freezing, high temperature
- Aerosolization
- Air drying, freeze-drying
- Sugars, salts
- Irradiation
- Osmotic shock
- Hydrostatic pressure
- Pulsed electric field, etc.

- Organic and inorganic acids
- Chlorine, detergent
- Mercuric, chloride
- Natural antimicrobial compounds
- Heavy metals such as copper, zinc, lead, nickel
- Electrolyzed water
- Essential oils, etc.

Normal foodborne bacterial cells

Physical stressors
Chemical stressors

Dead
Alive
Injured Normal
Dead Survive
<table>
<thead>
<tr>
<th>Stress types</th>
<th>Microorganisms</th>
<th>Treatment conditions</th>
<th>Sublethal injured cells</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorine solution (pH 6.5)</td>
<td>Shiga toxin–producing <em>Escherichia coli</em> (STEC)</td>
<td>Chlorine concentration (10 – 40 ppm) for 60 s</td>
<td>At 40 ppm, sublethal injury in stationary phase cells were higher as compared to long-term survival-phase cells</td>
<td>Bhullar et al. (2021)</td>
</tr>
<tr>
<td>Peracetic acid, lactic acid, heating and benzalkonium chloride</td>
<td><em>Listeria monocytogenes</em> Scott A, <em>L. monocytogenes</em> EGDe</td>
<td></td>
<td>Highest injury levels were observed for all the stressors</td>
<td>Siderakou et al. (2021)</td>
</tr>
<tr>
<td>Mild heating</td>
<td><em>Staphylococcus aureus</em> CECT 4459</td>
<td>Heat treatment ranges from 10 to 42 °C</td>
<td>Increase heat resistance was observed in cells due to the repair of injured cells</td>
<td>Cebrián et al. (2019)</td>
</tr>
<tr>
<td>Blue (462 ± 3 nm) Light emitting diode illumination</td>
<td><em>E. coli</em> (ATCC 11775), <em>S. aureus</em> (ATCC 12600)</td>
<td>High intensity 462 ± 3 nm LED (13 J/cm²) and photosensitizer curcumin at 10 and 20 μM</td>
<td>&gt; 90% sublethal injury was observed for the cells treated with photosensitizer and blue light simultaneously</td>
<td>Bhavya and Hebbar (2019)</td>
</tr>
<tr>
<td>Mixed oxide nanoparticles (MONs, TiO$_2$–ZnO–MgO)</td>
<td><em>E. coli</em> (ATCC 8739), <em>S. Paratyphi</em> (ATCC 9150), <em>S. aureus</em> (ATCC 33862), <em>L. monocytogenes</em> (ATCC 15313)</td>
<td>Cells were exposed to mixed oxide nanoparticles at 100 μg/mL for 15 min</td>
<td>Sublethal injury was ranged from 17–98% depends on each bacteria and each treatment</td>
<td>Anaya-Esparza et al. (2019)</td>
</tr>
<tr>
<td>Ohmic cooking and water bath cooking</td>
<td><em>E. coli</em> O157:H7 (NCTC 12900)</td>
<td>Voltage gradients (5, 10, and 15 V/cm) for ohmic cooking, 80 °C for water batch cooking</td>
<td>Both treatment caused sublethal injury to the cells and recovery was observed during the storage of pork batter</td>
<td>Tian et al. (2019)</td>
</tr>
<tr>
<td>Ultraviolet Radiation</td>
<td><em>Salmonella enterica</em> serovar Typhimurium (ATCC 14028)</td>
<td>Cells in saline were exposed to UV radiation (80 μW/cm²) for upto 3 min, cells in apple juice were exposed to 1500 μW/cm² for upto 12 min</td>
<td>Long-term survival phase cells had the least sublethal injury in the surviving population as compared to exponential- and stationary-phase cells</td>
<td>Wang et al. (2018a)</td>
</tr>
<tr>
<td>Stress types</td>
<td>Microorganisms</td>
<td>Treatment conditions</td>
<td>Sublethal injured cells</td>
<td>References</td>
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<tr>
<td>Neutral electrolyzed oxidizing (NEO) water</td>
<td><em>Yersinia enterocolitica, E. coli O157:H7, S. enterica serovar Enteritidis</em></td>
<td>1–50% NEO solution was used for 5 min</td>
<td>Around 38% of <em>Yersinia</em> and 25% of <em>Salmonella</em> population became sublethally injured</td>
<td>Han et al. (2018)</td>
</tr>
<tr>
<td>High Hydrostatic Pressure (HHP)</td>
<td><em>E. coli</em> (ATCC 7839), <em>L. innocua</em> CIP80.11 T</td>
<td>300, 400, 500 MPa HPP at ~20 °C for 5 or 10 min, stored at 5 °C and 25 °C</td>
<td>HHP-sublethally injured <em>L. innocua</em> was observed in carrot juice but not in beet juice</td>
<td>Nasiłowska et al. (2018)</td>
</tr>
<tr>
<td>High-pressure processing (HPP)</td>
<td><em>L. monocytogenes</em> CECT 4031 (serotype 1/2a), <em>L. innocua</em> CECT 910, <em>L. monocytogenes</em> Scott A (serotype 4b)</td>
<td>HPP 300, 400, 500 and 600 MPa at 6 °C for 5 min</td>
<td>~2 log CFU/g sublethal injured cells of <em>L. monocytogenes</em> CECT 4031 in cheese were observed at 400 MPa</td>
<td>Evert-Arriagada et al. (2018)</td>
</tr>
<tr>
<td>Mild heating and sonication</td>
<td><em>S. aureus</em></td>
<td>Ultrasound = 600 W with 20 kHz, mild heat alone = 55 °C</td>
<td>0.06, 0.58 and 0.08 log CFU/mL sublethal injured cells were recorded for ultrasound, mild heat and combined, respectively</td>
<td>Li et al. (2017)</td>
</tr>
</tbody>
</table>
terms *stress* and *injury* have been used interchangeably to describe the effect of sublethal treatment (Wesche et al. 2009). However, the most preferable term to describe such effects is *injury* according to Hurst (1984). Interestingly, injured bacterial cells can repair themselves under an optimal growth environment that contains elements such as the necessary nutrients, pH, and temperature (Bozoglu et al. 2004). After cells are injured, different metabolic processes occur during the repair processes depending on the nature of the stressors. The repair processes involve the synthesis of DNA, RNA, ATP, and mucopeptides (Bozoglu et al. 2004; Han et al. 2018; Pagán et al. 2001; Pagán and Mackey 2000; Wouters et al. 1998). The sublethal stressors damage the structural and functional components of microbial cells, including DNA, RNA, cytoplasmic membrane, inner membrane, ribosomes, cell wall, and some enzymes (Fig. 18.2) (Bozoglu et al. 2004; Jay et al. 2005; Li et al. 2017; Pan et al. 2019; Shi et al. 2017; Wu 2008). Jay et al. (2005) reported that the bacterial component most affected by sublethal stressors is the cytoplasmic membrane. They further stated that not all bacterial cells in a population will undergo the same level of injury and that not all forms of stress produce identifiable injuries. In addition, the severity of stressors, which ranges from minor to moderate to severe to extreme to lethal, impacts cell growth (Fulda et al. 2010). Bacterial cells subjected to a minor/moderate stress may adapt to the new environment and grow normally (Ye et al. 2019). Sublethal stressors also lead to transient

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**Fig. 18.2** Flow cytometric analysis of the effects of a pulsed electric field (PEF) treatment on *Lactobacillus rhamnosus*. Reprinted from (Wang et al. 2018b), with the permission of ELSEVIER
adaptation (an adaptive response), which is accompanied by a temporary physiological modification that sometimes increases stress tolerance (Wu 2008; Yousef and Courtney 2003). Not all bacterial cells die when subjected to lethal stress, while unaffected bacterial cells undergo adaptive mutations at the genetic level, which may enhance the survival of the overall population (Archer 1996; Hengge-Aronis 2000). The association between different stressors and injury levels and the ability of bacterial cells to adapt under these conditions have not yet been well elucidated.

18.2 Molecular Mechanisms of Stress-Induced Sublethal Injury of Bacterial Cells

As occurs for other organisms, microorganisms experience stress as a result of changes in the environment. This phenomenon is very useful in food processing and preservation. However, some microbes become injured at sublethal levels only and develop resistance. This development of resistance in sublethally injured cells is a complex phenomenon and involves many biochemical changes. Stress-habituated microbes are influenced by several internal and external factors, such as the type of microorganisms, stage of development, and environment (Lehrke et al. 2011).

18.2.1 Habituated Microbes Response to Acid Stress

When surroundings change in response to an acidic environment, microorganisms become acid tolerant, i.e., acid habituated. The most promising approach to determine these responses is to consider the synthesis of acid shock proteins (ASPs) at a reduced pH. Approximately 60 ASPs produced during acid stress have been identified (Ramos-Morales 2012). Nevertheless, it has not been fully determined how ASPs are associated with most of the biochemical changes that occur during acid stress tolerance. One possible reason for this association is that some microbes develop new pH homeostasis. Initially, it was suggested that atp is responsible for this novel homeostasis (Foster and Hall 1990), while some claim that the lysine decarboxylase-dependent homeostasis system is responsible for this new pH homeostasis (Rowbury 2003). The second biochemical change with respect to adaptation during acid stress includes protection from acid attack and induction of a damage limitation system. The primary target that acid affects is DNA, and it has previously been shown that mutants that were altered during DNA repair are acid-sensitive (Sinha 1986). One important phenomenon is the improved ability of acid-tolerant cells in comparison to nonhabituated cells to repair acid-damaged DNA. The same scenario occurs with plasmid repair; a relatively large number of plasmid-containing (p+) transformants are induced with the transfer of acid-damaged plasmids into a habituated culture (Raja et al. 1991). It has been reported that ADA (adaptive
response) plays a key role in the repair of DNA damaged by acid stress, as *ada* mutants are not only acid sensitive but also alkali sensitive (Foster and Moreno 1999). Moreover, acid sensitivity was also reported for *recA*, *polA*, and *uvrA* mutants, suggesting that many genes are involved in the repair of acid-damaged DNA (Goodson and Rowbury 1991; Sinha 1986). A novel DNA repair system could be involved in the *polA*, *recA*, or *uvrA* mutants.

### 18.2.2 Habituated Microbes Response to Alkali Stress

When microorganisms are transferred to environments that have pH values of 7 to 9, they become alkali tolerant (Goodson and Rowbury 1990, 1991). There are two possible explanations for the adaptation of cells to alkali environments: first, there is little damage to DNA by alkali conditions due to the induction of NhaA, and cells become alkali tolerant (Rowbury 1997); second, this cell tolerance involves exhibiting an improved repair system of alkali damage to DNA (Rowbury 1997), where NhaA is likely not involved and the novel repair system is apparently independent of RecA and PolA (Rowbury 2003).

### 18.2.3 Habituated Microbes Response to Heat Stress

It has been reported that heat shock proteins (HSPs), such as GroEL/GroES, are responsible for the heat tolerance of microbes and allow their growth at maximum temperatures (Kusukawa and Yura 1988). Several HSPs have been identified as key heat-tolerant factors, whose chaperone functions are vital through the synthesis, transport, folding, and degradation of proteins (Nicolau et al. 2010). Almost all microorganisms have the ability to synthesize HSPs as a quick response to elevated temperatures (Gao et al. 2016). HSPs such as Dnal, GrpE, and DnaK are recognized to regulate the heat shock transcription regulator σ^{32}, followed by the induction of HSP synthesis. Additionally, trehalose accumulates largely during heat shock and plays an effective role in heat tolerance (Reina-Bueno et al. 2012).

### 18.2.4 Habituated Microbes Response to Cold Stress

RNA chaperones (RNA-binding proteins) are found in all organisms and are involved during stress conditions. During cold stress, cold-induced proteins (CIPs), including cold shock proteins (CSPs) and the RNA helicase CsdA, are synthesized (Hébraud and Potier 1999; Yamanaka et al. 1998). The numbers of CSPs vary among different microbes, such as 9 CSPs in *E. coli*, 3 CSPs in *Bacillus*, 5 CSPs in *Lactococcus lactis*, and 3 CSPs in *Lactococcus plantarum* (Rajasheker
et al. 2019; Yamanaka et al. 1998). CSPs protect cells by allowing mRNAs to efficiently translate during stress, among which CspA is the most induced and accounts for 13% of total cellular protein (Goldstein et al. 1990; Hofweber et al. 2005). Both CspA and CspB are important for increasing protein translation during cold stress (Graumann and Marahiel 1998; Jiang et al. 1997; Wang et al. 1999). CspA may function with CsdA, which is also a cold-shock protein associated with ribosomes (Jones et al. 1992).

18.2.5 Habituated Microbes Response to Other Stressors

Stressors such as high-pressure carbon dioxide (HPCD), ohmic heating, and PEF have been reported to cause sublethal injury to microorganisms. Rivas et al. (2013) performed a two-dimensional electrophoresis (2-DE) proteomic analysis of PEF-induced *E. coli* DH5α and reported that structural and metabolic proteins such as OmpA, GmhA, ClpA, RS6, Dut, FtnA, TufB, FtsH, PutA, ATPA, and SdhA were differentially expressed in sublethally injured cells. In another study, Bi et al. (2017) utilized an isobaric tag for a relative and absolute quantification (iTRAQ) for proteomic analysis of HPCD-induced *E. coli* O157:H7. The results indicated that out of a total of 2446 proteins identified, a total of 93 proteins were differentially expressed in the sublethally injured cells compared to those in the control. Among these, 65 proteins showed downregulation and 28 showed upregulation, indicating that sublethally injured cells survived under HPCD by accumulating cell protective agents such as carbohydrates and amino acids and decreasing transcription and translation activities. In a recent study, ohmic heating and water bath-heating treatments induced *S. aureus* cells to show a total of 145 and 250 differentially expressed proteins, respectively, with the major portion being cell membrane proteins (Shao et al. 2021).

18.3 Recovery of Sublethally Injured Foodborne Pathogens

In the food industry, many treatments are applied to kill pathogens; however, among such populations, sublethally injured cells have the ability to be repaired and function normally in favorable environments (Espina et al. 2016; Kell et al. 1998; Wu 2008). Detection of these injured cells is important with respect to the safety of the final product. Compromised safety can result in severe consequences, such as reduced shelf lives and outbreaks of foodborne diseases (Bevilacqua et al. 2020; Silva et al. 2012; Wesche et al. 2009). Although rapid detection methods have been developed to determine bacterial populations, it is also important to detect live, injured, and dead cells separately to ensure the validity of the results, reducing the incidence of false-positive or false-negative results (Wu 2014). Resuscitation of injured cells initially requires cell-specific repair temperatures and times in
non-selective media with the addition of chemical stimuli to regain growth capabilities (Jay et al. 2005; Zhao et al. 2017). Regeneration of ribosomes (Tomlins and Ordal 1971); synthesis of phospholipids, cell walls, and proteins (Busta 1976); and resynthesis of RNA (Ray 1986) are essential for the recovery of injured cells (Jay et al. 2005). Moreover, once the injured cells are restored to their original functionality, their pathogenicity is also restored (Meyer and Donnelly 1992). Different repair mechanisms have been identified and are shown in Fig. 18.3.

It has been well documented that irrespective of the injury pathogenic bacterial cells experience, they tend to repair when appropriate conditions occur, including the optimum temperature and time required by a particular organism (Yamamoto et al. 2021). Moreover, after recovering normal growth functionalities, resuscitated cells tend to regain their normal resistance in selective media and multiplication processes (Wu 2008). For the repair and resuscitation of injured cells, both liquid and solid media can be used as mentioned earlier. Both of these methods have several advantages and disadvantages. Liquid repair methods are good for isolation, most likely number (MPN) enumeration, and further plating. However, these methods have not been validated for regulatory purposes, and the rapid growth of both normal and injured cells can hinder the recovery of the population of interest. On the other hand, solid repair methods are advantageous from an economical point of view, result in less variability, and involve less time. Conversely, solid repair methods can further injure a population, as most of these methods use molten agar at a high temperature (Wu 2014). Moreover, in overlay methods, colonies are usually small and can be difficult to isolate (Wu 2008). A comparison of these repair methods and the requirements of optimum repair conditions with reference to the recovery and selective growth of injured cells are summarized in Table 18.2.

### 18.3.1 Action of Chemical Compounds on the Modification of Repair Methods

The modification of a resuscitation medium with various chemical agents in combination with many of the abovementioned liquid and solid repair mechanisms was shown to improve the resuscitation of sublethally injured microbial cells. Many studies have been conducted to evaluate the resuscitation of sublethally injured cells through the addition of various compounds, viz. catalase (Andrews and Martin 1979; Rayman et al. 1978), pyruvate (Busch and Donnelly 1992; Izumi and Inoue 2018), 3,3'-thiodipropionic acid (McDonald et al. 1983), Tween 80 with magnesium chloride (Murthy and Gaur 1987), sorbitol (Kang and Siragusa 1999; Ma et al. 2019), bile salt, antibiotics (Hara-Kudo et al. 2000), oxynase (Adler and Spady 1997; Knabel and Thielen 1995; Schottroff et al. 2018; Wu et al. 2004), Sabouraud broth and citrate–phosphate buffer (Somolinos et al. 2008), phosphate buffer saline (Ma et al. 2019), magnesium, potassium, lithium, calcium, and zinc cations (Heinis
Fig. 18.3 Recovery methods most commonly in use for the resuscitation of injured bacteria
Table 18.2  Comparison between repair methods

<table>
<thead>
<tr>
<th>Repair method</th>
<th>Non-selective repair</th>
<th>Optimum repair conditions</th>
<th>Recovery and selective growth</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple Liquid-Repair Methods</td>
<td>Blended samples in non-selective broth.</td>
<td>Temperature = 25–37 °C, Temperature = 1 – 5 h</td>
<td>Directly plated or using MNP. MNP can take 48 – 96 h</td>
<td>Wu (2008), Wu and Fung (2001)</td>
</tr>
<tr>
<td>Twofold Dilution (2FD) Method</td>
<td>Cell suspension was diluted 2-folds with buffered peptone water in a 96-well microtiter plate.</td>
<td>Temperature = 37 °C, Time = 3 h</td>
<td>Addition of equal volume of twofold concentration of the selective broth. Incubated at 37 °C for 11 h</td>
<td>Kang and Siragusa (2001)</td>
</tr>
<tr>
<td>Ten-fold Dilution Method (10DF)</td>
<td>Cell suspension was diluted ten-fold with BHI, NB, TSB, and TSB with 0.6% yeast extract (TSBYE).</td>
<td>Temperature = 37 °C, Time = 3 h</td>
<td>After incubation, serial dilution with 0.2% peptone water was performed and 100 μL aliquots were plated onto XLD and SMAC. Incubated at 37 °C for 24 hrs.</td>
<td>Han et al. (2019)</td>
</tr>
<tr>
<td>Pour-Overlay Plating Method</td>
<td>Samples were blended with phosphate buffers and immobilized with pour-plate method with 5 mL of Trypticase Soy Agar (TSA) or Plate Count Agar (PCA).</td>
<td>Temperature = 25 °C, Time = 1 h</td>
<td>Overlaid plates with 10–12 mL of selective media and allowed to solidify for 15 min. Incubated at 35 °C for 24 h</td>
<td>Ray (1979)</td>
</tr>
<tr>
<td>Surface-Overlay Plating Method</td>
<td>Cells were suspended in 12 mL of non-selective media (e.g., TSA).</td>
<td>Temperature = 35 °C, Time = 2–4 hrs</td>
<td>Selective agar (7–12 mL) was overlaid on top of repaired cells and allowed to solidify. Incubated at 35 °C for 21 – 24 h</td>
<td>Hartman et al. (1975), Speck et al. (1975)</td>
</tr>
</tbody>
</table>

(continued)
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<thead>
<tr>
<th>Repair method</th>
<th>Non-selective repair</th>
<th>Optimum repair conditions</th>
<th>Recovery and selective growth</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thin-Agar Layer Method</td>
<td>Selective Medium</td>
<td>Temperature = 37 °C</td>
<td>Injured cells were repaired on non-selective medium and later on interact with selective medium in lower layer resulted in color development and selective differentiation on same plate</td>
<td>Qiu and Wu (2007), Wu (2008), Wu and Fung (2001)</td>
</tr>
<tr>
<td></td>
<td>(25 mL) was added in petri-plate, solidified, and layered with 14 mL of non-selective medium with direct inoculation of injured bacterial cells</td>
<td>Time = 24 hrs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solid Agar Overlay Method</td>
<td>Slight modification of TAL method was also found useful viz. separately solidifying 14 mL of both selective and non-selective medium followed by aseptic removal and over-layering of non-selective medium on selective medium</td>
<td>Temperature = 37 °C</td>
<td>Injured cells were repaired on non-selective medium and later on interact with selective medium in lower layer resulted in color development and selective differentiation on same plate</td>
<td>Yan et al. (2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time = 24 h</td>
<td></td>
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<tr>
<td>Four compartment Thin Layer (4-TAL) method</td>
<td>Non-selective medium overlaid on four different selective agars solidified in four-compartment petri plate</td>
<td>Temperature = 37 °C</td>
<td>Four different injured cells were recovered simultaneously</td>
<td>Wu and Fung (2003), Wu and Fung (2006)</td>
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<tr>
<td></td>
<td></td>
<td>Time = 24 h</td>
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<tr>
<td>Agar underlay method (Lutri plate recovery method)</td>
<td>In two-chambered Lutri Plate (LP), non-selective agar was added along with</td>
<td>Temperature = 37 °C</td>
<td>Selectivity in repair mechanism was introduced when the selective agents from lower LP</td>
<td>Chang et al. (2003), Kang and Siragusa (1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time = 24 h</td>
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During resuscitation of sublethally heat-injured *E. coli* O157:H7, the use of sorbitol-MacConkey agar (SMAC) with the agar underlay method showed

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### Table 18.2 (continued)

<table>
<thead>
<tr>
<th>Repair method</th>
<th>Non-selective repair</th>
<th>Optimum repair conditions</th>
<th>Recovery and selective growth</th>
<th>References</th>
</tr>
</thead>
</table>
| Membrane/ Solid Support-based Method | injured bacterial cells. After two hours of incubation, lower chambers of LP were introduced with selective agar | (a) Temperature = 37 °C
Time = 4 h
(b) Temperature = as per cell requirement
Time = 3 h | compartments were diffused into the non-selective one and help the repair mechanism | (a) Blackburn and McCarthy (2000)
(b) Kang (2002) |
| | (a) On non-selective medium, e.g., TSA plate surface, a membrane was placed and injured pathogenic cells were spread and allowed to resuscitate.
(b) A membrane filter holder (containing two chambers which were separated with a stainless steel net as solid-based support method was also applied by using a special equipment. First compartment A was filled with 5 mL of non-selective agar which was inoculated with injured bacterial cells to resuscitate. | | | |
| | (a) Membrane after resuscitation, was placed on selective medium such as sorbitol MacConkey Age for 20 h for selective growth which can be visible as colored colonies.
(b) The selective medium was added in chamber B. As the net between chambers is water-diffusible, the selective agar was diffused into chamber A to allow selective growth. | | | |

et al. 1977; Zhang et al. 2021), glucose, lactose, sucrose, and yeast extract (Busch and Donnelly 1992).
promising recovery (Kang and Siragusa 1999). Another study that added sodium novobiocin to bile salt in both modified E. coli broth and TSB enhanced the selective recovery of sublethally freeze-injured E. coli O157:H7 with a greater rate of resuscitation at 42 °C than at 37 °C (Hara-Kudo et al. 2000). In another study (Ma et al. 2019), both SEM and TEM analysis indicated intracellular and extracellular damage to HPP-injured E. coli O157:H7 cells, which were then repaired by using PBS. This recovery process was facilitated by PBS, resulting in a gradual return of the leaked ion concentration to the normal level, indicating the structural repair of sublethally HPP-injured cells. Moreover, a gradual increase in the activities of Na⁺K⁺-ATPase and Ca²⁺Mg²⁺-ATPase also accompanied the recovery process (Ma et al. 2019). The addition of sodium chloride in the recovery medium for sublethally PEF-injured S. cerevisiae cells also indicated the involvement of cytoplasmic membrane instability, which can then be recovered by the addition of Sabouraud broth with citrate–phosphate buffer (Somolinos et al. 2008). Carbohydrates such as glucose, lactose, and sucrose provide energy during repair mechanisms. Studies have suggested that RNA synthesis is required for the repair of sublethally injured cells, which is dependent upon the repair of the cell membrane and reconstruction of amino acids, both of which depend upon the energy supplied by these carbohydrates (Busch and Donnelly 1992; Iandolo and Ordal 1966).

A cytokine acting as a resuscitation-promoting factor (Rpf) is also crucial in the resuscitation process (Mukamolova et al. 2003). 3,3'-Thiodipropionic acid (TDPA) can neutralize the toxic effect of hydrogen peroxide, allowing increased viability of injured cells (Gram et al. 1984; McDonald et al. 1983). Moreover, the addition of agents such as catalase and pyruvates to reduce the incidence of reactive oxidative species such as hydrogen peroxide is also very important (Foegeding 1992; Izumi and Inoue 2018; McDonald et al. 1983). The introduction of iron supplementation in both direct-plating and broth-enrichment cultures increased the recovery and growth of injured cells (Gast and Holt 1995). Tween 80 has also been shown to resuscitate and protect bacterial cells injured by protecting the functionality of crucial redox enzymes through the use of the HHP technique and might also be associated with reduced membrane permeability and elevated conjugated fatty acids, which may be responsible for increased cell viability due to their intervention with ROS (Reitermayer et al. 2018). Previously, it was also concluded that adding magnesium chloride to Tween 80 enhanced repair mechanisms due to reduced calcium loss during membrane-specific injury (Murthy and Gaur 1987). Moreover, the magnesium required to repair sublethally heat-injured S. aureus has also been demonstrated to be influenced by the instability of ribosomes with leakage of magnesium during membrane loss (Hughes and Hurst 1976; Hurst and Hughes 1978).

Oxygen-reducing membranes, such as oxyrase, can help remove dissolved oxygen within a medium, thus improving the growth of both noninjured and injured microbial cells (Ali and Fung 1990; Knabel and Thielen 1995; Patel and Beuchat 1995; Schottroff et al. 2018; Tuitemwong et al. 1994; Yu and Fung 1990). A similar effect of the modification of an oxygen-rich environment was also observed in heat-injured L. monocytogenes with the use of N₂ purging, cysteine, oxyrase, and lactate enrichment (Knabel and Thielen 1995). Wu and Fung (2001) used the TEL method
with oxyrase and found it effective for the detection of injured and noninjured cells. Such oxygen-reducing membranes stimulate the recovery of injured cells by providing suitable conditions, especially for facultative anaerobes (Adler and Spady 1997). TSB enriched with yeast extract, glucose, ferrous and magnesium sulfate and pyruvate has also shown to be an effective repair broth for sublethally injured *L. monocytogenes* (Donnelly 2002).

### 18.4 Conclusions

Injured microorganisms in foods have received substantial interest, and their importance cannot be ignored. After a sublethal injury, microorganisms may be present but avoid detection by selective media. A number of stressors (heat, cold, freeze-drying, irradiation, antimicrobials, etc.) have been identified and discussed as capable of causing sublethal injury in microorganisms. These stressors induce different degrees of sublethal injuries depending on the physiological state of individual microbial cells within a population. Injured microorganisms adapt to stress by altering cellular metabolism and morphology, membrane composition, and degree of virulence, which help them produce stress proteins and provide more protection against other stressors. Injured microorganisms undergo a series of complex biochemical events to be resuscitated in foods depending on the microbial cells and the medium present. The early detection of injured cells in food products is crucial and requires more investigation to safeguard food safety.

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Bhullar MS, Shaw A, Mendonca A, Monge A, Nabwire L, Thomas-Popo E (2021) Shiga toxin–producing *Escherichia coli* in the long-term survival phase exhibit higher chlorine tolerance and
less sublethal injury following chlorine treatment of romaine lettuce. Foodborne Pathog Dis 18:276–282


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Chapter 19
Bacterial Programmed Cell Death

Jiao Li, Xiangzhao Mao, Xiaonan Lu, and Jinsong Feng

Abstract Programmed cell death (PCD) was long thought to be a unique characteristic of multicellular organisms, but a growing body of evidence has revealed the existence of PCD in bacteria. Similar to the requirement for their eukaryotic counterparts, being part of a community seems indispensable for bacterial survival in different environments. Different mechanisms underlying bacterial PCD have been identified, among which the most well-studied is the toxin-antitoxin (TA) system as a regulable “addiction module.” Moreover, phenotypic characteristics of apoptosis are also observed in bacteria and are related to ordered cellular breakdown in eukaryotes. This chapter mainly focuses on the genetically regulated systems that initiate PCD in bacteria, and the detailed evidence and reasons for PCD in the context of bacterial communities are also summarized.

Keywords Programmed cell death · Toxin-antitoxin system · Apoptotic-like death · Bacterial community

19.1 Introduction

Programmed cell death (PCD) is an active behavior mediated by intracellular genetic changes. It is an essential mechanism by which multicellular organisms remove unwanted structures and eliminate nonfunctional or harmful cells to ensure their survival (Fuchs and Steller 2011; Rantong and Gunawardena 2015). PCD was long
thought to be a unique characteristic of multicellular organisms, as a single cell cannot benefit from its own demise. However, according to recent studies, PCD systems have also been observed in unicellular eukaryotes and even in bacteria under stress conditions (Cheng and Hardwick 2007). The question, therefore, exists: why does PCD exist in bacteria? To answer this question, one has to look at accumulating examples where bacteria can behave as multicellular organisms. Similar to PCD in multicellular organisms, PCD in bacteria seems to have the characteristics of altruism, with some bacteria sacrificed to benefit the remaining subpopulation to ensure the survival of the whole population (Ramisetty and Sudhakari 2020; Lee and Lee 2019). Moreover, there is sufficient evidence that bacteria can form complex structures (e.g., biofilms) to colonize food as a group or use cooperative defense strategies against predators, usually working together to ensure the survival of the population (Claessen et al. 2014). This chapter provides a systematic review of the mechanisms underlying the induction of PCD in bacteria, and the detailed evidence and reasons for PCD in bacteria are also summarized.

19.2 The Induction of PCD in Bacteria by Stressors in Food Processing

Studies on the activation of the bacterial PCD system as a novel mechanism have mostly focused on the action of antibiotics (Dwyer et al. 2012; Elnakady et al. 2016), and increasing attention has started to be paid to the induction of PCD in bacteria by food-associated stressors. Hazan et al. (2004) showed that several stress conditions, including high temperatures, DNA damage, and oxidative stress, were able to induce mazEF-mediated cell death, and this process was shown to take place only during logarithmic growth and require an intact relA gene. Kim and Lee (2021) also reported that DNA fragmentation and membrane depolarization appeared in H$_2$O$_2$-treated cells, suggesting that H$_2$O$_2$ causes apoptosis-like PCD in *E. coli*. Interestingly, a subpopulation of *Salmonella enterica* sv. Typhimurium, *Bordetella bronchiseptica*, *Xanthomonas campestris*, and *Bacillus subtilis* were found by Wadhawan et al. (2014) to undergo PCD upon exposure to gamma radiation, with phosphatidylserine externalization (PS). It was further found that a PCD-negative mutant of *Xanthomonas* lacking caspase-3-like activity and displaying reduced PS externalization was comparatively less susceptible to radiation than its wild-type counterpart, indicating that PCD activity is caspase dependent upon radiation exposure. Lunov et al. (2016) discovered that short-term plasma exposure could induce the exposure of phosphatidylserine on the cell surface of *E. coli*, *P. aeruginosa*, and *S. aureus*, as measured by annexin-V-FITC labeling, without an increase in membrane permeability to propidium iodide (PI), indicating the apoptotic-like cell death. In addition, the expression of proteins with caspase-like substrate specificity increased significantly, as detected by V-D-FMK-mediated fluorescence. Li et al. (2018) reported that ultrasound-induced *E. coli* O157:H7 cell death exhibited
biochemical hallmarks of apoptosis, including exposed phosphatidylserine and activated caspases. It was also inferred that the accumulation of ROS and decrease in adenosine triphosphate might be related to the physiological changes in *E. coli* O157:H7. Thus, the occurrence and potential risks of PCD in bacteria should be considered highly important during food processing (Table 19.1).

19.3 The Mechanism Underlying Programmed Cell Death in Bacteria

Different pathways mediating bacterial PCD have been identified. The most well-studied system is the toxin–antitoxin (TA) system, which widely exists in bacteria and archaea. In addition, the morphological features that are associated with apoptosis have been found during evolution for the purpose of stress management. These genetically regulated mechanisms contribute to protective functions, permitting bacterial communities to eliminate damaged cells efficiently while preserving metabolic resources and avoiding an immune response.

19.3.1 Toxin-Antitoxin (TA) System Mediated PCD

In bacteria, the most studied PCD mechanism is the TA system. It is mediated through a pair of genes, which is known as the “addiction module” (De Bast et al. 2008; Engelberg-Kulka and Glaser 1999). One gene is responsible for encoding an unstable antitoxin, and the other gene encodes a stable toxin. The cell dies due to the action of the stable toxin when the unstable antitoxin is degraded. The first addiction
module carried on a bacterial chromosome was the *Escherichia coli* mazEF module (Fig. 19.1). It consists of two adjacent genes, *mazE* and *mazF*, coexpressing MazE and MazF (Sat et al. 2001). The crystal structures of MazE and the TA complex MazE-MazF have been determined, and the interaction between MazE and MazF is primarily mediated by the C-terminal domain of MazE (Lah et al. 2003). Although the same amount of MazE and MazF is produced from the transcript, it takes two MazF dimers to neutralize one MazE dimer by forming a linear heterohexamer complex composed of alternating toxin and antitoxin homodimers (MazF$_2$-MazE$_2$-MazF$_2$) (Kamada et al. 2003). Therefore, under normal growth conditions, the production of MazE is assumed to be enough to secure complete compression of the toxicity of MazF.

When bacteria encounter environmental stresses, MazEF-mediated cell death is triggered (Fig. 19.2). Correspondingly, MazE is a labile antitoxin that can be degraded by the ATP-dependent serine protease ClpPA, whereas MazF is a long-lived toxin (Zhang et al. 2003). In addition, the combined action of MazE and MazF proteins also negatively regulates the expression of mazEF at the level of transcription. MazF is able to inhibit protein synthesis through its endoribonucleolytic effect on mRNAs, which preferentially cleaves single-stranded mRNAs at ACA sequences (Zhang et al. 2003; Simanshu et al. 2013). It tags the corresponding newborn polypeptide chains with degradation signals. Cleavage of mRNAs can reduce translation rates and terminate essential protein synthesis needed for bacterial metabolism and survival. This phenomenon is referred to as “mazEF-mediated PCD.”

Initially, mazEF transcription was found to be inhibited by guanosine 3′,5′-bispyrophosphate (ppGpp), which is synthesized by the RelA protein under severe amino acid starvation (Aizenman et al. 1996). Under those conditions, labile MazE cannot be expressed continuously to maintain the concentration required to
antagonize the MazF toxin, resulting in cell death. Subsequently, \textit{mazEF}-dependent death was broadened to conditions under which transcription and/or translation were inhibited by antibiotics such as rifampicin and chloramphenicol. In contrast, in the mutant-type cells in which \textit{mazEF} was deleted from the bacterial chromosome, cell death was prevented in the presence of the above antibiotics. Engelberg-Kulka et al. (2009) also showed that the inhibition of transcription and/or translation caused \textit{mazEF}-mediated cell death by forming reactive oxygen species (ROS). Moreover, it was found that without the presence of a communication signaling peptide called extracellular death factor (EDF), the \textit{mazEF} system could not be activated, and the formation of ROS was thus inhibited by rifampicin (Engelberg-Kulka et al. 2009). Beyond that, another \textit{mazEF}-mediated cell death pathway was found under the stress of trimethoprim and sulfonamide, which caused thymine starvation (Sat et al. 2003). Furthermore, the DNA damage caused by thymine starvation might be responsible for the reduction in the activity of \textit{mazEF} promoter \textit{P}$_{2}$. In addition, it was also reported that the reduction in \textit{P}$_{2}$ transcription might also be indirectly induced by ppGpp synthesis (Aizenman et al. 1996). In general, \textit{mazEF}-mediated PCD usually occurs in bacteria upon severe amino acid and thymine starvation, DNA damage, or other oxidative stresses. The MazF toxin cleaves mRNAs at specific sites, reducing

Fig. 19.2  Schematic model of \textit{mazEF}-mediated PCD under stress conditions
the transcription and translation of essential proteins, eventually resulting in cell suicide.

In addition to the familiar *mazEF* module, there are other types of TA operons found in bacteria. In *Staphylococcus aureus*, two homologous operons, *cidA* and *lrgA*, have been shown to regulate cell death and lysis by affecting murein hydrolase activity, antibiotic tolerance, and biofilm formation (Rice et al. 2005, 2007). The *cidA* and *lrgA* operons were thought to be analogous to bacteriophage-encoded holins and anti-holins, respectively (Ranjit et al. 2011). The CidA protein has an enhancing effect on bacterial death and lysis, while the LrgA protein is able to inhibit death and lysis. The *cidA* gene has been demonstrated to control the activity of membrane-associated murein hydrolase or mediate the translocation of murein hydrolase across the membrane. After treatment with kendomycin, the expression of *cidA* was significantly upregulated, and the transcription of *lrgA* was downregulated. However, no significant increase in bacterial cell lysis was found, and it was assumed that the viability of *S. aureus* was affected by kendomycin primarily through holin formation rather than the control of murein hydrolase activity (Elnakady et al. 2016). Furthermore, the *cidA* mutant with decreased murein hydrolase activity was shown to acquire resistance to a series of antibiotics, including penicillin, rifampin, and vancomycin (Rice and Bayles 2008). The classification of CidA/LrgA suggests that bacteria have evolved with a means to control phage infection through controlled cell suicide by expressing these genes.

### 19.3.2 Apoptotic-Like Bacterial PCD

In mammalian cells, PCD is classically known as apoptosis, a term that was originally used to define morphological changes that characterize cell death. The morphological modifications of apoptotic cells include membrane depolarization, extracellular exposure to phosphatidylserine, chromatin condensation, and DNA fragmentation. The above hallmarks were found to be attributed to the activation of cysteine proteases, known as caspases, which can regulate a series of metabolic pathways to induce cell apoptosis (Van De Water et al. 2004; Thornberry and Lazebnik 1998). Del Carratore et al. (2002) first demonstrated apoptosis in yeast cells caused by UV-induced DNA damage. A TUNEL-positive phenotype was observed, and a dose-dependent increase in the sub-G₁ population was found by flow cytometry analysis, showing condensed chromatin in the nucleus and cell shrinkage. Although Bax or Bcl-2 homologs that regulate UV-induced mammalian-cell apoptosis have not been found in yeast, yeast may harbor proteins that perform similar tasks by as yet undiscovered pathways (Zha et al. 1996). The physiological characteristics of PCD were also assessed in *Candida albicans* when exposed to various stresses, including acetic acid, hydrogen peroxide, and amphotericin B. Nonviable cells that excluded propidium iodide displayed apoptotic markers with phosphatidylserine eversion, as shown by annexin-V-FITC labeling, and nuclease-mediated double-strand DNA was found to break, as shown by
TUNEL staining. Apoptosis in *C. albicans* was associated with an accumulation of G2/M phase cells, and under some conditions, significant proportions of cells switched to hyphal growth before dying (Phillips et al. 2003). This is also a demonstration of apoptosis in a unicellular eukaryote.

In recent years, the phenotypic traits of apoptosis, including cell shrinkage, DNA condensation, DNA fragmentation, and calcium-dependent membrane depolarization, have been observed in bacteria (Peeters and de Jonge 2018; Bayles 2014). This phenomenon was named “apoptotic like death,” and it resembles eukaryotic apoptosis. The dynamic balance between apoptosis and proliferation plays an important role in maintaining the stability of bacterial populations under stress conditions. Prodigiosin, the bacterial pigment, was tested against the foodborne pathogens *Bacillus cereus*, *S. aureus*, *Pseudomonas aeruginosa*, and *E. coli*. The biochemical and morphological features of apoptosis-like death, including DNA fragmentation, generation of ROS, and expression of a protein with caspase-like substrate specificity, were observed in bacterial cells (Darshan and Manonmani 2016). It was also observed that antibiotic treatment resulted in signs of apoptosis in *E. coli* cells, including phosphatidylserine exposure, chromosome condensation, and DNA fragmentation. Proteomic and genetic analysis showed that the multifunctional RecA-binding peptide sequences served as substrates for eukaryotic caspases, which orchestrated apoptosis in eukaryotic cells. Further analysis indicated that RecA was cleaved by the protease ClpXP, resulting in phenotypic changes (Dwyer et al. 2012). These findings illustrated that prokaryotes engage in a series of regulatory mechanisms to mark and clear dying cells in response to diverse environmental stimuli. Evidence for apoptosis-like processes in bacteria after physical treatments has also accumulated in recent years.

The study of Erental et al. (2012) proposed two PCD pathways in *E. coli*, one that was mediated by the *mazEF* system and another that exhibited the characteristics of apoptosis. This not only suggested that apoptotic-like events occurred in bacteria but also suggested that these processes were linked to the SOS response (Fig. 19.3). Apoptosis-like death was shown to be mediated by two proteins, RecA and LexA. LexA is an inhibitor of the SOS response, a global response to DNA damage (Janion 2008). Apoptosis-like death of *E. coli* was inhibited by the *mazEF*-mediated cell death pathway under conditions of severe DNA damage. The results showed that the *recA* mRNA level was reduced by MazEF and its downstream components, indicating that the apoptotic-like death pathway in *E. coli* is a system complementary to the traditional *mazEF*-mediated cell death pathway. When the components of the *mazEF* pathway were inactivated, bacterial cell death occurred through the apoptotic-like death pathway (Erental et al. 2012).
What Is the Benefit of Bacterial PCD?

Although knowledge of the presence of PCD in bacteria has been established, it still raises some intriguing questions. In multicellular organisms, PCD is critical for morphological development and host survival. As individual cells, bacteria cannot benefit from the PCD process, while microorganisms are almost invariably studied as a group. Therefore, when encountering environmental stresses, the bacterial community might act as a multicellular organism that sacrifices part of itself to ensure the survival of the population (Engelberg-Kulka et al. 2006). Interestingly, the hypothesized function of bacterial PCD shares many similarities with PCD in eukaryotic cells. One hypothesis is that dead cells could provide nutrients for survivors under starved conditions and thus enhance community perseverance, referred to as “nutritional altruism” (Peeters and de Jonge 2018). In addition, biofilms are thought to be a setting for PCD as a means to increase the amount of nutrients per cell through the control of population density. The formation of bacterial biofilms is similar to the morphological development of eukaryotic tissue, as a group of cells form a fairly fixed unit that performs certain functions together. In bacterial PCD, the released free DNA also serves a function for biofilm formation and structural stability (George and Halami 2019; Sena-Velez et al. 2016). When treated with DNase, the biofilm can be weakened as the structural component of the biofilm matrix is destroyed. Therefore, the advantages of bacterial PCD mostly benefit cell populations as a whole but not for individual bacterial cells, and cell clusters benefit from PCD as a defense mechanism in addition to immunity.
19.5 The Potential Risks/Pathogenesis of PCD in Pathogenic Bacteria

There are potential risks of PCD in bacteria that might threaten food safety and public health. The TA system has been reported to be associated with biofilm formation, persister cell formation, and the release of virulence factors from pathogenic bacteria (Wang and Wood 2011). Persister cells are bacterial cells that remain viable after treatment with lethal concentrations of antibiotics, and these cells arise primarily in biofilms and in stationary phase cultures. Moreover, the phenotype contributes to the resistance of biofilm bacteria to antibiotics, which is responsible for the recalcitrance of human infections (Singh et al. 2009). McFarland et al. (2015) also considered that both the host and pathogen might use PCD as a survival-promoting strategy during infection. Alp-mediated lysis in a subset of cells was activated in response to DNA damage in Pseudomonas aeruginosa, thereby limiting the spread of phage or promoting biofilm formation and in turn enhancing the survival of the population as a whole. Although PCD is lethal to the individual cell in which it occurs, it truly enhances the ability of the bacterium to cause disease.

19.6 Perspectives

Multidrug resistance among pathogens has become a global challenge to public health. The pathways involved in bacterial PCD may result in the identification of novel and unexpected targets for antimicrobial strategies. Current studies have shown that antibiotics lead to intracellular accumulation of ROS, which then activates MazEF and RecA. Thus, these studies revealed that existing antibiotics do not target these pathways directly but cause cell death through the activation of PCD pathways (Engelberg-Kulka et al. 2006). Analogs of crucial PCD-related proteins can be designed as antimicrobial compounds to initiate bacterial cell death, which helps to solve this major public health problem.

References

Chapter 20
Cross-Protection Response

Ricardo A. Wu, Hyun-Gyun Yuk, Xinyu Liao, Jinsong Feng, and Tian Ding

Abstract  Foodborne pathogens are able to adapt to many stresses throughout the food chain. They are highly adaptable microorganisms and have developed coordinated molecular mechanisms which allow them to survive and adapt to stresses. When a foodborne pathogen encounters stress at sub-lethal levels, it may adapt and develop tolerance to the that particular stress and even more, it may develop cross-protection to different stresses through many molecular mechanisms. Cross-protection may help foodborne pathogens to survive hurdles, posing a threat to food safety. In this chapter, stresses that may induce cross-protection and the known molecular mechanisms are discussed.

Keywords  Foodborne pathogen · Stress · Cross-protection · Molecular mechanism

20.1 Introduction

Stress is any harmful factor or condition (physical, chemical, or biological) that negatively affects the growth or survival of foodborne pathogens (Rodriguez-Romo and Yousef 2005). When associating this definition to food processing, it can be realized that preservation treatments are stressful, which include physical treatments such as heat, pressure, or osmosis; chemical treatments such as acidification or alkalization; and biological stress such as antibiotics or bacteriocins (Begley and

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T. Ding et al. (eds.), Stress Responses of Foodborne Pathogens,
https://doi.org/10.1007/978-3-030-90578-1_20
Different magnitudes of the stresses may have different effects on foodborne pathogens. Sub-lethal stress may not kill pathogens but may injure bacterial cells to an extent that induces metabolic changes (Hu et al. 2020), while lethal stress causes irreversible damage to bacterial cells (Rodriguez-Romo and Yousef 2005).

Foodborne pathogens are able to adapt to different stress conditions encountered at all stages of the food chain through coordinated molecular mechanisms. When adaptation occurs during exposure to sub-lethal stress conditions, foodborne pathogens can develop higher resistance to the same stress or to different stressors, a phenomenon known as cross-protection (Haberbeck et al. 2017). This protection may help pathogens to survive hurdle conditions, which are believed to be effective in controlling pathogen growth (Isohanni et al. 2013). Therefore, cross-protection could have serious implications for food safety.

Since different sub-lethal stresses may induce different mechanisms of cross-protection, the entirety of this knowledge is still far from being elucidated. However, it is now clear that many factors determine the fate of stress-adapted cells, such as physicochemical properties of foods and cellular molecular mechanisms triggered by the changing environment (Maserati 2017). Hence, it is known that during cross-protection, genes belonging to different stress response pathways are activated.

20.2 Stresses that Induce Cross-Protection in Foodborne Pathogens

Foodborne pathogens are prone to face various environmental conditions at any stage of the food chain, which may render them suitable circumstances to become adapted to such stresses. Bacteria that have survived through stress adaptation usually become stronger and may even develop tolerance to other stresses (Table 20.1).

20.2.1 Heat Stress-Associated Cross-Protection

Heat is commonly used to inactivate foodborne pathogens in food processing (Koga et al. 1999). Bacterial cells pre-exposed to sub-lethal stresses may develop stress adaptation and may become more resistant to subsequent lethal challenges, including heat (Bucur et al. 2018). Moreover, heat adaptation may also induce cross-protection to other stresses. Cross-protection between heat and other stresses has been reported in many studies. Heat adaptation may induce cross-protection to other stresses (Isohanni et al. 2013), and vice versa, adaptation to other stresses may also induce cross-protection to heat (Browne and Dowds 2001; Álvarez-Ordóñez et al. 2009b; Kang et al. 2018).
<table>
<thead>
<tr>
<th>Sub-lethal stress</th>
<th>Cross-protection</th>
<th>Species</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Heat</td>
<td>Heat</td>
<td>Arcobacter butzleri</td>
<td>Isohanni et al. 2013</td>
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<td></td>
<td>+</td>
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<tr>
<td></td>
<td>Cold</td>
<td>Acinetobacter baumannii</td>
<td>Ebinesh et al. 2018</td>
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<tr>
<td></td>
<td>+</td>
<td></td>
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<tr>
<td>Cold</td>
<td>+</td>
<td>Listeria monocytogenes</td>
<td>Pittman et al. 2014</td>
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<tr>
<td>Acid</td>
<td>+</td>
<td>Salmonella enterica</td>
<td>Xu et al. 2008</td>
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<td></td>
<td>+</td>
<td>Salmonella Enteritidis</td>
<td>Ye et al. 2019</td>
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<td></td>
<td>+</td>
<td>Escherichia coli O157:H7;</td>
<td>Leenanon and Drake 2001</td>
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<td>Escherichia coli</td>
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<td>Listeria monocytogenes</td>
<td>Al-Nabulsi et al. 2015</td>
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<td></td>
<td>+</td>
<td>Listeria monocytogenes</td>
<td>Faleiro et al. 2003</td>
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<td></td>
<td>+</td>
<td>Vibrio parahaemolyticus</td>
<td>Chiang et al. 2014</td>
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<td>Salmonella Typhimurium</td>
<td>Greenacre et al. 2006</td>
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<tr>
<td>Osmotic</td>
<td>+</td>
<td>Salmonella Typhimurium</td>
<td>Fletcher et al. 2001</td>
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<td>Faleiro et al. 2003</td>
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<td>Escherichia coli</td>
<td>Zhu and Dai 2018</td>
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<td></td>
<td>+</td>
<td>Staphylococcus aureus</td>
<td>Shebuski et al. 2000</td>
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<tr>
<td></td>
<td>+</td>
<td>Vibrio vulnificus</td>
<td>Rosche et al. 2005</td>
</tr>
<tr>
<td>Desiccation</td>
<td>+</td>
<td>Salmonella enterica</td>
<td>Fong and Wang 2016</td>
</tr>
</tbody>
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(continued)
### Table 20.1 (continued)

<table>
<thead>
<tr>
<th>Sub-lethal stress</th>
<th>Cross-protection</th>
<th>Species</th>
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<tbody>
<tr>
<td></td>
<td>Heat +</td>
<td><em>Salmonella Typhimurium</em>; <em>Salmonella Enteritidis</em>; <em>Salmonella Newport</em>; <em>Salmonella Infantis</em></td>
<td>Gruzdev et al. 2011</td>
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<tr>
<td>Heat +</td>
<td>Cold + Acid + Osmotic Desiccation Antibiotic Ethanol Starvation</td>
<td>+ + +</td>
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<tr>
<td>Antibiotic +</td>
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<td><em>Vibrio vulnificus</em></td>
<td>Dombroski et al. 1999</td>
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<td>Ethanol +</td>
<td></td>
<td><em>Salmonella Enteritidis</em></td>
<td>He et al. 2018</td>
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<td></td>
<td>+</td>
<td><em>Salmonella Enteritidis</em></td>
<td>He et al. 2016</td>
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<td></td>
<td>+ + +</td>
<td><em>Cronobacter sakazakii</em></td>
<td>Huang et al. 2013</td>
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<tr>
<td>Starvation +</td>
<td></td>
<td><em>Escherichia coli O157:H7</em></td>
<td>Gawande and Griffiths 2005</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td><em>Escherichia coli O157:H7</em></td>
<td>Arnold and Kaspar 1995</td>
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<td></td>
<td>+</td>
<td><em>Escherichia coli O157:H7</em></td>
<td>Zhang and Griffiths 2003</td>
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<td></td>
<td>+</td>
<td><em>Escherichia coli</em></td>
<td>Jenkins et al. 1990</td>
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Isohanni et al. (2013) reported that heat adaptation at 48 °C for 2 h induced cross-protection to lethal acid stress (pH 4.0) in Arcobacter butzleri. Non-adapted cells showed increased susceptibility to lethal acid stress. Reciprocally, Browne and Dowds (2001) found that cross-protection of Bacillus cereus between stresses displayed a “hierarchy” of resistance, which means that salt protected against hydrogen peroxide, which protected against ethanol, which protected against heat. Exposure to stressors induced synthesis of stress-specific proteins and accumulation of proteins was found between heat- or salt-stressed cells. The chaperone GroEL, which participates in protein folding, was induced by heat shock. Other studies have shown that adaptation to other stress, may also induce cross-protection to heat. For example, Fletcher et al. (2001) reported that high osmotic stress (0.3 M NaCl) increased the thermotolerance (53 °C) of Salmonella Typhimurium. Fong and Wang (2016) found that desiccation adaptation (a_w 0.52 ± 0.00) of Salmonella enterica strains induced cross-protection to heat (70 °C).

20.2.2 Cold Stress-Associated Cross-Protection

Foods are commonly stored at low temperatures to prevent the activity of enzymes, chemical reactions, and growth of spoilage and pathogenic microorganisms (Erkmen and Bozoglu 2016). In a food, when the temperature is reduced below −2 °C, the free water starts to freeze and form ice crystals (Erkmen and Bozoglu 2016). Solute concentration increases as temperature drops and more ice crystals form, which increases the freezing point. The increase in solute concentration (salts, proteins, nucleic acids, ions, etc.) may cause migration of water molecules from inside to outside the bacterial cell, causing dehydration in bacterial cells (Erkmen and Bozoglu 2016). Foodborne pathogens may encounter cold environments throughout the food processing chain, such as chilling, cold chain distribution, and cold storage; thus, low temperatures may become a common stress. Moreover, the physiological challenges that cold stress represents induces adaptation and may also initiate cross-protection to other stresses (Ricke et al. 2018).

The development of cross-protection in foodborne pathogens may occur in the presence of cold temperatures. In a study conducted by Pittman et al. (2014), L. monocytogenes cells that were exposed to cold (4 °C) up to 6 h developed increased cross-protection to osmotic stress (3% NaCl). The longer cells were exposed to cold, and the stronger was their osmotic stress resistance. In contrast, Shen et al. (2014) reported that pre-exposure to cold (4 °C) did not induce cross-protection to acid stress in L. monocytogenes. On the other hand, Xu et al. (2008) found that acid-adapted S. enterica cells developed cross-protection to cold stress. Similarly, Ye et al. (2019) reported that acid adaptation in stationary phase S. Enteritidis induced cross-protection to cold stress at 4 °C but not at −20 °C as compared to log-phase. The reason may be because at 4 °C, more cold-related genes were upregulated compared to −20 °C. Leenanon and Drake (2001) found that acid adaptation enhanced the freeze-thaw (−20 to 21 °C) resistance of Escherichia coli.
O157:H7 and nonpathogenic *Escherichia coli*. These outcomes may represent important implications for food safety as refrigeration and acidification are commonly used for food preservation.

### 20.2.3 Acid Stress-Associated Cross-Protection

Acid is a common stress that foodborne pathogens may encounter in foods, during food processing, or in the gastrointestinal tract of humans (Álvarez-Ordóñez et al. 2012). A stable pH is indispensable for bacteria to survive, as the optimal pH for foodborne pathogens is around neutral (pH 7.0). Low pH threatens microbial growth by triggering cellular damages such as impairments of essential enzymes and electron transport chain and protein denaturation (Zhou and Fey 2020). However, sub-lethal levels of acidity (pH 5.4–4.5) could have a different effect in most foodborne pathogens, inducing what is known as acid adaptation (Calvo et al. 2017). Numerous studies have demonstrated that acid adaptation response increases the resistance of foodborne pathogens to extreme acid conditions (Chiang et al. 2014). Furthermore, this acid adaptation response might induce cross-protection to other stress or multiple stresses such as heat, cold, osmosis, antibiotics, oxidation, sanitizers, or non-thermal technology (Lou and Yousef 1997; Faleiro et al. 2003; Tetteh and Beuchat 2003; Ritter et al. 2014; Chiang et al. 2014; Al-Nabulsi et al. 2015; Liao et al. 2018).

The cross-protection effect of acid to various stresses in different foodborne pathogens has been reported previously. Álvarez-Ordóñez et al. (2009a) reported that acid adaptation at pH up to 4.5 with different acids (acetic, citric, hydrochloric acid) in *S. Typhimurium* and *S. Senftenberg*, induced subsequent resistance to extreme pH (2.5) and cross-protection to heat (58°C) in apple and orange juice. Vice versa, adaptation to other stresses may also induce cross-protection to lethal acid challenge. Isohanni et al. (2013) reported that heat adaptation (48°C for 2 h) in *A. butzleri* induced cross-protection against lethal acid stress (pH 4.0). Faleiro et al. (2003) found that osmotic adaptation (3.5% NaCl) induced cross-protection against acid challenge (pH 3.5), and acid adaptation (pH 5.5) induced cross-protection against lethal osmotic shock conditions (20% NaCl) in *L. monocytogenes*.

### 20.2.4 Osmotic Stress-Associated Cross-Protection

Salts are widely used in the food industry. High salt concentration may cause water loss from bacteria and impact cellular activities (Liu et al. 2018). Foodborne pathogens may commonly encounter high salt concentrations in foods (Zhu and Dai 2018). Bacterial cells respond to osmotic stress because it drastically affects their physical and chemical stability (Altendorf et al. 2013). Osmotic stress is associated with the content of available water for physiological functions. For instance, the
osmotic pressure of an aqueous solution increases as water activity (aw) decreases, an inverse proportion (Altendorf et al. 2013).

The effects of osmotic stress on the cross-protection of foodborne pathogens have been reported in previous studies. High salt conditions (0.1–0.4 M NaCl) may induce cross-protection to antibiotics tetracycline (1–4 μM) and chloramphenicol (2–8 μM) in E. coli (Zhu and Dai 2018). It has also been demonstrated that osmotic stress-induced thermotolerance cross-protection in S. Typhimurium (Fletcher et al. 2001) and Staphylococcus aureus (Shebuski et al. 2000). In B. cereus, osmotic stress (1% NaCl) was found to protect against hydrogen peroxide (5 mM/L) (Browne and Dowds 2001). Rosche et al. (2005) found that osmotic shock (artificial seawater microcosms, 920 mOsm) induced Vibrio vulnificus to develop cross-protection to high temperature (45 °C) and oxidative challenge (0.2 mM H2O2). Inversely, other stresses have also been shown to induce cross-protection to osmotic stress. The acid tolerance response of S. Typhimurium induced cross-protection to heat and osmotic stress (Greenacre and Brocklehurst 2006). Cold stress (4 °C) response in L. monocytogenes has also induced cross-protection to osmotic stress (3% NaCl) (Pittman et al. 2014).

20.2.5 Desiccation Stress-Associated Cross-Protection

Drying is commonly used in the food industry as a preservation technique in which the primary aim is to prevent the microbial proliferation of spoilage bacteria and foodborne pathogens (Corry 1975). This technique is based on the reduction of the water content in foods, which described using the concept of aw (Maserati 2017). One of the main challenges that foodborne pathogens encounter when exposed to dry environments is desiccation (Maserati et al. 2018). However, some foodborne pathogens such as Salmonella, L. monocytogenes, and C. sakazakii have developed resistance to desiccation stress, which also induced cross-protection to other stresses.

Salmonella enterica is one of the most studied pathogens for its ability to resist desiccation. Some important salmonellosis outbreaks have been associated with low aw foods, such as raw almonds (Isaacs et al. 2005), peanuts (Kirk et al. 2004), cereal (Russo et al. 2013), and infant dried milk (Rowe et al. 1987). It has been demonstrated that desiccation stress adaptation of Salmonella enterica induces cross-protection to multiple stresses. Gruzdev et al. (2011) found that desiccation stress adaptation of several Salmonella serotypes, namely, S. Typhimurium, S. Enteritidis, S. Newport, and S. Infantis developed cross-protection to lethal treatments of ethanol, sodium hypochlorite, dodecyl dimethyl ammonium chloride, hydrogen peroxide, NaCl, bile salts, dry heat, and UV irradiation (Gruzdev et al. 2011). However, desiccation-adapted Salmonella serotypes cells were susceptible to acetic and citric acid (Gruzdev et al. 2011). Shiga toxin-producing E. coli has also been associated with outbreaks in low aw foods, such as in-shell hazelnuts (Miller et al. 2012) and deer jerky (Keene et al. 1997). Other important foodborne pathogens have
shown desiccation resistance, including *Cronobacter* spp., *L. monocytogenes*, and *Campylobacter* spp. (Burgess et al. 2016).

### 20.2.6 Antibiotic Stress-Associated Cross-Protection

The use of antibiotics is a common practice in agriculture for the control of bacteria that attack crops and animals (Liao et al. 2020). This gives the opportunity to pathogens to develop resistance and to grow in the intestine, animal excreta and surrounding environment. Antibiotic-resistant bacteria can then be transmitted throughout the food chain until reaching the human body (Liao et al. 2020). Antibiotic resistance is acquired by pathogens, like for the other stresses, by undergoing various physiological changes in an adapted response mediated through chromosomal mutations, gene expression, gene transfer, or cross-protection response (Oniciuc et al. 2019).

Antibiotic resistance could enhance the resistance of foodborne pathogens to other stresses, such as heat, cold, osmosis, acid, sanitizers, and non-thermal treatment (Liao et al. 2020). Likewise, these other stresses may also provide cross-protection to antibiotic stress (Liao et al. 2020). Previous studies have reported the effect (positive and negative) of antibiotic resistance on other stress tolerance. For example, Komora et al. (2017) found that the tolerance to heat did not differ significantly between antibiotic-susceptible and antibiotic-resistant *L. monocytogenes*. Dombroski et al. (1999) found that wild-type *V. vulnificus* displayed lower D-values (47 °C) than nalidixic acid-resistant mutants did (2.2 versus 3.0 min, respectively). In contrast, Duffy et al. (2006) found that multi-antibiotic resistant *E. coli* O157:H7 presented a lower D-value (55 °C) than antibiotic-susceptible strains did (0.90–1.1 versus 8.18–13.42 min, respectively). Reciprocally, heat stress adaptation also provides cross-protection of foodborne pathogens to antibiotics. Ebinesh et al. (2018) found that the heat adaptation (45 °C) of *Acinetobacter baumannii* induced cross-protection to amikacin, norfloxacin, piperacillin, tazobactam, imipenem, and meropenem.

### 20.2.7 Ethanol Stress-Associated Cross-Protection

Ethanol 70% is a traditional and agreed-to-use disinfectant in the food industry for decontaminating surfaces after cleaning (Graziano et al. 2013). It is also extensively used as a preservative. Various studies have reported that ethanol is effective for the control of foodborne pathogens (Seo et al. 2019). Early studies have shown that the growth of bacteria, particularly foodborne pathogens, was prevented with around 8% ethanol (citations). Additionally, the use of 2% ethanol has been used as a preservative in pizza in the USA and in sealed food in Japan (Kalathenos and Russell 2003). Shibasaki (1982) has reported the efficacy of low concentrations of ethanol
(2–5%) in the preservation of various foods, including hamburger, fish paste, sponge cake, miso, soy sauce, bean paste noodle, and packed egg-tofu. Kalathenos and Russell (2003) reported that the use of ethanol for rapid cool down in noodle production prevented the microbial growth in noodles for 2 weeks at 30 °C. However, recent studies have demonstrated that foodborne pathogens may develop the resistance to ethanol and ethanol adaptation may induce cross-protection to other stresses (He et al. 2018).

He et al. (2018) found that Salmonella could develop ethanol adaptation, which resulted from the treatment with 5% ethanol for 1 h. Subsequently, ethanol-adapted cells developed cross-protection to malic acid (20 μL/mL twofold increase in minimum bactericidal concentration), but not to acetic (2.5 μL/mL), ascorbic 20 μL/mL), lactic (5 μL/mL), citric (5 μL/mL), and hydrochloric acid (10 μL/mL). Similarly, He et al. (2016) found that ethanol adaptation induced cross-protection in S. Enteritidis against −20 °C. Chiang et al. (2008) found that ethanol shock (5%) increased survival of Vibrio parahaemolyticus to hydrogen peroxide (20 ppm) but decreased their survival to crystal violet, high NaCl, and organic acids. Huang et al. (2013) reported that ethanol adaptation enhanced survival of C. sakazakii to heat (51 °C), cold (−20 °C), and acid (pH 3.3).

### 20.2.8 Starvation Stress-Associated Cross-Protection

Bacteria, like any other living organism, need nutrients for growth and reproduction. In food, bacteria may find a rich source of nutrients. However, the nutrient content is generally low in some food materials such as water, which is used in the food industry for cleaning or rinsing food contact surfaces or even incorporated directly into the food (Gawande and Griffiths 2005). Previous studies have shown that due to exposure to starvation, several foodborne pathogens develop protective mechanisms that allow the cells to survive to other multiple stresses (Gawande and Griffiths 2005).

E. coli O157:H7 is resistant to starvation stress, and it has been shown that starvation stress induces cross-protection to subsequent stresses (Arnold and Kaspar 1995; Gawande and Griffiths 2005; Zhang and Griffiths 2003). Starved log-phase E. coli O157:H7 cells may develop cross-protection to acid stress (pH 2 with 5 N HCl) as compared with non-starved stationary phase cells (Arnold and Kaspar 1995). Starvation of E. coli O157:H7 also induces cross-protection to temperature-related stresses (Gawande and Griffiths 2005). In the case of cryotolerance, starvation has shown to increase cryotolerance of E. coli O157:H7 in storage at −18 °C for 24 h (Gawande and Griffiths 2005). Thermotolerance of E. coli O157:H7 has been associated with the cross-protection effect of starvation stress, which promoted the expression of heat shock protein genes (Zhang and Griffiths 2003). Non-toxigenic E. coli has also been shown to develop cross-protection to other stressors following starvation stress. Stationary phase E. coli may develop osmotolerance following exposure to starvation stress (Jenkins et al. 1990). Increased radiation resistance
(up to 2.5 kGy) of *L. monocytogenes* has also been linked with cross-protection resulting from the preexposure of cells to starvation for 12 days in physiological saline (0.85%) (Mendonca et al. 2004).

### 20.3 The Cellular and Molecular Bases of Cross Adaptation Response in Foodborne Pathogens

#### 20.3.1 Sigma Factors

Bacterial RNA polymerase (RNAP) requires a specialized reversibly-binding subunit called the sigma factor (Davis et al. 2017). σ factors mediate all gene expression in bacteria (Fig. 20.1). Thus, the expression of stress-related genes is also mediated by sigma factors in response to but not limited to heat, cold, acid, osmolarity, oxidative stress, ethanol, etc. (Cebrián et al. 2009). A protein to be classified as a sigma factor must perform two indispensable functions: directing the catalytic core of the RNAP to transcription sites and initiating separation of DNA strand as the first step of the transcription (Feklístov et al. 2014). In Gram-negative bacteria, the general stress-responsive alternative sigma factors are called RpoS (σ^S_), while in Gram-positive bacteria are SigB (σ^B_). (Boor 2006). Both sigma factors belong to the σ^70 family, which is the largest and most ubiquitous family of σ factors (Davis et al. 2017).

Previous studies have confirmed the regulatory effect of sigma factors under the exposure of various stresses. Schulthess et al. (2009) reported that the σ^B_ of *S. aureus* mediates the transcription of antibiotic resistance genes. *S. aureus* cells defected in σ^B_ exhibited decreased resistance to methicillin, teicoplanin, and vancomycin. Thus, σ^B_ may regulate the transcription of a series of stress-related genes,
which may provide cross-protection to other stresses. Kindrachuk et al. (2011) found that exposure of *Pseudomonas aeruginosa* to tobramycin (an aminoglycoside) induced the overexpression of heat shock sigma factor RpoH. This sigma factor was involved in the expression of the efflux pump genes *mexXY* and the heat shock gene *asrA*, which in turn conferred resistance to tobramycin and cross adaptation to heat. Mitosch et al. (2017) found that trimethoprim treatment (0.5 μg/mL) to *E. coli* induced cross-protection to acid stress. The mechanism of this cross-protection response might be regulated by the upregulation of RpoS, which is involved in the expression of GadB and GadC proteins. These two proteins are part of the Glutamate decarboxylase (GAD) system (see subsection 20.3.2), which is an important system for acid resistance in foodborne pathogens (Liu et al. 2015).

In a study reported by Rosche et al. (2005), *V. vulnificus* was treated with osmotic shock (1% artificial seawater). Log-phase *V. vulnificus*, after osmotic shock, developed cross-protection to heat (45 °C) and oxidative stress (0.2 mM H₂O₂). The alternative sigma factor RpoS was required for the cross-protection mechanism of log-phase osmotic-shocked cells to oxidative stress but not to heat stress. Rosche et al. (2005) found that expression of RpoS in log-phase *V. vulnificus* was very low compared to stationary phase *V. vulnificus*, but after 1.5 h of osmotic shock (1% artificial seawater), RpoS was induced. Kang et al. (2018) discovered that *S. Enteritidis* exposed to simultaneous acid-salt treatment developed heat resistance at 60 °C. Heat shock sigma factor (*rpoH*) and heat shock genes (*dnaK* and *groEL*) were upregulated following acid-salt stress and found to be associated with the heat resistance mechanism of *S. Enteritidis*.

The role of sigma factors in the mechanism of cross-protection induced by acid adaptation has been reported previously. Wemekamp-Kamphuis et al. (2004) reported that σB was essential for the expression of GAD genes *gadCB* and *gadD*, and nine proteins including SigB, Pfk, ClpP, Lmo1580, GalE, GadD, GadC, PykA, and Lmo1339, which were involved in stress protection and metabolism in *L. monocytogenes*. In the cells with active σB, exposure to pH 4.5 provided cross-protection against high hydrostatic pressure (350 Mpa) and freezing (−20 °C). In other study, acid adaptation of *E. coli* O157:H7 under cold conditions overexpressed the levels of *rpoS* and also *gadA* and *adiA* genes (Kim et al. 2016). RpoS is a general stress-responsive sigma factor mediates the expression of specific stress-related genes, including *gadA* and *adiA*, which are involved in pH homeostasis in foodborne pathogens (Kim et al. 2016). However, contradictory results have also been reported. In a study by Fong and Wang (2016), the alternative sigma factor gene *rpoE* in *S. enterica* was downregulated following desiccation or sub-lethal heat treatment. It is known that RpoE plays an important role in the survival of foodborne pathogens at low temperatures and hyperosmotic stress (McMeechan et al. 2007). Thus, it seems that RpoE was not indispensable for cross-protection between heat and desiccation stress resistance. Instead, two desiccation stress-related genes *fadA* and *otsB*, and a heat resistance gene *dnaK* were upregulated following desiccation and sub-lethal heat treatment, respectively.
### 20.3.2 Glutamate Decarboxylase (GAD) System

The glutamate decarboxylase (GAD) system is an important mechanism for keeping pH homeostasis in the cellular cytoplasm and is present in a variety of bacteria such as *E. coli*, *Shigella flexneri*, and *L. monocytogenes* (Liu et al. 2015). The GAD system is probably the most efficient system of acid resistance in *E. coli* and *L. monocytogenes* (Karatzas et al. 2012). The GAD system is composed of one to three decarboxylases (enzymes) (e.g., GadA, GadB, GadD1, GadD2, GadD3), which are the key of the GAD system, and one or two antiporters (e.g., GadC, GadC1, GadC2, GadT1, GadT2); the architecture of the system varies between species (Feehily and Karatzas 2013). When a bacterium (e.g., foodborne pathogen) is exposed to low pH, the decarboxylase, which is localized in the cytoplasm at neutral pH, is recruited to the membrane. One molecule of extracellular glutamate is transported to the cell through the antiporter. Then the decarboxylase catalyzes the molecule of glutamate to one molecule of γ-aminobutyrate (GABA), which is further expelled from the cell through the antiporter (Fig. 20.2). This process consumes one proton, so the main objective of the GAD system is to reduce the proton concentrations in the cell, retaining pH homeostasis (Alonso-Hernando et al. 2009; Feehily and Karatzas 2013).

![Fig. 20.2](image)

**Fig. 20.2** The principle of the GAD system. This figure reprinted from Liu et al. (2015), with the permission of ELSEVIER
Previous studies have demonstrated the role of the GAD system in the cross-protection of some foodborne pathogens. *L. monocytogenes* is by far, the species with the most “complete” GAD system, as it possesses three decarboxylases and two antiporters (Feehily and Karatzas 2013); however, it also depends on the strains, as some strains possess a defective GAD system (Boura et al. 2020). Following exposure to pH 4.5, *L. monocytogenes* was able to develop cross-protection to high hydrostatic pressure and freezing (Wemekamp-Kamphuis et al. 2004). The GAD system was associated with this cross-protection response through the overexpression of *gadCB* and *gadD* genes, which was σ^B-dependent (Wemekamp-Kamphuis et al. 2004). The other way around, *L. monocytogenes* treated with other stresses (trisodium phosphate, acidified sodium chloride, citric acid, chlorine dioxide, peroxo acids) induced cross-protection against acid stress (Alonso-Hernando et al. 2009). However, in some cases, the GAD system was found to be less related to the survival of *L. monocytogenes* at low pH (Alonso-Hernando et al. 2009). It seems that preexposure of the aforementioned stresses did not induce overexpression of GAD proteins, and *L. monocytogenes* might conduct another mechanism for the cross-protection response, which remains to be further explored.

The GAD system is important for the resistance response to nisin in *L. monocytogenes* (Begley et al. 2010). The gene *gadD1* of the GAD system contributes to the tolerance of *L. monocytogenes* to the antibiotic nisin (300 μg/mL) under low pH (presence of lactic acid bacteria *L. lactis*) conditions. Moreover, the GAD system also involves in the oxidative stress response of *L. monocytogenes* and occurs partly through catalase activity (Boura et al. 2020). Results of Boura et al. (2020) suggest that all GAD genes in *L. monocytogenes* (i.e., *gadD1*, *gadD2*, *gadD3*) are associated with increased oxidative stress. The upregulation of one or more of these genes may increase the acid and oxidative tolerance in *L. monocytogenes*.

### 20.3.3 Prevention or Repair of Damages in Macromolecules or Cellular Structures

Several proteins in foodborne pathogens are induced following exposure to certain stress. These proteins are involved in the mechanisms of stress adaptation in foodborne pathogens and are grouped according to the stress they are related to, such as acid shock proteins (He et al. 2018; Kim et al. 2005), heat shock proteins (Narberhaus and Balsiger 2003), osmotic shock proteins (Finn et al. 2013a, b), and so on. The main function of these proteins is to prevent or repair damages in macromolecules or cellular structures, such as DNA, proteins, membrane, organelle, etc., caused by any stress. Generally, these proteins are regulated by sigma factors that are actively involved in the transcription of related genes through the RNAP in bacterial cells following induction by exposure to stress. Several molecular chaperones (a group of ATP-dependent proteins) are involved in protein protection and
repair of misfolded proteins as a consequence of cellular stress such as acid, heat, oxidation, osmosis, etc., by stabilization of folding intermediates and prevention of protein misfolding and aggregation (Wickner et al. 2017). While chaperonins are a group of molecular chaperones and are large protein complexes that facilitate the folding of unfolded polypeptides in an ATP-dependent mechanism (Wickner et al. 2017).

### 20.3.4 Heat Shock Proteins (HSPs)

Some cellular stress responses of foodborne pathogens utilize heat shock proteins (HSPs), which are molecular chaperones that play an essential role in refolding damaged proteins caused by various stresses (Fig. 20.3). HSPs may be induced by various stresses, and expression of heat shock protein genes is mostly regulated by RpoH in Gram-negative bacteria and \( \sigma^B \) in Gram-positive bacteria, respectively (Nicolaou et al. 2010). The \( \text{gro}E \) genes, which encode GroE proteins in \( E. coli \), are the first chaperonin genes to be discovered (Nicolaou et al. 2010). While chaperonins are a group of molecular chaperones required for the folding of some cellular proteins and are defined by sequence similarity (Lund 2009). \( E. coli \) is the most widely studied bacteria in regard to chaperonins. Multiple chaperonin systems have been discovered in \( E. coli \), such as DnaK (HSP70), DnaJ, GrpE, GroEL (HSP60), and GroES (Chung et al. 2006). Most chaperonins are heat inducible, but there is evidence that other stresses such as acid may also induce the expression of chaperonin genes (Ye et al. 2019). Moreover, many foodborne pathogens such as \( S. Typhimurium \), \( E. coli \), and \( L. monocytogenes \) may express two or more chaperonin genes.

GroEL and DnaK are class I heat shock proteins or chaperonins involved in the protection of proteins from heat shock (Nicolaou et al. 2010). Under stress conditions, heat shock proteins such as GroEL and DnaK prevent aggregation and assist in protein refolding of damaged proteins (Nicolaou et al. 2010).

### 20.3.5 Cold Shock Proteins

In response to a sudden temperature drop, foodborne pathogens rely on cold shock proteins. Temperature drop affects the stabilization of RNA secondary structure, hence interrupting transcription and translation of genes. Foodborne pathogens respond to this stress by synthesizing cold shock proteins (Fig. 20.4). The major cold shock protein in \( E. coli \) is CspA. CspA is an RNA chaperone that facilitates transcription and translation during cold shock in foodborne pathogens (Ray et al. 2020). They are found in several bacteria such as \( E. coli \) (Yamanaka et al. 1998), \( S. Typhimurium \) (Ray et al. 2020), \( Clostridium perfringens \) (Talukdar and Sarker 2019).
Several osmoprotectant transport systems in foodborne pathogens have been identified. Under osmotic stress conditions, *S. Typhimurium* utilizes four osmoprotectant transport systems to transport glycine betaine into the cytoplasm, namely ProP,
ProU, OsmU, and YehZYXW (Kim et al. 2013). While E. coli K-12 utilizes ProP and ProU (Haardt et al. 1995). Glycine betaine is an important osmoprotectant, not only for bacteria but also for many species in all domains of life while facing osmotic stress (Wargo 2013). Glycine betaine is transported into the cytoplasm of foodborne pathogens by osmoprotectant transporters that possess high affinity to it. ProP is a proton symporter and member of the major facilitator superfamily of permeases (Frossard et al. 2012). The ProU system, an ATP-dependent transport system, is composed of ProV, ProW, and ProX, which function as ATPase, membrane pore, and periplasmic substrate-binding protein, respectively (Fig. 20.5). While OsmU is composed of OsmV (ATPase), OsmX (substrate-binding protein), OsmW and OsmY (membrane permeases) (Kim et al. 2013). ProP, ProU, and OsmU are encoded by the genes proP, proU, and OsmU, respectively, and regulated by the alternative sigma factor RpoE (Finn et al. 2013a). Osmoprotectant transporters are believed to be induced by osmotic stress (Frossard et al. 2012). However, previous studies have reported that other stresses such as acid stress may also induce osmoprotectant transporters regulated by the sigma factor (Ye et al. 2019), making them a mechanism of cross-protection in foodborne pathogens. For instance, OmpR, an acid shock protein, is a member of the two-component system EnvZ/OmpR, which is involved in osmoregulation and is important in acid and osmotic stress response (Jaworska et al. 2018).
20.3.7 Alteration of Cell Membrane Composition

The cell membrane is a structure of the bacterial cell and consists of a lipid bilayer. The main function of the cell membrane is to regulate the transportation toward the cytoplasm by transport proteins that bind specific substrate (Bhunia 2018). In Gram-negative bacteria, the cell envelope is surrounded by an outer membrane, which is absent in Gram-positive bacteria and consists of phospholipids, proteins, and lipopolysaccharides (Bhunia 2018).

It has been shown that membrane fatty acids are involved in the stress adaptation and cross-protection of foodborne pathogens (Yuk and Marshall 2003). Some studies have been done to understand the role of membrane fatty acids in these responses (Álvarez-Ordóñez et al. 2008). It has been demonstrated that variations in growth temperature, pH, ethanol concentration, and external osmolality, and even transition to stationary phase lead to changes in membrane fatty acids composition of foodborne pathogens (Álvarez-Ordóñez et al. 2008). Acid adaptation in S. Enteritidis, a Gram-negative pathogen, decreased the ratio of unsaturated fatty acids to saturated fatty (UFA/SFA) acids and also increased the content of cyclic fatty acids (CFA) in the cell membrane (Álvarez-Ordóñez et al. 2008). These changes in fatty acid composition decreased the membrane fluidity because SFA has a higher melting point than USFA, and the increase of CFA could also be
associated with the reduction in membrane fluidity (Yuk and Marshall 2004). Moreover, acid-adapted S. Enteritidis cells exhibited heat resistance (54–60 °C) (Yang et al. 2014). The authors concluded that the mechanism of acid adaptation and heat resistance was attributed to the decrease of membrane fluidity without the upregulation of RpoS and RpoH (Yang et al. 2014). In another serotype of Salmonella, S. Typhimurium exposure to different acids and growth temperatures also induced modifications in membrane composition, which was associated with the cross-protection to heat (Álvarez-Ordóñez et al. 2008). Acid-adapted cells showed low UFA/SFA ratio, high CFA content, low C18:1 and high methylenoctadecanoic acids relative concentration. Low UFA/SFA is linked to decreased membrane fluidity (Álvarez-Ordóñez et al. 2008). Ethanol shock has also induced changes in membrane fatty acids profile in V. parahaemolyticus. Following ethanol shock, the proportion of vaccenic acid in cell membranes increased, while the proportion of palmitic acid and SFA/UFA ratio decreased. Ethanol shock induced cross-protection only to hydrogen peroxide, but not NaCl, crystal violet, and organic acids (Chiang et al. 2008). The different ratio of SFA/UFA (0.90–1.05) of ethanol-shocked may explain why ethanol-shocked cells did not develop cross-protection to the other stressors besides hydrogen peroxide. In Gram-positive pathogen L. monocytogenes, adaptation to organic acid salts induced cross-protection to antimicrobials, specifically potassium lactate induced cross-protection to ε-polylysine. The cross-protection response was associated with alteration of membrane fatty acid composition, which decreased membrane fluidity (Kang et al. 2015).

Changes in the membrane protein of Gram-negative pathogens have also been associated with cross-protection mechanisms. Koga et al. (1999) found that acid adaptation of V. parahaemolyticus induced cross-protection to heat, crystal violet, bile, and deoxy cholic acid. Acid-adapted cells showed an increase in outer membrane proteins, which was linked to the cross-protection mechanism (Koga et al. 1999)). The outer membrane, which is only found in Gram-negative bacteria, contains nonspecific channel-forming proteins called “porins,” which allow the influx of nutrients and extrusion of water products; thus, porins are intrinsically associated with antibiotic resistance (Nikaido 2003). The synergistic effect of the multidrug efflux complex extrudes drugs through the outer membrane, making it effective in preventing the influx of antibiotics (Nikaido 2003).

### 20.4 Conclusions

Foodborne pathogens are prone to face multiple stresses throughout the food processing chain. Their high adaptability is demonstrated by the adaptation to certain stresses and by more complex adaptations such as cross-protection. This phenomenon may bring serious implications for food safety since it could be a mean for pathogens to survive through hurdle technologies. However, the exact mechanisms of cross-protection are still unknown. Therefore, efforts should be contributed to understand its exact underlying mechanisms and to provide effective strategies to
counterattack. Future research direction would be focused on the study of the exact mechanisms of cross-protection. Traditional detection methods offer a limited ability to inquire in-depth. Novel techniques such as omics, are believed to be a pivotal tool to decipher all the questions surrounding cross-protection.

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Chapter 21
In Situ Analytical Tools to Resolve Stress Response Mechanisms of Foodborne Pathogens

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Abstract In situ analytical techniques hold great potential for the investigation of stress response of foodborne pathogens, as they can provide unprecedented spatial, temporal, and single-cell resolved information of the process. In this chapter, the basic features and methods of in situ analysis will firstly be presented, followed by in situ imaging strategies using advanced microscopy. Particularly, we focus on in situ analytical techniques for gene or gene expression both in fixed and living cells and bacteria, which can shed light on the molecular response of foodborne pathogens towards stress. Finally, we draw on some key applications of in situ analysis in stress response research and propose the further development of in situ analytical tools facilitating the elucidation of stress response mechanisms of foodborne pathogens.

Keywords In situ imaging · Foodborne pathogens · Stress response · Food safety · Gene expression

21.1 Introduction

Foodborne diseases caused by pathogens rank as one of the most critical food safety issues. The broad spectrum of foodborne infections has changed dramatically over time. Foodborne pathogens with the resistance against stressors may emerge because of the changing ecology or technology in the food chain. New pathogens can also emerge by the transfer of mobile virulence factors, often through bacteriophage (Dangl and Jones 2001; Tauxe 2002). In such a backdrop, the development of tools to resolve the stress response mechanisms of foodborne pathogens is highly demanded. The stress response is triggered through the various stresses that bacteria encountered in the natural environment (Chowdhury et al. 1996; Gandhi and Shah 2017). Exposure to stress, which is defined as some environmental factors, can
induce the initiation of stress response pathways and result in profound changes in
gene expression and physiology. These environmental factors are detrimental to
bacterial growth rate or survival (Guest et al. 2017; Lee et al. 2009). Under the action
of stress response, foodborne pathogens can improve the survival ability and
enhance the pathogenicity, such as the formation of biofilms and transform into a
viable but nonculturable (VBNC) state (Gao et al. 2021). Meanwhile, the stress
response of foodborne pathogens is closely related to gene expression. For example,
\( \text{oxyR} \) gene in bacteria can be a regulator of oxidative stress response (Hausladen et al.
1996). There is an intricate relationship between the gene expression, spatial distri-
bution, and stress response of foodborne pathogens. The analysis of spatial structure
is necessary for revealing the development mechanism of the resistance of foodborne
pathogens towards various stressors (Nadell et al. 2016). The emergence of in situ
analytical technology provides an opportunity for investigating the gene expression
and spatial structure of foodborne pathogens (Wagner and Haider 2012).

Conventional analytical techniques are based on bulk samples, mainly to obtain
information about the predominant bacteria in the population. However, based on
this approach, the characteristics of a small number of bacteria in the population
would be masked. Thereby, it is difficult to obtain the gene expression characteristics
of a few key strains in the bacterial population by conventional methods (Riley 2020;
Van ‘t Veer et al. 2002). Foodborne pathogens contain different genotypes and
phenotypes. These differences are also attributed to various stress response mecha-
nisms and virulence of pathogens, which ultimately affect the diagnosis (Zhao et al.
2017a, b). The development of single bacterial analysis methods is crucial for the
further study of gene regulation pathways in foodborne pathogens. Apparently, in
situ analysis allows obtaining single bacterial information. Due to the heterogeneity
of microbial cells, it is significant to study the differences between each bacterium
through single bacterial analysis. Unlike bulk population analysis, single bacterial
analysis allows in-depth profiling of populations at single-cell resolution and
uncovering of rare key bacteria subpopulations (Liu et al. 2020; Ji et al. 2020).

In this chapter, we will present the features and applications of a variety of in situ
techniques in foodborne pathogens (Table 21.1). Differed from conventional anal-
ysis methods, the most prominent advantages of these in situ methods lie in the
availability of colony spatial information and single bacterium information (Barra
Caracciolo et al. 2005; Cardinale et al. 2008).

### 21.2 Advanced Microscopy

Benefited from the development of microscope technologies, such as scanning
electron microscope (SEM), confocal laser scanning microscope (CLSM), and
other advanced microscopy, the morphology of bacterial surface can be imaged.
Further, even the differences between various bacterial structures can be distin-
guished. Thereby, these technologies have emerged as powerful and precise tools
for in situ analysis of foodborne pathogens (Carpenter et al. 2013; Richards and
Turner 1984). SEM utilizes a beam of electrons to form an image of a specimen, which allows to image and quantify the topographic features of the surface (Surman et al. 1996). Further, CLSM can also be used to probe the three-dimensional (3D) structure of biofilm and to realize the visualization of the biofilm ultrastructure (Falsafi et al. 2020).

### Table 21.1 Summary of in situ techniques for detection of foodborne pathogens

<table>
<thead>
<tr>
<th>Analysis target</th>
<th>In situ techniques</th>
<th>Features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial biofilm</td>
<td>Scanning electron microscope (SEM)</td>
<td>Image and quantify of surface topographic features</td>
<td>(Sangetha et al. 2009; Hochbaum and Aizenberg 2010)</td>
</tr>
<tr>
<td></td>
<td>Confocal laser scanning microscope (CLSM)</td>
<td>Probe the three-dimensional (3D) structure and ultrastructure of biofilm</td>
<td>(Lawrence et al. 2003; Yan et al. 2016)</td>
</tr>
<tr>
<td>Gene expression imaging in fixed cell and bacteria</td>
<td>Fluorescence in situ hybridization (FISH)</td>
<td>Obtain the spatial information of RNA expression and cell heterogeneity information</td>
<td>(Almeida et al. 2013; Rocha et al. 2019)</td>
</tr>
<tr>
<td></td>
<td>Single molecule FISH (smFISH)</td>
<td>Quantification of the copy number of RNA in single cells</td>
<td>(Femino et al. 1998; Raj et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>In situ polymerase chain reaction (PCR)</td>
<td>Detection of low abundant RNA or detect genes</td>
<td>(Bagasra 2007; Ebina et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>In situ isothermal amplification</td>
<td>Can be performed without high temperature and is fit for detecting short RNA or mutations</td>
<td>(Deng et al. 2014; Larsson et al. 2010; Li et al. 2016)</td>
</tr>
<tr>
<td></td>
<td>Highly multiplexed RNA imaging</td>
<td>Combinational labeling can quantitatively analyze the behavior and mechanism of pathogenic bacteria</td>
<td>(Lubeck and Cai 2012)</td>
</tr>
<tr>
<td>Gene expression imaging in living cell and bacteria</td>
<td>Light-up RNA imaging</td>
<td>Allow to investigate how coding and non-coding RNAs in living bacteria are changed in response to stress in the environment</td>
<td>(Bertrand et al. 1998; Forrest and Gavis 2003; Hocine et al. 2013; Tutucci et al. 2018a, b; Paige et al. 2011; Pothoulakis et al. 2014)</td>
</tr>
<tr>
<td></td>
<td>CRISPR-Cas system</td>
<td>Dynamically image RNA localization to obtain more spatial information</td>
<td>(Chen et al. 2013; Yang et al. 2019)</td>
</tr>
<tr>
<td></td>
<td>Nanomaterial imaging system</td>
<td>Dynamically image endogenous genes, study the stress response mechanisms of foodborne pathogens</td>
<td>(Wang et al. 2017; Wu et al. 2015)</td>
</tr>
</tbody>
</table>
Some outbreaks of foodborne diseases have been found to be associated with biofilms. The formation of multicellular microbial communities, called biofilms (Giaouris et al. 2014; Srey et al. 2013; Zhao et al. 2017a, b). The induction process of biofilm is a typical response of pathogenic bacteria to environmental stress (Baker et al. 2010). Bacterial biofilm is a kind of agglomeration caused by bacteria adhering to contact surface (Berne et al. 2018; Hall-Stoodley et al. 2004; Teschler et al. 2015). It is evident that biofilm formation is an ancient and integral process of prokaryotic life cycle, and is a key factor for bacteria survival in diverse environments (Hall-Stoodley et al. 2004). In food processing, the metabolic activities of biofilm can corrode the surface of metal equipment and metal pipes, which is more likely to cause food contamination and eventually lead to foodborne diseases. The existence of biofilm might lead to a huge loss of financial and human resources and has become a serious public health problem. The number of cases and deaths caused by biofilm infections worldwide is up to millions each year (Dosler and Karaaslan 2014). Hence, the efficient inhibition of biofilm formation and eradication of microbial biofilm along the food processing are urgent problems to be solved at present.

Sangetha et al. (2009) applied SEM to explore the antimicrobial activity and prevention of Candida albicans (C. albicans) biofilm induced by Cassia spectabilis (C. spectabilis) extract at a concentration of 6.25 mg/mL. As shown in Figs. 21.1a, b, SEM revealed the reduction in C. albicans biofilm after 36 h treatment. The antifungal activity indicated that extract of C. spectabilis possessed its potential to against infections caused by C. albicans. Hochbaum et al. (2010) utilized SEM to compare Pseudomonas aeruginosa (P. aeruginosa) adhesion on structured and unstructured regions of the growth substrates. Substrates with varying dimensional parameters, such as nanopost diameter, height, pitch, and array symmetry were constructed to systematically investigate the substrate effect on bacterial growth. P. aeruginosa was cultured for 22 h in a rocking LB culture. The fluorescence image in Fig. 21.1c(A) showed the interface between a flat region (upper) and one of patterned posts (lower) on the same substrate. SEM images (Fig. 21.1c) also showed cross-sectional views of the different bacterial conformations in a biofilm grown on a flat substrate. Meanwhile, the extreme ordering case where cells are oriented normally to the substrate was imaged (Fig. 21.1c(C)). This study further demonstrated that SEM is an important approach for in situ analysis of bacteria morphology and biofilm.

Yan et al. (2016) used a single-cell imaging method to reveal the biofilm growth program and architecture of Vibrio cholerae (V. cholerae). They utilized microscopy techniques to enable single-cell resolved imaging of a V. cholerae biofilm as it develops from one single founder cell to a mature biofilm of 10,000 cells. The images indicated the forces underpinning the architectural evolution (Fig. 21.2a). This study discovered that directional proliferation of rod-shaped bacteria played a dominant role in shaping the biofilm architecture of V. cholerae, and the growth pattern was controlled by a single gene, named rbmA. Lawrence et al. (2003) mapped the exopolymeric matrix of microbial biofilm using CLSM. In this study, combinations of nucleic acid, protein, lipid, and glycoconjugate staining revealed a complex microbial community composition. Figure 21.2b showed a three-channel
series. The results illustrated the colocalization of nucleic acid-specific, protein-specific, and lipid-specific probes that bind to regions enriched in their target molecules. As shown in Fig. 21.2c, it is demonstrated that both localization and

Fig. 21.1 SEM-based in situ analysis for bacterial biofilm. (a) The control group of scanning electron micrographs showing a reduction in Candida albicans biofilm after 36 h treatment. One milliliter C. albicans cell suspension (1 × 10^6 cells/mL) was inoculated on a Sabouraud dextrose agar plate and incubated at 37 °C. Reprinted with permission from Sangetha et al. (2009). Copyright 2009 Elsevier; (b) The C. spectabilis extract treatment group of scanning electron micrographs showing reduction in C. albicans biofilm after 36 h treatment. Reprinted with permission from Sangetha et al. (2009). Copyright 2009 Elsevier; (c) Comparison of Pseudomonas aeruginosa adhesion on structured and unstructured regions of the growth substrates. (A) Fluorescence microscopy showed the localized effect of substrate topography on bacterial adhesion as compared to flat surfaces. The image showed the interface between a structured and unstructured region on the same substrate. The interface between the flat (upper) and structured (lower) areas is abrupt. The result is the transition from ordered packing to random microcolony aggregates, which lack long-range cell order. The cells were stained with SYTOX green nucleic acid stain. (B, C) Cross-sectional SEM images of P. aeruginosa cultured on flat and periodically structured epoxy surfaces, respectively, showing the stark difference in attachment morphology. The aligned cells in (C) are false-colored to highlight their orientation. Scale bars are 10 μm in (A) and 1 μm in (B) and (C). Reprinted with permission from Hochbaum and Aizenberg (2010). Copyright 2010 American Chemical Society.
Fig. 21.2  (a) Single-cell imaging of a growing *Vibrio cholerae* biofilm cluster. Cross-sectional images of the bottom cell layer at (A) 1 h, (B) 7 h, (C) 12 h, and (D) 18 h. (Scale bars: 3 μm.) (E) Segmenting the 3D biofilm cluster in (D) into 7199 cells, color-coded according to z position (0–21 μm). (F) Spatial distribution of the z and radial components of each cell’s orientation director $e_z$ and $e_r$, volume fraction $\phi$, and alignment order parameter $\zeta$ for the 18 h cluster in E. Reprinted with the permission from Yan et al. (2016). Copyright 2016 National Academy of Sciences. (b) Three-channel imaging of biofilm material stained with (A) Sypro Orange (protein), Syto9 (nucleic acids), and (B) Nile Red (hydrophobic-lipid rich), and (C) the three-color combination of the channels showing the localization and (D) colocalization of protein (red), nucleic acids (green) and lipid-hydrophobic regions (blue). Arrows indicate (a) protein plus DNA, (b) protein plus lipid, and (c) regions rich in lipid alone in the biofilm. Reprinted with permission from Lawrence et al. (2003). Copyright 2003 American Society for Microbiology. (c) Triple-labeled confocal images of different biofilm samples illustrating the distribution of protein, nucleic acids, lipid, and polysaccharide regions labeled with N-acetylglucosamine and N-acetyllactosamine-sensitive lectins. Colors denote the following: (A) protein (red), nucleic acids (green), polysaccharide (blue) (arrow indicates filamentous structure with protein plus nucleic acids); (B) lipid (red), nucleic acids (green), polysaccharide (blue); (C) lipid (red), protein (green), and polysaccharide (blue) (arrow indicates cyan region where colocalization of protein and polysaccharide was detected); (D) polysaccharide (red), nucleic acids (green), and polysaccharide (blue) in this case, the two lectins are localized in distinct polysaccharide regions. Reprinted with permission from Teschler et al. (2015). Copyright 2015 Springer Nature
colocalization of the probes were associated with cells and their exopolymers. The utilization of CLSM makes it possible to delineate regions of space, cells, and exopolymeric materials. It also provides the potential to construct optically sectioning and create 3D images of biofilms.

The combination of multi-microscopy technologies has been developed as an important approach for in situ analysis of bacteria and can be used to create a detailed correlative map of biofilm structures and compositions (Wahab et al. 2020; Neu et al. 2010; Fröjd et al. 2011). These correlative techniques could improve our understanding of the biochemical basis for foodborne pathogens biofilm organization. Furthermore, the approach can assist studies which intended to investigate and optimize biofilms for environmental remediation applications. The exploration of pathogenic bacteria biofilms will be an essential approach to study stress response mechanisms. Multi-microscopy techniques can also be used to image changes in bacterial morphology when exposed to different environmental stresses, such as heat, cold, osmosis, and oxidation. Besides, bacterial persisters are considered to be an important cause of biofilm formation and refractory infection. The phenotypic characteristic of bacterial persister cells is to enter a state of no growth or slow growth. By means of phenotypic analyses via imaging, bacterial persister cells can be detected (Defraine et al. 2018; Fisher et al. 2017).

21.3 Gene Expression Imaging in Fixed Cell and Bacteria

21.3.1 Fluorescence In Situ Hybridization (FISH)

During the early 1990s, fluorescence in situ hybridization (FISH) has gained increasing importance as a common technique to detect and identify microorganisms. The analysis of ribosomal-RNA (rRNA) allows to investigate the evolution and toxicology of foodborne pathogens (Amann and Fuchs 2008). FISH has become a tool for rapid and direct single-cell identification of microbes by recognizing signature regions of rRNA (Wagner and Haider 2012). FISH is easy to operate which only mainly involves only a DNA probe hybridization (Amann and Fuchs 2008). FISH can obtain the spatial information of RNA expression and cell heterogeneity. The FISH technique is mainly performed by the following steps: (1) fixation of cell or tissue samples; (2) design of nucleic acid probes with fluorescence tags complementary to the target sequence, and labeling the target sequence by in situ hybridization; (3) in situ imaging (Wagner et al. 2003; Rohde et al. 2016). FISH can not only provide insights into the cell structure but can also be combined with CLSM to reconstruct the spatial arrangement of habitat microbial communities (Daims et al. 2001).

FISH has been used to study the relevant pathogenic bacteria which cause foodborne infections, such as Salmonella enterica (S. enterica), Escherichia coli (E. coli), Listeria monocytogenes (L. monocytogenes), and Bacillus cereus (B. cereus) (Rohde et al. 2015). Fang et al. (2003) detected Salmonella spp. in...
foods using FISH with optimized 23 rRNA probes. The authors selected two species-specific 23S rRNA-targeted oligonucleotide probes (Sal-1 and Sal-3) and newly designed one (Sal-544). This method has been successfully applied in a variety of food samples including meat, fish, milk products, eggs, cheese, lettuce, and butter. Almeida et al. (2013) proposed a new peptide nucleic acid fluorescence in situ hybridization (PNA-FISH) method for the rapid detection of *E. coli* O157:H7. The method was verified in 54 strains, which demonstrated the probe is highly sensitive and specific for *E. coli* O157:H7. The authors also developed the method to reduce the autofluorescence. Two different approaches have been tested: a centrifugation step (to remove some autofluorescence food particles) and the use of a detergent (1% Triton X-100) to emulsify the fatty compounds. As shown in Fig. 21.3a, both methods decreased the autofluorescence signal, but the detergent presented a stronger reduction and also seems to improve the fluorescence signal. Strong autofluorescence signal that is presented from some food components can be avoided through this procedure. Rocha et al. (2019) explored a developed PNA-FISH method for the detection of *L. monocytogenes*. The method is able to analyze *L. monocytogenes*. The limit of detection was 0.5 CFU/25 g or mL in food samples and presented 100% specificity and sensibility. After inoculating artificial beef with $\approx$1 CFU/25 g of sample, the images of *L. monocytogenes* were exhibited in Fig. 21.3b. As can be observed from the images, *L. monocytogenes* did not present the typical small rod-shaped bacilli but present a filamentous form in beef. This phenomenon may be influenced by the composition of the One Broth Listeria (OBL) broth (Giotis et al. 2007). The utilization of FISH technology may also be a tool for the investigation of biofilm mechanisms. For example, the recognition and identification of microbial strain can be achieved by this approach. Key genes associated with bacterial persisters could also be detected coupling with FISH, such as the *hipA* gene of *E. coli* (Moyed and Bertrand 1983).

### 21.3.2 Single Molecule Fluorescence In Situ Hybridization (smFISH)

The sensitivity of FISH is low, resulting in the difficulty to detect low concentrations of RNA in foodborne pathogens. In fact, most intracellular RNAs are low-level expressed. For example, more than 80% of mRNAs in yeast are expressed in less than two copies (Larson et al. 2009). With the introduction of peptide nucleic acid probes or locked nucleic acid probes (Silahtaroglu et al. 2007; Thomsen et al. 2005), the recognition and binding ability of the probes to target sequences can be improved. However, due to the high autofluorescence signal in cells and unavoidable nonspecific attachment of probes, it is difficult for these developed FISH technologies to guarantee specificity for RNA imaging at single-molecule level (Itzkovitz and van Oudenaarden 2011). Attributed to the development of nucleic acid synthesis and labeling technologies, single-molecule fluorescence in situ hybridization (smFISH)
has recently been developed. The key point of the smFISH method is to hybridize multiple nucleic acid probes modified with one single fluorescent group or one single nucleic acid probe modified with multiple fluorescent groups onto the target RNA. smFISH provides spatial information of each RNA in cells (Crosetto et al. 2015).

Femino et al. (1998) firstly achieved cellular single-molecule mRNA imaging coupling FISH in 1998. In this study, the long nucleic acid probe was replaced by a...
probe with a length of only 50 bases complementary to the target mRNA sequence. The key design lies in the utilization of probes labeled with multiple fluorescent groups (Fig. 21.4a) to improve the signal strength. Raj et al. (2008) further improved the method. They designed multiple short-chain probes (17–22 bases) labeled with single or multiple fluorescent groups complementary to the target mRNA. This method can greatly improve the signal intensity of the marker RNA. A diffraction fluorescence bright spot of corresponding single-molecule RNA can be obtained.

**Fig. 21.4** (a) Principles of single RNA molecules by single-molecule fluorescence in situ hybridization (smFISH). (A) One optical plane of a normal rat kidney (NRK) cell after in situ hybridization with calibrated CY3 probes to β-actin mRNA shows a number of bright foci superimposed on a diffuse background arising from out-of-focus light. Red arrow, bead = 0.099 μm; yellow arrow, two transcription sites. (B) The distribution of fluorescent probes after image restoration with an iterative constrained algorithm. The restored image revealed discrete points of light, which allow accurate measurement of the total fluorescence intensity (TFI) emanating from each point source (bead is restored to a point source). (C) The image was rendered so that the cell was viewed from the bottom looking upward into the nucleus. The red surface portrayed the boundary of the nuclear envelope. The opening in the center of the nuclear surface was a result of truncating the upper optical sections. (D) Enlargement of (C) showing the dimensions of a restored transcription site in the nucleus (cross-hairs), in contrast to a 100-nm bead (yellow arrow), the most intense object in the image, restored to a point source. Reprinted with permission from Femino et al. (1998). Copyright 1998 The American Association for the Advancement of Science. (b) Simultaneous detection of unique 128 probes sequences and a repeated sequence in individual mRNA molecules. (A) Schematic of the construct used. The 48 probes used to detect the GFP coding sequence were labeled with Alexa 594, and the four different probes used to detect the tandem repeat in the 3’ UTR were labeled with TMR. (B) Maximum intensity merges of a pair of z-image stacks of fluorescent images of CHO cells taken in the Alexa 594 channel (left) and the TMR channel (right). (C) False-color merge of the boxed regions in b, with circles representing computationally identified mRNA particles. Scale bars, 5 mm. Reprinted with permission from Raj et al. (2008). Copyright 2008 Springer Nature
through fluorescent imaging (Fig. 21.4b). The smFISH can generate the RNA expression and spatial information in a single cell by fluorescence dot counting. In addition, the interference of non-specific adsorption signals can be avoided because the signal was generated by multiple fluorescent-labeled probes hybridized with target RNA. The most prominent advantage of smFISH over FISH is that gene expression can be analyzed at single bacteria level. With the introduction of smFISH, the comparison of gene expression differences and phenotypes of single pathogenic bacteria could be conducted. Thus, it can be used to study stress response mechanisms at single bacteria level.

### 21.3.3 In Situ Polymerase Chain Reaction (In Situ PCR)

The single-molecule RNA imaging based on FISH enhances the fluorescence signal by hybridizing high-density fluorescent-labeled probes on target RNA. Further, the signal amplification of target RNA or DNA can be realized by nucleic acid amplification technologies to accomplish gene expression investigation. The polymerase chain reaction (PCR), as a mature amplification technique, can also be used for in situ analysis. PCR is a common amplification strategy for achieving highly sensitive nucleic acid detection. In situ PCR utilizes PCR or reverse transcriptase-PCR (RT-PCR) to amplify target gene in fixed cell and bacteria, thus allowing to highly sensitive image of gene or RNA.

Bagasra (2007) established in situ PCR method for the amplification of both DNA and mRNA targets. Amplicons of targets can be detected by monitoring the hybridization and labeled probes. The frozen or paraffin-fixed tissue sections, cell culture, and other single-cell suspensions can be performed in this method. The technique has the ability to identify gene expressing of individual cells in a tissue section coupling with a microscope. This study has completed the detection of human herpesvirus type 8 (HHV8) DNA in the sperms of an HIV-1 infected man by in situ PCR (Fig. 21.5a) and the detection of hZIP1 (human zinc transporter 1) mRNA in normal and malignant prostate sections by in situ reverse transcriptase-PCR (RT-PCR) (Fig. 21.5b). These two examples represented the two extreme ranges of interpretations, which become significant when carrying out in situ PCR. In situ PCR can be widely used in medical diagnosis, detection of foodborne pathogens, environmental analysis, and other fields. Ebina et al. (2001) analyzed the topographic distribution of p53 mutations in cells including non-small cell lung cancers (NSCLCs) using in situ PCR. The authors designed primers to detect mutant mRNA through RT-PCR in the individual tumor cells. They found that the distribution of mutant p53 mRNA coincided with that of immunohistochemical overexpression of p53 protein in each case. The results indicated that the regulation of mutant expression was heterogeneous in the neoplastic cells but not the genotype. The photomicrographs of the squamous cell carcinoma operated by in situ PCR were showed in Fig. 21.5c. In addition, in situ PCR technology can also be used to identify different strains and identify gene mutations. Thus, in situ PCR can be applied in the
investigation of stress response mechanisms of various kinds foodborne pathogens under different environments. In particular, mutations of pathogenic bacteria genes that result from environmental stress responses can be detected.

21.3.4 In Situ Isothermal Amplification

The high temperature required in the PCR process may destruct cellular structure and lead to the degradation of RNA molecules. The emerge of isothermal nucleic acid amplification technologies allows the image of gene expression at constant temperature meanwhile keeps high sensitivity due to their high amplification efficiency (Xia et al. 2020a, b; Van Ness et al. 2003). Isothermal amplification includes rolling circle amplification (RCA) (Ali et al. 2014), loop-mediated isothermal amplification (LAMP) (Wong et al. 2014), hybridization chain reaction (HCR) (Huang et al. 2001).
exponential amplification reaction (EXPAR) (Van Ness et al. 2003), catalytic hairpin assembly (CHA) (Jiang et al. 2013), strand displacement amplification (SDA) (Shi et al. 2014) and helicase-dependent amplification (HDA) (Vincent et al. 2004). Among them, RCA and HCR often used for in situ analysis. Compared to PCR, isothermal amplification can be performed without precise control of temperature cycling and is well fit for detecting short RNA or DNA. The procedure can be performed inside fixed cells or even living cells, which is hardly feasible by PCR. Further, isothermal amplification technology has the characteristics of mild reaction conditions and easy design. Isothermal amplification is thus a versatile in situ amplification strategy, which may enable the quantification and imaging of RNA or DNA in single cells.

RCA technology is an early isothermal amplification strategy in intracellular RNA imaging. The design of RCA uses circular DNA as a template and then extends the primers in the action of phi29 DNA polymerase to amplify the long DNA strands. The long DNA and circular DNA templates are complementary. Larsson et al. (2010) achieved in situ detection of single-molecule mRNA by RCA strategy. The target mRNA was reversely transcribed into cDNA, then the RNA was digested by RNase I. Next, cDNA was used as the primer for in situ RCA amplification, so as to achieve in situ detection of single-molecule mRNA (Fig. 21.6a). In this study, four different target mRNAs were simultaneously detected by four different fluorescent-labeled nucleic acid probes. Attributed to the high specificity of the padlock probe, the method allowed to detect a somatic point mutation. This method can make distinctions between members of a gene family and perform multiplex detection of transcripts in human and mouse cells and tissue. Nevertheless, due to the complex cell environment, the efficiency of in situ amplification reaction is not satisfactory.

Deng et al. (2014) introduced a toehold-initiated rolling circle amplification (TIRCA) for visualizing individual microRNAs in situ in single cells. This assay can identify miRNAs at physiological temperature with high specificity. The TIRCA method is a potent strategy that enables both stringent recognition and in situ amplification of the target sequences. High specificity for miRNA detection inside the lung cancer cell line A549 cells has been achieved (Fig. 21.6b). It was observed that a large number of bright spots appeared in A549 cells when miRNA let-7a was detected using a matched seal probe. TIRCA has become thus a competitive candidate technology for the identification of miRNAs. This method may help us to understand the role of non-coding RNAs in stress response of foodborne pathogens.

The efficiency of enzyme-dependent amplification reaction is affected by enzyme activity and intracellular environment. Thus, enzyme-free isothermal amplification may improve the efficiency of imaging and may be applied to observe RNAs in living cells. The HCR method contains two stable hairpin probes and can avoid the involvement of enzymes. Only when the target sequence is hybridized with probes to open the hairpin structure, polymerization reaction could be initiated. Li et al. (2016) recently constructed a nano-delivery system based on graphene oxide as the carrier of HCR probes. The results provided recognition and signal enhancement of specific miRNAs in living cells (Fig. 21.7). The amplification of miRNA-based HCR would
Fig. 21.6  (a) In situ imaging of intracellular mRNA based on rolling circle amplification (RCA) amplification and padlock probes. (A) Schematic diagram of mRNA imaging principle based on RCA amplification technology and padlock probe; (B) fluorescence imaging of mRNA (single base difference) in co-cultured fibroblasts of human and mouse. Scale bars: 20 μm. Reprinted with permission from Larsson et al. (2010). Copyright 2010 Springer Nature. (b) Schematic of toehold-initiated rolling circle amplification (TIRCA) for visualizing individual miRNAs in situ inside cells. After binding to the toehold domain of the seal probe, the target miRNA switches the seal probe to the “activated” circular form through a strand-displacement process, thereby initiating RCA. When there is a sequence mismatch between the miRNA and the seal probe, the miRNA fails to branch migrate through the probe and there is no amplification. TIRCA was applied to visualize individual miRNAs in A549 cells. Scale bars: 10 μm. Reprinted with permission from Deng et al. (2014). Copyright 2018 Wiley
finish at a time of 8 h. Imaging of miRNA in living cells could facilitate monitoring dynamic expression of miRNA and research on miRNA-related cellular processes. Profited from the mild reaction condition (room temperature) of isothermal amplification, the morphological integrity of cells or bacteria can be well retained. In situ amplification methods allow to resolve the bacterial structure and gene information in reserved cellular states, such as biofilm. Particularly, non-coding RNAs or RNA mutations can be analyzed to explore the relationship between dark gene information and stress response at the transcriptional level. Thus, the mechanisms of foodborne pathogens resistant towards harsh conditions could be inferred.

Fig. 21.7  (a) Scheme of amplification and two-color imaging of miRNAs in living cells based on HCR and graphene oxide. (b) Fluorescence images of miR-21 in MCF-7 cells after different incubation times. Reprinted with permission from Li et al. (2016). Copyright 2016 Royal Society of Chemistry
21.3.5 Highly Multiplexed RNA Imaging

The detection flux of in situ fluorescence imaging poses a major challenge by the limitation of non-spectral overlapping fluorescence species that can be used for labeling. Exploring the expression differences of a large number of RNAs at single-cell level can understand the correlation of different gene expressions. This approach can also discover new gene regulatory networks and gene functions, and obtain the spatial information of different genes at subcellular levels. Hence, the development of intracellular highly multiplexed single-molecule RNA imaging can help us to quantitatively analyze the behaviors and mechanisms of pathogenic bacteria.

Combinational labeling can effectively improve the flux of FISH. Levsky et al. (2002) firstly proposed the combinatorial labeling technique in 2002 and examined the expression of 11 genes simultaneously in cells. Combinational labeling is to carry out collocation labeling through a variety of fluorescent molecules. Different channels were used to identify the target RNA (Walter et al. 2006). Lubeck and Cai (2012) proposed a simple but general strategy to drastically increase the capacity for multiplex detection of molecules in single cell using optical super-resolution microscopy (SRM) and combinatorial labeling. In this study, high-resolution fluorescence imaging of the target mRNA was completed, allowing the spatial information of fluorescence markers to be used as a distinguishable method (Fig. 21.8a). At the same time, the target mRNA was encoded by the combined fluorophore, and the labeling accuracy of this method was high. This super-resolution imaging enables combinatorial labeling of individual transcripts (Fig. 21.8b). The introduction of highly multiplexed imaging methods has become an increasing tool for both disease analysis and clinical research (Baharlou et al. 2019). Highly multiplexed RNA imaging can profile the stress response mechanisms at the level of the whole bacteria community. Highly multiplexed RNA imaging will permit comprehensive explorations of microbial community structure, functional state and cell-cell interactions. Furthermore, this method holds the potential to be applied in the study of pathways of the stress response in complicated environments.

21.4 Gene Expression Imaging in Living Cell and Bacteria

The introduction of optical microscopy to observe cells has fascinated biologists since the early days of the study of cell structures (Stephens and Allan 2003). Fluorescence imaging technology of living cells and living bacteria can provide information about the dynamic changes of gene expression as well as cellular structure. These information will facilitate our understanding of stress response of foodborne pathogens (Xu et al. 2004; Sun et al. 2019). The disorder of RNA-protein interactions and RNA localization is closely related to the occurrence of disease. In this section, we will introduce the emerging tools allowing to image living
foodborne pathogen, including the synergistic application of fluorescent protein, light-up aptamer, CRISPR system, and nanomaterials.

### 21.4.1 Light-Up RNA Imaging

In situ RNA imaging could be realized by coupling with a fluorescent protein and RNA aptamer. The MS2 system is a genetically encoded reporter derived from the bacteriophage MS2 (Tutucci et al. 2018a, b). It has been widely used in organisms ranging from bacteria to higher eukaryotes. MS2-fluorescent protein system is often used for living cells imaging. The system consists of two parts: (1) expression of the fusion protein of MS2-fluorescent protein; (2) expression of multiple copies of MS2 target RNA in the stem-loop region (LeCuyer et al. 1996). Bertrand et al. (1998)
firstly visualized real-time localization of the mRNA in living yeast (Fig. 21.9a). Forrest et al. (2003) used this technique to accomplish the mRNA imaging in living oocytes of Drosophila (Fig. 21.9b). The results revealed a diffusion-based, late-acting posterior localization mechanism for long-range transport of nanos mRNA. Attributed to its high sensitivity, MS2-GFP system has been used for single-molecule detection. Hocine et al. (2013) demonstrated a two-color imaging system with single-molecule resolution using MS2 and PP7 RNA labeling (Fig. 21.9c). Tutucci et al. (2018a, b) proposed a re-engineered MS2 system to visualize single mRNAs in living Saccharomyces cerevisiae (S. cerevisiae) (Fig. 21.9d). MS2-fluorescent protein imaging technology has the problem of high background value caused by free MS2-fluorescent protein. The optimization of the ratio of MS2-fluorescent protein to target RNA expression is required when RNA labeling is performed. The advantage of single-molecule imaging lies in the ability to detect single transcripts and distinguish multiple mRNA species using various labeled probes.

Light-up RNA aptamer system usually refers to an RNA-based fluorogenic module consisting of an aptamer, which is able to specifically interact with a fluorophore to form a fluorescent complex (Sim et al. 2019). Light-up aptamers include Spinach (DasGupta et al. 2015; Paige et al. 2011), Broccoli (Chandler et al. 2018), Mango (Dolgosheina et al. 2014), BHQ apt (Murata et al. 2011), etc. Some of these modules are significantly brighter than the widely used GFP, and compared with protein counterparts, they do not require a translation process (Bouhedda et al. 2018). Attributed to the RNA’s ability to fold into a complex three-dimensional structure, Paige et al. (2011) screened an RNA aptamer that can form a specific structure and produced a small molecule named DFHBI (3,5-difluoro-4-hydroxybenzylidene imidazolinone). DFHBI does not emit light, but when it was bound to the RNA aptamer, a green fluorescence can be emitted. The brightness of the complex is comparable to that of fluorescent proteins. The RNA aptamer-DFHBI complex was named “Spinach”. The Jaffrey group not only developed Spinach and Broccoli but also converted the light-up aptamers into in vivo imaging sensors. The fluorescence signal of Spinach was detected in living cells, promising its use in situ imaging (Fig. 21.10a). In addition, Spinach was also found to be suited for imaging messenger RNAs in foodborne pathogens. Pothoulakis et al. (2014) explored the co-expressing of RNA aptamer and a red fluorescent protein (mRFP1). Simultaneous measurement of mRNA and protein levels from engineered constructs can be realized. After 2 h post-induction, living cells were assayed for Spinach and mRFP1 expression by a fluorescence microscopy (Fig. 21.10b). This investigation accomplished the utilization of the Spinach aptamer sequence as a tool to characterize mRNA expression in E coli.

The utilization of fluorescent proteins and light-up RNA aptamer makes it possible to detect and image RNAs in living bacteria. This technique will allow us to explore how coding and non-coding RNAs in living bacteria are changed in response to stress in the environment.
**Fig. 21.9** (a) Visualization of mRNAs in living cells. (A) Schematic of the constructs used MS2 and green fluorescent protein system. (B) Expressing the GFP-MS2 fusion protein in living cells and the reporter mRNA. Arrows indicate some of the particles, usually in the bud. Scale bars: 5 μm. Reprinted with permission from Bertrand et al. (1998). Copyright 1998 Elsevier. (b) Fluorescent RNA accumulates in early oocytes. (A-C) Confocal images of living egg chambers expressing both mRNA and MCP-GFP. Reprinted with permission from Forrest and Gavis (2003). Copyright 2003 Elsevier. (c) (A) Co-expression of PCP-2yEGFP and MCP-mCherry results in allele-specific labeling of mRNA. (B, C) Transcription of the indicated allele produces green or magenta fluorescent spots. (D) Merge of mRNA fluorescence for both alleles. Reprinted with permission from Hocine et al. (2013). Copyright 2012 Springer Nature. (d) Schematic representation of the DOA1 locus tagged with 24 × MBSV6 inserted in the 3′ UTR. Two-color smFISH for DOA1 mRNAs tagged with 24 × MBSV6 in cells expressing MCP. Reprinted with permission from Tutucci et al. (2018a, b). Copyright 2018 Springer Nature.
Fig. 21.10  (a) Living cell imaging of Spinach-tagged 5S RNA. Fluorescence and phase images of HEK293 T cells expressing 5S tagged with either Spinach or Lambda, a control RNA. Reprinted with permission from Paige et al. 2011. Copyright 2011 The American Association for the Advancement of Science. (b) Living cell fluorescence (GFP/Green and Cy3/Red) and phase images of Escherichia coli cells carrying either the pET28c-Spinach (T7-Spinach) or pGPR01 (T7-mRFP1-Spinach) plasmid in the presence or absence of 200 μM 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI). White arrows highlight off-population cells not showing any fluorescence. Reprinted with permission from Pothoulakis et al. (2014). Copyright 2014 American Chemical Society
21.4.2 CRISPR-Cas System

Clustered regularly interspaced short palindromic repeats-CRISPR-associated proteins (CRISPR-Cas) system is promising for tracking and labeling nucleic acids in foodborne pathogens (Cox et al. 2017). The spatial and temporal organizations of DNA and RNA are important for maintaining and regulating bacterial functions, such as gene expression, DNA replication, and repair (Wang et al. 2018; Hao et al. 2020). RNA localization information is necessary because the functions of RNA are associated with unique subcellular localization. CRISPR-Cas system has been used for dynamically imaging DNA and RNA in living cells.

Chen et al. (2013) proposed a method for imaging repetitive elements in telomeres and coding genes in living cells with Cas9 protein and a structurally optimized small guide RNA (sgRNA). The schematic of optimized CRISPR/Cas system for visualizing nucleic acids in living cells is shown in Fig. 21.11a(A). Sequence-specific enrichment of fluorescence signals by sgRNA-directed dCas9-EGFP allows the imaging of genomic elements in living cells. The CRISPR imaging of telomeres in cells is shown in Fig. 21.11a(B). This CRISPR-based imaging method allows to easily and reliably track dynamic information during the elongation and disruption process of telomeres. Dreissig et al. (2017) also revealed dynamic telomere movements for living cells imaging in plants. They demonstrated reliable imaging of telomere repeats in living cells of *Nicotiana benthamiana* and realize the potential visualization of multiple genomic loci. In order to effectively label other types of RNAs, Yang et al. (2019) proposed a system to visualize specific RNAs for living cells in a user-friendly manner. In this way, the dynamic imaging of RNAs in living cells by CRISPR-Cas13 has been accomplished (Fig. 21.11b). Particularly, the authors suggested that future studies may identify additional Cas13 proteins to further enhance the utility of this system in real-time RNA imaging in cells. The CRISPR-Cas-based technique could be applied in real-time RNA imaging of foodborne pathogens in the future. For example, the dynamic information of RNA mutations and gene expression changes of bacterial persister cells may be obtained coupling with CRISPR-Cas imaging system. Furthermore, real-time in situ imaging has potential in the study of drug efflux process of pathogenic bacteria under antibiotic stress. The regulatory genes of multiple drug efflux pumps could be tracked and detected, such as *mexR*, *nalC*, and *nalD* of *Pseudomonas aeruginosa* (*P. aeruginosa*) (Sader et al. 2017; Kiratisin et al. 2012).

21.4.3 Nanomaterials Imaging System

The accomplishment of above in situ imaging methods mostly relies on the introduction of foreign genes and genetic modification. Nevertheless, this situation can be avoided with the help of nanomaterials. Nanomaterials have become efficient tools for intracellular delivery of nucleic acid reagents to achieve sensitive detection and
Fig. 21.11 (a) A CRISPR/Cas9 protein system for visualizing genomic sequences in living cells. (A) Schematic of CRISPR imaging. Sequence-specific enrichment of fluorescence signals by small guide RNA (sgRNA) directed dCas9-EGFP allows the imaging of genomic elements in living cells. (B) CRISPR imaging of telomeres in cells using different sgRNA designs. Reprinted with permission from Chen et al. (2013). Copyright 2013 Elsevier. (b) CRISPR-dCas13 systems enabled visualization of NEAT1 in living cells with single gRNAs. (A) Illustration of gRNAs used in labeling NEAT1 by CRISPR-dCas13. (B) Overview of CRISPR-dCas13-mediated labeling. (C) Representative images of different dCas13 subtype proteins (including 2 dCas13a, 3 dCas13b, and 3 dCas13d) used in NEAT1 labeling in the NONO-mRuby3 knocked-in Hela cell line. Reprinted with permission from Yang et al. (2019). Copyright 2019 Elsevier.
regulation of gene expressions (Kanasty et al. 2013). The superiority of nanomaterials-based methods lies in that can directly image endogenous genes, thus avoiding the process of genetic modification. The emergence and development of nanomaterials offered an opportunity for in situ analysis owing to these appealing attributes (Xia et al. 2020a, b). By coupling with nanocarriers, such as Au nanoparticles (Wu et al. 2015), graphene oxides (GO) (Chen et al. 2015), and MnO2 (Fan et al. 2015) nanosheets, it has the potential for probing DNA and RNA in living cells and bacteria (Dong et al. 2020). These probes are amenable to dynamically investigate foodborne pathogens processes, such as apoptosis and stress response. Some other nanomaterials have catalytic activity, named nanozymes, including Fe3O4, CeO2, MnO2, and V2O5 nanoparticles (Zhou et al. 2017; Liu and Liu 2017). The combination of nanomaterials and nucleic acids probes may be an effective approach for in situ analysis (Zhu et al. 2020).

Wu et al. (2015) developed an electrostatic DNA nano-assembly for living cells imaging. Hybridization chain reaction was introduced for signal amplification. The key design of the intracellular analysis was a core gold nanoparticle, an interlayer of cysteine-terminated cationic peptides, and an outer layer of fluorophore-labeled nucleic acid probes (Fig. 21.12a). The ultrasensitive imaging of mRNA expression in living Hela cells was showed in Fig. 21.12b. Bright and faint fluorescence images were obtained through the nano-assembly carrying probes of H1 and H2. The images exhibited clear cytosolic localization in the orange (575–610 nm) and the green (510–550 nm) channels under the excitation at 488 nm. Wang et al. (2017) proposed a multiple-targeted GO nanocarrier for mRNAs imaging and expression monitored (Fig. 21.12b). The results indicated that the method coupling with ssDNA/GO nanocarrier can be used to specifically probe mRNAs in cancer cells. This nanomaterials-based method has provided an opportunity for the simultaneous imaging of multiple biomarkers in living cells and bacteria. In fact, nanomaterials can be even more useful. Xu et al. (2004) utilized Ag nanoparticles for real-time probing of membrane transport in living microbial cells. The authors demonstrated that these Ag nanoparticles could transport through the inner and outer membrane of the cells.

In recent decades, nanomaterials have been intensively investigated in the field of diagnosis and imaging (Aouidat et al. 2019). For imaging and drug delivery, these nanomaterials are often tagged with some fluorescent agents. The efficient delivery capacity of nanomaterials could transmit the probes into bacteria; thus, it does not require the expression of genes or translation process of proteins inside microbial cells. This approach could be used to study the stress response mechanisms of foodborne pathogens without damaging cells. The ability to obtain dynamic information enables the analysis of regulatory genes of E. coli persister cells, such as dskA, relA, spoT, ygfA, and yigB (Lewis 2010). Through the dynamic analysis of gene expression, the mechanism of the formation of bacterial persister cells can be better revealed.
21.5 Application of In Situ Analysis in Stress Response

Foodborne pathogens may be spread through food chain and cause adverse effects on human health. In situ analysis can be introduced to construct sensing strategies for stress response mechanisms of foodborne pathogens and cells. Cui et al. (2019) realized the mapping of oxidative stress response to lithium cobalt oxide nanoparticles in single cells with the utilization of multiplexed in situ analysis. They developed an approach for directly visualizing the antioxidant state at single-cell resolution. The authors reconstructed color-coded maps containing gene expression information (Fig. 21.13). These strategies can be applied to monitor the oxidative stress response of foodborne pathogens in the same principle.

The biofilm under antibiotics stress has been investigated via multiplexed FISH. Valm et al. (2011) proposed a combinatorial labeling and spectral imaging FISH (CLASIFIISH) strategy for the structural analysis of a microbial biofilm, human dental plaque. They reported that 15 taxa in the plaque community could be
simultaneously imaged, including species of *Streptococcus, Prevotella, Actinomyces*, and *Veillonella* (Sutrave et al. 2019). This technique allowed to assess the effect of daptomycin and vancomycin on *Staphylococcus epidermidis* biofilms coupling with FISH. This investigation could in situ visualize and quantify biofilms upon the exposure of the antibiotics daptomycin and vancomycin. These results provide an opportunity for structural analysis of biofilm organizations under different stress conditions.

In addition, foodborne pathogens can enter the viable but nonculturable (VBNC) state upon stress conditions. The VBNC refers to a special state in which a number of bacteria respond to some environmental stress, such as cold, heat, osmosis, and high pressure (Zhao et al. 2017a, b). Xu et al. (1982) firstly discovered and presented VBNC in 1982. It is difficult for conventional culture-dependent methods to detect foodborne pathogens in the VBNC state. Hence, the prevention and control of VBNC bacteria in food processing and the environment is of great importance (Ding et al. 2017). For example, *E. coli* O157:H7 was found to express virulent genes *stx1* and *stx2* in the VBNC state (Lothigius et al. 2010). The production of virulent factors has a potential health risk for humans. Aurass et al. (2011) developed in situ analysis to investigate the influence of copper-ion stress on *E. coli*. A Nikon Eclipse fluorescence microscope and two fluorescent probes were used in this

![Fig. 21.13 Visualizing the compositions of three oxidative stress response groups in single cells using reconstructed red, green, and blue color map images. Reprinted with permission from Cui et al. (2019). Copyright 2019 American Chemical Society](image)
method. Viable *E. coli* stained with SYTO9 emitted green fluorescence, while dead *E. coli* stained with propidium iodide (PI) stains emitted red fluorescence. The results indicated that the majority of cells remained culturable for at least 40 days without copper-ion stress. In addition, about 95% of cells were membrane-damaged at day 40 under the copper ion-stressed microcosm at 23 °C (Fig. 21.14a). Zhao et al. (2013) observed the characteristics of *E. coli* in different states through scanning electron micrograph (SEM). As shown in Fig. 21.14b(A), the morphology of the exponential-phase cells of *E. coli* O157:H7 was rod-shaped with a smooth surface. In the VBNC state, cells were mostly curved rods (Fig. 21.14b(B), arrow) with 1.45 ± 0.11 μm length, and the cell surface was relatively rough. After 6 h of resuscitation, most of the cells changed from curved to long rods, which were longer than the exponential-phase cells (Fig. 21.14b(C)). Ten milliliter of the four HPCD-treated samples with less than 0.1 cfu/mL culturable cells were centrifuged at 8000 g for 10 min. Next, the pellets were resuspended in the same volume of TSB, and then 10 mL of these cell suspensions and their ten-fold serial dilutions in TSB were transferred to 50 mL sterile glass tubes. Finally, the tubes were kept at 37 °C. Servais et al. (2009) proposed a procedure called direct viable count-FISH (DVC-FISH) for estimating *E. coli* in freshwater. The investigation indicated that the ratio of culturable to viable *E. coli* was close to 1 in highly contaminated water, while decreasing drastically in weakly contaminated samples. It is further proved that in situ analysis can explore the influence of environmental factors on the VBNC formation of foodborne pathogens.

### 21.6 Outlook

In situ analytical techniques rapidly emerge due to the development of biomolecular technologies and advanced microscopy in the last two decades. Ongoing improvements in imaging technologies result in both higher time and spatial resolution. The presence of nucleic acid probes coupling with fluorescent proteins or nanomaterials enables to identify genes and RNAs with even single-nucleotide resolution in either fixed or living cells. Particularly, in situ analysis enables imaging genes of interest while preserving the morphology of foodborne pathogens. Gene silencing or overexpression triggered by stress response could be explored by in situ imaging. Sequentially, the information of gene mutations in response to stress under various environments could be recognized. In addition, noncoding RNA of foodborne pathogens may be explored coupling with in situ techniques. These techniques can allow to dynamically image RNA localization and endogenous genes. Both single-molecular imaging and highly multiplexed imaging can be achieved.

Benefited from the advantages of in situ analysis, further researches on the stress response of foodborne pathogens will be expected. In the future, how to simplify the sample pretreatment and obtain more spatial information is worthy of attention. Meanwhile, enriching the molecular markers associated with stress response of foodborne pathogens is also worthy to comprehensively understand the pathogen
Fig. 21.14  (a) Copper ion stressed, nonculturable *Escherichia coli* O104:H4 populations contain viable bacteria. Reprinted with permission from Aurass et al. (2011). Copyright 2011 Wiley. (b) Scanning electron images (magnification of ×10,000) of *E. coli* O157:H7. (A) The exponential-phase cells; (B) VBNC cells induced by high-pressure CO₂ (HPCD) treatment at 5 MPa and 25 °C for 40 min; (C) Resuscitated cells cultured in tryptic soy broth (TSB) for 6 h; (D) Resuscitated cells cultured in TSB for 24 h. Reprinted with permission from Zhao et al. (2013). Copyright 2013 PLoS
behavior. At present, single-molecule imaging by in situ analysis will remain important in ongoing researches. Super-resolution fluorescence imaging promises to directly reveal single bacteria organizations. Techniques for in situ analysis would help us to elucidate the relationship between genotype, cellular molecular network, and phenotype of stress response of foodborne pathogens.

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Part V

Concluding Remarks and Future Prospect
Chapter 22
Control of the Stress Response of Foodborne Pathogens

Shigenobu Koseki

Abstract  Bacteria can change responsively adapt to various environmental stresses during food processing. Detection and evaluation of physiological changes of microbial cells play a key role in the appropriate processing control. The appropriateness of the processing magnitude will be expected to enable minimum or at least mild treatment of food. This chapter reviews useful culture-independent methods for the detection and identification of bacterial injury due to environmental stress. In addition, a stress combination that enables control of the recovery of injured cells is discussed.

Keywords  Stress response · Detection method · Processing control · Bacterial injury

22.1 Culture-Independent Methods for the Quantitative Evaluation of Bacterial Injury

22.1.1 Ion Flux-Based Techniques

The change of net ion fluxes is a remarkable characteristic of cellular response to environmental stress. The measurement of net ion fluxes has been used to demonstrate the response of different organisms towards the external stressors (Macpherson et al. 2005; Shabala et al. 2006). As a non-invasive and high-resolution technology, the microelectrode ion flux estimation technique (MIFE) has been well recognized. Pioneering attempts have applied MIFE to evaluate adaptive responses of bacteria to food preservation-associated stresses, including cold (Shabala and Ross 2008), osmotic pressure (Shabala et al. 2009), and acidity (Shabala et al. 2002a, b). These attempts showed that the application of MIFE can cover a board of cases of induced

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T. Ding et al. (eds.), Stress Responses of Foodborne Pathogens,
https://doi.org/10.1007/978-3-030-90578-1_22
bacterial injury. However, MIFE has not been applied for physically induced bacterial injuries (e.g., thermal processing and high-pressure treatment).

Thermal treatment can inactivate bacteria mainly by denaturing membrane proteins (Marquis et al. 1994; Wesche et al. 2009). The release of functional macromolecules (e.g., lipopolysaccharides, lipids, phospholipids) was caused by the injury on the outer membrane, which subsequently induced the disruption of membrane permeability (Boziaris and Adams 2001; Wuytack et al. 2003). Thermal treatment clearly can influence the ion flux. Hence, understanding the heat-induced variations of ion flux through the cell membrane might provide an insight into the mechanism underlying the inactivation of thermal treatment on bacteria. Koseki and colleagues investigated the responsive change of K⁺ flux of *Escherichia coli* K12 to a series of thermal conditions (from 55 to 70 °C for 15 min) using MIFE (Koseki et al. 2012). They observed no responsive change of K⁺ fluxes that were induced regardless of different thermal conditions. Interestingly, hyperosmotic stress (3% NaCl w/v) can elicit a remarkable loss of net K⁺ flux in thermal-treated *E. coli* cells. However, this phenomenon was only applied to cells treated with a thermal condition below 65 °C. As hyperosmotic stress would result in a significant random flux noise when the thermal condition was above 65 °C. When using ion flux to predict bacterial injury, there is always a delay effect. For example, when the thermal conditions reach 62 °C, no recovery or growth of *E. coli* cells are observed, and the K⁺ flux disruption started to show at 68 °C. In addition, enrichment culture for the viability loss test can well correlate with the disruption of K⁺ fluxes in thermal-treated *E. coli* cells. In general, measurement of ion flux can evaluate the loss of bacterial viability in a rapid manner. The testing duration can be as short as 2 h. Ion flux measurement is a nonculture-dependent method provide an alternative for bacterial injury test.

### 22.1.2 PCR

Injured pathogenic bacteria can recover and pose potential food safety risks (Miller et al. 2006; Chambliss et al. 2016). Therefore, understanding the bacterial recovery from thermal treatment is critical for food manufacturers to modify processing procedures to eliminate the potential food safety risk.

Culture-based cell counting is the conventional method to evaluate the inactivation effect against pathogens. By comparing the cell counts on non-selective and selective media, the injury level of bacteria can also be reflected (Hara-Kudo et al. 2000). Because injured cells cannot recover and form typical colonies on the selective media (Smith and Archer 1988). Bacterial staining by LIVE/DEAD BacLight™ bacterial viability kit (Molecular Probes) is an alternative method to discriminate bacterial viability (Berney et al. 2007). However, this method is inapplicable for food samples due to the interference of food components and the presence of food microflora to dyes (Elizaquível et al. 2012). The dyes-propidium monoazide (PMA) and ethidium bromide monoazide (EMA) can selectively penetrate the dead cell membrane and subsequently prevent the detection of DNA amplification by PCR.
technique (Barbou-Piednoir et al. 2014). Hence, these dyes coupled PCR techniques can evaluate viable cells from a population containing dead cells (Soejima et al. 2011; Wang and Mustapha 2010). However, since this PCR-based method needs to design staining procedures for targeted food samples, it might not be applicable to determine injured bacterial cell numbers in all kinds of food.

Real-time PCR technique has been widely used to quantify bacterial counts in various food systems due to its ultra-specific and sensitive characteristics (Kimura et al. 2001; Botteldoorn et al. 2008; Josefsen et al. 2010; Kawasaki et al. 2014; Hong et al. 2016; Noviyanti et al. 2018, 2020). To evaluate the levels of injured cells, the growth delay time (GDT) in the recovery medium was introduced into a real-time PCR assay (Kawasaki et al. 2018). Kawasaki and colleagues have applied this method for ground beef samples. They spike *Salmonella* Enteritidis in ground beef and applied thermal treatment on the spiked sample. Real-time PCR was used to monitor the recovery of *S*. Enteritidis to grow, and the DNA copy numbers targeted on *invA* gene were subsequently applied for the construction of the growth recovery curve. The growth recovery kinetics were analyzed for GDT in which intensive thermal treatment increased GDT. Compared to traditional culture methods, real-time PCR is more applicable for the evaluation of the recovery of injured *S*. Enteritidis from food samples.

### 22.1.3 Dielectrophoresis (DEP)

In the 1950s, Pohl (1951) made the first use of dielectrophoresis (DEP) to describe the polarization-induced movement of neutral particles in a non-uniform electric field. It has been developed for the manipulation and characterization of microbes (Pohl et al. 1978; Pethig and Markx 1997; Pethig 2010; Pohl and Kaler 1979). A DEP force is associated with the size of the targeted particle, the strength of the electric field, and the dielectric properties of the surrounding environment. DEP-based separation approaches have been developed on a lab-on-a-chip device. Subsequent modifications enable this microfluidic device to efficiently manipulate biological matter by DEP force (Tuval et al. 2006; Gascoyne and Vykoukal 2002; Whitesides 2006). In addition, there are some devices functionalized for the discrimination of cell viability (Lapizco-Encinas et al. 2004; Hakoda et al. 2010). Kikkeri et al. (2018) developed a microfluidic-based test platform coupled with impedance sensing for the determination of the live/dead bacterial cells. Currently, the application of DEP mainly focuses on the healthcare area, DEP techniques also show great potential in other public health-associated areas (Martinez-Duarte 2012; Razak et al. 2013; Rahman et al. 2017). A rapid bacteria detection instrument based on the DEP technique is a successful application in public health areas (Wakizaka et al. 2020).

The DEP-based tools have also been developed in the food industry for the quick detection of bacteria in food. Betts proved the thermotical possibility of DEP application in complex food matrices (Betts 1995). Brown et al. (1999) developed
an automated continuous-flow DEP-based system to enumerate the suspended bacteria in a real-time manner (Brown et al. 1999). In the 2000s, more and more efforts have been specified in the investigation of DEP techniques for the identification of foodborne pathogens (Fernandez et al. 2017). However, studies of the DEP-based method on the enumeration of microorganisms in real food samples are still limited (Ogawa et al. 2021).

22.2 Hurdle Strategies to Completely Eradicate Foodborne Pathogens Without Triggering Cross-Adaptive Response

22.2.1 HPP and Mild Heat-Treatment

High-pressure processing (HPP), a promising non-thermal technique, can be applied for the post-processing of thermally processed foods. The inactivation effect of HPP against bacteria on sliced cooked ham has been explored in the previous studies (Aymerich et al. 2005; Cheftel and Culioli 1997; López-Callero et al. 1999; Hayman et al. 2004; Morales et al. 2006). Hayman et al. (2004) found that 3-min HPP exposure can inactivate up to $10^4$ CFU/g of *Listeria monocytogenes* cells onto sliced cooked ham at 20 °C. HPP treatment did not kill all *L. monocytogenes* cells but achieve a long-lasting bacteriostatic effect (Ellenberg and Hoover 1999; Bull et al. 2005; Koseki and Yamamoto 2006; Chilton et al. 2001). Accurate evaluate the HPP effect and control the recovery of bacterial recovery on the treated sample is of importance for ensuring food safety. Treated *L. monocytogenes* cells cannot grow until 42 days post-treatment, and cell replication proceeded slowly to $10^6$ CFU/g at 84 days post-treatment (Aymerich et al. 2005). However, the bacteriostatic effect of HPP varied among different bacteria. For example, it is found that after exposure of 20-min HPP under 7 °C and 400 MPa, the lactic acid bacteria regrew on the sliced cooked ham at 21 days post-treatment (López-Callero et al. 1999). HPP with a pressure of over 550 MPa can result in up to a reduction of 7 log$_{10}$ CFU/mL *L. monocytogenes* cells in milk at 25 °C within 5 min (Koseki et al. 2008). However, *L. monocytogenes* recovered within 7 days when stored at 25 °C post-HPP. A combined treatment of HPP at 550 MPa for 5 min and mild thermal exposure ranging 37 to 50 °C can be extended the bacteriostatic effect for up to 70 days when milk is stored at 25 °C.

The recovery of bacteria post-HPP was accelerated when the temperature was slightly below the optimum growth temperature. Accordingly, modifying storage temperature post-HPP was critical for the control of bacterial recovery. Koseki et al. (2008) applied the successive treatment of HPP at 550 MPa for 5 min and mild heat for the control of *L. monocytogenes* recovery of in milk. The authors used a mathematical model to assess the necessity of the additional mild heat exposure to retard *L. monocytogenes* cells to regrow in HPP-treated milk during storage. The
model could predict the performance of various combined treatment factors, such as temperature-holding time and temperature, on inhibiting the capacity of bacteria to recover from the injury. The mechanism underlying the post-treatment recovery asserts that warm temperature impaired the intermolecular forces among the molecules in the bacterial cell membrane, compromising the repair of destructed membrane induced by pressure (Russell 2002). However, the detailed mechanisms are not clearly demonstrated, and further studies should aim to decipher the mechanism at a molecular level. Such work would benefit the application of HPP as a practical microbiological control technique. Although the capacity of HHP-treated bacteria to achieve recovery is unsettled, proper temperature management as an additional constraint may prevent bacteria to recover during post-HHP storage.

22.2.2 Osmotic and/or Chill Stress with D-Tryptophan Treatment

To maintain intracellular hemostasis in osmotic stress, bacteria would take in compatible solutes (e.g., glycine-betaine). The effect of incompatible solutes as structural homologs of compatible solutes on bacterial physiology is still unknown. Koseki et al. (2015) evaluated the effects of various amino acids on bacterial growth in peptone-yeast-glucose (PYG) broth using optical density assay. The influence of 23 amino acids in the forms of both L and/or D isomers on bacterial growth were estimated. Among these amino acids, D-tryptophan at a concentration of around 40 mM showed an inhibitory effect on the growth of *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes*. In the presence of 3% salt, D-Tryptophan ranging from 30 to 40 mM exhibited a complete inhibition on the growth of *Salmonella* and *E. coli* O157:H7, but not *L. monocytogenes*. In contrast, when salt concentrations were decreased to the concentrations of 0–2% NaCl, it was found that D-tryptophan could retard *L. monocytogenes* to grow but not other bacteria. The inhibitory effects on bacterial growth of D-tryptophan varied among different bacterial strains. The above results suggest that the D-tryptophan as an incompatible solute posed an osmotic pressure to inhibit the bacterial growth.

D-tryptophan exhibited maximum inhibitory activity against *E. coli* when coupled with NaCl compared with other salts, including potassium chloride (KCl) and sucrose (Kan et al. 2018). A logistic regression model has been developed to characterize the boundary conditions of NaCl and D-tryptophan densities for the inhibition of bacterial growth. According to this model, the minimum concentration of NaCl to generate an inhibitory effect against *E. coli* with D-tryptophan at a concentration of 40 mM was 2.5% (w/v). In addition, a higher concentration of NaCl can promote an inhibitory effect. The logistic regression model also points out that incubation temperatures have an impact on the bactericidal performance of D-tryptophan. When the incubation temperature is increased, D-tryptophan reduces viable *E. coli* at a higher speed. This phenomenon might be attributed that optimum
growth temperature can promote physiological metabolism and subsequently increase the uptake of D-tryptophan.

The inhibitory effectiveness of D-tryptophan against Vibrio spp. was also affected by the concentrations of NaCl (Chen et al. 2018). NaCl at a concentration of 4.0% with D-tryptophan at a concentration of 20 mM led to intensive and persistent blocking on the growth of V. vulnificus and V. parahaemolyticus. It is found that the exposure of peptone water containing NaCl (4.5%) containing D-tryptophan (40 mM) for 48 h could efficiently retard V. vulnificus and V. parahaemolyticus to grow in shucked oysters at 25 °C. D-tryptophan demonstrated a lower inhibitory activity on the growth V. parahaemolyticus and V. vulnificus in TSB than in artificial seawater with the same salinity level under the temperature of 25 °C. These evidences listed above suggest that D-tryptophan can be a prominent food preservative for the control of Vibrio spp. in fresh oysters.

The combination treatment of D-tryptophan and the chilled temperature has been previously reported. D-tryptophan retarded the growth of L. monocytogenes at 4 °C for a 30-day period. Compared with the absence of the D-tryptophan condition, addition of 30 mM D-tryptophan prolonged the lag phase of L. monocytogenes to a maximum value of 400 h (Chen et al. 2020). Particularly, D-tryptophan treatment at 40 mM and 4 °C could achieve a 3-log reduction of L. monocytogenes during incubation for 30 days. When combined with over 3.0% NaCl, 40 mM D-tryptophan could lead to an overall stronger bacteriostatic effect. Overall, D-tryptophan demonstrates great potential as an effective food preservative.

22.3 Conclusions

The occurrence of injured bacterial cells may be unavoidable during food processing. However, detection of injured bacterial cells would be realized not only by conventional culture-dependent methods but also by emerging culture-independent methods that are introduced in this chapter. In addition, some combinations of processing techniques will prevent the recovery of injured bacteria during storage. These technologies will contribute to assuring safe and high-quality foods.

References


