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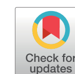
Application of atomic force microscopy in food microorganisms



Qin Liu[a,b], Hongshun Yang[a,b,*]

[a] Food Science and Technology Programme, C/o Department of Chemistry, National University of Singapore, Singapore 117543, Singapore
[b] National University of Singapore (Suzhou) Research Institute, 377 Lin Quan Street, Suzhou Industrial Park, Suzhou, Jiangsu 215123, PR China

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ABSTRACT

Background: Microorganisms play an important role in the food industry. Knowledge of the surface structural and physical properties of food microorganism cells is crucial to gain a detailed understanding of their functions in the natural environment and to explore them efficiently in various food processes. Atomic force microscopy (AFM), as a non-invasive examination tool, has been widely used to image the surface ultrastructure and to probe the physical properties of food microorganisms.
Scope and approach: In the current review, detailed applications of AFM in various food microorganisms are outlined, including surface imaging, biomolecular interactions, surface stiffness, and physicochemical properties, which have contributed to our knowledge of cell surface functions. The study emphasises the combination of AFM imaging with force determination, which added a new feature to the AFM technique; i.e., mapping of specific interactions. The combined use of AFM with other complementary techniques for a comprehensive description of cell surface is also reported.
Conclusions: and key findings: AFM has given promising results and thus could be a powerful technique for surface characterisation at nanoscale resolution and could provide new insight into the structure-function relationship of microbial surfaces.

1. Introduction

Microbes are ubiquitous and can be found in many food products and food contact surfaces. Within industries such as the production and delivery of meat, poultry, vegetables, and seafood products, microbial contamination can be detrimental to the process because they can cause product deterioration and decreased process efficiency (Garrett, Bhakoo, & Zhang, 2008). Microbial contamination is increasingly becoming a cause of concern for food safety and human health (Van Boxstael et al., 2013). Foodborne illness is mainly caused by ingestion of food spoiled by harmful pathogens or their toxins. Foodborne infectious disease, whether caused by bacteria, viruses, or fungi, are easily spread, and consequently continue to be a major public health problem, with associated social and economic costs, worldwide.

A distinct feature of microbial cells is their viability and mechanical properties in a wide range of environmental conditions. Understanding the surface properties of microorganisms will increase our knowledge of cell structural and physical functions, and behaviour at interfaces, such as cell adhesion. The physical properties of the surfaces have been difficult to elaborate because of the small size of microorganisms.

Atomic force microscopy (AFM) provides a new opportunity to study the surface structures of cells *in situ* at nanoscale resolution (Yang, 2014, chap. 1). AFM has emerged in the past few years as an important addition to traditional methods for high-resolution imaging and analysis of microbiological systems, because of its inherent advantages of simple sample preparation and its capability for imaging in air or an aqueous environment continuously, and manipulating the interaction between macromolecules (Liu, Tan, Yang, & Wang, 2017a,b; Yang et al., 2007). The optical microscope is a useful tool to identify cells, but the resolution is limited by the wavelength of the light source. Scanning electron microscopy and transmission electron microscopy can provide high-resolution images of cells; however, the complex sample preparation and vacuum conditions required could cause substantial distortion of the sample. AFM could complement the data obtained by electron microscopy and light microscopy techniques by providing high spatial resolution and real-time monitoring information. Minimum or even no sample preparation is required for imaging; therefore, AFM can provide real 3-dimensional (3D) topological structures. Furthermore, AFM sample observation can be conducted in aqueous solution, which provides opportunities for biological applications to monitor surface properties of live cells in real-time, and to non-destructively examine the membrane and protein leakage in an ambient environment. AFM can qualitatively probe the interaction based on the tip-sample distance and the cell elasticity of bacteria.

* Corresponding author. Food Science and Technology Programme, C/o Department of Chemistry, National University of Singapore, Singapore 117543, Singapore.
E-mail address: chmynghs@nus.edu.sg (H. Yang).

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This review introduces the fundamentals of AFM and provides a survey of the latest applications offered by AFM in food microbiology, demonstrating the unique attributes of AFM for studying the molecular mechanisms at the nanoscale and unravelling key microbial problems from the single cell through to the biofilm.

2. Principle of AFM and imaging methods

2.1. Principles of AFM

The AFM technique is based on the interaction between a sharp cantilever tip and the sample surface. The tip is moved across the surface at a certain distance (non-contact) or directly in contact with it (contact mode), depending on the surface properties to be determined. This technique is able to measure microscopic features of a sample and is not a 'conventional microscope' that collects and focuses light. The most characteristic property of AFM is that the real-time images are acquired by feeling the sample surface without using light. Images of surface topographies up to a resolution of several tenths of a nanometre can be obtained. In this way, besides surface topography with good resolution, and in contrast to other microscopically techniques, AFM is able to reveal various material characteristics, e.g. stiffness, hardness, friction, or elasticity, and the strength of the interaction between the sample surface and the cantilever tip. Therefore, AFM offers a powerful way to examine surface properties of different microorganisms.

The contact mode is the simplest and original AFM imaging mode, which is widely used to measure the topography of cells (Aguayo, Strange, Gadegaard, Dalby, & Bozec, 2016; Yun et al., 2014). In contact mode AFM, the deflection of the flexible cantilever is sensed while the sample is raster-scanned horizontally by a piezoelectric scanner. The image is generated by mapping the vertical distance during scanning, because the deflection remains constant at each lateral data point.

The non-contact mode is a more advance development in which the integrated cantilever vibrates at or near its resonance frequency, which is driven by a piezoelectric actuator (Rogers, Manning, Sulchek, & Adams, 2004). Tapping mode imaging can be implemented in ambient air or fluid. When the tip approaches towards the surface, the interaction forces (impulsive force) induce amplitude changes of the cantilever's oscillation. This amplitude acts as a control factor that adjusts the height of the cantilever above the sample. The electronic servo (feedback loop) maintains the oscillation at a constant amplitude during imaging. Topographical images are produced by mapping the vertical distance that the scanner moves as it maintains a constant oscillation amplitude at every lateral data point. Tapping mode significantly reduces problems associated with adhesion, electrostatic forces, friction, and lateral shear forces present in conventional AFM techniques by lifting the tip off to avoid dragging the tip across the surface (Boussu et al., 2005).

2.2. Force spectroscopy

Cell force spectroscopy is a recently established technique to measure the strength of cell adhesion at the single-molecule level by generating force curves. The curves are acquired by performing controlled vertical tip-sample interactions. The deflection of the cantilever is recorded as a function of its piezoelectric displacement (i.e., the tip-sample distance) as the tip approaches toward and retracts from the sample to generate a force-distance curve. Microbial cell force spectroscopy allows the probing of the interfacial behaviour at the nanoscale, including specific and nonspecific forces that are involved between cell-cell surfaces and nanomechanical properties; e.g., the elasticity of living cells (Harimawan, Rajasekar, & Ting, 2011). Microbial cell force spectroscopy allows the detection of the different kinds of cell-surface interaction forces, quantitation of cell adhesion, and molecular details of bacterial interactions (Beaussart et al., 2013; Helenius, Heisenberg, Gaub, & Muller, 2008; Schaer-Zammaretti & Ubbink, 2003).

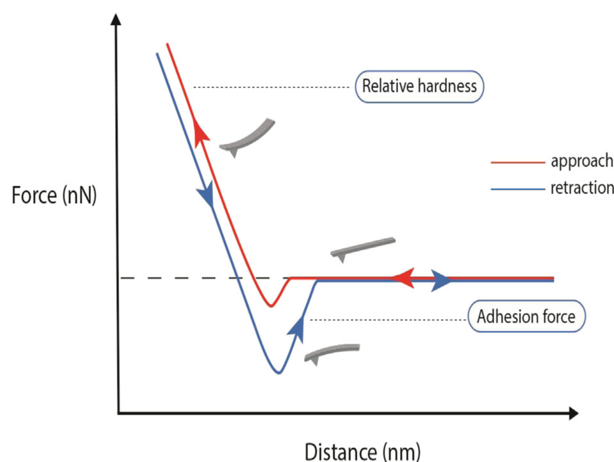

Fig. 1. Schematic force-distance curve of AFM force spectroscopy (Adapted from Wright, Powell, Johnson, & Hilal, 2014).

The recording of force-distance curves is one of the most versatile tools provided by AFM instrumentation and is the basis of microbial cell force spectroscopy. Force-distance curve-based AFM is a valuable tool to simultaneously characterise the morphology and furnish the mechanical properties of biological samples in terms of interaction and adhesion forces at the nano- or subnanoscale (Alsteens, Trabelsi, Soumillion, & Dufrêne, 2013).

Typical approach and retraction force-distance curves are shown in Fig. 1. Fig. 1 demonstrates a single approach-retract cycle of AFM mapping. At the beginning of the AFM approach (red curve), the tip is completely retracted from the sample, a long distance separates the tip from the sample surface, and no interaction force exists. The tip then moves towards the sample surface controlled by piezoelectric element, which oscillates the cantilever and facilitates every tiny but accurate movement on command. During the initial phase of the approach, the probe is mediated by long-range attractive (van der Waals) or repulsive (electrostatic) interaction forces, which are measured simultaneously by AFM cantilever deflection. Further movement towards the surface causes the cantilever to bend upward in response to repulsive forces. Subsequently, the tip tries to retract from the surface. The adhesion force between the AFM tip and sample obstructs tip retraction. When the tip overcomes the unbinding force, the cantilever is further retracted from the surface; only the large-distance tip-sample forces are experienced until the default starting separation is reached.

3. Application in food microorganisms

To improve the surface analyses by AFM within food microorganisms, more insight is needed into the application of different types of food microbes: food borne pathogens, spoilage microorganisms, and beneficial bacteria. AFM can be used to quantify the dimension alterations and surface features of food borne pathogens and spoilage microbes to elucidate bactericidal mechanisms and cellular responses under adverse environments. AFM analyses of the adhesion capacity of food borne pathogens, such as *Listeria monocytogenes*, *Salmonella enterica*, and *Bacillus subtilis* (Deza, Araujo, & Garrido, 2005; Jonas et al., 2007; Park, Haines, & Abu-Lail, 2009; Pesce et al., 2014), contribute to the biocontrol of biofilm in food-processing surfaces, the elimination of disease transmission, and the prolongation of product shelf life. In addition to pathogen and spoilage microbes, probiotic bacteria, such as *Lactobacillus* sp. are being increasingly exploited by AFM to understand their adhesive interactions toward inert and living surfaces. AFM applications have been increasingly applied for the multiparametric analysis of microorganism cell surfaces, offering novel insights into their structure-function relationships.

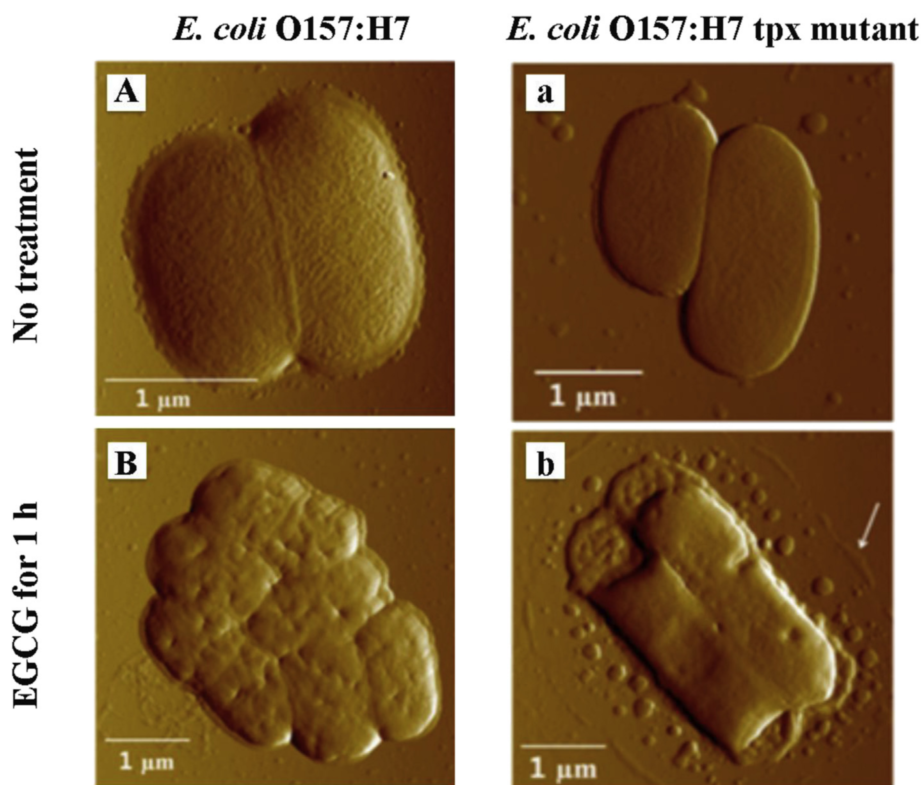

Fig. 2. Topological images of *E. coli* O157:H7 (A, B) and *E. coli* O157:H7 *tpx* mutant (a, b) treated with epigallocatechin-3-gallate (EGCG) (60 mg/L). The mutant is more susceptible to EGCG than the wild-type (Cui et al., 2012).

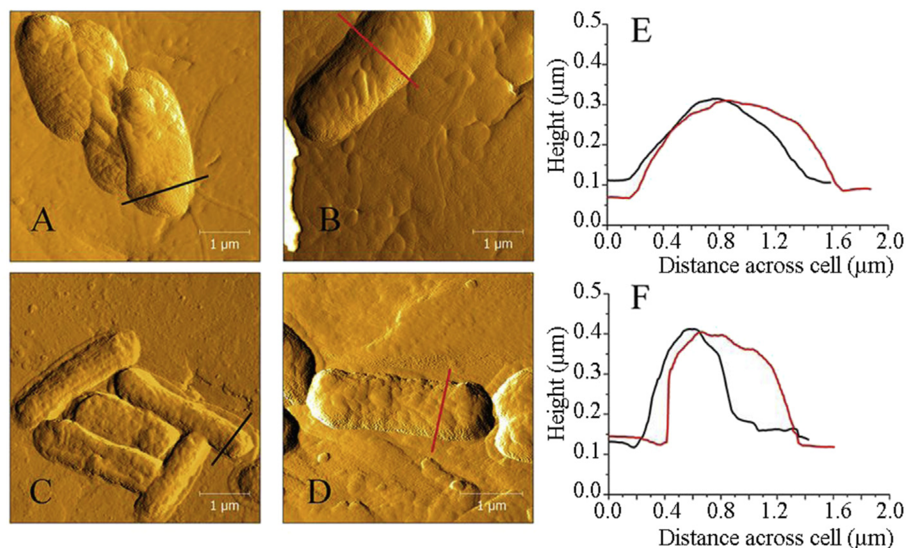

Fig. 3. AFM topographical images of untreated and treated *E. coli* by low concentration electrolysed water (EW; free available chlorine 4 mg/L) for 5 min: untreated (A–B), EW treated (C–D). The profiles of the black and red lines were shown in the figures: untreated (E), EW treated (F) (Liu et al., 2017b). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.1. Single cell imaging

Over the past decade, rapid progress has been made in applying AFM imaging to single microorganism cells, thereby complementing information obtained using light microscopy and electron microscopy. Conventional microbiological investigations provide information on large numbers of cells. By contrast, single-cell microscopy provides new opportunities to analyse a dynamic biosystem whose surface constituents unremittingly interact with the environment, enabling investigators to determine cellular interactions and interaction features in a way that was not possible before.

AFM has been used widely to evaluate the morphological effects of treatments on food microorganisms. Cui et al. (2012) compared morphological alterations of *Escherichia coli* and *Staphylococcus aureus* induced by a component of tea catechins (Epigallocatechin-3-gallate (EGCG)) at sub-minimum inhibitory concentrations (Fig. 2). AFM imaging revealed that EGCG initially induced aggregates in the cell envelopes of *S. aureus* and ultimately induced cell lysis. It also caused microscale grooves in the cell envelope and nanoscale perforation of *E. coli* O157:H7. An *E. coli* O157:H7 *tpx* mutant, with a defect in thioredoxin-dependent thiol peroxidase (Tpx), showed more severely damaged by EGCG when compared with its wild type. Rougher surfaces and cell debris were observed using AFM after the treatments. In the presence of peroxidase (10 units/mL), EGCG could not cause damage to

the membrane of the mutant cells, suggesting that the oxidative stress exerted by EGCG was relieved by peroxidase (see Fig. 2).

AFM is widely used to examine the microorganism cells qualitatively and quantitatively after treatment to reveal the bactericidal activities of various sanitisers (Yang & Wang, 2008; Zhang & Yang, 2017; Zhao, Zhang, & Yang, 2017) as well as adverse environmental conditions (Kuda, Shibata, Takahashi, & Kimura, 2015). Liu et al. (2017b) revealed that oxidative stress altered the morphological structure of *E. coli* and caused the intracellular material effusing from the cell. Quantitative morphological changes including height, width, and root-mean-square roughness (RMS) also demonstrated that oxidative stress produced by electrolysed water caused morphological changes in *E. coli* (Fig. 3). Hyldgaard, Sutherland, Sundh, Mygind, and Meyer (2012) explored the antimicrobial effect of monocaprylate on the cell structure of *Staphylococcus xylosus* and *Zygosaccharomyces bailii*. Treatment with monocaprylate at two times the minimal inhibitory concentration resulted in the appearance of indentations in *S. xylosus* and a rougher surface of *Z. bailii.* Bhat et al. (2015) examined the toxicity and adaptation mechanisms of *E. coli* exposed to xenobiotic chemicals. AFM was used to characterise the cell surface of *E. coli* remodelled after 2,4-dichlorophenoxyacetic acid (2,4-D) exposure. The roughness of the cell surface was calculated for all cells. The surface features of the control cells were identical and well ordered. In the treated cells, surface roughness increased as a function of the 2,4-D level, along with irregularity. When exposed to concentrations greater than 0.0021 mM 2,4-D, no regular surface subunit features were observed. More importantly, multiparametric AFM enables us to observe the surface structure properties, such as peptidoglycan, with high resolution and sensitivity (Dover, Bitler, Shimoni, Trieu-Cuot, & Shai, 2015). The studies mentioned above are summarised in Table 1.

AFM is therefore a promising approach to investigate dynamic topographical changes after different treatments. In the future, nanoscale analyses by AFM could have a great impact on food sanitiser development, because they may help identify the mode of bactericidal action of novel antimicrobial agents.

3.2. Biofilm and adhesion on food contact surfaces

AFM is also widely used to observe the formation and adhesion of biofilms. The adhesion of bacteria to solid surfaces and the formation of biofilms are important concerns in the food industry. The adhesion process of bacteria to surfaces has been measured previously. However, experimental data still have not provided a full comprehension for presenting the formation and adhesion of biofilm because of discrepancies from theoretical models. Many recent studies have shown that AFM is a useful tool to characterise the adhesion of microorganisms to food contact surfaces. Biofilm is a general term referring to complex clusters of microorganism cells and extracellular substances in association with the microorganisms that attached to the surface of a solid material. Biofilms may be detrimental to the environment. Biofilm formation on food surfaces or food-contact surfaces can lead to food spoilage, economical losses, and transmission of diseases because of food deterioration. Food-contact surfaces can harbour a variety of microorganisms, including food-borne pathogens, because food preparation surfaces are usually covered with organic matter, which serve as nutrients for microorganisms. In general, extracellular polymeric substances (EPS) excreted by bacteria promote cell aggregation and contribute to their adhesion to contact surfaces. Cells also communicate between themselves to initiate antibiotic biosynthesis, and extracellular enzyme biosynthesis, which result in increased resistance to disinfection treatments that can lead to substantial health and economic concerns (Luppens, Reij, van der Heijden, Rombouts, & Abee, 2002).

AFM studies on biofilms usually focused on acquiring topographical biofilm and extracellular material features present on the surface. However, because of the difficulties associated with working with biofilms, particularly their surface gelatinous and soft nature within a

Table 1
AFM imaging of the surface structure of living cells.

Food microorganism	Operation mode	Immobilization procedure and imaging environment	Observations	Reference
Staphylococcus aureus	Tapping mode	Adsorption on glass cover slip, air	Perforation and grooves on surface; cell lysis; cell debris	(Cui et al., 2012)
Escherichia coli	Tapping mode	Adsorption on stainless steel coupon, air	Dimension characteristic parameters	(Zhang & Yang, 2017)
Escherichia coli	Non-contact mode	Adsorption mica sheet, air	Different dimension characteristic parameters between the strains	(Yang & Wang, 2008)
Pichia pastoris	Tapping mode	Adsorption on stainless steel coupon, air	Destruction of protective barriers; leakage of intracellular contents	(Zhao et al., 2017)
Aureobasidium pullulans	Tapping mode	Adsorption on stainless steel coupon, air	Disordered conidia structure; wrinkled surface with breaches	(Zhao et al., 2017)
Salmonella Typhimurium	Dynamic force mode	Adsorption on a cover slip, air	Flattened morphology by desiccation	(Kuda et al., 2015)
Escherichia coli	Tapping mode	Adsorption on mica sheet, air	Dimension characteristic parameters; leakage of intracellular contents	(Liu et al., 2017a)
Staphylococcus xylosus	Intermittent contact mode	Adhesion to cell-tak-coated coverslips, air	Surface indentations	(Hyldgaard et al., 2012)
Zygosaccharomyces bailii	Intermittent contact mode	Adhesion to cell-tak-coated coverslips, air	Surface indentations; imaging artifacts (rough surface)	(Hyldgaard et al., 2012)
Escherichia coli	Contact mode with silicon nitride (Si_3N_4) cantilevers	Chemical fixation on coverslips, air	Variable roughness; dimension characteristic parameters	(Bhat et al., 2015)
Streptococcus agalactiae	Tapping mode	Chemical fixation on mica sheet, air; Attached to a metal disc using adhesive tab, liquid	Peptidoglycan architecture; cell-wall elasticity	(Dover et al., 2015)

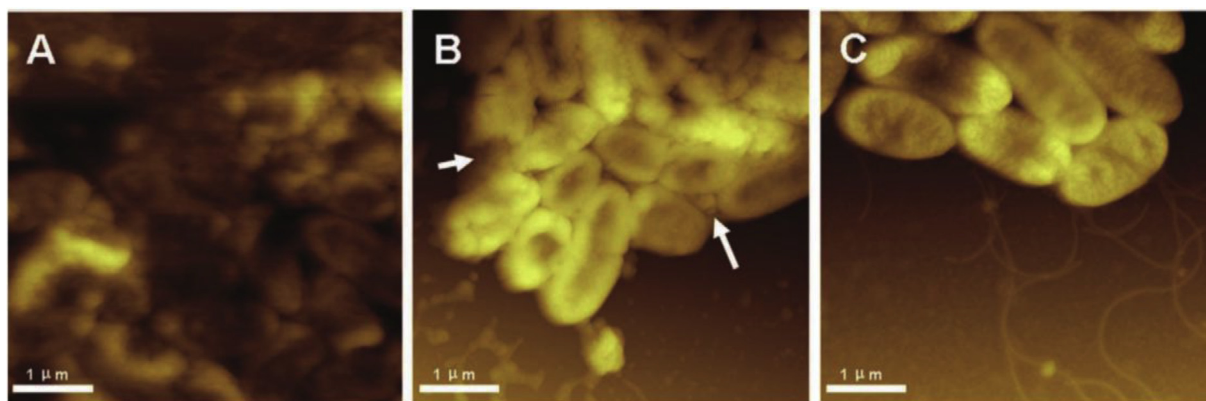

Fig. 4. AFM images of biofilms formed by *Pseudomonas aeruginosa* PAO1 strain (A), PAO1 ΔpilA (B) and PAO1ΔpilAΔpelAΔpslBCD strains (C) after static growth for 24 h. There is a large amount of matrix-like materials among the cells in the biofilms formed by PAO1 and the PAO1ΔpilA mutant, while this is not the case for the PAO1ΔpilAΔpelAΔpslBCD mutant (Yang et al., 2011).

liquid environment, most biofilms have been dried first before AFM scanning and examined in an air-environment to obtain high-resolution images.

Yang et al. (2011) used high-resolution AFM to study how different EPS materials affect structural differentiation during biofilm development of *Pseudomonas aeruginosa*. *P. aeruginosa* has a high tendency to form biofilms on food equipment surfaces and biofilm development is very rapid. When in the biofilm state, *P. aeruginosa* cells are more resistant to stress conditions, such as heat or sanitiser, than their planktonic counterparts (Deza et al., 2005). AFM provided detailed surface propertied of biofilms formed by *P. aeruginosa* wild-type, ΔpilA (pili-defective), and ΔpelAΔpslBCD mutants (defective for Pel and Psl polysaccharide biogenesis). The study provided direct evidence that both Psl and Pel polysaccharides are required for type IV pilus-independent cell aggregation in the early stages of biofilm formation by *P. aeruginosa* PAO1 (Fig. 4). Jonas et al. (2007) investigated the roles of cellulose, curli, and surface proteins in the topography of bacteria grown within biofilms on mica slides. The results showed that AFM could be used as an efficient technique to determine the topographical changes of microbes grown within biofilms formed in a liquid. The wild-type biofilm and those developed by the curli, cellulose, and BapA mutants were compared to better elaborate how BapA, cellulose, and curli lead to biofilm formation on submerged mica slides (Fig. 5). AFM revealed that the global regulator CsgD mutant and curli mutant were both severely impaired in biofilm formation. The cellulose mutant (*bcsA*) preserved some capability for cell aggregation and wild-type levels of extracellular material could be observed. By contrast, the surface protein mutant (*bapA*) developed a thick dense biofilm within 24 h and secreted an extensive extracellular matrix of the same size as the wild type. Overproducing of CsgD displayed more fibril structures at the edges of the cells than in the wild-type and induced a denser and thicker biofilm compared with the wild-type. This provided direct evidence that cellulose and curli, but not surface protein BapA, have a great impact on the morphology and formation of a biofilm. In addition, curli appear to be more significant for the development of biofilms than cellulose.

3.3. Surface structure visualisation

The unique ability of AFM to image the subcellular specimens at the nanoscale has been intensively exploited to reveal the structure of the cell surface layer (S-layer). Studies on the S-layer have provided powerful insights into the functionality of the cellular surface and the life of microorganisms. A survey of reports on cell surface layers using AFM is given in Table 2.

Surface protein layers are common constituents of microorganism cell protective barriers, which are thought to play pivotal roles in bacterial biology. Surface layers are commonly monomolecular arrays or double layers of single proteins, glycoproteins (MW 40 to 200 kDa), or more complex lattices (Pum & Sleytr, 2014). Many isolated surface layer proteins can reassemble into stable crystalline structures on a solid surface; therefore, they are particularly well suited for AFM analysis. Surface layer protein A (SlpA) of *Propionibacterium freudenreichii* was extracted and recrystallised spontaneously to investigate the assembly and organization of proteins using AFM (de sa Peixoto et al., 2014). SlpA was found to maintain the monolayer hexagonal lattice architecture, but the structure was softened upon acidification or mild heating. S-layer proteins derived from many *Bacillus* species revealed the capability to reassemble the lattice structure at solid surfaces, whereby the size of the coherent lattice array is only dependent on the size of the solid substrate (Györvary, Stein, Pum, & Sleytr, 2003; Pleschberger et al., 2003; Toca-Herrera et al., 2005). Glycoprotein subunits are also highly porous protein mesh with a unit cell size in the range of 3–30 nm. The oblique lattice symmetry with identical orientations of *Geobacillus stearothermophilus* was visualised on gold sensor surfaces (Schuster & Sleytr, 2015).

The porin protein from *E. coli* is another well-studied example. Gram-negative bacteria are protected by an outer barrier in which the porins facilitate the passage of small solutes. Intact porins from the outer membrane maintain the fluxes and permeability of live cells. Large aggregates induced by adverse environment or biocidal action can obscure surface porins, causing disruption of the outer membrane (Edens, Wang, Whitten, & Keller, 2014).

3.4. Biomolecular interaction investigation

In addition to morphological observation, AFM can also be exploited to characterise the interaction between nanometre scale biological objects, macromolecular assemblies, and the dynamic behaviour of biological systems. The interaction force between the AFM tip and a sample can be determined by moving the tip perpendicular to the surface while recording the force on the tip in force spectroscopy mode. The detection of cellular interaction or molecular bonds is straightforward: the force probe, attached to microbial cells, or functionalised with a biomolecule, is brought to a substrate that is densely packed with the counter-molecule. The force-distance approach is a valuable tool to image the structure while simultaneously mapping the biophysical features of the samples at the nanoscale and enables researchers to correlate structural images of complex biological systems with quantitative maps of their biophysical properties. There has been considerable recent research progress in the biological application using force spectroscopy to quantify either continuous or discrete cellular interactions of food microorganisms.

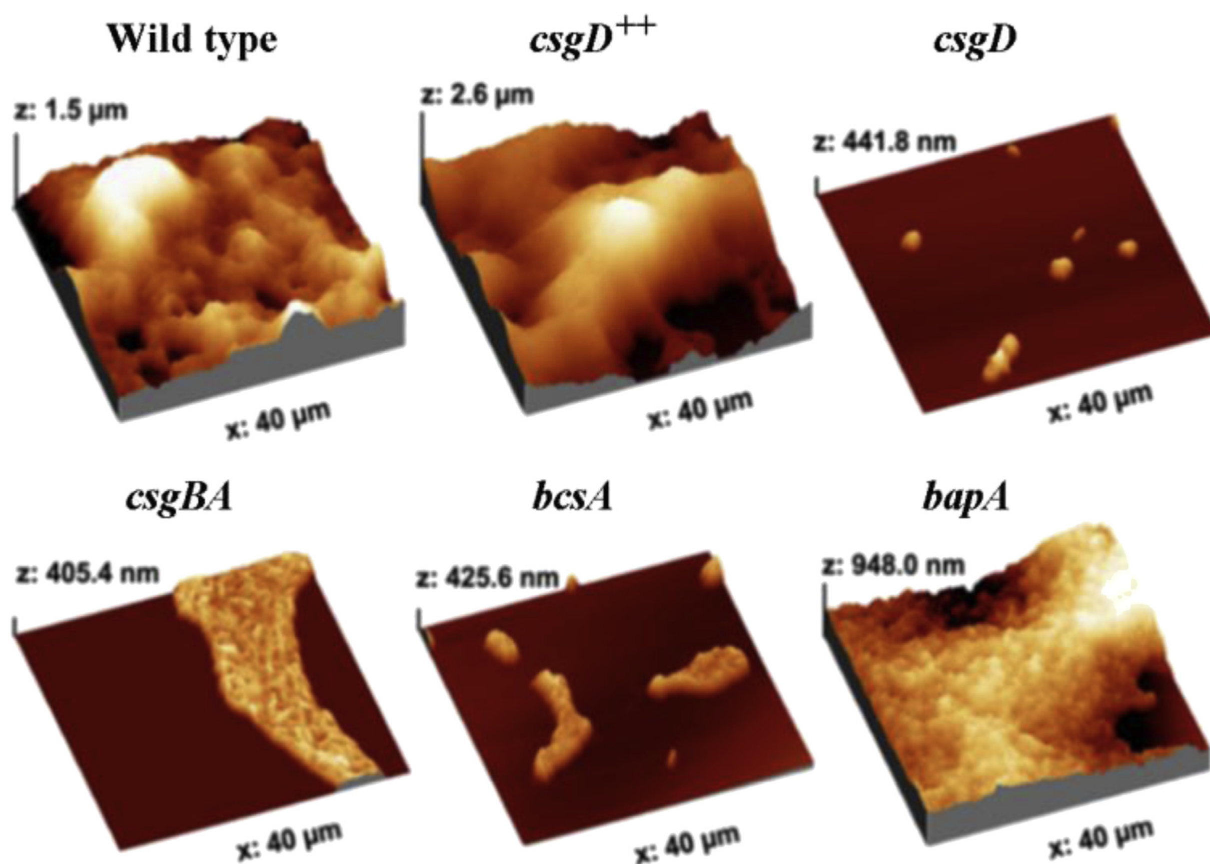

Fig. 5. 3D AFM images showing the topography of *Salmonella* biofilms formed by UMR1 (wild-type), MAE52 (*csgD*$^{++}$), MAE51 (*csgD*), MAE14 (*csgBA*), MAE222 (*bcsA*) and MAE619 (*bapA*) after growth for 24 h on mica surfaces (Jonas et al., 2007).

3.4.1. Adhesion forces

The process of surface adhesion of bacteria to a solid surface is a crucial step in the formation of a biofilm. AFM is a powerful tool to investigate the mechanical and nanometric physicochemical properties of cell surfaces, including the quantitative determination of the periphery of the cell-substratum contacting the surface, the cell-cell interface, and the adhesion forces on a single bacterium cell surface. For example, Fang, Chan, and Xu (2000) quantified the AFM cellular interaction of a sulphate-reducing bacteria surface over the various sections and various interfacial regions of a biofilm. The surface elasticity was quantified from the slope of the repulsive section of the force–-distance curve (Fig. 6). The cell-cell and cell-substratum periphery forces were considerably higher than the force between the tip and cell surface, which could be contributed by the accumulation EPS. The elasticity information may help to better understand the complex matter of biofilm formation.

The force-distance curves can record the variations of interaction forces and provide valuable information on cellular interaction, which is sensitive to the biochemical nature of the cell surface (van der Aa & Dufrêne, 2002). Zeng et al. (2015) exploited the effect of functional amyloid on the stiffness and robustness of the amyloid-producing *Pseudomonas* sp. UK4. The study observed that both the wild-type and amyloid mutant *fap* cells showed compromised surface morphology after desiccation in air, while amyloid fibrils overexpressing pFAp cells did not collapse and were able to maintain the rod-like shapes, indicating that amyloid rendered cells more resistant to desiccation. To better understand the adhesion forces pattern conferred by amyloid on the surface of *Pseudomonas* sp. UK4, a colloid AFM probe was used after UV-oxidation or silanisation to make it hydrophilic or hydrophobic. The hydrophilic or hydrophobic probe first approached the cells and was then retracted while the cantilever's deflections and path were recorded. The retraction force curves for both the wild-type and amyloid fibrils overexpressing pFAp strain directly provided the maximal adhesion force. The AFM force curves demonstrated that amyloid made *Pseudomonas* more hydrophobic and increased biofilm stiffness 20-fold.

The adhesion ability of lactic acid bacteria to human gastro-intestinal epithelium cells is one of the most crucial factors determining the probiotic ability of a strain. This ability is considered a prerequisite for enteropathogenic bacteria exclusion and immunomodulation of the host (Bernet, Brassart, Neeser, & Servin, 1993). Adhesion force analysis is an important and widely used tool to select adhesive probiotic strains, because it provides information on the binding ability of probiotic bacteria to intestinal cells at the molecular level, as well as on sample elasticity. Schaer-Zammaretti and Ubbink (2003) exploited the possibility to use AFM to differentiate various *Lactobacillus* sp. on the basis of their elasticity, interactions, and surface morphologies. Elasticity and adhesion force maps of different bacterial strains were calculated from force volume images, which showed that *L. crispatus* and *L. helveticus* had a surface with a homogeneous stiffness, with no adhesion events. It is inferred that the absence of adhesion was caused by the S-layer, which completely covered the surface of the bacteria. However, the polysaccharide monomer-coated surface was detected to have relatively strong adhesion forces of 0.1–0.3 nN.

3.4.2. Specific interactions during microbial cell aggregation

Currently, AFM is frequently applied to determine interaction forces between biological molecules. Microbial aggregation is one of the most crucial cellular events in biotechnological processes and the natural environment. Touhami, Hoffmann, Vasella, Denis, and Dufrêne (2003) used AFM to determine individual discrete lectin-carbohydrate interactions involved in the reversible flocculation of *Saccharomyces carlsbergensis* cultures, an aggregation event of significant importance in the

Table 2
Imaging the structure of cell surface layers.

Microorganism	Operation mode	Object	Immobilization procedure and imaging environment	Observations	Reference
Propionibacterium freudenreichii	AC mode (dynamic mode)	Protein	Recrystallisation on fresh mica, under liquid	Hexagonal lattice; thickness value around 10 nm; monolayer organization	(de sa Peixoto et al., 2014)
Bacillus sphaericus	Contact mode	Protein	Recrystallisation on hydrophobic/hydrophilic silicon, under liquid	Square lattice; monolayer (9 nm in height) at the hydrophobic silicon; bilayer (15 nm in height) at the hydrophilic silicon	(Györvary et al., 2003)
Bacillus sphaericus	Contact mode	S-layer	Recrystallisation on Flat Silicon Polyelectrolyte Multilayers, under liquid	Square lattice (a = b = 13 nm, and γ = 90°)	(Toca-Herrera et al., 2005)
Bacillus sphaericus	Contact mode	S-layer protein	Recrystallisation on silicon, under liquid	Square lattice structure	(Pleschberger et al., 2003)
Geobacillus stearothermophilus	Contact mode	Glycoprotein	Recrystallisation on gold-coated surface, under liquid	Oblique lattice	(Schuster & Sleytr, 2015)
Escherichia coli	Tapping mode	Porin protein	Adsorption on glass, air; Adhesion to Cell-Tak-coated coverslips, under liquid	Circular structure	(Edens et al., 2014)

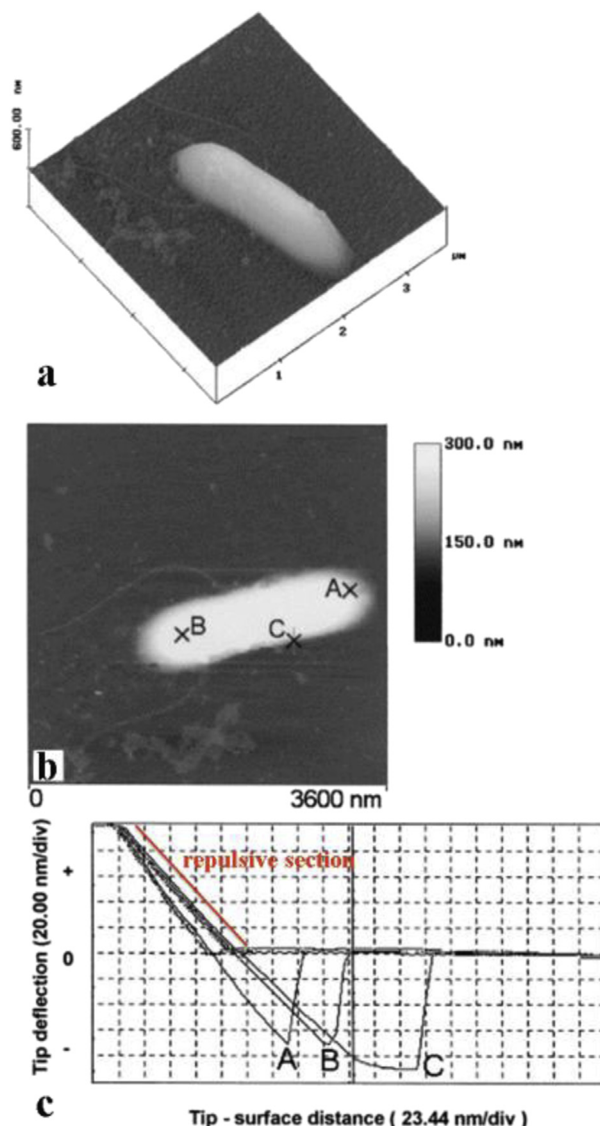

Fig. 6. AFM images of sulphate-reducing bacteria in (a) 3D display, (b) 2D display, (c) the corresponding force curves at locations A, B, and C (Fang et al., 2000).

food fermentation process, such as wine making and brewing. Biochemical investigations have illustrated the specific interactions between mannose and lectins on the cell surface, which were observed directly using AFM measurement. Biofunctionalised probes with oligoglucose carbohydrates and lectin concanavalin A (Con A) were applied to generate the force-distance curves on yeast cells. In the flocculation process, adhesion forces of 117 ± 41 and 121 ± 53 pN were measured with Con A- and oligoglucose carbohydrate-terminated probes, respectively, illustrating the specific interaction with individual cell-surface mannose and lectins residues. Furthermore, these adhesion forces were not detected in non-flocculating yeast cells.

The functionalization strategy of probes combined with AFM imaging has great potential in cell biology and food microbiology to interpret the specific interaction at various locations and to map the distribution of specific sites at cell surfaces. AFM has evolved into a multifunctional tool, which, together with biofunctionalised probes, makes it possible to characterise microbial cells and cell-wall materials. This approach also allows the development of new applications for a better understanding of biochemical and physical interactions, such as molecular-recognition (Dufrêne, 2004), electrostatic (Dufrêne, 2000), and specific intermolecular forces of biointerfaces (Alsteens et al.,

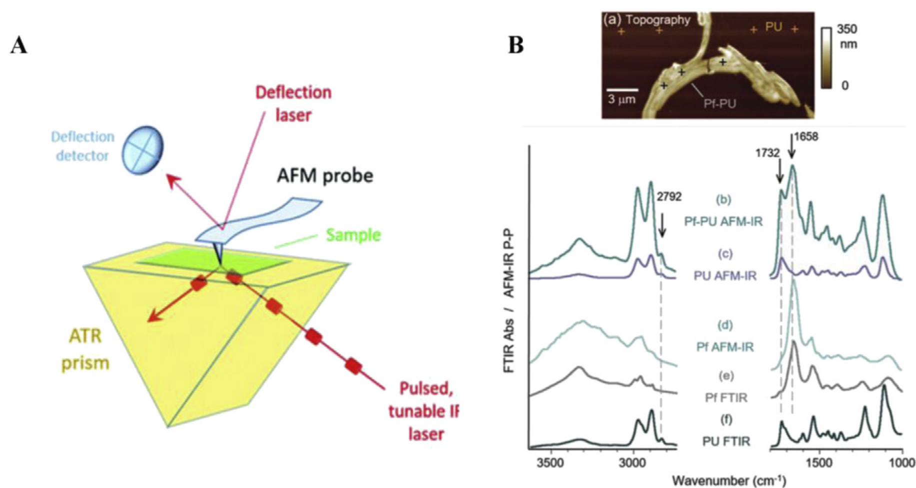

Fig. 7. Diagram of AFM-IR working principle (A) and an AFM-IR topographic image of a *P. protegens* monolayer cluster on 300 nm polyether-poly-urethane/ZnSe (B). The "+" marks identify polyether-poly-urethane and *P. protegens* – polyether-poly-urethane regions where the spectra were collected (Barlow et al., 2016).

2017).

3.4.3. Antibody-antigen interactions

Recent advances in the development of nanomaterial have stimulated research interest in their application for bioanalysis of food pathogen detection. Antibody-antigen adsorption is one of the strategies for specific recognition between target bacterial cells and biofunctionalised materials. Development of sensitive biosensors to detect food-borne pathogens is one of the major prospects for nanotechnology application in food safety. The investigation of antibody and antigen adsorption on surfaces in under biological conditions is a fundamental and critical procedure to improve the specificity and sensitivity of a biosensor. AFM has been used frequently to investigate the interaction between biological objects at the nanometre scale and to investigate the dynamic behaviour in a biological environment (Chen et al., 2005).

The investigation of protein A, a cell wall protein, and antibody adsorption on a surface is an essential and important step to biosensor application. AFM can be used to measure biomolecular interactions between antibodies and protein A. The protein layer features and properties of a biosensor system can be instructed through AFM high-resolution imaging (Kim et al., 2004). Lee, Pillai, Singh, and Willing (2008) used AFM to investigate the attachment of protein A and its subsequent binding to a *Salmonella* antibody on gold surface, utilizing a combination of contact and tapping mode. The layer step height was determined based on the concept that small areas of the protein A layer could be moved by exerting a high force between the sensor surface and a contact mode tip that can be controlled through AFM. Changing the exerted force to known surface sections and determining the difference of step height between the exerted force sections and the surrounding sections allowed the researchers to examine not only the presence of a protein layer, but also the thickness and attachment quality to a gold quartz crystal surface. They found that the average step height alteration between the solid gold surface and the protein A layer was 3.0 ± 1.0 nm, while the average step height alteration between the solid gold surface and protein A with the attached antibody was 6.0 ± 1.0 nm, indicating the single monolayer structure of the protein A and antibody adsorption.

Dammer et al. (1996) investigated the specific interaction between biotinylated bovine serum albumin (BBSA) and immunopurified polyclonal goat IgG antibodies (anti-biotin antibodies). The rupture forces between individual antibody-antigen complexes were measured directly by AFM, and it was suggested that only very few antigen/antibodies complexes contributed to the determined force.

3.5. Combined use of AFM with other techniques

AFM has become an invaluable tool to explore the morphology of the micro- and nanoworlds with many advantages. However, the AFM technique as a stand-alone tool has its own limitations. Combining AFM with other complementary techniques to establish hybrid instruments is a promising way to obtain more comprehensive information in one experiment.

3.5.1. Combining AFM with infrared (IR) spectroscopy

Several AFM-based vibrational spectroscopies have been established with spatial resolutions that can be used for chemical identification and nanoscale quantitative analysis below the diffraction limit of light. AFM-IR is a combined method that shows promise to characterise the nanoscale chemical features of biological-materials, and is capable of producing sub-micrometer spatial resolution IR spectra as well as absorption images. This new method enables chemical mapping at levels not previously possible. It is based on detecting the thermal expansion of the infrared-pulsed region of the sample.

In AFM-IR, an IR transparent prism (e.g. ZnSe) is used as sample substrate. The sample is irradiated with internal reflection illumination and the absorbed light induces a photothermal response when the IR laser is tuned to the wavelength corresponding to the absorption of the sample. The absorption is converted into heat, which causes in rapid thermal expansion pulse, which is then detected with the tip of the AFM. Thus, the AFM cantilever is used to detect the rapid thermal expansion, which is caused by absorption of the tuned pulses of IR radiation at a given wavelength (Dazzi et al., 2012). Using an AFM tip to sense and map the thermal expansion pulse is the key for measuring IR absorption below the conventional diffraction limit. Due to the rapid expansion of biological material, the AFM cantilever will 'ring' at the resonance characteristic frequencies of the cantilever (Fig. 7). AFM-IR performs well for samples with high expansion coefficients and low thermal conductivities, and for micro- and nanoscale chemical analysis of microorganisms. Vitry et al. (2016) presented a study using AFM-IR to detect triglyceride vesicles in *Streptomyces* bacteria, an important microorganism producing bioactive secondary metabolites, such as antifungals, antivirals, and especially, antibiotics. The AFM-IR microscope allowed the localization of triglyceride vesicles in the bacteria by their specific IR absorption at 1740 cm^{-1}. Barlow et al. (2016) assessed features of AFM-IR analysis on monolayer cluster *P. protegens* Pf-5 bacteria, a known polyurethane degrader, cast on a 300 nm thick polyether-poly-urethane (PU) film (resistant to bacteria degradation). Good agreement was found between the AFM-IR and Fourier transform infrared (FTIR) spectra. Local AFM-IR spectra of *P. protegens* on PU

exhibited bands from both constituents, indicating that AFM-IR is highly sensitive to chemical components at or below the bacteria surface (Fig. 7). Using this approach, it is possible to resolve the chemical difference on the scale as low as 100 to 200 nm, which enables the information of specific marker bands and characteristic frequency ranges that could not have been gained with conventional infrared microspectroscopy.

3.5.2. Combining AFM with raman spectroscopy

Raman, a vibrational spectroscopy, is a label-free chemical characterisation technique (Kurouski, 2017). The Raman signal can be enhanced by the coherent oscillation of conduction band electrons in metal nanostructures, which are strongly dependent on nanoparticle shape, size, and material (Jia, Adam, Marks, & Ionescu, 2015; Kleinman, Frontiera, Henry, Dieringer, & Van Duyne, 2013; Rippa et al., 2017; Wang et al., 2017). Such nanoparticles can be lithographed or electrochemically etched (deposition of Ag on silicon cantilevers) at the apex of an AFM scanning probe. The major advantages of this method, known as tip-enhanced Raman spectroscopy (TERS), are that the metallic tip acts as an antenna that improves the electrical field and can be precisely curbed over the substrate's surface (Verma, Ichimura, Yano, Saito, & Kawata, 2010). The illumination of the rough metallic surface with electromagnetic radiation originates surface plasmons, which lead to a manipulated local electromagnetic field and Raman signals from molecules over a very narrow area (Stadler, Schmid, & Zenobi, 2010). Therefore, combining AFM with TERS enables the chemical fingerprint recognition provided by the surface-enhanced Raman spectroscopy with a spatial resolution down to a few tens of nanometres.

Recently, the simultaneous recording of both morphological and chemical fingerprints produced by the AFM and TERS approach has allowed a deeper insight into the surface structures of many systems at the micro- and nanoscale (Liu et al., 2012; Rusciano, Zito, Pesce, & Sasso, 2017), including microbiology systems. Rusciano et al. (2014) applied TERS-based images correlated with AFM topographical maps for surface heterogeneity analysis of *Bacillus subtilis* spores, which is of concern to the food industry because of their ability to survive under adverse environmental conditions during processing (Murashita, Kawamura, & Koseki, 2016; Setlow & Johnson, 2013). Understanding the physicochemical nature of spore surfaces could lead to the more appropriate techniques for food decontamination (Pesce et al., 2014). Micro- and nanoscale AFM-phase maps have been produced for several spore strains (Janganan et al., 2016; Plomp et al., 2007). However, this was the first experimental application of tip-enhanced Raman spectroscopy on *B. subtilis* spores. The acquired spectra reflected the heterogeneous and complex chemical environment explored by the plasmonic tip along a row of a 120 nm raster scan (Fig. 8A), which exhibited marked point-to-point variation at the nanoscale. By mapping the oscillating cantilever, AFM imaging simultaneously detected spatial variations in surface properties, such as friction, viscoelasticity, and adhesion, thereby differentiating positions of high and low surface hardness or adhesion (Fig. 8B), providing particular insights on the surface ridge region. Neugebauer et al. (2006) reported topographic information of *Staphylococcus epidermidis* cells with significant locations of the corresponding TERS measurement. These studies represent advanced but crucial steps toward correlative surface distribution of chemicals of bacteria via nanoscale spectroscopy, which could be an attractive approach to display surface-chemistry characterisation of a bacterial system. The combined use of AFM and TERS analysis has focused on the investigation of bacteria cell-wall systems (Budich, Neugebauer, Popp, & Deckert, 2008), as well as DNA and RNA systems (Neugebauer et al., 2007).

4. Conclusions and future trends

Characterising the surface properties of food microorganisms is of great importance to efficiently exploit them in biotechnology. This review discussed recent progress in the use of AFM for food microbiology.

AFM is more than a surface analysis tool, because force measurements can be used to quantify the physical properties of the surface specimen, such as adhesion behaviour, molecular interactions, and mechanical properties, complementing other physiochemical and biochemical methods. Through tip functionalization with biomolecules, it is possible to provide further details on the various specific interactions and biomolecular recognitions. According to the present review, it is noticeable that AFM coupled with other complementary tools has great potential and may not only meet the needs for morphological and interaction studies for food microorganisms, but also offers new insights into nanoscale monitoring of chemical reactions that have not been investigated before. Combining AFM with other complementary tools could extend the probing analysis of biomolecular processes at the subcellular level, allowing significant leverage of existing techniques.

Future studies should focus on refinements in the use of AFM with other techniques, including spectroscopy and other microscopy techniques, and the development of suitable functionalised tips for desired specific interaction analyses. As such, the broad utility of AFM is expected to make it a unique and useful tool to achieve unique insights into the structure and function of microbial cell surfaces.

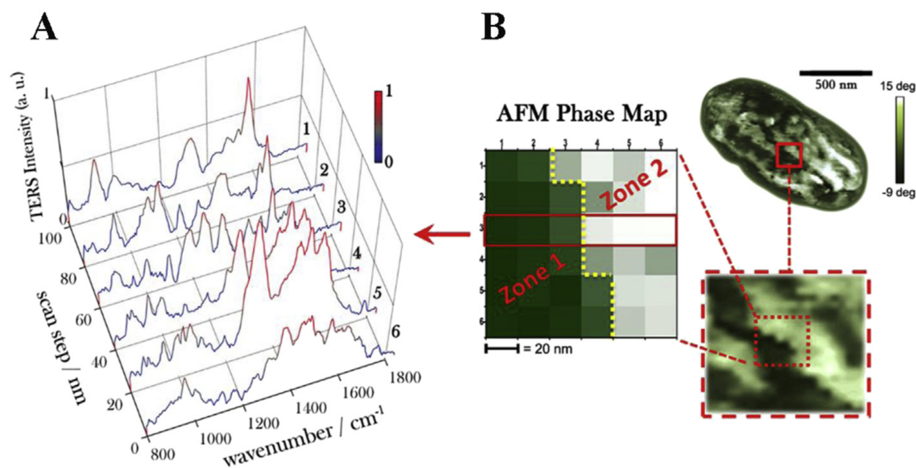

Fig. 8. (A) Tip-enhanced Raman spectroscopy spectra acquired with a 20 nm step along the horizontal line; (B) Phase map of AFM and detailed zoom of a selected spore region across a spore ridge (Rusciano et al., 2014).

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