Effects of electrolysed water combined with ultrasound on inactivation kinetics and metabolite profiles of *Escherichia coli* biofilms on food contact surface

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**ABSTRACT**

This study aimed to better understand the overall metabolic responses of *Escherichia coli* biofilms to the combined stresses of ultrasound and low concentration acidic electrolysed water (LcAEW, free available chlorine: 4 mg/L). The inactivation kinetics of all *E. coli* strains (ATCC 25922, ATCC 35150 and ATCC 43895) were simulated well by the modified Weibull model ($R^2$: 0.81–0.97; RMSE: 0.04–0.71). By analysing metabolite profiles, ultrasound mainly disrupted nucleotide metabolism in *E. coli* cells within biofilms, as most intracellular nucleotide-related compounds (e.g., uridine, ATP, ADP) showed decreased trend especially in ATCC 25922 and ATCC 35150. Increased contents of most amino acids and decreased contents of most carbohydrates were shown in all strains after LcAEW treatment. Under combined treatment, adaptive strategies like glutamate decarboxylase system and mixed acid fermentation were activated to different extents among the three strains. These findings revealed that NMR-based metabolomics technology is promising in identifying strain-specific metabolic responses of biofilms to different antimicrobial treatments, providing guidance for future mechanism studies related to food contact surface sanitisation.

1. Introduction

Biofilms can be defined as aggregates of microbial cells embedded within a self-secreted matrix called extracellular polymeric substances (EPS) (Cui, Li, Abdel-Samie, Surendhiran, & Lin, 2021). In the food industry, once foodborne pathogens attach to food contact surfaces (e.g., work benches, storage tanks, kitchen wares), they are very likely to form biofilms as their survival strategy, leading to subsequent food spoilage and even foodborne diseases (Tan & Karwe, 2021; Yemmireddy & Hung, 2015). Considering an estimated 65–80% of human microbial infections are caused by biofilms (Jamal et al., 2018), finding an effective biofilm removal strategy is of great significance in food contact surface sanitisation.

Many chemical sanitisers are applied in the food industry for sanitising food contact surfaces currently. However, fewer chemical sanitisers can be used for organic food production due to the strict rules (Adhikari, Syamaladevi, Killinger, & Sablani, 2015). For example, U.S. National Organic Program regulates that the residual chlorine levels should not exceed 4 mg/L when chlorine-based materials are used to sanitise organic food contact surfaces (NOP 5026, 2011). Compared to the conventional chemical reagents, low concentration acidic electrolysed water (LcAEW, free available chlorine (FAC): 4 mg/L), yielded from water and salt electrolysis, can meet organic operation standards due to its environmentally friendly nature and safe characteristic. As one of the emerging sterilisation agents in food industry in recent decades, LcAEW exhibited significant antimicrobial effect on planktonic cells in our previous study, but when used on biofilms, it might become less effective like other chemical sanitisers due to its limited penetration into deep layers of biofilm (Zhao, Zhang, & Yang, 2017; Zhao, Zhao, Phey, & Yang, 2019). Therefore, to achieve an effective detachment and inactivation of microbial biofilms from food contact surfaces, another method needs to be combined with LcAEW and ultrasound could be one choice.

Ultrasound-involved emerging strategies have gained increasing attention recently. As a strong physical processing method, ultrasound can destroy the structure of microbial communities effectively and remove the biofilms from food contact surfaces. However, the...
bactericidal effect of ultrasound alone is very limited, as the thick and soft capsule layer of bacteria can dampen the mechanical effects of ultrasound (Gao, Lewis, Ashokkumar, & Hemar, 2014). Fortunately, when combined with chemical disinfectants to treat biofilms, ultrasound can expose inner cells to the disinfectants and promote the permeability of chemical disinfectants into the biofilms by mechanical oscillation, helping chemical sanitisers to achieve an enhanced bactericidal effect on biofilms (Bang, Park, Rahman, & Ha, 2017). In previous studies, commonly used sanitisers like sodium hypochlorite, benzenalkonium chloride and quaternary ammonium were combined with ultrasound to treat biofilms formed on different food contact surfaces like stainless steel, polystyrene and polyvinyl chloride, causing 60–90% reduction of viable cells and achieving synergistic bactericidal efficacy than each used alone (Berrang, Frank, & Meinersmann, 2008; Lee, Kim, & Ha, 2014; Torlak & Sert, 2013). However, although the combination of electrolysed water and ultrasound has been widely used in food preservation as an effective sanitising method in recent years (Ding et al., 2015; Wu et al., 2018), there are few available information about their effects on the inactivation kinetics and metabolic changes of biofilms on food contact surface. A variety of mathematical models have been evaluated to describe the antibacterial effect over the treatment time, such as first-order kinetic model fitting linear reduction curve and Weibull, Baranyi, Gompertz and Huang models fitting nonlinear reduction curve (Baranyi & Roberts, 1995; Huang, 2009). However, the inactivation kinetics of ultrasound and LcAEW combination against biofilms on food contact surface has not been generally characterised by mathematical models. Moreover, beneath the reduction surface, the overall metabolic changes of biofilms after this combined treatment have not been investigated so far. Metabolomics, as supplementary to proteomics and genomics, can help people figure out how a series of actual physiological processes change in living organisms, considering it is able to focus on low-molecule-weight metabolites produced by the cells (Smart, Aggio, Van Houtte, & Villas-Boas, 2010). Although there are multiple studies on the comparison of metabolite profiles between biofilm and their planktonic counterparts during biofilm formation process (Hamilton et al., 2009; Lu, Que, Wu, Guan, & Guo, 2019), the metabolic changes within established biofilms during external sanitising treatments have not been fully understood, let alone the strain-specific biofilm metabolic responses to ultrasound and LcAEW. Therefore, in this study, the inactivation kinetics of three separate strains of Escherichia coli biofilms attached on stainless steel coupons were described quantitatively after ultrasound and LcAEW combined treatment. Moreover, the metabolic variations of each biofilm induced by the two stresses were also identified by using nuclear magnetic resonance (NMR) spectroscopy, providing more fundamental understanding of cellular behaviours to hostile environments. Based on the dynamics study and the metabolic profiling analysis, this study would expand our knowledge on the biofilm-specific response mechanisms and provide effective antibiofilm strategies to the organic food industry.

2. Materials and methods

2.1. Bacterial strain and culture conditions

Three *E. coli* strains (*E. coli* O157:H7 ATCC 35150, *E. coli* O157:H7 ATCC 43895 and their non-pathogenic counterpart, *E. coli* ATCC 25922), obtained from Department of Food Science & Technology, National University of Singapore, were used in this study. After taken out from −80 °C freezer, the lyophilised culture of each strain was transferred to 5 mL of sterile tryptic soy broth (TSB, Oxoid, UK) for an 18–24 h resuscitation in a 37 °C incubator with 100 rpm rotation, following a streak plating on tryptic soy agar (TSA, Oxoid, UK) to get a single colony. After an overnight incubation at 37 °C, the individual colony was sub-cultured in TSB again and the working suspension of each strain was obtained after two consecutive transfers.

2.2. Biofilm formation

Preparation of *E. coli* biofilms was conducted as described previously with some modifications (Ayebah, Hung, Kim, & Frank, 2006; Yang, 2012). The working suspension at a concentration of 107 CFU/mL in 5 mL TSB was centrifuged (8000 × g, 20 °C, 5 min), and the obtained pellets were added 5 mL 0.1% peptone water (PW) and centrifuged again. After this operation was repeated twice, the final bacteria cells were obtained and resuspended in 5 mL PW. One millilitre of the suspension was mixed well with 9 mL diluted TSB (dTSB, 1:10) in a petri dish, with sterilised stainless steel coupons (diameter: 1 cm; thickness: 0.7 mm; type: 430) immersed inside. The bacteria on the surface of coupons were cultured in an incubator at 25 °C for 4 h to allow adhesion, after which the suspensions were discarded and the coupons were gently rinsed in 10 mL PW to remove the loose cells. Then the coupons were transferred to 10 mL new dTSB for further biofilm growth at 25 °C. After a 48-h incubation, the spent medium was discarded, and the coupons were submerged in 10 mL fresh dTSB for another 24-h incubation to allow further biofilm growth. After above incubations, the initial biofilm populations of each strain on stainless steel coupons could reach ~7.0 log CFU/coupon, which were ready for following treatments.

2.3. Treatment of biofilms on the coupons

After the rinsed coupons were dried for 2 h in a laminar flow biosafety cabinet, 20 pieces of them were separately immersed in sterile 25 mL beakers containing 10 mL LcAEW (FAC: 4 mg/L), which was obtained by diluting anolyte from a 0.9% NaCl-electrolysis device (ROX-10WB, Hoshizaki Singapore Pte Ltd). The pH and oxidation reduction potential (ORP) values of used LcAEW were measured in a range of 4.0 ± 0.1 and 940.5 ± 10.0 mV, respectively based on previous methods (Chen, Chen, Chang, Huang, & Chen, 2021). A chlorine test kit (Merck, Darmstadt, Germany), pH meter (Thermo Orion pH meter, Waltham, USA) and ORP meter (HM Digital ORP-200, Culver City, USA) were used to test FAC, pH and ORP value, respectively. The same steps were applied for the control group of using 10 mL sterile deionised water (DW). Ultrasound alone or combined treatments were applied by putting the beakers (containing DW or LcAEW) in an ultrasonic cleaning unit (Elmasonic S 30 H, Siegen, Germany), with a settled frequency of 37 kHz and effective power of 80 W. After 0–5 min treatment, the collected coupons at different time points were immersed in 5 mL neutralising buffer (containing 5 g/L sodium thiosulfate) for 5-min neutralisation, to neutralise residual chlorine before microbial analysis (Luu, Chhetri, Janes, King, & Adhikari, 2021). Then the adherent cells on coupons were knocked down by 2 g glass beads (0.2 mm) on a vibrating vortex at maximum speed in 5 mL phosphate-buffered saline (PBS, pH 7.2) for 1 min. This method was proved to be effective to detach biofilm cells from stainless steel coupons previously (Kang, Lee, & Kang, 2021).

2.4. Cell enumeration

The detached cells from coupons were decimally diluted by PBS and 0.1 mL of each diluent was spread onto TSA plates. The colonies on the plates were enumerated after incubating at 37 °C for 24 h and expressed as log CFU/coupon. For microorganisms transferred to suspension after each treatment, 0.5 mL of the suspension in the beakers was mixed well with 0.5 mL neutralising buffer for 5-min neutralisation. The survival population in the suspension was counted following the same steps of dilution and plating as mentioned above and shown as log CFU/mL.

2.5. Mathematical modelling of antibiofilm effect

Three inactivation models were employed to describe the removal kinetics of *E. coli* biofilms on coupons under different treatments:

Linear model: 

\[ y = kx \]

where \( y \) is the antibiofilm effect in log CFU/coupon, \( x \) is the time in min, \( k \) is the linear regression parameter.
Modified Weibull model: 
\[ y = \frac{1}{\alpha} \left( \frac{t}{\alpha} \right)^{\beta}, \]
where \( \alpha \) is the scale factor, \( \beta \) is the shape parameter. Moreover, Tq (the time needed for 90% biofilm reduction) was calculated based on the following equation: 
\[ Tq = \frac{T_{\text{R}}}{\alpha(2.303)^{\beta}}, \]
to evaluate Weibull parameters (Liu, Jin, Feng, Yang, & Fu, 2019).

Reduced Baranyi model: 
\[ y = \mu_{\text{max}} + \ln(\exp(-\mu_{\text{max}}) + \exp(-h) - \exp(-\mu_{\text{max}} - h)), \]
where \( \mu_{\text{max}} \) is the maximum specific growth rate, \( h \) is the physiological state of the bacterium in log CFU/coupon.

The goodness of each model fitting was evaluated by the coefficient of determination (R²) and the Residual Mean Square Error (RMSE), which were obtained from MATLAB R2018a (The Mathworks, Inc., Natick, USA). In addition, the parsimony of each model was judged by Akaike Information Criterion (AIC), which was used for an overfitting determination and calculated as below:
\[ \text{AIC} = n \ln(\text{SSE}) + 2p \]
where \( n \) is the number of data points used for modelling, \( \text{SSE} \) is the Sum of Squares for Error and \( p \) is the number of parameters used in each model.

### 2.6. Confocal laser scanning microscopy (CLSM) analysis

To visualise the biofilm structure after each treatment, CLSM analysis was conducted based on a reported method (Pang & Yuk, 2019). The biofilms on stainless steel coupons were stained with a LIVE/DEAD BacLight Viability Kit L-7007 (Molecular Probes, Eugene, USA). After incubation at dark for 15 min, the coupons were examined immediately by an inverted FLUOVIEW FV 1000 Laser Scanning Confocal Microscope (Olympus, Tokyo, Japan) with a 60x objective accompanied by a 488 nm and 543 nm argon laser for green and red excitation, respectively, in which BA 505–525 and BA 560 IF were two filters used for the green and red channel, respectively. Representative 3D CLSM images from each treatment group were processed using IMARIS 7.6 software (Bitplane AG, Zurich, Switzerland).

### 2.7. Extraction of biofilm metabolites

The biofilms on coupons after 5-min treatment were used to do the metabolomics analysis, according to a method described by Zhao, Zhao, Wu, Lou, and Yang (2019) with some modifications. Once the cells were washed by PBS twice and the collected pellets were dissolved in a mixture of equal volumes of PBS and acetonitrile, which was served as a metabolite extraction solution. Then the bacteria cells were broken down by an ultrasonic crusher with 25 cycles and each cycle included 5 s pulses and 10 s stops. After centrifugation (12,000 \( \times \) g, 4 °C, 10 min), the supernatant with metabolites was obtained and the solid residues were used for collecting a secondary supernatant by homogenising again in the same extraction solution with a vortex. The two supernatants were then combined and both the acetonitrile and water in it were removed by a rotary evaporator and then the metabolite extracts from the lysed cells were obtained. The dried samples were stored at −20 °C for further NMR analyses.

### 2.8. NMR spectroscopic analysis

The solid samples prepared for NMR analysis was dissolved in deuterated water (D₂O, 99.9%), and sodium 3-trimethylsilyl [2,2,3,3-d⁴] propionate (TSP, Sigma-Aldrich, USA) was added as an internal reference with a 0.005% concentration ratio, to help quantify the concentration of biofilm metabolites and calibrate the chemical shift. The mixture was centrifuged (12,000 \( \times \) g, 4 °C, 10 min) and 600 μL supernatants were transferred into 5-mm NMR tubes by pipette. A Bruker DRX-500 NMR Spectrometer (Bruker, Rheinstetten, Germany) set at 25 °C was applied to conduct \(^{1}H\) NMR measurements, with a use of Triple Inverse Gradient probe running at 500.23 MHz. The \(^{1}H\) spectrum was obtained after setting the parameters needed in a first increment of NOESY pulse sequence, which were set as follows according to a previous report (Wu, Zhao, Lai, & Yang, 2021): recycle delay (2 s) – 90° (10 μs) – t₁ (6.5 μs) – 90° – t₂ (100 ms) – 90° – acquisition (1.36 s). A continuous weak wave irradiation was joined during the period of both recycle delay and t₂ to do a water suppression. Each spectrum consisted of 64 transients of 32 k data points with a 20-ppm spectral width. Furthermore, before Fourier transformation, an exponential window function with a 1-Hz broadening factor was used to transform all free induction decays. After 1D spectrum was gained via 4 dummy scans and 128 scans, a series of 2D \(^{1}H–^{1}C\) heteronuclear single quantum coherence spectra (HSQC) of selected samples were obtained to identify the metabolite signal assignment. The F1 channel was assigned to test the \(^{13}C\) spectra with a 180-ppm spectral width while the F2 channel was used to collect the \(^{1}H\) spectra with a 10-ppm spectral width (Lavoine et al., 2014). All assays were done in triplicate.

### 2.9. Spectral processing and analysis

The obtained NMR spectra were subject to software TopSpin 4.0.3 (Bruker) for baseline correction and phase adjustment. The 2D \(^{1}H–^{13}C\) NMR spectrum was used as an auxiliary means of metabolite identification cooperatively with 1D \(^{1}H\) spectra. The chemical shift assignments were determined by referring to several databases, such as the E. coli Metabolome Database (http://www.ecmdb.ca/) and the Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu/). Furthermore, the spectra were integrated into regions (0.5–10.0 ppm) with water region (4.74–4.78 ppm) excluded by using the software Mestrenova (Mestreab Research SL, Spain), which can normalise the peaks to the sum intensities with equal width of 0.004 ppm. The data sets were then subject to multivariate analysis using SIMCA software (version 14.0, Umetrics, Sweden), which can carry out the principal component analysis (PCA) and the orthogonal projection to latent structure discriminant analysis (OPLS-DA) sequentially, showing the separation of each data set and identifying the differences between control and combined treatments, respectively. All OPLS-DA models were further evaluated by the variable importance in projection (VIP), the fold change (FC) and the related \( P \) values in each pairwise comparison, to distinguish significantly changed metabolites that were susceptible to external treatments (Wang, Wu, & Yang, 2022). Lastly, metabolites with VIP > 1 were imported into MetaboAnalyst 5.0 (https://www.metaboaanalyst.ca/) for metabolic pathway analysis, by referring to Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/pathway.html) accordingly.

### 2.10. Statistical analysis

All microbiological assays were conducted in triplicates and the bacterial count was calculated from duplicate plating at each data point. The results were expressed as mean value ± standard deviation. The data were analysed statistically by using analysis of variance (ANOVA) (\( P < 0.05 \)) and the means comparisons among different treatment groups were achieved through the Duncan’s multiple range test with an IBM SPSS statistical software (version 24; IBM Co., Armonk, NY, USA).

### 3. Results and discussion

#### 3.1. Eradication effect of ultrasound and LcAEW on coupon and in suspension

The biofilm reduction curves fitted with different models on three E. coli strains under different treatments are shown in Fig. 1. There was little difference in reduction amount between LcAEW and control group for all E. coli biofilms, which is as expected since the cells in the inner
layer of biofilm are well-protected by the EPS structure against disinfectant penetration. However, through CLSM examination, a large quantity of cells of three strains under 5-min LcAEW treatment were stained red as shown in Fig. S1, indicating that the cells lost their membrane integrity to a certain extent but were still cultivable (Zhang, Pang, Seck, & Zhou, 2021). On the other hand, the loose cells of ATCC 25922 and ATCC 43895 transferred to suspension in LcAEW group could not be detected after 5-min treatment as shown in Table 1, indicating effective sanitising effect of LcAEW on planktonic cells as the free chlorine content of LcAEW remained within the range of 2–4 mg/L when we covered the container by aluminium foil to avoid volatilisation (Liu et al., 2020). It was worth noting that under the same condition, the survival population of ATCC 35150 remained at around 3.76 log CFU/mL in suspension after LcAEW treatment, which might be due to its higher initial biofilm amount formed on stainless steel coupons (Table 1). Abu-Ali, Ouellette, Henderson, Whittam, and Manning (2010) reported that different strains even in the same E. coli O157:H7 lineage could exhibit different gene expression during their colonisation process, leading to different biofilm-forming ability. Another study found that E. coli ATCC 25922 could be served as a representative surrogate for

![Fig. 1. Modelling of biofilm reduction on coupons under different treatments. A: ATCC 25922; B: ATCC 35150; C: ATCC 43895. A1, B1, C1: Linear model; A2, B2, C2: the modified Weibull model; A3, B3, C3: the reduced Baranyi model.](image)

| Table 1 | Survival of three E. coli strains on coupon and in suspension after each 5-min treatment. |
|-----------------|-------------------------------------------------|-------------------------------------------------|
| **Survival populations on coupon** | **Survival populations in suspension** | **Log CFU/coupon** | **Log CFU/mL** |
| ATCC 25922 | ATCC 35150 | ATCC 43895 | ATCC 25922 | ATCC 35150 | ATCC 43895 |
| **Untreatment** | 7.12±0.15<sup>2</sup>| 7.79±0.10<sup>2</sup> | 7.09±0.24<sup>2</sup> | ND | ND | ND |
| DW | 6.53±0.04<sup>2</sup> | 7.56±0.11<sup>2</sup> | 6.47±0.19<sup>2</sup> | 5.81±0.08<sup>2</sup> | 5.57±0.29<sup>2</sup> | 5.64±0.15<sup>2</sup> |
| Ultrasound | 5.10±0.15<sup>2</sup> | 4.52±0.11<sup>2</sup> | 4.37±0.19<sup>2</sup> | 5.92±0.08<sup>2</sup> | 6.71±0.29<sup>2</sup> | 5.85±0.15<sup>2</sup> |
| LcAEW | 6.33±0.11<sup>2</sup> | 7.40±0.15<sup>2</sup> | 6.05±0.03<sup>2</sup> | ND | 3.76±ND | ND |
| Combination | ND | 3.18±0.17<sup>2</sup> | ND | ND | ND | ND |

Within each column, values with different letters are significantly different (P < 0.05). ND: Not detectable: the survival population on coupon < 2 log CFU/coupon and the survival population in suspension < 2 log CFU/mL.
E. coli O157:H7 due to similar attachment characteristics on food surface (Kim & Harrison, 2009). Our results are consistent with these previous findings showing respective differences and similarities among serotypes and/or strains.

As a comparison, the ultrasound alone treatment resulted in the reduction of three E. coli strains by around 2.02–3.27 log CFU/coupon, suggesting that ultrasound could detach the biofilms from coupons effectively through mechanical oscillation. The CLSM images were consistent with the enumeration data, as a significant decrease in cells was observed on coupons, with partly compromised cell membrane structure under cavitation bubble’s attack (Fig. S1). However, the population of survival cells in suspension increased after 5 min, which verified again that ultrasound has limited antimicrobial effect but is mainly used for removal purpose (Ishibashi et al., 2010). It was notable that the highest reductions of three E. coli strains both on coupons and in suspension were observed after exposure to combined treatment of ultrasound and LcAEW, causing 4.61–6.42 log reductions from coupons and no detectable survival cells in suspension for all strains (Fig. 1 and Table 1). The CLSM results also showed an increase in the proportion of red cells on coupons after this combined treatment, which was quite different from those in control group with much thicker structure (Fig. S1). By collaborating with each other, ultrasound could damage the biofilm structure first and the detached cells from coupons to the suspension were inactivated effectively by LcAEW. However, although this combined method achieved best eradication effect on all E. coli biofilms, ATCC 35150 exhibited stronger adhesion ability and removal resistance compared to others, which might be attributed to some differences in its EPS structure and composition. Thus, the overall metabolic profiles of each strain were further evaluated in our study.

3.2. Modelling of antimicrobial effect

The fitting curves of three inactivation models showed similar reduction trends in Fig. 1; however, to determine which one was more suitable to describe the biofilm behaviour under each treatment, the goodness-of-fit of each model is shown in Table S1, as represented by R², RMSE and AIC values.

Overall, the modified Weibull model provided the highest R² (0.81–0.97), lowest RMSE (0.04–0.71) and AIC (−27.75–8.18) values for all E. coli strains after different treatments, demonstrating its universal applicability to describe the inactivation kinetics of E. coli biofilms on coupons. When focusing on the combination groups specifically, the reduced Baranyi model was more suitable for describing these groups of data than the modified Weibull model as reflected in its higher R² and lower RMSE and AIC values, which was more obvious on the ATCC 35150 strain (Table S1). However, this good appropriateness was reduced in other treatment groups among all strains. The linear model, on the other hand, showed worst fitness by evaluating these parameters, indicating the biofilm removal process induced by these treatments was not linear. These findings were supported by Josewin, Ghate, Kim, and Yuk (2018), who indicated that both the Weibull and Baranyi models could produce a suitable fit for inactivation curves of Listeria monocytogenes, which were inoculated on the smoked salmon and treated by the light emitting diode (LED). Comparing the overall results, the modified Weibull model best fitted the data in current study.

To further understand the sensitivity of each E. coli biofilm against different treatments, the fitting parameters of the modified Weibull model were determined and are shown in Table 2. The value of the shape parameter, β, was smaller than 1 in all treatment groups among all strains, indicating all inactivation curves displayed downward convexity without inflexion points and shoulders. According to a previous report by Liu et al. (2019), an upwardly survival curve means over the inactivation time, the decline of the population quantity tends to an equilibrium situation. Similarly, the downwardly reduction curve here also indicated the longer the treatment time, the more difficult to detach remaining sturdy cells from coupons. Although the shape parameters did not change regularly among different treatments, the T₀ and α values were dependent on the treatment, as the lowest values of them were obtained in the strongest treatment groups (the combined) while the highest values of them were observed in the control groups, which applied to all E. coli strains. The minimal T₀ values for non-pathogenic E. coli and E. coli O157:H7 were 0.04 and 0.01 min, respectively, showing ultrasound and LcAEW combination could detach the biofilms from coupons immediately and efficiently. Moreover, additional data (treatment time: 4 min) were used to validate the model performance, which confirmed good predictability of the model with bias percentages smaller than 10% in all treatment groups (data not shown).

Mathematical models can help us predict the inactivation effect through statistical analysis. In previous studies, the Weibull model also proved the most suitable model for describing antimicrobial effects of nisin and grape seed extract against L. monocytogenes on shrimps, with good consistency between predicted and observed values (Zhao, Chen, Zhao, He, & Yang, 2020). Moreover, Ghate et al. (2017) found that the Weibull model could fit well the inactivation kinetics of Salmonella on fresh-cut pineapples under LED illumination, with its unique compatibility with the biphasic characteristic of LED’s antimicrobial effect. In addition to providing relatively accurate quantifications, model fitting could also be used as a tool for comparing antimicrobial kinetics under different conditions, providing detailed information about sensitivity and resistance of microorganisms against different treatments during inactivation process.

3.3. Metabolite profiling of E. coli biofilms after treatments

The typical ¹H NMR spectra of three E. coli biofilms after each treatment are shown in Fig. S2. In total, 50 metabolites were identified and their detailed resonance assignments are shown in Table S2. The two strains of E. coli O157:H7 displayed high similarity in their metabolite variety, with 19 amino acids, 9 organic acids, 7 sugars, 7 nucleotide-related compounds and 8 other metabolites observed in the region of 0.5–9.5 ppm. Different intensity and diversity of some metabolite signals were found in the non-pathogenic E. coli spectra. For instance, the intensities of ¹H resonance at 2.21, 5.12 and 8.84 ppm, which was assigned as acetoin, phosphoenolpyruvic acid (PEP) and nicotinamide adenine dinucleotide (NAD), respectively, were stronger in ATCC 25922 than those in other two counterparts (Fig. S2).

One previous study suggested that substantial metabolic differences could exist in E. coli strains even they are closely related phylogenetically (Van Der Hooft, Goldstone, Harris, Burgess, & Smith, 2019). On the other hand, Hancock, Vejborg, and Klemm (2010) found the distance between pathogenic and non-pathogenic E. coli strains sometimes could be very short, as they might be distinguished by just a few hundred
genes. These seeming conflicting opinions might be derived from different studied strains. In this study, serovar-specific metabolic traits were observed among three *E. coli* biofilms, along with sharing many kinds of metabolites in common.

Furthermore, to identify the metabolite level changes in each *E. coli* biofilm under the stresses, a heat map of 32 metabolites with no overlapping chemical shifts was plotted for relative quantification (Fig. S3). Based on the z-score values, the deeper green and red colour represent lower and higher level of metabolites, respectively. In ATCC 25922, the levels of most amino acids (e.g., Leu, Val, Thr, Arg) were increased after single ultrasound treatment, showing opposite trends compared to their changes in planktonic cells as observed in our previous research (Zhao, Zhao, Wu, et al., 2019), and indicating biofilm-associated resistance and survival strategy to ultrasound stress. However, when combined with LcAEW, a marked depletion of most amino acids and nucleotide-related compounds as well as all organic acids and sugars was observed. This was like the metabolite changes in planktonic cells after same combined treatment, indicating mature non-pathogenic *E. coli* biofilm failed to produce effective adaptive responses when facing excessive stresses and shared many similarities in the stress response with bacteria in stationary growth phase (Beloin et al., 2004). Whereas in the *E. coli* O157: H7 strains, the levels of most metabolites showed a trend of fluctuation compared with their respective control groups after the combined treatment. Álvarez-Ordóñez et al. (2013) found that the *E. coli* strains belonging to the serogroup O157 exhibited more tolerance to environmental stresses (e.g., acid, heat and high pressure), while the non-pathogenic strains tended to be most sensitive. Our results were in accordance with these findings, as *E. coli* O157:H7 biofilms we studied here showed some efforts for maintaining the levels of a series of metabolites to improve resistance. Therefore, to further investigate which metabolites played important roles in modulating the stress response, following analyses were carried out by screening the principal metabolites.

### 3.4. Principal components analysis

The model quality parameters, $R^2_X$ and $Q^2$ shown in Fig. 2A1–C1, were used to evaluate PCA model’s interpretative and predictable abilities, respectively. For *E. coli* O157:H7 strains, the first three PCs of ATCC 35150 and ATCC 43895 explained 97.0% (PC1: 63.9%; PC2: 22.2%; PC3: 10.9%) and 97.8% (PC1: 64.8%; PC2: 24.2%; PC3: 8.8%) of the total data, respectively, whereas in the non-pathogenic strain, PC1, PC2 and PC3 explained 60.7%, 18.1% and 9.6%, respectively, showing the fitness of the model might be compromised relatively. Moreover, the

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**Fig. 2.** Principal component analysis (PCA) of three *E. coli* strains under different treatments. A1, B1, C1: The principal components explaining variances used in the PCA models; A2, B2, C2: the 3D score plot of PCA; A3, B3, C3: The 3D loading plot of PCA. Note: $R^2_X$: explained variable value; $Q^2$: model predictability value; I: ATCC 25922; II: ATCC 35150; III: ATCC 43895; 1: deionised water (DW) treatment; 2: ultrasound treatment; 3: low concentration acidic electrolysed water (LcAEW) treatment; 4: combination of ultrasound and LcAEW treatment.
\( Q^2 \) values of three strains lied within the range of 0.73–0.96, which were more than 0.5 indicating good model predictability (Wiklund, 2008).

The samples of each strain after different treatments were well separated into several clusters, as shown in the score plots (Fig. 2A2–C2). The most distinctive separation of ATCC 25922 was observed between the control and combined group, suggesting the synergic stress of ultrasound and LcAEW could cause the largest changes of metabolites. However, in \( E. coli \) O157:H7 strains, the samples after being treated by LcAEW alone or combined with ultrasound showed similar distances from their respective control group, by calculating the given score variables shown in Table S3. Moreover, the relative squared cosines of the three PCs characterising each group are displayed in Table S4, which could help us find which component played a more significant role in interpreting the variables (Abdi & Williams, 2010).

For example, ultrasound alone and combined with LcAEW groups in ATCC 25922, single ultrasound and LcAEW groups in ATCC 35150, and all four groups in ATCC 43895 were affected mainly by PC1, while other groups were influenced more by other two PCs.

Moreover, the discriminative metabolites that contributed to these group separations of each strain are presented respectively in the loading plots (Fig. 2A3–C3). In ATCC 25922, Glu, acetoin, fumaric acid, acetic acid and succinic acid were mainly characterised by PC1 and PC2, PC1 and PC3, PC1 and PC2, PC1 and PC3, PC2 and PC3, respectively. Similar metabolites with distinctive loadings on the three PCs were observed in \( E. coli \) O157:H7 strains, along with some strain-specific metabolites (e.g., \( \gamma \)-aminobutyric acid (4Abu)) as shown in Table S5. These metabolites could be recognised as potential biomarkers that responded to different treatments by comparing their \( \cos^2 \) values (Table S6) with those of score variables in Table S5. For instance, Val, Glu, \( \beta \)-D-glucose, glucose-6-phosphate and PEP might be closely related to the groups containing ultrasound in ATCC 25922, while the combined group of ATCC 35150 could be characterised by Asp, UMP and acetamide specifically. The results suggested that \( E. coli \) biofilms exhibited a diversity of metabolic responses under stresses, making their different removal kinetics from coupons reasonable.

3.5. Alternative metabolites during the combined treatment

To further study the metabolic response of \( E. coli \) biofilms following the combined treatment of ultrasound and LcAEW, OPLS-DA was conducted to visualise separations between the control and combined groups. As shown in the left sides of Fig. 3A–C, all pairwise comparisons

![Fig. 3. Orthogonal projection to latent structure discriminant analysis (OPLS-DA) score plots (left side of A, B, C) and coefficient-coded loading S-lines (right side of A, B, C) for the control and combined treatment groups. I: ATCC 25922; II: ATCC 35150; III: ATCC 43895; 1: DW treatment; 4: combination of ultrasound and LcAEW treatment.](image-url)
demonstrated clear separations with good model fitness and predictability (ATCC 25922: $R^2 = 0.874$, $Q^2 = 0.990$; ATCC 35150: $R^2 = 0.764$, $Q^2 = 0.985$; ATCC 43895: $R^2 = 0.818$, $Q^2 = 0.992$). The loading S-line in the right side showed major metabolites that resulted in these pairwise differentiations, with downward and upward peaks illustrating lower and higher levels of metabolites in the combined group compared to those in the control group, respectively. In ATCC 25922, except Arg, Glu, Asp, uridine, adenosine, putrescine and NAD, decreased contents of other metabolites were detected in the combined group. For *E. coli* O157:H7 strains, higher concentration of around half of metabolites was associated with the combined stimulation.

Moreover, volcano plot of each strain was generated based on the metabolite’s correlation coefficient, VIP, FC, and P values, as shown in Fig. 4A1–C1. Metabolites with a FC > 1.5 and $P < 0.05$ were considered as statistically significant (Wu et al., 2021). In ATCC 25922, acetic acid, α-D-glucose, uracil and G-1-P coloured in blue were located in the negative side of the x-axis, indicating these metabolites were most susceptible to the combined treatment compared to other reduced counterparts. On the other hand, Glu was most closely related to the combined stress among the seven increased metabolites as mentioned above. The metabolites significantly influenced by the combined treatment were different in *E. coli* O157:H7 strains, as Glu, acetic acid, Leu,
Phe and AMP were highlighted with significant increases in ATCC 35150 whereas this significantly increasing trend happened to putrescine, 4Abu, nicotinic acid, fumaric acid, Arg and Tyr in ATCC 43895. These statistically significant metabolites can be seen as potential biomarkers to characterise injured cells during this combined stress.

A schematic model illustrating the antibiofilm mechanism of ultrasound and LcAEW from multiple attacking targets is proposed in Fig. 5. Composition of EPS is complex and may vary between different strains of E. coli, but in general, polysaccharides are prominent components in EPS that provide structural support for the biofilm, accompanied by other substances such as proteins, lipids and extracellular DNA (eDNA) (Koo, Allan, Howlin, Stoodley, & Hall-Stoodley, 2017). These biomolecules in EPS can get hit first under external treatments.

Previous studies showed that the antibiofilm mechanism by ultrasonic waves was mainly attributed to their cavitation effect, which can destroy EPS structure through the implosive collapse of cavitating bubbles with the help of shock waves and microstreaming (Vyas et al., 2019). This mechanical oscillation could destroy polysaccharides in the EPS by breaking their glycosidic bonds, making increased content of glucose in ATCC 25922 and ATCC 43895 reasonable after ultrasound treatment (Nachtigall et al., 2019). However, the opposite trend observed in ATCC 35150 might be due to its different polysaccharide structure, as polysaccharide with longer side chain might exhibit less sensitivity to external stress (Nachtigall, Rohn, & Jaro, 2021). Besides mechanical oscillation, free radicals (e.g., H- and OH) and strong oxides (e.g., HO2) generated by the hydrolysis of water during cavities’ implosion also played an important role in damaging EPS components. For example, some specific amino acid residues on extracellular proteins might be attacked and inactivated by the generated oxides, which could explain why most amino acid levels (Iso, Leu, Val, Thr, etc.) in E. coli O157:H7 strains were decreased under ultrasound stress (Yu et al., 2020). On the other hand, the channels that transport nutrients and oxygen to the deeper layers of the biofilm could be activated by ultrasound, stimulating bacterial metabolism from starved state and supporting biosynthesis of some components in the EPS, which might be an explanation for increased levels of some amino acids in ATCC 25922 (Erru et al., 2014).

The interaction between LcAEW and macromolecules in EPS could change EPS content and composition as well. For example, Han et al. (2017) found that after AEW treatment on E. coli biofilms, the C-O-C bonds of polysaccharides in the EPS and the aromatic rings in tyrosine and phenylalanine of EPS protein were deformed, accompanied by the destruction of the ring structure of the eDNA. Similar results were observed in our study, as the decreased levels of most carbohydrates and nucleotide-related compounds occurred in all E. coli biofilms after LcAEW treatment. It has been believed that the eradication effect of LcAEW on biofilm could be attributed to its chlorination and oxidation properties, as its chlorine compounds (e.g., HOCl, OCI), free radicals (e.g., OH) or the combination reasonable as shown in Table 1. However, aromatic and sulfurous amino acids such as Phe, Tyr, Met and Cys were reported to be more sensitive to oxidative stress (Yang et al., 2019), which might cause corresponding effect on the content of their precursors and products.

Multiple stresses (e.g., oxidative, acidic pH, osmotic and heat stresses) brought by ultrasound and LcAEW treatments can have a cross-impact on E. coli pathway alterations, while simultaneously, E. coli biofilms can activate multiple regulators as adaptive responses toward these stresses, and usually the induction of one regulator due to one particular stress tends to show cross-protection against other stresses concurrently (Fernández et al., 2018). The up- and down-regulation of some genes might be responsible for the increased resistance of biofilms to sanitisers compared to their planktonic counterparts.

It has been reported that HOCl can activate soxRS and oxyR expression in E. coli to produce SoxS, SoxO and OxyR protein, which can act as antioxidant systems and protect the cell from attack of superoxide anion and hydrogen peroxide, respectively (Lushchak, 2011). Therefore, the elevated levels of some amino acids like Iso, Leu and Val in ATCC 35150 biofilm after treatments containing LcAEW might imply some strain-specific protein biosynthesis responses as its adaptation to oxidative stress, indicating its better adaptive ability during oxidative challenge than the other two strains and making its only survival in suspension reasonable as shown in Table 1. However, aromatic and sulfurous amino acids such as Phe, Tyr, Met and Cys were reported to be more sensitive to oxidative stress (Yang et al., 2019), which might cause corresponding effect on the content of their precursors and products.

On the other hand, glutamate decarboxylase (GAD) system can be upregulated in acidic condition and help remove intracellular protons by converting Glu to 4Abu through decarboxylation reaction. As many oxidative reactions are affected by the pH, this GAD acid-resistance system can also provide oxidative stress protection by maintaining intracellular pH homeostasis (Chen et al., 2022). However, the higher contents of Glu in all treated E. coli strains in our study indicated that the conversion rate of Glu to 4Abu was low. Pearson, Lee, and Casey (2009) demonstrated that the induction of GAD activity required low pH such as
Fig. 5. Hypothetical target sites and mechanisms of action of ultrasound and LeAEW on E. coli biofilm. The extracellular polymeric substances (EPS) structure and macromolecules (e.g., extracellular protein, polysaccharide, eDNA) within mature biofilms established on the food contact surface can be disrupted and degraded by ultrasound and LeAEW, which can be regarded as first targeting stage of the treatments. Under the combination, the biofilm structure is damaged and the cells in the deep layers of biofilm are exposed and attacked, experiencing a series of intracellular metabolite changes.
2.5, so the pH 4.0 of LcAEW used in this study might situate GAD in a non-optimal status for providing sufficient protection against acidic and oxidative stresses. Moreover, some amino acids (e.g., Pro, Val) can act as osmoprotectants to maintain the stabilisation of cytoplasm osmolality when faced with osmotic stress, which could also be one possible reason for increased levels of some amino acids (e.g., Arg, Val) under the combined treatment (Byrne & Booth, 2002; Valle et al., 2008).

However, the multiple stress-response regulators can only partially compensate the impaired pathways when microbes face compounding and excessive stresses. In this study, lower content of glucose and higher content of PEP (an intermediate can be converted to pyruvic acid) indicated an unbalanced glycolysis of E. coli strains under combined treatment, which in further repressed tricarboxylic acid (TCA) cycle as many TCA cycle related metabolite levels (e.g., succinic acid, fumaric acid and NAD) were decreased. Koebmann, Westerhoff, Snoep, Nilsson, and Jensen (2002) demonstrated glycolysis in E. coli can be controlled in an ATP-dependent manner, in which increased ATP hydrolysis and lower ATP/ADP ratio could result in an increased glycolytic flux. Therefore, the reduction of ATP level mentioned in Section 3.5 could induce more glucose consumption in stressed cells, while an increase of ATP level in ATCC 43895 might imply an energy conservation strategy for survival. On the other hand, as a central metabolic pathway providing many precursors and intermediates for other processes, the disturbance of TCA cycle could make many related metabolic networks affected (Zhang et al., 2018), such as Ala- and Asp-dependent protein metabolism and α-ketoglutaric acid-associated Glu metabolism, which were subject to various degree changes in our study.

As alternative shunts for sustaining glycolysis concomitantly, the pentose phosphate pathway (PPP) and the mixed acid fermentation could also be affected under stresses (Bertels, Fernandez Murillo, & Heinisch, 2021; Vidra & Németh, 2018), with depleted level of ribose-5-phosphate (R-5-P) and increased level of acetic acid observed more apparently in ATCC 25922 than in the pathogenic strains in our study. Considering R-5-P is an important precursor for nucleotide biosynthesis, its diminishment indicated impaired DNA replication and cell proliferation. Moreover, the metabolic switch from oxidation to mixed acid fermentation producing lactic acid, acetic acid and ethanol could be recognised as an adaptive strategy of bacteria, when they were situated in a low oxygen environment under LcAEW and ultrasound treatments (Schalenbach, Zeradjanin, Kasian, Cherevko, & Mayrhofer, 2018).

4. Conclusion

The antibiofilm effects and mechanisms of ultrasound and LcAEW combination against different E. coli strains were investigated. Specifically, ultrasound mainly damaged the EPS and cell wall structure by mechanical oscillation, whereas LcAEW attacked intracellular metabolic flux as a leading antimicrobial mechanism. This study suggested that NMR-based metabolomics strategy could be used to explain antibiofilm mechanisms of disinfection interventions on biofilm removal process, providing guidance for further mechanism research related to food contact surface sanitisation. Meanwhile, the combination of ultrasound and LcAEW is worthy of utilisation as an effective biofilm control measure in food processing plants where biofilms are a problem on food contact surface, especially in organic food industry.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.


