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Comparison of the metabolic responses of eight *Escherichia coli* strains including the "big six" in pea sprouts to low concentration electrolysed water by NMR spectroscopy

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ABSTRACT

The antimicrobial effects of low concentration electrolysed water (LcEW) on common foodborne pathogens have been well documented; however, the effects on six emerging *Escherichia coli* serotypes (the "big six") remained unknown. Using pea sprouts as the food matrix, this *in vivo* study examined the sanitising efficacy of a 10-min LcEW treatment against eight *E. coli* strains (non-pathogenic *E. coli* ATCC 25922, pathogenic *E. coli* O26:H11, O45:H2, O103:H11, O111, O121:H19, O145 and O157:H7). The metabolic responses of the strains were further analysed using nuclear magnetic resonance (NMR) spectroscopy. An overall negative correlation between the resistance to LcEW and the magnitude of metabolic variation was observed. The metabolic status and pathways of the most resistant O103:H11 and O45:H2 strains were the least affected. The O26:H11 and O145 strains which demonstrated the highest sensitivity to LcEW had an array of metabolites depleted and had multiple pathways involved in amino acid metabolism, energy metabolism as well as osmotic and oxidative protection perturbed. Distinctive metabolic responses were detected in ATCC 25922, suggesting that alternative stress defence mechanisms may be present in the non-pathogenic *E. coli* strain. Amongst the pathogenic strains, the most unique metabolic responses were illustrated by O121:H19. Overall, the study showed that the NMR-based metabolomics is promising in elucidating the metabolic changes of various *E. coli* strains in pea sprouts during an antimicrobial process. It also provides clue for controlling "big six" contamination in fresh produce.

1. Introduction

Since *Escherichia coli* O157:H7 raised public awareness during an outbreak investigation of haemorrhagic colitis in the 1980s, reported cases of Shiga toxin-producing *E. coli* (STEC) have been increasing yearly (Beier et al., 2016). According to World Health Organisation (WHO), the current annual occurrence of foodborne STEC diseases is 1.2 million globally; while diarrhoea is the most common symptom, serious conditions, such as haemolytic uremic syndrome, kidney failure and even death, are also occasionally reported (Centers for Disease Control and Prevention, 2016). E. coli factsheet. ; Hald et al., 2016). Although *E. coli* O157:H7 is to blame for causing 36% of the total STEC infections, hundreds of non-O157 serotypes are also culprits for the severe issue; amongst them, the top six dominant serotypes are O26, O103, O111, O121, O45 and O145, combinedly called as "big six" (Centers for Disease Control and Prevention, 2016). In the past decade, Centers for Disease Control and Prevention (CDC) has reported 12 outbreaks associated with the "big six", among which one third were linked to consumption of fresh produce, such as sprouts and lettuce (https://www.cdc.gov/ecoli/outbreaks.html). Considering the high rate of both O157 and non-O157 outbreaks related to fresh produce, a sanitising approach generic to these major STEC serotypes is strongly demanded by the fresh produce industry.

Electrolysed water (EW) is a new potential food sanitiser highlighted in recent years (Liu et al., 2017a, 2017b; Zhao, Zhao, Wu, et al., 2019). It is a water-based sanitiser produced by electrolysing diluted salt to generate high oxidation-reduction potential (ORP), low pH and different forms of chlorine at the anode side, which thus exposes microbial cells to a combination of acidic, oxidative and potentially osmotic stresses. The application of EW in food is under strict regulation; in the case of organic

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produce, the residual free available chlorine (FAC) in the washing water must not exceed 4 mg/L, which is the upper limit stated in the Safe Drinking Water Act of the U.S. Environmental Protection Agency (National Organic Program, 2011, p. 5026). However, a low FAC (<1 mg/L) in the washing water can be quickly depleted via reacting with the organic matter of food matrices (Shen et al., 2013). As a result, low concentration EW (LcEW) at 4 mg/L FAC may be the most appropriate choice to apply to a range of fresh produce. The sanitising efficacy of LcEW has been documented in previous works. For instance, Liu, Wu, et al. (2017) reported that a 5-min LcEW treatment on the non-pathogenic surrogate of *E. coli* O157:H7, ATCC 25922, led to a significant cell reduction of 0.78 log CFU/mL. In their other study, the effectiveness of LcEW against *E. coli* O157 and *Listeria monocytogenes* in organic broccoli was also recorded (Liu, Tan, et al., 2017). The sanitising efficacy was further confirmed when treating *E. coli* and *Listeria* residing in organic lettuce (Zhao, Zhao, Phey, & Yang, 2019).

Although LcEW has demonstrated effectiveness in reducing bacterial populations, a complete elimination of bacterial cells cannot be achieved. In fact, cells surviving LcEW would initiate a series of metabolic alterations to adapt to the sublethal stress as an instinct to enhance their chance of survival during subsequent travel in the human gastrointestinal tract (Zook et al., 2001). Emerging metabolomic techniques have made it feasible to acquire "snapshots" of the comprehensive bacterial metabolic profiles under external stresses (Chen et al., 2020). Among them, nuclear magnetic resonance (NMR) spectroscopy is applied with increasing popularity as its rapid generation of informative and reproducible data eases the determination and quantitation of microbial metabolites (Zhao et al., 2020). On this basis, a metabolomic study facilitated by NMR spectroscopy can provide insights in understanding LcEW's antimicrobial mechanisms as well as the corresponding defence mechanisms employed by different bacterial strains.

As was mentioned above, the existing studies which used LcEW to treat *E. coli* were mainly conducted on *E. coli* O157:H7 or on its surrogate. The only study that addressed the effectiveness of LcEW against various STEC, to the best of our knowledge, reported a complete elimination of the "big six" strains at as low as 1.50 mg/L of FAC in LcEW (Jadeja et al., 2013). The *in vitro* study, however, did not seem to take into consideration the rapid exhaustion of the low FAC via interacting with the organic food matters, which cast doubt on the performance of LcEW in actual food treatment in real life. In light of this, the entire work of the current study was conducted *in vivo*, where LcEW's sanitising efficacy against eight *E. coli* strains, including the "big six", was holistically assessed in pea sprouts, a typical fresh produce. Besides, with the facilitation of NMR-based metabolomics, the study also aimed to take one step further by analysing the metabolic changes of each strain during the LcEW antimicrobial process so as to provide more convincing scientific basis for adopting this approach in future fresh produce sanitisation.

2. Materials and methods

2.1. E. coli strains and culture condition

Eight *E. coli* strains from different serotypes [non-pathogenic *E. coli* ATCC 25922, and pathogenic O26:H11 (ATCC BAA-2196), O45:H2 (ATCC BAA-2193), O103:H11 (ATCC BAA-2215), O111 (ATCC BAA-2440), O121:H19 (ATCC BAA-2219), O145 (ATCC BAA-2192) and *E. coli* O157:H7 (ATCC 43895)] were obtained from Department of Food Science and Technology, National University of Singapore. Since it is impossible to perform an exhaustive experiment of every strain of a serotype, the eight strains were used as representatives of their respective serotypes to study the different responses of different *E. coli* serotypes to the LcEW treatment. The strains were resuscitated from their respective glycerol stock solutions by separately inoculating into 10 mL of tryptone soya broth (TSB, Oxoid, Basingstoke, UK) and incubated overnight at 37 °C. The cultures were then acclimatised to 100 μg/mL of nalidixic acid (Sigma-Aldrich, St. Louis, MO, USA) via consecutive transfers with stepwise increments in nalidixic acid concentration. All media used in this study were supplemented with 100 μg/mL nalidixic acid to eliminate any effects that naturally existing microbes in pea sprouts might exert (Kharel et al., 2018). The adapted cultures were individually inoculated into 10 mL of fresh TSB and incubated at 37 °C overnight. The cultures with around 8 log colony forming units (CFU)/mL were centrifuged at 4500×*g* for 10 min (24 °C), washed twice with 0.1% peptone water and resuspended in 100 mL of peptone water to achieve a final concentration of approximately 7 log CFU/mL.

2.2. Pea sprouts preparation and inoculation

Pea sprouts were purchased from a local supermarket in Singapore within 24 h before use and kept at 4 °C. They were gently rinsed for 60 s by cold tap water to remove undesired residues and those with no visible damage were weighed into 10-g portions.

Dip inoculation was applied to simulate the immersion process which is a suspected point of contamination in food production (Ruiz-Cruz et al., 2007). Specifically, each pea sprouts sample (10 g) was submerged in the 100 mL of prepared *E. coli* suspension for 10 min, followed by air-dried for 30 min in a laminar flow biosafety cabinet (Chen et al., 2019, 2020; Liu, Tan, et al., 2017; Zhao, Zhao, Phey, & Yang, 2019). The eight *E. coli* strains were inoculated separately in this way. Samples inoculated with each strain were randomly assigned to the LcEW-treated group and the deionised water (DW) control group before proceeding to sanitisation.

2.3. Sanitising treatment

LcEW was prepared by electrolysing dilute sodium chloride solution using an electrolysed water generator (ROX-10WB3, Hoshizaki Electric Company, Aichi, Japan) and diluting with DW to reach a FAC level of 4 mg/L. The ORP and pH of the prepared LcEW were determined by an ORP meter (Metrohm 713 pH Meter, Herisau, Switzerland) and a pH meter (Orion 410, Thermo Scientific, Waltham, MA, USA), respectively. The LcEW was used within 2 h after preparation since the rapid loss of FAC at low pH would have a direct impact on the bactericidal activity (Chhetri et al., 2019; Wang et al., 2009).

Samples assigned to the LcEW-treated group and the control group were immersed into 200 mL of LcEW or DW simultaneously (Liu et al., 2019; Zhao, Zhao, Phey, & Yang, 2019). Based on our preliminary study where inoculated sprouts were treated for different amounts of time (3, 10 and 20 min), those treated for 10 min led to comparable *E. coli* reduction as those treated for 20 min, suggesting that 10 min in LcEW was sufficient for completing the potential sanitisation (data not shown). Hence, a 10-min treatment time was adopted for this work. After treating, samples were air-dried for 30 min in the laminar flow biosafety cabinet.

2.4. Microbiological analysis

Each sample was transferred into a stomacher bag containing 90 mL of 0.1% peptone water aseptically. The mixture was then homogenised for 180 s using the Masticator Stomacher (IUL Instruments, Germany). Serial dilution was prepared and 100 μL diluent was plated on tryptic soy agar (TSA, Oxoid Limited, Hampshire, UK) with incubation at 37 °C overnight. The results were expressed as log CFU/g of the fresh weight. For each strain, the difference in reducing populations between the LcEW-treated group and the control group was used to represent the efficacy of LcEW against the specific strain.

2.5. Metabolites extraction

Extraction of the eight *E. coli* strains was conducted individually. Two hundred grams inoculated pea sprouts were used in each group for

metabolic analysis. After treating the sprouts with LcEW or DW for 10 min, the treatment solutions were collected immediately and centrifuged at 500×*g* for 3 min (4 °C) to precipitate pea sprouts debris (Zhao et al., 2020). *E. coli* cells were harvested from the solutions by centrifugation at 12,000×*g* for 10 min (4 °C) and then washed with 0.1% peptone water twice. The collected cell pellets were mixed with 1 mL of ice-cold methanol-d4 (Cambridge Isotope Laboratories, Tewksbury, MA, USA) immediately. The mixture was frozen in liquid nitrogen and thawed on ice for three cycles to destroy the membrane structure (Winder et al., 2008). Overnight extraction at – 20 °C was subsequently applied to maximise metabolites yield, followed by centrifugation at 12,000×*g* for 20 min (4 °C) (Chen et al., 2020). Trimethylsilyl propanoic acid (TSP, dissolved in methanol-d4, 10 mM) was added to the obtained supernatant as an internal reference at a final concentration of 1 mM. After vertexing, 600 μL of the mixture was transferred into a 5 mm NMR tube (Sigma-Aldrich, St. Louis, MO, USA) and directly subject to NMR analysis.

2.6. NMR analysis

All NMR measurements were performed at 298 K using a Bruker DRX-500 NMR spectrometer (Bruker, Rheinstetten, Germany) with a Triple Inverse Gradient probe. The ^{1}H spectra with a 10.0 ppm spectral width were obtained for all samples using the standard Bruker NOESY pulse sequence (noesypr1d). The free induction decays were multiplied by an exponential function equivalent to a 1-Hz line-broadening factor before Fourier transformation. The 2D ^{1}H–^{13}C heteronuclear single quantum coherence spectroscopy (HSQC) of two representative samples was acquired to facilitate metabolites identification. With the Bruker hsqcedetgpsisp2.3 pulse sequence applied, the ^{1}H spectra with a width of 10.0 ppm and the ^{13}C spectra with a width of 180.0 ppm were collected in the F2 and F1 channels, respectively (Zhao et al., 2019 ab).

2.7. Spectral analysis

Baseline correction and phase distortions adjustment of all spectra were manually conducted on TopSpin 4.0.9 (Bruker). 1D ^{1}H and 2D ^{1}H–^{13}C spectra were used cooperatively for metabolites identification. The chemical shifts were verified using the Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu/metabolomics), the Madison Metabolomics Consortium Database (http://mmcd.nmrfam.wisc.edu) as well as the Human Metabolome Database (http://www.hmdb.ca/). Relevant studies which identified metabolites from NMR-spectra were also used as references (Chen et al., 2020; Liu, Wu, et al., 2017; Ye et al., 2012). After excluding the water (4.50–5.10 ppm) and methanol (3.30–3.35 ppm) regions, the resulting spectra (0.5–10.0 ppm) were normalised to the sum intensities and binned into baskets with 0.02 ppm integral width using the software Mestrenova (Mestreab Research SL, Santiago de Compostela, Spain) (Zhao et al., 2020).

Based on the binned data, a heatmap was plotted using ClustVis (https://biit.cs.ut.ee/clustvis/) for preliminary and intuitive presentation of all strains' metabolic profiles. The data were then subject to the calculation of Euclidean distances among the eight strains and hierarchical clustering was generated accordingly. Furthermore, orthogonal projection to latent structures-discriminant analysis (OPLS-DA) was applied to each strain using SIMCA software (version 13.0, Umetrics, Umeå, Sweden) for pairwise comparison between the LcEW-treated sample and the control. Collectively based on the criteria of |correlation coefficient| > 0.602, VIP value > 1 and $P < 0.05$, the most influential discriminative metabolites were screened (Chen et al., 2020; Liu et al., 2018; Zhao, Zhao, Wu, et al., 2019). The screened metabolites were then used for pathway analysis on MetaboAnalyst 5.0 (http://www.metaboanalyst.ca/) to diagnose the main pathways disturbed in the sanitisation process.

2.8. Statistical analysis

All tests were performed on at least three replications where each replication was treated as an independent and autonomous experiment using separately inoculated cultures and plating media. One-way analysis of variance (ANOVA) and the least significant difference (LSD) were conducted in the software SAS 9.4 (Statistical Analysis System, Cary, NC, USA) to compare the sanitising efficacy of LcEW against each strain in the pea sprouts samples. The significance of difference was defined at $P < 0.05$.

3. Results and discussion

3.1. Sanitisation efficacy of LcEW against E. coli strains in pea sprouts

The LcEW used in the study had a stable ORP of 1021.1 ± 9.9 mV and a stable pH of 3.5 ± 0.1. These properties were well controlled to ensure that the inter-strain differences unveiled in this work were not due to variations in the treatment solutions. DW was employed simultaneously with LcEW as the control solution and the difference in viable populations from the two treatments was calculated to indicate LcEW's sanitising efficacy. Adopting a control could avoid mistakenly attributing the number of cells remaining in the treatment solution to the sanitising efficacy. Meanwhile, DW has no sanitisation power, so with all other factors (e.g. temperature, solution volume and treatment time) controlled, the cell count difference between the two treatments could be solely attributed to the antimicrobial effects of LcEW.

The efficacy of LcEW against eight *E. coli* strains [S1: ATCC 25922; S2: O26:H11 (ATCC BAA-2196); S3: O45:H2 (ATCC BAA-2193); S4: O103:H11 (ATCC BAA-2215); S5: O111 (ATCC BAA-2440); S6: O121:H19 (ATCC BAA-2219); S7: O145 (ATCC BAA-2192); S8: O157:H7 (ATCC 43895)] in pea sprouts is shown in Fig. 1. In general, the 10-min LcEW treatment effectively reduced all strains by over 0.7 log CFU/g, with the lowest cell reductions observed in S3 and S4, which were 0.73 ± 0.04 and 0.72 ± 0.05 log CFU/g, respectively. The two strains demonstrated significantly higher LcEW resistance than even the most prominent Shiga toxigenic foodborne pathogen, O157:H7 (S8) (1.02 ± 0.08 log CFU/g), and its non-pathogenic surrogate, ATCC 25922 (S1) (0.86 ± 0.08 log CFU/g) ($P < 0.05$). The effects of LcEW were more pronounced against S5 and S6, diminishing them by 1.23 ± 0.02 and 1.17 ± 0.06 log CFU/g, respectively. Moreover, with cell reductions doubling that of the most resistant strains, S2 (1.44 ± 0.15 log CFU/g)

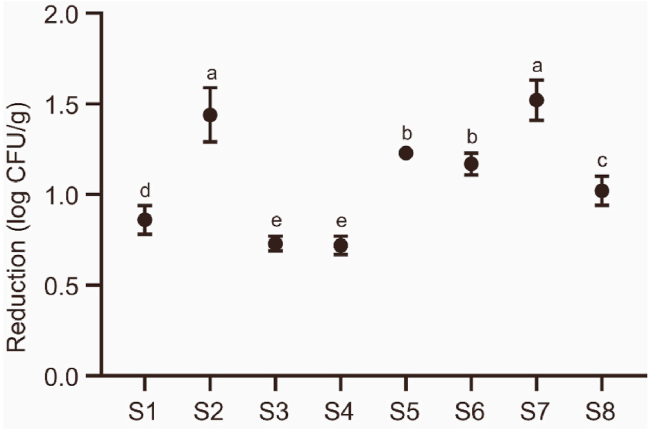

Fig. 1. Reduction of *E. coli* cells under 10-min low concentration electrolysed water (LcEW) (free available chlorine = 4 mg/L) treatment in pea sprouts. Data are presented as means ± standard deviation (n = 3). Means with different letters are significantly different ($P < 0.05$). Note: S1: ATCC 25922; S2: O26:H11 (ATCC BAA-2196); S3: O45:H2 (ATCC BAA-2193); S4: O103:H11 (ATCC BAA-2215); S5: O111 (ATCC BAA-2440); S6: O121:H19 (ATCC BAA-2219); S7: O145 (ATCC BAA-2192); S8: O157:H7 (ATCC 43895).

and S7 (1.52 ± 0.11 log CFU/g) were identified as the least resistant strains to the LcEW stress. Overall, the observations demonstrated that the eight *E. coli* strains could be reduced by LcEW to varying extents; however, the sensitivity or resistance to LcEW was not related to pathogenicity. To unravel the underlying reasons, metabolic changes occurred within the *E. coli* cells during LcEW application must be investigated.

3.2. Metabolic profiles of E. coli strains in pea sprouts

The ^{1}H NMR spectra of eight *E. coli* strains in pea sprouts with and without LcEW treatment are shown in Fig. S1. Despite the varying peak intensities, similar patterns were observed in these spectra. For instance, all spectra showed a preponderance of peaks clustered in the region of 0–5 ppm, which were assigned to amino acids and sugars in previous studies on *E. coli* metabolome (Liu, Wu, et al., 2017; Ye et al., 2012). Peaks at around 4.0–4.5 ppm and 5–10 ppm previously designated to nucleotide-related compounds were also detected in the spectra (Liu, Wu, et al., 2017; Planchon et al., 2017). Cooperatively referring to the 2D ^{1}H–^{13}C spectra, the identity of a total of 40 metabolites in the *E. coli* strains were readily confirmed (Table S1). Abundant amino acids (e.g. leucine, valine, isoleucine, alanine and arginine), nucleotides (e.g. cyclic AMP, ADP and ATP) as well as multiple sugars and sugar phosphates (e.g. α-D-Glucose, β-D-glucose, glucose-1-phosphate, ribose-5-phosphate) were recorded; the metabolites coincided with those identified from the same eight strains (untreated) isolated from TSB (Chen et al., 2020), which suggested that media and treatments did not affect the overall metabolic constituents of the strains.

Within each group (LcEW or DW), the intensity of metabolites' signals varied among the eight strains. For quantification purpose, the signals of 36 metabolites without overlapping chemical shifts were analysed and a heatmap was plotted in a blue-red scale to aid visualisation of the relative metabolite abundance in each of the strains (Fig. 2). Taking a horizontal view of the heatmap, threonine and lactic acid, presented in a spectrum of red colours, were the metabolites detected in the highest amounts in all strains. In contrast, a range of nucleotide-related compounds, including ATP, ADP, cyclic AMP, NAD, NADP and uracil, are presented in bluish colours, representing the rarest metabolites in the strains. Interestingly, in both the LcEW-treated group and the control group, S1 and S7 (O145, ATCC BAA-2192) demonstrated relatively low contents of uracil but high contents of cyclic AMP as compared to the others, which suggested that these two metabolites may not be markedly affected by LcEW sanitisation.

Viewing the heatmap vertically can help identify the metabolites differentiating the eight strains (Fig. 2). For instance, with colours transiting from dark orange to light orange along the column, the possession of glutamic acid decreased in the sequence of S4, S5, S2, S8, S7, S3, S1 and S6 within the control group and in the sequence of S4, S6, S8, S1, S5, S3, S7 and S2 within the LcEW-treated group, which highlighted glutamic acid as a metabolite responsible for the inter-strain difference. Likewise, fumaric acid, with colour variations from light blue to dark blue along the column, may be another representative for the inter-strain difference. Contrarily, the metabolites shown in semblable hues, such as valine and pyroglutamic acid, were likely present in a constant level among different strains, suggesting that their concentrations cannot be used as indicators to diagnose a specific *E. coli* strain. Based on the heatmap, S8 (O157:H7, ATCC 43895) had no such indictor metabolites and showed no unique features on its metabolome, which potentially revealed that *E. coli* O157:H7 is an ordinary STEC at the metabolic level.

Euclidean distances and hierarchical cluster analysis (HCA) in Fig. 3 offered more sophisticated classification of the strains. In the control group (Fig. 3A), the Euclidean distances ranged from 11.55 to 30.26 among the eight strains. S7 and S1, which successively seceded from the main cluster in HCA, showed the longest overall Euclidean distances to the other strains; this may be ascribed to their distinctive nucleotide

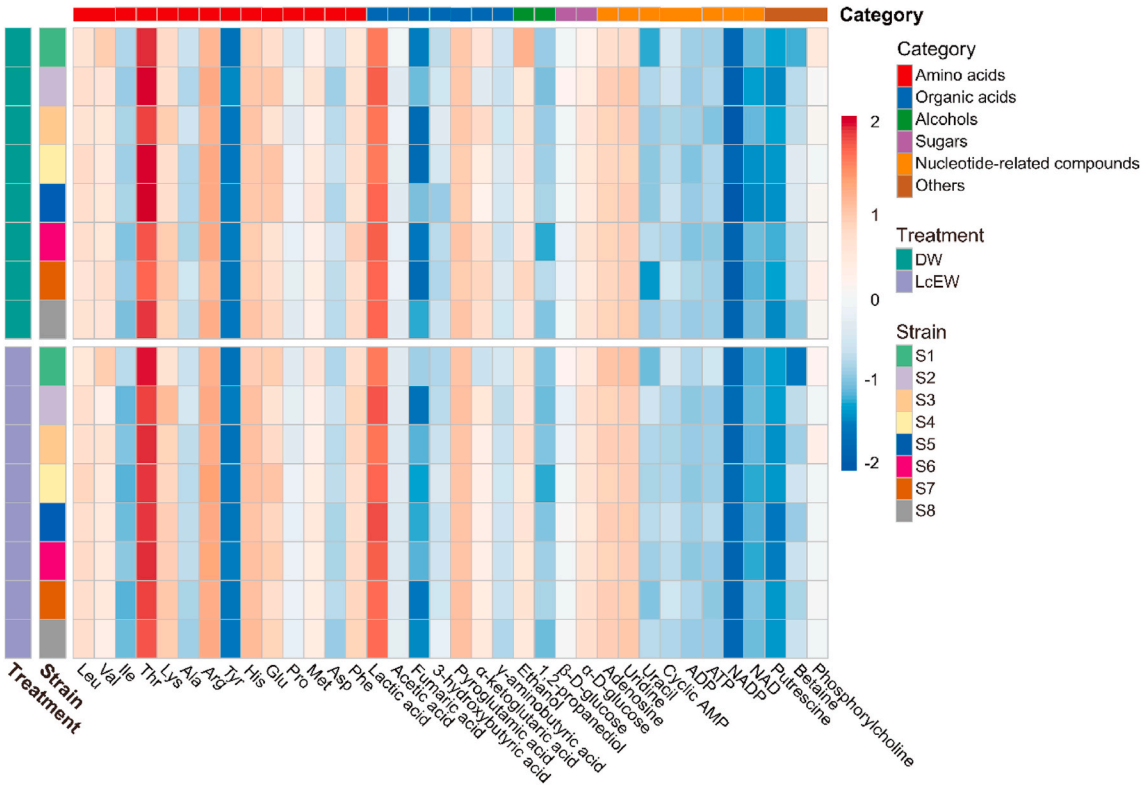

Fig. 2. Heatmap of identified metabolites in *E. coli* strains from deionised water (DW)-treated and low concentration electrolysed water (LcEW)-treated pea sprouts. Note: S1: ATCC 25922; S2: O26:H11 (ATCC BAA-2196); S3: O45:H2 (ATCC BAA-2193); S4: O103:H11 (ATCC BAA-2215); S5: O111 (ATCC BAA-2440); S6: O121:H19 (ATCC BAA-2219); S7: O145 (ATCC BAA-2192); S8: O157:H7 (ATCC 43895).

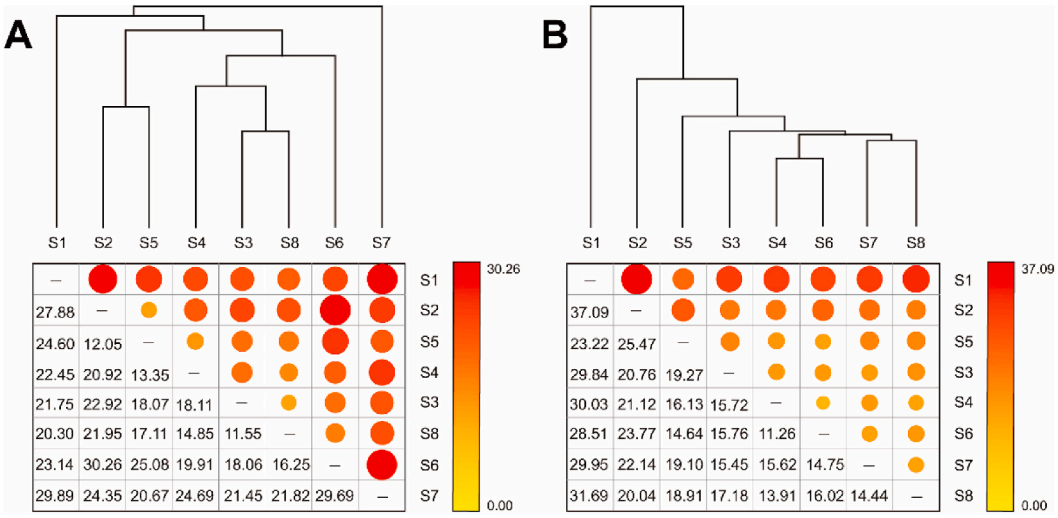

Fig. 3. Euclidean distances and hierarchical cluster analysis (HCA) among eight *E. coli* strains from deionised water (DW)-treated pea sprouts (A) and low concentration electrolysed water (LcEW)-treated pea sprouts (B). Note: S1: ATCC 25922; S2: O26:H11 (ATCC BAA-2196); S3: O45:H2 (ATCC BAA-2193); S4: O103:H11 (ATCC BAA-2215); S5: O111 (ATCC BAA-2440); S6: O121:H19 (ATCC BAA-2219); S7: O145 (ATCC BAA-2192); S8: O157:H7 (ATCC 43895).

contents (e.g. cyclic AMP and uracil) mentioned above. Interestingly, the three pathogenic strains, S3 (O45:H2, ATCC BAA-2193), S4 (O103:H11, ATCC BAA-2215) and S8, which were found in the same cluster, were also those demonstrating relatively high resistance to LcEW in *3.1*; the similar metabolic compositions might have offered them similarly high level of protection against LcEW.

The Euclidean distances range from 11.26 to 37.09 in the LcEW-treated group (Fig. 3B). The observations that S1 showed the longest overall Euclidean distance to the others and was separated from the main cluster in HCA implied the huge metabolic differences between pathogenic and non-pathogenic *E. coli* strains. Similar results were also documented in previous studies. Chen et al. (2020)' s study using the same eight strains illustrated clear separation of *E. coli* ATCC 25922 from the others in metabolomic analysis. In addition, Monk et al. (2013) reported markedly different metabolic capabilities between non-pathogenic *E. coli* K12 and pathogenic serotypes according to their simulated growth phenotypes. Unlike that in the control group, no obvious sub-clustering was observed in strains underwent LcEW exposure, which revealed that the varying defence mechanisms to antagonist the stress might have induced diverse metabolic alterations to differentiate strains that were originally closely clustered.

3.3. Alterative metabolites in E. coli strains in pea sprouts under LcEW stress

The acquired metabolic profiles of each *E. coli* strain in LcEW-treated and control samples were subject to pairwise comparison using OPLS-DA to determine the biochemical alterations induced by LcEW. All OPLS-DA models established demonstrated good predictability and interpretability based on the R^2X and Q^2 values (Table S2). Each pair was clearly separated on the score plots (Fig. 4 A1-H1), suggesting that the antimicrobial effects of LcEW were visible at the metabolic level (Zhao et al., 2020). The loading S-lines (Fig. 4A2-H2) plotted based on the correlation coefficient values highlighted the potential discriminative metabolites in warm colours. Upward and downward peaks indicated higher relative contents in the LcEW-treated strain and the control strain, respectively.

Within this pool of discriminative metabolites, the most critical ones with an absolute correlation coefficient >0.602, a VIP >1 and a P value < 0.05 were screened out and summarised in Table 1 and Table S3, which represented the significantly altered metabolites under the LcEW stress. In total, 7, 14, 9, 5, 13, 9, 15 and 16 metabolites were significantly altered in S1 – S8, respectively. As can be seen, LcEW primarily led to declined metabolite contents in *E. coli*. Specifically, except for S1 (ATCC 25922) and S6 (O121:H19, ATCC BAA-2219), most strains demonstrated a decreasing trend in the level of amino acids (e.g. isoleucine, methionine, proline, tyrosine, glutamic acid and aspartic acid) after LcEW treatment, with just variations in which amino acids decreased. Moreover, following LcEW, most strains were also highlighted with significantly lowered amounts of organic acids [e.g. fumaric acid, acetic acid, α-ketoglutaric acid and γ-aminobutyric acid (GABA)]. As for the variation in alcohols, except for the elevated 1,2-propanediol content in S6, the level of 1,2-propanediol and ethanol either decreased or remained unaffected in the other strains. Besides, in concord with the mainstream metabolites' declination tendency, β-D-glucose, secondary metabolites (e.g. phosphorylcholine, betaine, putrescine) as well as a group of nucleotide-related compounds also underwent significant reduction in most strains whereas their contents were reserved in S6 and the non-pathogenic S1.

S6 demonstrated the most unique pattern of metabolite variation, opposing the metabolites decreasing pattern observed in most other strains (Table 1). Among the nine metabolites significantly varied after the LcEW treatment, only two organic acids (acetic acid and α-ketoglutaric acid) were down-regulated whereas the other seven metabolites, mainly amino acids (leucine, isoleucine, glutamic acid, proline, histidine), were uniformly up-regulated. This echoed what are shown in Fig. 2 that the level of isoleucine, glutamic acid and 1,2-propanediol in S6 raised from the lowest in the native environment to about the average under the LcEW stress among the eight strains. Research has shown that when confronted with detrimental stresses, many metabolites in *E. coli* cells are consumed at an accelerated rate to support normal physiological functions (Ye et al., 2012). Therefore, we hypothesize that an initial accumulation step may be present which allows the metabolites to mount to a threshold level for joining the defence mechanism. It was likely that S6 cells were at this accumulation step when they were subject to metabolites extraction.

Strain S1 also showed a distinctive metabolite variation pattern. Unlike most other strains, it retained the level of sugars, nucleotide derivatives, putrescine and phosphorylcholine during LcEW sanitisation while accumulating the intracellular amino acids. The huge disparity with other strains at the metabolic level was not beyond expectation since S1 was the only non-pathogenic strain analysed in the study. In addition, as the difference in metabolomic alteration pattern did not make S1 more vulnerable to LcEW attack (Fig. 1), presence of alternative

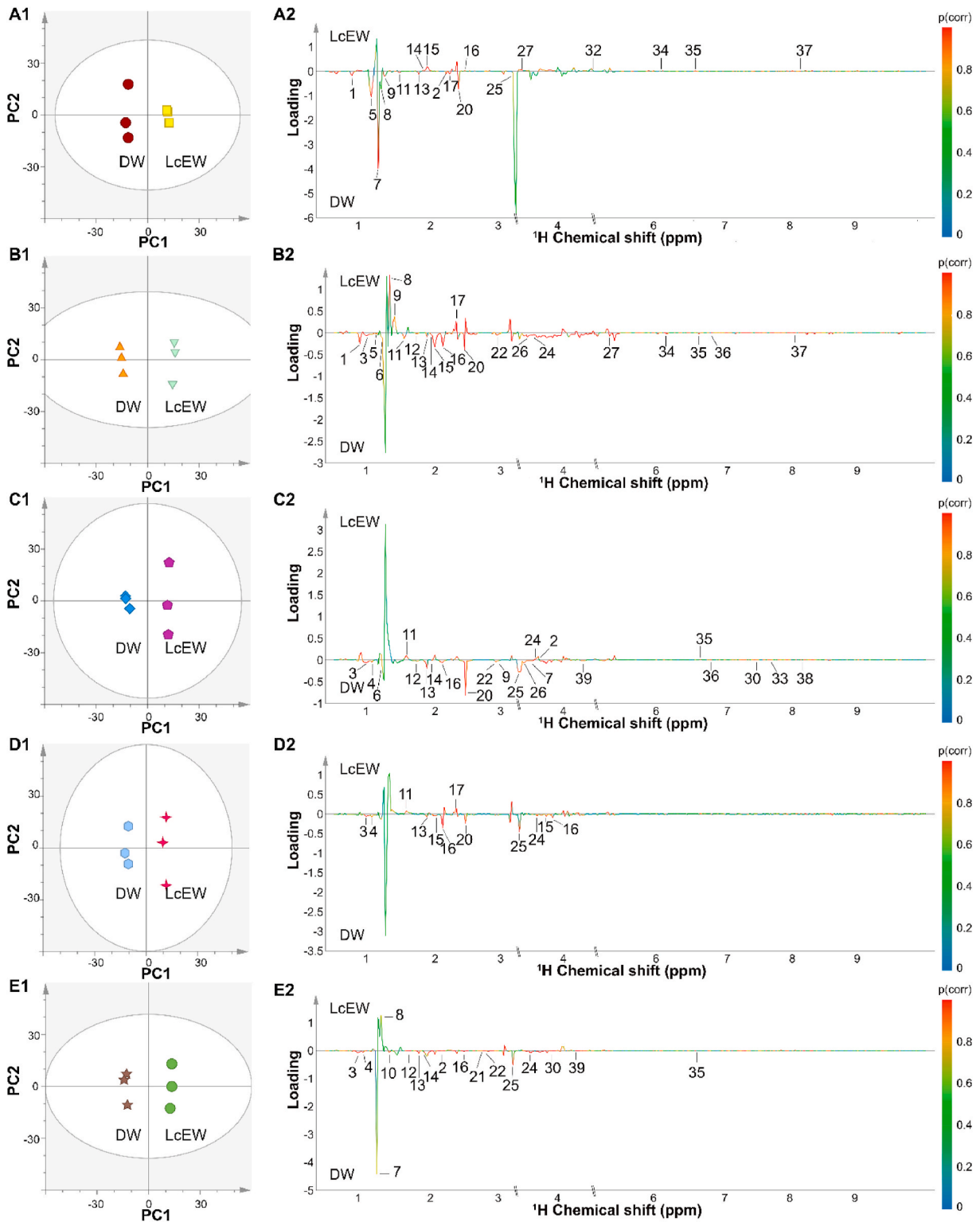

Fig. 4. Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) comparison results of each *E. coli* strain from deionised water (DW)-treated and low concentration electrolysed water (LcEW)-treated pea sprouts. Score plot (A1) and coefficient-coded loading plot (A2) of S1 (ATCC 25922); Score plot (B1) and coefficient-coded loading plot (B2) of S2 (O26:H11, ATCC BAA-2196); Score plot (C1) and coefficient-coded loading plot (C2) of S3 (O45:H2, ATCC BAA-2193); Score plot (D1) and coefficient-coded loading plot (D2) of S4 (O103:H11, ATCC BAA-2215); Score plot (E1) and coefficient-coded loading plot (E2) of S5 (O111, ATCC BAA-2440); Score plot (F1) and coefficient-coded loading plot (F2) of S6 (O121:H19, ATCC BAA-2219); Score plot (G1) and coefficient-coded loading plot (G2) of S7 (O145, ATCC BAA-2192); Score plot (H1) and coefficient-coded loading plot (H2) of S8 (O157:H7, ATCC 43895).

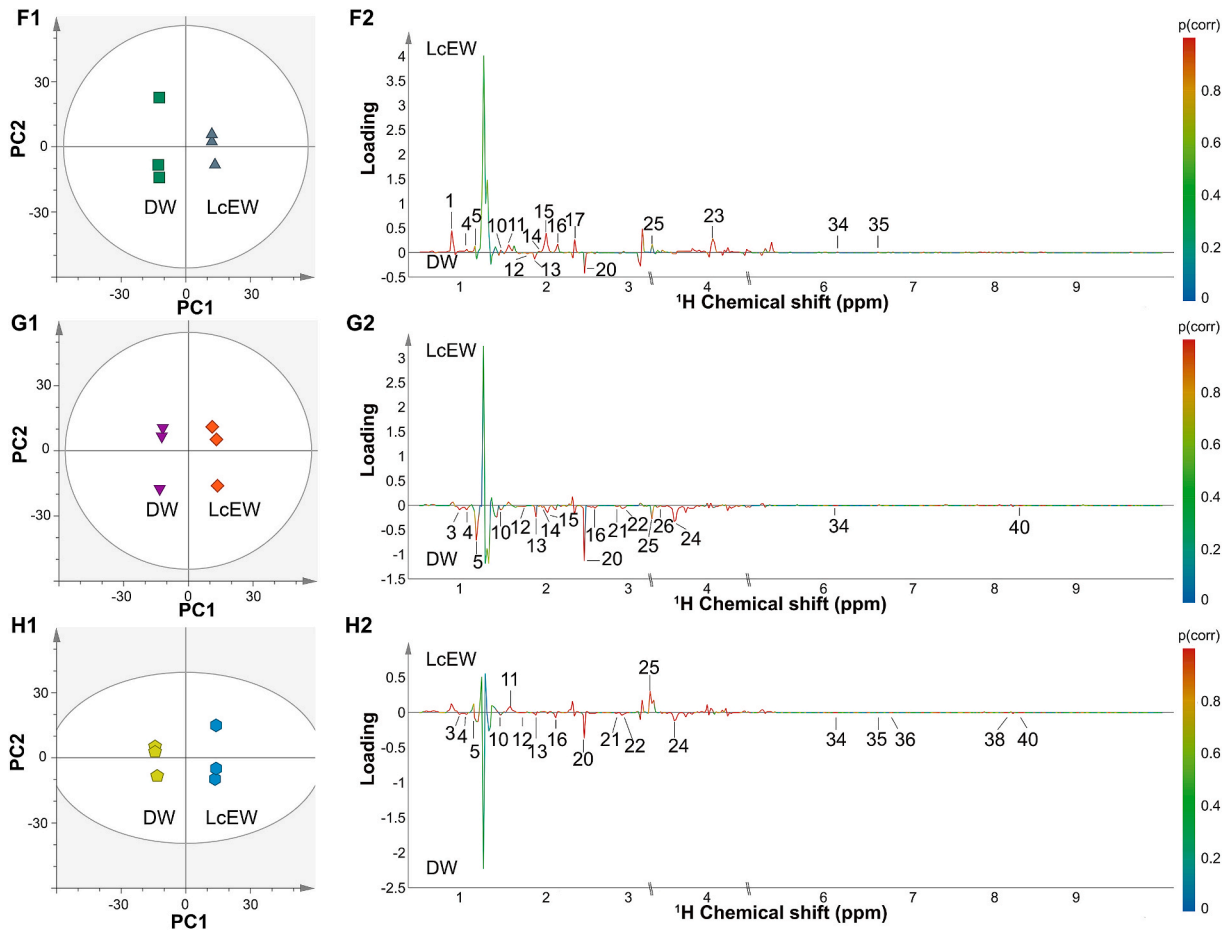

Fig. 4. (*continued*).

stress defence mechanisms in S1 was highly suspected. It is well-known that non-pathogenic *E. coli* serotypes constitute part of the mammalian gut microbiota and can protect the host from pathogen colonisation (Stromberg et al., 2018). Moreover, they also play a role in providing humans with life-long vitamin K and B_{12} as they are amongst the initial colonising gut microbiota for infants (Blount, 2015; Eggesbø et al., 2011). In this case, an effective yet distinctive stress defence system is of priority to assist the non-pathogens survive and prosper when exposed to antimicrobial treatments so that they can continue exerting health benefits to humans.

It is also noteworthy that S4 (O103:H11, ATCC BAA-2215) illustrated minimal metabolic changes, with only five compounds (isoleucine, methionine, α-ketoglutaric acid, betaine and phosphorylcholine) varied during sanitisation. The low extent of metabolic changes reflected low sensitivity of S4 to LcEW as compared to the others. Hence, it was unsurprising that S4's cell reduction was the mildest among the eight strains (Fig. 1). A previous study which challenged the same eight serotypes at pH 3.7 and 3.2 showed that *E. coli* O103 was the least affected at low pH (Kim et al., 2015); this low sensitivity to acidic stress could at least partially explain for S4's low susceptibility to LcEW. Analogously, LcEW elicited high magnitude of metabolic responses in S2 (O26:H11, ATCC BAA-2196), S5 (O111, ATCC BAA-2440), S7 (O145, ATCC BAA-2192) and S8 (O157:H7, ATCC 43895), as indicated by the remarkable variation of an array of metabolites, which uncovered at the metabolic level why the four strains were associated with high extent of cell reduction of over 1.0 log CFU/g. In general, a negative correlation between the resistance to LcEW and the magnitude of metabolic change could be concluded.

3.4. Alterative metabolic pathways of *E. coli* in pea sprouts

Pathway analysis was performed to excavate the interference in each strain's metabolic networks under LcEW stress. Based on the significant discriminative metabolites screened out in *3.3*, all affected pathways were predicted by MetaboAnalyst 5.0 and those showing $P < 0.05$ were regarded to be key in the antimicrobial process, summarised in Table S4 and marked as circles in Fig. 5. With few metabolites altering, S4 had only one pathway significantly affected, being aminoacyl-tRNA biosynthesis, a pathway in amino acid metabolism responsible for correct pairing of an amino acid with its cognate tRNA. Significant alteration of this pathway was also observed in the rest of strains except for S3. Apart from aminoacyl-tRNA biosynthesis, two more pathways, alanine, aspartate and glutamate metabolism and arginine biosynthesis, were also perturbed uniformly in seven strains (S1, S2, S3, S5, S6, S7 and S8), followed by arginine and proline metabolism and butanoate metabolism, which were significantly influenced in six and five strains, respectively (S1, S2, S5, S6, S7 and S8; S2, S5, S6, S7 and S8). Additionally, glycolysis/gluconeogenesis, pyruvate metabolism as well as citrate cycle (TCA cycle) were each significantly affected in four strains (S1, S2, S3 and S7; S2, S3, S5 and S6; S2, S3, S6 and S8).

Based on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database, an assumptive schematic map showing the metabolic changes of all eight strains under LcEW exposure are summarised in Fig. 6. Metabolites with arrows pointing upwards or downwards represent higher or lower contents in the LcEW-treated strain compared with the control strain, respectively. The disturbance in metabolic status and relevant pathways among S2 (O26:H11, ATCC BAA-2196), S5 (O111, ATCC BAA-2440), S7 (O145, ATCC BAA-2192) and S8 (O157:H7, ATCC 43895) were found to be relatively consistent.

Table 1
Metabolites significantly altered in each *E. coli* strain after 10-min low concentration electrolysed water (LcEW) (free available chlorine = 4 mg/L) treatment. Note: S1: ATCC 25922; S2: O26:H11 (ATCC BAA-2196); S3: O45:H2 (ATCC BAA-2193); S4: O103:H11 (ATCC BAA-2215); S5: O111 (ATCC BAA-2440); S6: O121:H19 (ATCC BAA-2219); S7: O145 (ATCC BAA-2192); S8: O157:H7 (ATCC 43895).

Metabolite category	Metabolite	Strains showing significant increase (coefficient > 0.602; VIP > 1; $P < 0.05$)	Strains showing significant decrease (coefficient < -0.602; VIP > 1; $P < 0.05$)
Amino acids	Ile	S6	S2; S3; S4; S5; S7; S8
	Met	S1; S4	S2; S5; S7; S8
	Pro	S1; S6	S2; S5; S7
	Tyr		S2; S3; S8
	Glu	S1; S6	S2; S7
	Leu	S6	S1; S2
	Asp		S5; S8
	Val	S3	S5
	Ala		S7
	His	S6	
	Arg	S8	
Organic acids	Acetic acid		S1; S2; S3; S5; S6; S7; S8
	α-ketoglutaric acid	S2	S1; S3; S4; S6; S7; S8
	Fumaric acid	S3; S6	S2; S5; S8
	γ-aminobutyric acid		S5; S7; S8
	3-hydroxybutyric acid		S3
Alcohols	1,2-propanediol	S6	S3; S5; S7; S8
	Ethanol		S1; S7
Sugar	β-D-glucose		S2; S3; S7
Nucleotide-related compounds	ATP		S2; S7; S8
	NAD		S3; S8
	ADP		S7; S8
	NADP		S2
	Cyclic AMP		S5
Others	Phosphorylcholine		S2; S4; S5; S7; S8
	Betaine	S8	S4; S5; S7
	Putrescine		S5; S8

Concomitant with the observed amino acids exhaustion, amino acid metabolism was no doubt the most influenced pathways in S2, S5, S7 and S8. Under LcEW sanitisation, amino acids catabolism was more favoured than anabolism in these strains; this might be due to that the amino acid anabolic process is very sensitive towards hostile factors, such as oxidation, acid and heat (Jozefczuk et al., 2010). Besides, some enzymatic reactions in amino acid catabolism are likely promoted in acidic environments, since the reactions are effective in consuming protons and producing alkaline compounds so as to buffer the low pH (Richard & Foster, 2004). Decarboxylation is one of such reactions; catalysed by decarboxylases, free amino acids are converted into biogenic amines to counter acidity and increase survival (Ferrario et al., 2014; Kanjee & Houry, 2013; Álvarez-Ordóñez et al., 2010). qRT-PCR experiments have confirmed that the expression of genes encoding lysine decarboxylase (*ldc*), histidine decarboxylase (*hdc*) and glutamic acid decarboxylase (*gadA*) were highly induced at acidic pH (Ferrario et al., 2014; Liu et al., 2020; Park et al., 2017). Deamination reactions catalysed by deaminases are also vital for withstanding acidic stress, through which NH_3 is released from amino acids into the cytoplasm and the intracellular pH is raised (Pennacchietti et al., 2018; Sun et al., 2012). In addition to the low pH which required enzymatic catalysation of amino acids, the FAC led to further exhaustion of amino acids in the strains. Hypochlorous acid (HOCl), for instance, is especially potent against the sulphur-containing amino acids (da Cruz Nizer et al., 2020), which resulted in the reinforced reduction of methionine in S2, S5, S7 and S8. Due to the fact that multiple amino acids are natural osmotic regulators, such as alanine, proline, glycine and glutamic acid (Khan et al., 2010; Wiesenthal et al., 2019; Wu et al., 2021), as a consequence of the expedited amino acid depletion, there could be insufficient osmolytes in maintaining the cytoplasmic osmolality, possibly progressing to subcellular structures breakdown and even cell death.

Contrary to the majority, S6 (O121:H19, ATCC BAA-2219) and S1 (ATCC 25922) illustrated more defined amino acid anabolism than catabolism during LcEW sanitisation. As were discussed in *3.3*, possible rationales were that S6 may have to make up for the originally low amino acid contents first before expending them in defence whereas S1 may not rely on mechanisms that depleted amino acids to resist the LcEW stress. Amino acid metabolism in the other two strains, S4 (O103:H11, ATCC BAA-2215) and S3 (O45:H2, ATCC BAA-2193), were barely affected, with only one and two amino acids significantly decreased, respectively. The high retention of amino acids in the two strains guaranteed the sufficiency of osmolytes for osmolality maintenance. Meanwhile, as LcEW did not initiate the amino acids catabolism-based defencing mechanisms, the high LcEW resistance of S3 and S4 was once again evidenced, which offered metabolic basis for their low cell mortality shown in *3.1*.

Amino acid metabolism provides carbon skeletons entering the TCA cycle. After the LcEW treatment, significant depletion of fumaric acid or α-ketoglutaric acid was observed in all strains, which implied that varying extent of TCA cycle impairment occurred. Simultaneously, a marked decline of GABA was observed in S5, S7 and S8. As GABA can be converted to succinic acid via the GABA shunt (Watanabe et al., 2002), its loss could result in a lower amount of succinic acid fed into the TCA cycle, which further attenuated the pathway, causing inadequate energy supply and even cell death. Consequently, measures had to be taken by the strains to compensate for the short of energy during LcEW sanitisation.

On the one hand, strains may turn to non-TCA pathway for ATP replenishment. S2 and S7 were two representative strains adopting this approach. Evidenced by the parallel reduction in ATP and β-D-glucose contents, they were believed to use glucose as the core energy source and glycolysis as the main energy production pathway. *E. coli*'s using glycolysis as the principal pathway for energy release under acidic environments has been diagnosed previously; in the polymerase chain reaction (PCR)-based study (Zhang et al., 2020), the transcription of the glycolysis genes, involving *glk*, *pgi*, *pgk* and *pyk*F, which encode hexokinase, glucose-6-phosphate isomerase, phosphoglycerate kinase and pyruvate kinase I, respectively, was found to be significantly up-regulated in acid-stressed *E. coli*, whereas the transcription of genes involved in fatty acid oxidation, oxidative phosphorylation and oxidation of other organic compounds were down-regulated. On the other hand, strains may compensate for the energy loss by strengthening the TCA cycle. In facultative anaerobes, the end-product of glycolysis, pyruvic acid, can be metabolised downwards either to the aerobic TCA cycle or to the anaerobic mixed acid fermentation (Zhao et al., 2020). From the reduced level of ethanol or acetic acid production in S1, S2, S3, S5, S6, S7 and S8, it can be interpreted that the mixed acid fermentation was repressed and likely switched to the TCA cycle which is considered to be more energy efficient.

Besides the perturbations on energy metabolism, LcEW also exerted strong oxidative stress on the *E. coli* strains, as manifested by the high positive ORP value (Liao et al., 2007). The oxidative stress disequilibrates the intracellular redox balance maintained by intracellular redox couples, such as the glutathione/glutathione disulphide couple, and therefore generates cytotoxicity (Murray et al., 2006). Putrescine, with possible effects of relieving oxidative stress, may be closely related to *E. coli*' s survival under LcEW treatment. According to Tkachenko et al. (2001), through regulation of the key adaptive response regulators (e.g. OxyR, SoxR and SoxS), putrescine indirectly enhances the activity of hydroperoxidase I, alkyl hydroperoxide reductase and glutathione reductase so as to eliminate reactive oxygen species and restore the reduced form of the redox couples in the cell. Elevated expression of

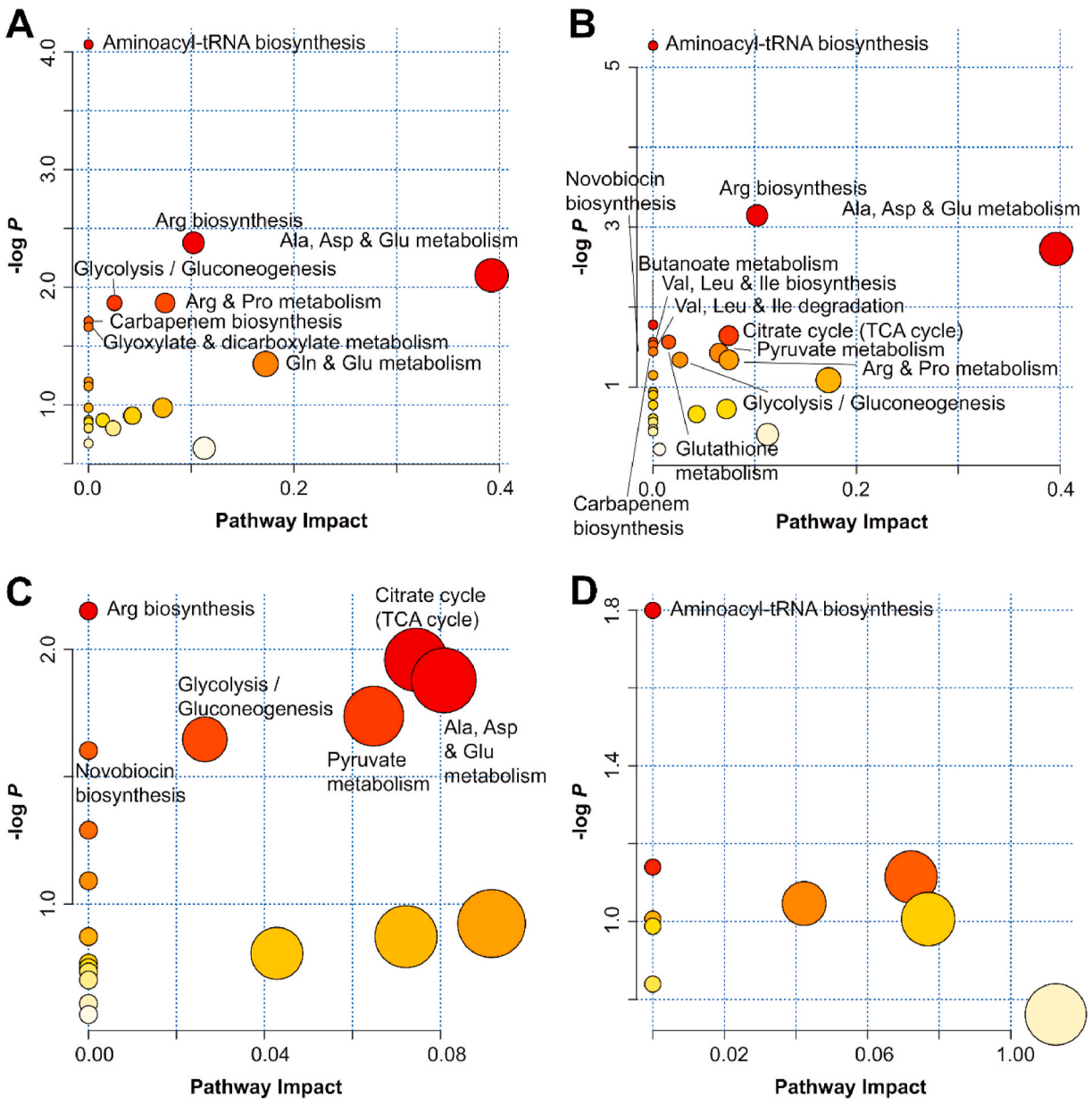

Fig. 5. Metabolic pathways altered by low concentration electrolysed water (LcEW) in S1 (ATCC 25922) (A); S2 (O26:H11, ATCC BAA-2196) (B); S3 (O45:H2, ATCC BAA-2193) (C); S4 (O103:H11, ATCC BAA-2215) (D); S5 (O111, ATCC BAA-2440) (E); S6 (O121:H19, ATCC BAA-2219) (F); S7 (O145, ATCC BAA-2192) (G) and S8 (O157:H7, ATCC 43895) (H).

these regulator genes was previously observed in *E. coli* O157:H7 EDL 933 treated with EW (Liu et al., 2020), which provided additional evidence for the importance of putrescine in coping with oxidative stress in *E. coli*. The lowered putrescine level observed in S5 and S8, thus, may indicate a compromised protection against the oxidative stress. In addition, because putrescine plays a role in cell division and is deemed as a growth indicator in bacteria (Ye et al., 2012), the marked reduction in putrescine level also illuminated an inhibited growth of the strains during LcEW treatment. More importantly, putrescine is also the precursor of GABA, which metabolised to GABA via the transaminase pathway and the glutamylated putrescine pathway (Schneider & Reitzer, 2012). The decrease in putrescine, therefore, would lead to the decrease in GABA, which finally progressed to the impaired TCA cycle that was mentioned above.

Phosphorylcholine, a key membrane phospholipid precursor (Geiger et al., 2013), experienced significant decline in S2, S4, S5, S7 and S8 during the LcEW application. Being involved in bilayer formation and membrane protein folding, phosphorylcholine is crucially responsible for maintaining cell membrane integrity (Bogdanov et al., 1999; Sohlenkamp et al., 2003); its depletion, thus, indicated a weakened membrane structure of specific *E. coli* strains. Such membrane damage could be a joint consequence of the FAC and the high ORP within LcEW; FAC was found to exert its effect by eliciting blebs and breaks in the outer membrane of bacteria (Kiura et al., 2002), while a high ORP further enhances the permeability of the outer and inner cell membranes by oxidation so as to penetrate the protective sphere of bacteria (Liao et al., 2007). In a previous study based on atomic force microscopy (AFM), morphological changes of *E. coli* cells, including traumas on the membrane structure and roughened membrane surface, were observed after the LcEW treatment (Liu, Wu, et al., 2017), which ulteriorly evidenced the destruction of LcEW on *E. coli* membranes. Besides, phosphorylcholine can be metabolised to betaine, the most active naturally

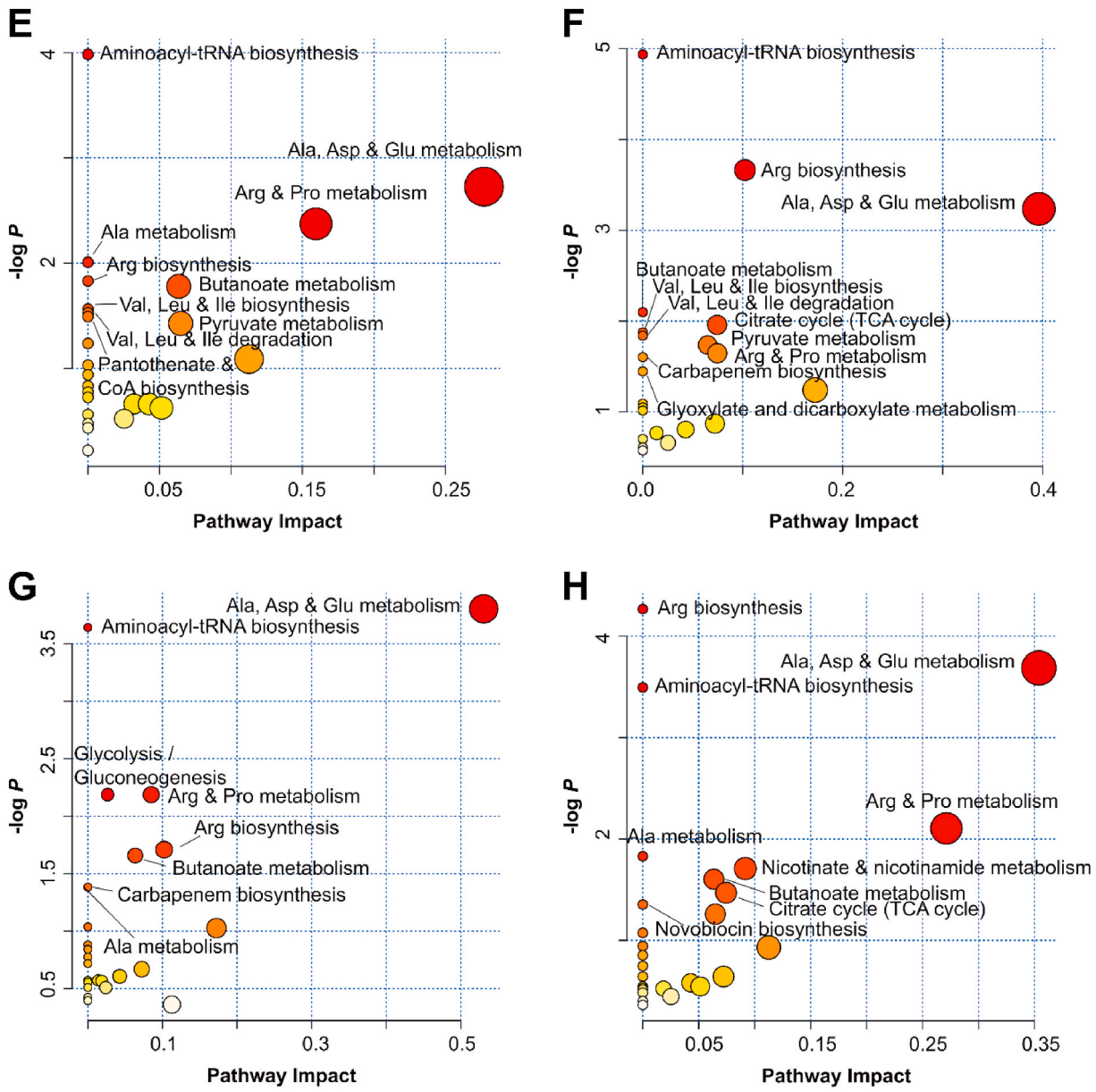

Fig. 5. (*continued*).

occurring osmoprotectant molecule in *E. coli*, via formation of choline as intermediate (Ly et al., 2004; Zhao, Zhao, Phey, & Yang, 2019). In consequence of phosphorylcholine deficiency, production of betaine in S2, S4, S5 and S7 was significantly or insignificantly stagnated. Except for S4 which retained sufficient amino acids as alternative osmolytes for osmotic homeostasis maintenance, the loss of betaine severely diminished the osmoprotection ability in the other strains, which consequently led to the vast cell reduction observed in S2, S5 and S7.

In contrast to the pathogenic strains, S1 maintained the level of all beforementioned compounds relevant to oxidation protection, osmoprotection and membrane formation during the LcEW treatment. This once again confirmed the presence of inherent distinctive stress defence mechanisms in the non-pathogenic strain to help it survive stresses intended to kill pathogens.

4. Conclusion

In LcEW, FAC, low pH and high ORP collaborate with each other to launch a powerful attack against *E. coli* cells. In this work, the metabolic responses of eight *E. coli* strains in pea sprouts underlying LcEW's sanitisation efficacy were studied and compared. S4 (O103:H11, ATCC BAA-2215) and S3 (O45:H2, ATCC BAA-2193) showed the lowest cell reduction under LcEW exposure; their low sensitivity to the antimicrobial process was also indicated by the mild metabolic profile variations. At the opposite extreme, S2 (O26:H11, ATCC BAA-2196) and S7 (O145, ATCC BAA-2192) represented the most sensitive strains to the LcEW treatment, which were characterised by depleted levels of a wide range of metabolites. Further analysis revealed that the metabolite changes were associated with perturbed amino acid metabolism, weakened TCA cycle, expedited glucose catabolism as well as impaired protection against oxidative and osmotic stresses. Similar patterns of metabolic alterations were also observed in S5 (O111, ATCC BAA-2440) and S8 (O157:H7, ATCC 43895). The uniformity in metabolic responses suggested the potential of using a universal sanitiser to inhibit several *E. coli* strains simultaneously. The only exceptions were S6 (O121:H19, ATCC BAA-2219) and the non-pathogenic S1 (ATCC 25922), which presented strain-specific metabolic variation patterns under LcEW. Nonetheless, a negative correlation between the resistance to LcEW and the magnitude

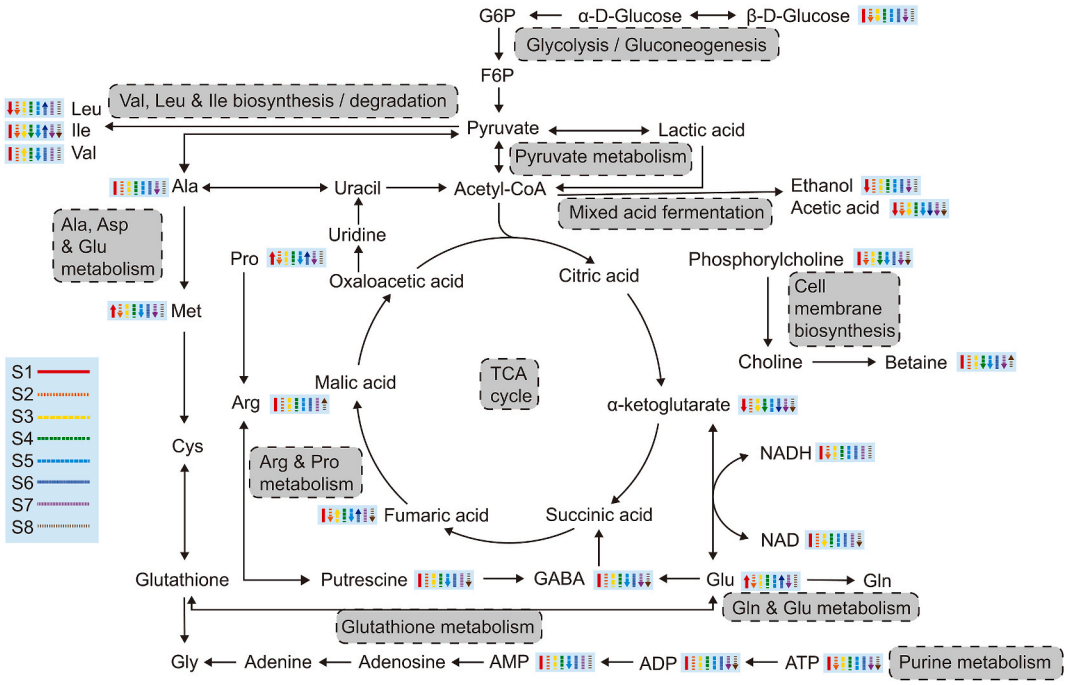

Fig. 6. Proposed schematic of metabolic alterations by low concentration electrolysed water (LcEW). Note: S1: ATCC 25922; S2: O26:H11 (ATCC BAA-2196); S3: O45:H2 (ATCC BAA-2193); S4: O103:H11 (ATCC BAA-2215); S5: O111 (ATCC BAA-2440); S6: O121:H19 (ATCC BAA-2219); S7: O145 (ATCC BAA-2192); S8: O157: H7 (ATCC 43895).

of metabolic variation was well demonstrated in the study. Overall, the study sheds light on the effectiveness of NMR-based metabolomics to illuminate the antimicrobial mechanisms of LcEW on eight *E. coli* strains, which provides guidance for the food industry to control "big six" contamination in fresh produce.

CRediT authorship contribution statement

Yue Wang: Conceptualization, Methodology, Investigation, Software, Resources, Visualization, Writing – original draft. **Ji'en Wu:** Software, Validation, Writing – review & editing. **Hongshun Yang:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2021.108458.

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