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Sustainability spotlight

Fresh strawberries are prone to contamination and spoilage, and current washing practices still rely heavily on chlorine-based sanitisers that raise safety and environmental concerns. This work develops a mild, food-grade disinfection strategy that combines ultrasound with levulinic acid to inactivate multiple bacterial and viral surrogates on strawberries while preserving firmness, colour and antioxidant capacity, thereby extending shelf life and reducing food loss. Mechanistic insights from NMR based metabolomics and oxidative stress assays show the effective pathogen control can be achieved without intensive heating or high chemical loads. This approach aligns with the UN Sustainable Development Goals aiming for safe food, clean water use and responsible consumption and production.

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Insights into Pathogen Inactivation and Shelf-Life Extension of Strawberries via Combined Ultrasound and Levulinic Acid Treatment

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Abstract

This study investigated the bactericidal effects of levulinic acid (LVA) and ultrasound against *Listeria monocytogenes* and analysed the corresponding metabolic response posttreatment via nuclear magnetic resonance (NMR) spectroscopy. The results suggested that the combined LVA and ultrasound treatment affected amino acid metabolism, energy metabolism, and osmotic and oxidative stress defense mechanisms in *L. monocytogenes*. This finding was supported by the reactive oxygen species (ROS) assay, which confirmed that the combined treatment intensified oxidative stress, leading to disrupted membrane integrity. To assess the broader antimicrobial efficacy of this combination and application potential in food systems, the treatment was further evaluated on fresh strawberries using representative bacterial and viral surrogates. On fresh strawberries, the combined treatment reduced *L. monocytogenes* and all other tested bacterial groups to undetectable levels after washing (< 2 log CFU/g). Significant inactivation was also observed for Tulane virus and bacteriophage MS2 ($P < 0.05$). Additionally, the application of ultrasound and LVA improved the physicochemical quality of strawberries during storage and significantly enhanced their antioxidant enzyme activities. This study highlights the potential of this combined treatment approach as a promising disinfection and preservation method for strawberries.

Keywords: Fresh produce; strawberries; ultrasound; levulinic acid; metabolomics; food safety

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1. Introduction

Strawberries are highly popular on the market because of their vibrant color, sweet-tart flavour, and rich nutrient content, with the fresh strawberry market projected to reach $27.82 billion by 2031.[1] Strawberries are often consumed unwashed or lightly processed, with minimal mitigation of microbial contamination occurring during cultivation, harvest, and postharvest handling.[2] Poor worker hygiene, contaminated irrigation water, and close contact with soil are the primary sources of contamination by pathogens such as *Salmonella*, pathogenic *Escherichia coli*, *Listeria monocytogenes*, and human norovirus (hNoV).[3, 4] These factors contribute to their role as carriers of pathogens and the association of strawberries with disease outbreaks. In recent years, multiple outbreaks of foodborne diseases linked to strawberries have been reported. For example, an outbreak of *E. coli* O157:H7 in 2011 was attributed to contaminated strawberries in Oregon, resulting in 15 confirmed cases and 2 fatalities.[5] In 2012, a large-scale outbreak of hNoV gastroenteritis in Germany affected more than 10,000 children, with the source identified as contaminated frozen strawberries.[6]

Postharvest washing and disinfection are critical interventions throughout the farm-to-table process to remove field contaminants and pathogens from fresh produce. Notably, under practical postharvest conditions, washing often achieves limited pathogen reductions on strawberries.[7] Meanwhile, wash water has been identified as an important vehicle for the transmission of microbial contaminants in fresh produce.[8] Therefore, the incorporation of antimicrobial agents into washing water has been studied for commercial produce washing operations.[8] Chemical sanitizers such as chlorine-based

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disinfectants and peroxides have been proposed for such purposes.[9, 10] However, due to concerns about the potential adverse effects of chemical residues on strawberry quality, human health, and/or the environment, interest in exploring safer and more sustainable sanitization methods is increasing. Strategies are needed to better balance fruit decontamination, process water hygiene, and strawberry quality under practical operating conditions.

Ultrasound, an emerging nonthermal sterilization technology, offers several advantages, including high efficiency and environmental friendliness.[11] Cavitation is widely recognized as the primary mechanism through which ultrasound eliminates bacteria in liquids.[12] Studies have shown that ultrasound affects a variety of bacteria and is dependent on power intensity and treatment duration. However, higher power levels and longer treatment times may adversely impact food quality and incur higher costs.[13, 14] On the other hand, at lower intensities, the bactericidal effect of ultrasound is relatively limited.[15] Studies have shown that the limited sterilization efficacy of ultrasound is due to the hydrophobic cavitation bubbles generated during cavitation effects, which tend to detach from the hydrophilic bacterial surface upon collapse.[16] Therefore, combining ultrasound with chemical sanitizers might be an option to develop more sustainable, safe, efficient, and cost-effective methods for broader applications.[17, 18]

Organic acids are widely utilized in the food industry as safe and cost-effective sanitizers. Previous studies have demonstrated their efficacy in inactivating foodborne pathogens on fresh produce.[19] Levulinic acid (LVA), classified as "generally

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recognized as safe" (GRAS) by the U.S. Food and Drug Administration (FDA), has received relatively less attention. Most existing research has focused on the use of LVA in combination with sodium dodecyl sulfate (SDS) as an antimicrobial agent for food preservation, while studies exploring its combination with physical treatment methods are limited.[7, 20]

On fresh produce, treatment efficacy is strongly influenced by surface microstructure, as microorganisms can persist within crevices and other protected niches, thereby reducing the uniformity of antimicrobial exposure under quality-preserving conditions.[22] The antimicrobial activity of organic acids is generally attributed to membrane permeation of the undissociated form, followed by intracellular dissociation and cytoplasmic acidification, making their efficacy dependent on sanitizer transport and effective contact at the produce surface.[23] In this context, ultrasound may offer a practical approach to enhance LVA delivery to attached cells on the complex surface of strawberries; however, evidence supporting this specific combination, along with mechanistic insight and post-treatment quality performance during storage, remains limited.

This study aimed primarily to evaluate the disinfection efficacy of ultrasound in combination with LVA against various pathogens on strawberries. To elucidate the antibacterial mechanisms underlying this combined disinfection approach, *L. monocytogenes* was studied extensively by assessing its membrane permeability damage, enzymatic activity, and metabolic alterations. Additionally, during an 8-day storage period, we investigated the effects of these treatments on the physicochemical

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properties of strawberries and their intracellular enzyme activities. As an innovative food preservation technique, the findings of this study are expected to contribute to the development of a promising disinfection method to increase the safety of fresh produce.

2. Materials and methods

2.1 Bacterial and viral strain preparation

Generic *E. coli* (ATCC 25922), *E. coli* O157:H7 (ATCC 35150) and *L. monocytogenes* (serotype 4b) that were initially isolated from smoked salmon, were obtained from the Department of Food Science & Technology, National University of Singapore.[21] *S.* Typhimurium (ATCC 14028), and *S.* Enteritidis (ATCC 13076) were obtained from the American Type Culture Collection (ATCC; Rockville, Maryland, USA). The samples were stored in 15% (v/v) glycerol stocks at −80 °C. The bacteria were transferred to tryptic soy broth (TSB, Oxoid, UK) and incubated overnight at 37 °C for resuscitation. The bacteria were subsequently cultured overnight on tryptic soy agar (TSA, Oxoid, UK) at 37 °C to isolate single colonies, which were then passaged in TSB for subsequent use.

MS2 (*E. coli* bacteriophage 15597-B1TM) and *E. coli* 15597 were obtained from ATCC. The lyophilized MS2 was reconstituted according to the product instructions and propagated via *E. coli* 15597. Tulane virus, kindly provided by Professor Xi Jiang from Cincinnati Children's Hospital Medical Center (Cincinnati, OH, USA), was propagated and assayed in the monkey kidney cell line LLC-MK2 (ATCC® CCL-7™), following the method described by.[22]

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2.2 Effects of different antimicrobial treatments on *L. monocytogenes*

The bacterial pellets were washed and prepared to a final concentration of approximately 10^7 CFU/mL in deionized (DI) water. Ultrasound treatment was performed via an ultrasonic crusher (20 kHz, 950 W, Scientz-II D; Ningbo Scientz, Zhejiang, China) equipped with a 6 mm diameter probe. The bacterial suspension (10 mL) was added to a 50 mL Falcon tube, and the probe was immersed 1 cm below the suspension surface. Additionally, to standardize the probe's initial thermal condition and avoid heat accumulation from previous runs, the ultrasound probe was pre-cooled by immersion in an ice bath for 30 seconds prior to each treatment.

Ultrasound has been reported to exhibit antibacterial activity; however, prolonged exposure may adversely affect food matrices, leading to quality deterioration and nutrient loss.[23] For the ultrasonic instrument used in this study, preliminary experiments revealed that the firmness of strawberries remained comparable to that of the control group following up to 15 min of treatment, which was selected as the treatment condition for the following study. After more than 15 min of ultrasonic processing, the hardness of the strawberries decreased slightly (data not shown).

Previous studies have reported that high concentrations of LVA (>5%) may result in residues exceeding the threshold of toxicological concern (TTC) of 1.8 mg/day.[7] More recent investigations have therefore employed lower LVA concentrations, primarily focusing on antimicrobial efficacy. For instance, Zhao *et al*.[26] used a concentration of 3%, whereas Chen *et al*.[27] applied levels ranging from 0.5% to 3%. In the present study,

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preliminary experiments demonstrated that, relative to the control, the bactericidal effect of LVA against *L. monocytogenes* became evident at concentrations above 1% (data not shown). Based on these results, 1% LVA (pH 2.3 ± 0.1) was selected as the working concentration to achieve effective microbial inhibition while proactively minimizing the risk of chemical residues.

The ultrasound power intensity was set at 321 W/cm^2 for 15 min (5 s on; 5 s off). During the ultrasonic treatment, the sample container was immersed in an ice-water bath for cooling. After sonication under ice-bath conditions, the sample temperature was 4 ± 2 °C. For the LVA treatment group, LVA (Sigma−Aldrich, USA) was added to the bacterial suspension to achieve a final concentration of 1% (v/v) for 15 min. For the LVA and ultrasound combination treatment, LVA was added to the bacterial suspension at the specified final concentrations, followed immediately by ultrasound treatment as described above. After LVA or the combined treatment, the samples were immediately diluted with 0.1 M phosphate-buffered saline (PBS), a step previously validated to neutralize residual LVA and prevent carry-over inactivation. The neutralized samples were then plated on PALCAM agar (Difco ™ , France) supplemented with Bacto™ Palcam antimicrobic supplement and incubated at 37 °C for 48 h. All microbial counts are expressed as log CFU/mL.

2.3 Metabolomic analysis of *L. monocytogenes*

Metabolomic analysis was performed on *L. monocytogenes* following different treatments (control, US, LVA, and US_LVA) on the basis of the method described by

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Guo *et al.*[24], with minor modifications. The treated *L. monocytogenes* cells were harvested from the solutions via centrifugation at 12,000 × *g* for 15 min (4 °C) to obtain the cell pellets. The collected cell pellets were mixed with 1 mL of ice-cold methanol-d4 (Cambridge Isotope Laboratories, Tewksbury, MA, USA), lysed by three freeze thaw cycles, each consisting of freezing in liquid nitrogen until completely solid followed by thawing on ice until fully melted, and stored overnight at −20 °C. The supernatant, obtained by centrifugation (12,000 × *g*, 20 min, 4 °C), was prepared for NMR analysis by adding trimethylsilyl propanoic acid (TSP, 10 mM in methanol-d4) as an internal standard (final concentration of 1 mM). After vortexing, 600 µL of the mixture was transferred to an NMR tube for analysis. Metabolite extraction from different groups was performed in triplicate.

A Bruker DRX-500 NMR spectrometer (Bruker, Rheinstetten, Germany) was used to scan the samples via a triple inverse gradient probe. The standard Bruker NOESY pulse sequence was used to obtain the ^{1}H spectrum with a width of 10 ppm. The acquisition time was set at 3.3 s, the relaxation delay was set at 2 s, and the temperature was maintained at 25 °C. The obtained spectra of the control, US, LVA (1%), and US_LVA groups were processed via Mestre-nova (Mestreab SL, Santiago, Spain) to produce an organized database for the analysis of multiple variables. The qualitative and quantitative analyses of the metabolites were performed via Chenomx NMR suite software (Chenomx Inc., Canada).

Prior to statistical analysis, metabolite intensities were normalized to the internal standard signal to correct for between sample intensity variation. Univariate screening

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was performed using raw *p* values together with a fold change threshold, and the metabolomics results were interpreted conservatively as exploratory signatures in conjunction with multivariate models and phenotypic assays.

2.4 Determination of intracellular material leakage and enzyme activity of *L. monocytogenes*

The membrane integrity was evaluated according to the concentrations of cell constituents in the supernatant after being subjected to different treatments. The test was designed on the basis of Hai et al. (2022) with modifications. Each treated group of *L. monocytogenes* suspensions was centrifuged (8,000 × *g*, 4 °C, 10 min), and the supernatants were collected for measuring soluble protein and nucleic acid leakage. The leakages of nucleic acids and proteins across all treatment groups were quantified by measuring the absorbance of the supernatants at 260 nm and 280 nm, respectively, via a UV–visible spectrophotometer (UV-2600, Shimadzu, Tokyo, Japan).

Reactive oxygen species (ROS) levels in *L. monocytogenes* were determined using dichlorodihydrofluorescein diacetate (DCFH-DA) as a fluorescent probe.[25] Treated samples were incubated with 5 μM DCFH-DA in the dark for 1 h at 37 °C. After the samples were washed twice with PBS, the fluorescence intensity was recorded at excitation and emission wavelengths of 488 and 525 nm, respectively, via a microplate reader (SpectraMax M2, Molecular Devices, San Jose, CA, USA), and expressed as relative fluorescence units (RFU).

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2.5 Pathogen inoculation and sanitizing treatments of strawberries

Strawberries (Fragaria × ananassa Duch.) imported from Korea were purchased from a local supermarket in Singapore. A total of 20 g of fresh strawberry was inoculated with 200 μL of bacteria or virus suspensions from more than 20 spots and allowed to dry in a biosafety cabinet for 3 h, resulting in loads of approximately 7.0 log CFU/g bacteria or 7.0 log PFU/g virus on the strawberries.

The strawberry samples were treated separately via the following methods: control (immersing in DI water); ultrasound treatment (immersed in DI water, and the probe was immersed 1 cm below the water surface, 15 min with 5 s pulses followed by 5 s pauses); LVA treatment (immersed in LVA solution); and the combination of ultrasound and LVA treatment (immersed in LVA solution, 15 min of ultrasound treatment). Each treatment involved soaking the strawberries in beakers containing 180 mL of the corresponding treatment solutions for 15 min. To control the temperature, ultrasonic and combined treatments were carried out with the sample beaker immersed in an ice-water bath, resulting in a final solution temperature of 4 ± 2 °C.

Additionally, the microbial viability in each treatment mixture was assessed immediately after the experiment. After different treatments, the strawberries were air-dried at room temperature (25 °C) for 30 min, packed into perforated Ziplock bags, and stored at 4 °C for 8 days.

2.6 Bacterial and viral enumeration of spiked pathogens on strawberries

Immediately after the treatment, approximately 20 g of strawberry from each treatment

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group was homogenized in 180 mL of 0.1% sterile peptone water via a stomacher (Masticator Stomacher, IUL Instruments, Germany) within a sterile stomacher bag. The serial dilutions were prepared, and the diluent was aseptically spread onto the corresponding agar plates. The enumeration of *E. coli* was conducted on sorbitol MacConkey agar (SMAC, Oxoid, UK), whereas the enumeration of *Salmonella* was conducted on xylose lysine deoxycholate agar (XLD, Oxoid, UK) after the plates were incubated at 37 °C for 24 h. The enumeration of *L. monocytogenes* was performed on PALCAM agar with Bacto™ Palcam antimicrobic supplement and incubated at 37 °C for 48 h.

The enumeration of MS2 and Tulane virus followed the same procedure as described by Tan *et al.* [22]. For the detection of MS2, the recommended double-layer tryptone yeast glucose agar (TYGA) method was used, with *E. coli* 15597 included in the semisolid TYGA layer. The MS2 titre was quantified via a plaque assay and expressed as plaque-forming units (PFU/g). Conversely, the infectivity titre of Tulane virus was determined via the 50% tissue culture infectious dose ($TCID_{50}$) method. Serially diluted samples (10^{-1} to 10^{-7}) were inoculated onto LLC-MK2 cells in 96-well plates (200 µL/well, four replicate wells/dilution) and incubated for 7 days (37 °C). Visible cytopathic effects (CPEs), such as cell balling, were recorded. A negative control was included on each plate. Additionally, preliminary validation utilizing cytotoxicity and neutralization controls confirmed that the dilution process effectively neutralized residual LVA without inducing host cell damage or impairing viral recovery (Table S2). Based on the sample preparation and 0.1 mL plating volume, the limit of detection (LOD) for the

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strawberry samples was calculated to be 2.0 log CFU/g (for bacteria), 2.0 log PFU/g (for MS2), and 2.0 log $TCID_{50}$/g (for Tulane virus).

To assess the combined efficacy of US and LVA in inactivating *L. monocytogenes*, the synergy value (SV) was calculated as follows:

$$SV = I_{US\_LVA}/(I_{US} + I_{LVA} - I_{US} \times I_{LVA})$$

The interaction was classified into three categories: non-synergistic (SV < 0.85), weakly synergistic (SV ranging from 0.85 to 1.15), and strongly synergistic (SV > 1.15). In this context SV represents the synergy value, and I_{US}, I_{LVA}, and I_{US_LVA} denote the inactivation rates (%) of the US treatment alone, the LVA treatment alone, and their combined treatment, respectively.

2.7. Microbiological and quality analysis of the strawberries during storage

2.7.1. Microbiological quality

On each sampling day (days 0, 2, 4, 6, and 8), randomly selected strawberries (approximately 20 g) from each treated group were homogenized with 180 mL of 0.1% sterile peptone water within a stomacher bag with the use of a stomacher. To determine the aerobic mesophilic bacteria count on strawberries, dilutions were plated onto plate count agar (PCA, Oxoid, UK) and incubated at 37 °C for 48 h. Yeast and mold counts were performed separately on potato dextrose agar (PDA, Oxoid, UK) by incubation at 25 °C for 3 days and 5 days, respectively. All the results are expressed as log CFU/g of sample.

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2.7.2. Weight loss and firmness

The total weight of 10 strawberries from each replicate was measured on each sampling day. The weight loss was calculated as the percentage (%) reduction in weight relative to the original weight.

Additionally, 10 strawberries from each treated group were cut into 2 cm × 2 cm × 2 cm cubes and subjected to compression testing of firmness. A TA-XT2i texture analyser (Stable Micro Systems Ltd., Godalming, Surrey, UK) equipped with a P35 probe (6 mm) was calibrated and used for the measurements. The pretest, test, and posttest speeds were set to 5, 2, and 7 mm/s, respectively, with a trigger force of 0.1 N. The results were expressed as the maximum force encountered.

2.7.3. Enzyme activities

The activities of phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), and peroxidase (POD), as well as the content of malondialdehyde (MDA), were determined via their respective assay kits (Solarbio Science & Technology Co., Ltd., Beijing, China). All the experiments were performed in triplicate, and the average values are reported.

2.8. Statistical analysis

All experiments were performed with three independent biological replicates (n = 3). Data are presented as mean ± SD. A one-way ANOVA was performed for each sampling day to evaluate treatment differences. Analysis of variance (ANOVA) was

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performed via Duncan's multiple range test in SPSS Statistics (IBM Corp., Armonk, NY, USA) to determine differences between groups. Plate counts below the limit of detection (LOD) were computed as 2.0 log CFU/g. Statistical significance was defined as $p < 0.05$.

3. Results and discussion

3.1 Effects of different treatments against *L. monocytogenes* in suspensions

Compared with the control treatment (6.9 ± 0.1 log CFU/mL), individual treatments of ultrasound and 1% LVA reduced the number of viable *L. monocytogenes* by 1.0 ± 0.1 log CFU/mL and 2.1 ± 0.1 log CFU/mL, respectively (Fig. 1). Notably, the combined treatment resulted in the lowest surviving population (3.4 ± 0.1 log CFU/mL), achieving a significant reduction of 3.5 ± 0.1 log CFU/mL ($p < 0.05$). Based on the inactivation rates, the calculated SV was 1.25, classifying the interaction as strongly synergistic. The combined treatment showed a synergistic effect, which could be attributed to ultrasound-induced cavitation enhancing mass transfer and transiently increasing membrane permeability, thereby facilitating organic acid uptake and antimicrobial action.

3.2 Metabolic profiles of *L. monocytogenes*

Metabolomic analysis was performed on *L. monocytogenes* to determine the different responses of the bacteria to different treatments. A total of 116 metabolites were detected in *L. monocytogenes* via NMR metabolomic analysis. For multivariate analysis,

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the predictive factor for OPLS-DA is Q^2, whereas the quality factors are R^2X and R^2Y.[26] OPLS-DA was used to perform pairwise comparisons of the metabolic profiles obtained from *L. monocytogenes* under control, ultrasound, LVA (1%), and US_LVA treatments (Fig. 2A1-C1). In general, all the treatments could be distinguished by OPLS-DA (control *vs* ultrasound: $R^2X=0.844$, $R^2Y=1$, $Q^2=0.991$; control *vs* LVA (1%): $R^2X=0.938$, $R^2Y=1$, $Q^2=0.998$; control *vs* US_LVA: $R^2X=0.945$, $R^2Y=1$, $Q^2=0.998$) and the reliability of these models was supported by permutation tests with 200 iterations (Fig. S2). Each pair was distinguished on the score plot, suggesting that the antimicrobial effects of LVA and ultrasound treatments might be associated with metabolic responses in *L. monocytogenes*.

The volcano plots shown in Fig. 2 are based on correlation coefficients, *p* values, and fold changes (FCs). Differential metabolites (VIP > 1, $p < 0.05$) were defined as up-regulated (FC > 1) or down-regulated (FC < 1) as an exploratory screening. Notably, the metabolites that changed among the different treatment groups were located primarily in the upper regions of the volcano plots (Fig. 2A2-C2), indicating that various treatments potentially correlated with changes in *L. monocytogenes* metabolite levels. Treatment-associated metabolic alterations were observed across all treatment groups (Fig. 3A1-C1).

Compared with the control group, all the treatments were reflected in metabolic changes, with the ultrasound, LVA, and US_LVA treatments resulting in 15, 27, and 30 altered metabolites, respectively ($p < 0.05$). Notably, the US_LVA treatment had the most substantial impact, with 19 metabolites decreasing and 11 increasing. These findings

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highlight the distinct metabolic responses elicited by each treatment. Although the metabolomic analysis provides relative rather than absolute quantification, the magnitude of perturbation reflected by the number of these altered metabolites and the corresponding fold changes was consistent with the stronger antimicrobial outcome observed for the combined treatment.

Metabolite changes were interpreted in the context of injury phenotypes measured in this study, with particular attention to membrane-associated metabolites, energy metabolism, and redox-related pathways that are potentially related to antimicrobial outcomes. Compared with the control treatment, the ultrasound treatment was associated with metabolic profiles suggestive of severe dysregulation, as evidenced by the depletion of key membrane components, including glycerol, erythritol, xylitol, and D-threitol, indicating potential effects on membrane structural stability. Concurrently, the levels of oxidative stress-related metabolites such as homocysteine and glycolate showed alterations, which may reflect an intensified oxidative stress response.[27, 28]

Compared with those in the control group, the depletion of energy-related metabolites (succinate, acetone, and butanone), as well as organic acids and their derivatives (adipate, propionate, isopropanol, butanone, and valerate), was observed following LVA treatment. Given the essential role of fatty acids in maintaining membrane integrity and cellular functionality, their depletion is consistent with potential membrane destabilization and increased permeability.[29] Additionally, the levels of stress-responsive amino acids such as proline and arginine increased, which may indicate a bacterial adaptive response to osmotic stress. As osmoprotectants, these

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amino acids play crucial roles in maintaining intracellular homeostasis and supporting bacterial survival under environmental stress conditions.[30,31]

Notably, LVA and the combined treatment resulted in similar trends in major metabolite alterations. However, compared with the LVA treatment, the combined treatment led to changes in the levels of oxidative stress-related metabolites, including homocysteine, cystathionine, glutarate, and pyroglutamate. Additionally, the upregulation of carbohydrate-related metabolites (tartrate, xylose, cellobiose, and glucitol) and the depletion of amino acid-related metabolites (histidine, pipecolate, cystathionine, aspartate, and homocysteine) suggest metabolic changes. Since these amino acids are essential for bacterial protein and enzyme synthesis, their depletion is consistent with a potential impairment of protein biosynthesis, which could disrupt cellular functions and bacterial growth.[32] This observed metabolic shift suggests a possible disruption of amino acid metabolic pathways caused by the combined treatment, potentially contributing to the inhibition of bacterial growth and proliferation. Additionally, to generate exploratory hypotheses, pathways affected by these different metabolites (VIP > 1) were analysed via MetaboAnalyst 5.0, and pathways with raw $p < 0.05$ were highlighted as potentially key pathways involved in the antibacterial process (Fig. 3A2-C2). The results are consistent with treatment-associated metabolic perturbations in *L. monocytogenes*. Among the top 25 enriched pathways, 17 were shared across all three treatment groups. These pathways involved primarily amino acid metabolism, nucleotide metabolism, energy metabolism, and carbohydrate metabolism. The analysis of unique metabolic pathways suggested potential distinct metabolic

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disruptions associated with each treatment condition. In response to ultrasound treatment, five uniquely enriched pathways were identified, including pyruvate metabolism, glycerolipid metabolism, and taurine and hypotaurine metabolism, all of which are strongly associated with oxidative stress response activation.[33-35] This finding aligns with the metabolite profiling results, supporting the possibility that US treatment induces severe oxidative stress in *L. monocytogenes*. In the LVA treatment group, the uniquely enriched propanoate metabolism pathway was associated with changes related to fatty acid and short-chain organic acid metabolism. This finding aligns with the observed downregulation of lipid-related metabolites, suggesting that LVA treatment could be associated with disruptions to bacterial lipid synthesis and energy production, potentially compromising membrane integrity and metabolic homeostasis.

In the combined treatment, the enrichment of valine, leucine, and isoleucine degradation and riboflavin metabolism suggested a potential influence on branched-chain amino acid catabolism and redox-associated processes.[36, 37] The alterations in these pathways are indicative of potential disruptions in maintaining amino acid turnover and redox balance, which could contribute to extensive metabolic dysfunction and bacterial inactivation. These exploratory findings provide preliminary metabolic level insights into treatment-associated perturbations, highlighting distinct responses to US, LVA, and their combination.

3.3 Changes in oxidative stress and membrane integrity in *L. monocytogenes*

On the basis of the metabolic results, the combined treatment appears to induce

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metabolic disruptions in the cell membrane of *L. monocytogenes* and trigger internal oxidative stress responses. Therefore, further investigations into cell membrane integrity and oxidative damage were conducted by assessing intracellular ROS levels and leakage of intracellular contents (Fig. 4A-C).

In this study, compared with treatment with ultrasound or LVA alone, US_LVA treatment induced excessive ROS generation in *L. monocytogenes* suspensions (Fig. 4A). The ROS levels increased to 17.2 ± 0.4 RFU and 710.4 ± 66.9 RFU in response to the individual US and LVA treatments, respectively, whereas the US_LVA treatment resulted in a markedly elevated ROS level of 29,999.6 ± 12.1 RFU, which was greater than the other groups ($P < 0.05$). This increase in intracellular ROS was attributed to the ultrasound-induced generation of hydroxyl radicals. Previous studies have demonstrated that excessive ROS not only inhibit the expression of genes related to proton dynamics but also accelerate the Fenton reaction, resulting in oxidative damage to the cell membrane and biomolecules, ultimately leading to bacterial death.[13, 38]

The loss of cell membrane integrity was further confirmed by enhanced leakage of nucleic acid and protein under different treatments, as measured by the OD at 260 nm and OD at 280 nm, respectively (Fig. 4B-C). The amount of nucleic acid and protein leaked from *L. monocytogenes* cells was significantly greater under the US_LVA treatment than under each treatment ($P < 0.05$). This is consistent with the depletion of membrane-related metabolites in Section 3.2, supporting an associative link to the increased membrane permeability indicated by intracellular leakage. These results indicate that the combined US_LVA treatment caused greater oxidative stress and

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membrane disruption than the individual treatment did, leading to significant leakage of intracellular content and cellular damage in *L. monocytogenes*. During membrane damage, the cavitation bubbles generated by ultrasound cause shear forces and pressure fluctuations upon collapse, disrupting the cell membrane integrity and increasing its permeability. These damages triggered the leakage of cytoplasmic components including large amounts of proteins and nucleic acids, thereby enhancing bactericidal efficacies of the US_LVA treatment.

3.4 Inactivation effects of *L. monocytogenes* and other pathogens on strawberries

As this study aimed to increase the food safety of products via the combined treatment, a strawberry washing model was employed to validate the effectiveness of US_LVA in a food-based system.

Following the washing treatment, the population of *L. monocytogenes* on the strawberry surface in the control group was 6.0 ± 0.1 log CFU/g (Fig. 5A). The treatments with ultrasound and 1% LVA resulted in counts of 5.1 ± 0.1 and 4.4 ± 0.1 log CFU/g, respectively, both of which were significantly lower ($p < 0.05$). Notably, consistent with the findings in the broth, the combined treatment in the food matrix model completely reduced the population to an undetectable level (< 2.0 log CFU/g). The superior efficacy of this combined approach aligns with previous findings on metabolic disruption and intracellular content leakage.

To determine whether the reduction in *L. monocytogenes* loads on strawberries was primarily due to a bactericidal effect or a wash-off effect, the bacterial counts in wash

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water after treatment were analysed (Fig. 5B). The results revealed notable increases in bacterial counts in the wash water after ultrasound treatment, suggesting that US primarily removed bacteria alongside limited direct inactivation. In contrast, both the LVA (1%) and US_LVA treatments resulted in significant reductions in bacterial counts compared with those of the control group (5.6 ± 0.3 log CFU/mL), reducing the counts to 3.3 ± 0.1 log CFU/mL and 4.1 ± 0.1 log CFU/mL, respectively ($p < 0.05$). Importantly, the reduced counts in the wash water after US_LVA indicate its potential to mitigate cross-contamination risk associated with recirculating wash water in industrial strawberry flume tank operations.

Notably, while bacterial reduction in the *L. monocytogenes* suspension was driven primarily by direct inactivation, the mechanisms differed on the strawberry surface. The complex structure of the strawberry matrix provides a protective effect, which may limit direct lethality. In this context, the wash water data strongly suggest that US treatment promoted bacterial detachment alongside partial inactivation. Specifically, the cavitation effects and high-frequency vibrations of US primarily dislodged bacteria from the strawberry surface into the washing solution. Therefore, the overall reduction on the strawberries was a combined result of US-enhanced physical detachment and direct bactericidal effects, rather than lethality alone.

In contrast, LVA relies on chemical inactivation to reduce bacterial viability. In the combined treatment, the presence of bacteria in the washing mixture indicated that US transferred bacteria from the strawberry surface rather than directly eliminated them, whereas LVA further disrupted the cell structure and inhibited bacterial survival. The

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synergy between these two mechanisms enhances the overall decontamination efficiency by effectively removing the bacteria from strawberry surface meanwhile impairing their viability.

To further explore their inactivation effects on different bacteria and viruses, this study also tested on *S.* Typhimurium (ATCC 14028), *S.* Enteritidis (ATCC 13076), and *E. coli* O157:H7 (ATCC 35150). In addition, owing to the limitations of directly using the pathogens on an industrial scale, surrogate strains have been commonly employed to validate the applicability of laboratory findings in industrial processes. The non-pathogenic *Escherichia coli* strain (ATCC 25922) is widely used as a substitute for enteric bacterial pathogens, whereas the MS2 bacteriophage and Tulane virus are two commonly used surrogate models for hNoV, serving as indicators of foodborne viruses.[39] By using both bacterial and viral surrogates, this study aimed to provide comprehensive insights into the potential of ultrasound and LVA as a broad-spectrum disinfection strategy.

The inoculation level of the test strains on fresh strawberries was approximately 10^7 CFU/g. For bacteria, ultrasound treatment resulted in a reduction range of 0.8 to 1.2 log for all tested strains (Fig. 6A-D). For samples treated with US_LVA, all tested bacterial groups were reduced to undetectable levels (< 2 log CFU/g). The results for the non-pathogenic *E. coli* strain (ATCC 25922) were comparable to those for the pathogenic bacterial strains tested. These experimental results indicate that the combined treatment method has a broad-spectrum antimicrobial action against both pathogenic and non-pathogenic *E. coli* strains, thereby validating the stability and applicability of this

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disinfection approach. Additionally, LVA (1%) significantly reduced the number of pathogenic bacteria, especially *S*. Typhimurium and *S*. Enteritidis. This outcome may be attributed to different sensitivities of various bacterial strains towards acidity. Consistent with previous reports, *Salmonella* has been shown to be more susceptible to acidic treatments than *E. coli* O157:H7 and *L. monocytogenes* do, making it easier for inactivation.[40]

Tulane virus and MS2 are commonly used as surrogates for human norovirus in testing disinfectant efficacy. In this study, the inactivation trends of viruses and bacteria were generally consistent. According to the results shown in Fig. 6E-F, compared with the control, ultrasound treatment reduced Tulane virus and MS2 by 1.0 ± 0.8 and 0.8 ± 0.2 log PFU/g, respectively. In the combined treatment, Tulane virus was reduced to undetectable levels (< 2 log $TCID_{50}$/g), and MS2 was reduced by 3.3 ± 0.2 log PFU/g after sanitization. The different results for Tulane virus and MS2 likely reflect surrogate-dependent susceptibility, as MS2 has indeed been reported as a relatively conservative and disinfectant resistant viral surrogate or indicator.[41] Their intrinsic differences in virion composition and capsid properties may contribute to this, as Tulane virus is a nonenveloped calicivirus whereas MS2 is a nonenveloped single stranded RNA coliphage.[42, 43] In addition, surrogate-specific susceptibility is treatment-dependent because interventions differ in their dominant damage pathways, which can alter the relative inactivation observed across surrogates.[44-46] Previous studies on physical and chemical treatments have demonstrated that viruses are generally more resistant to inactivation than bacteria.[47-49] However, our results demonstrated that the

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combined treatment significantly reduced Tulane virus and MS2 ($p < 0.05$), indicating its effectiveness against viruses.

Overall, this combined treatment had both antiviral and antibacterial effects on most of the strains tested. Ultrasound enhanced the inactivation efficiency of LVA against pathogens, and compared with the individual treatments, the combined US_LVA treatment demonstrated superior effectiveness. This combined treatment may be useful in controlling the outbreaks associated with foodborne viruses.

3.5 Storage quality of strawberries

3.5.1. Appearance and quality analysis of strawberries

To further evaluate the effects of US_LVA treatment on the quality and shelf life of strawberries, a systematic assessment of changes in the appearance, weight loss, and firmness during storage was conducted.

The weight loss rate is an important indicator of the storage stability of food products such as fruits and vegetables. Fig. 7A illustrates the impact of different treatments on the weight loss rate of strawberries during storage. All the strawberry samples gradually increased in weight loss rate with increasing storage time. Throughout the storage period, the treatments with ultrasound, LVA (1%), and US_LVA significantly delayed the increase in the weight loss rate of strawberries ($p < 0.05$). Among these, the strawberries treated with LVA (1%) and US_LVA presented the lowest weight loss over the entire storage period. At the end of the storage period, the weight loss rates for the control, US, LVA, and combined treatment groups were 5.38 ± 0.13%, 3.82 ± 0.07%,

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3.07 ± 0.01%, and 2.49 ± 0.16%, respectively. The primary causes of weight loss in strawberries during storage are active water vapour pathways, moisture transfer, and transpiration processes.[50] LVA might interact with the fruit epidermis, reducing transpiration and respiration rates and thereby slowing the moisture loss.[51]

Although weight loss is an important indicator for evaluating the storage quality of strawberries, it primarily reflects the macrolevel loss of water and materials. In contrast, changes in firmness provide deeper insights into the stability of the cellular structure and internal tissue integrity. As shown in Fig. 7B, strawberry firmness decreased with prolonged storage across all treatment groups. However, the fruits subjected to the ultrasonic and US_LVA treatments exhibited greater firmness stability throughout the storage period. A comparison between the initial and final storage periods revealed that firmness decreased by 3.23 N, 2.51 N, and 2.40 N in the control, US, and LVA groups, respectively. Notably, the combined treatment group presented the smallest reduction in firmness, with a decrease of only 1.25 N.

In addition to effectively controlling firmness and weight during storage through US_LVA treatment, strawberries also exhibited significant differences in appearance throughout the storage period. As shown in Fig. 7C, during the storage period at 4 °C (0–8 days), no signs of decay were observed in any sample type treated with ultrasound, LVA (1%), or US_LVA. However, for the control group, the extent of decay significantly exacerbated after Day 4. Overall, the treated strawberries maintained good visual acceptability during the 8-day storage period.

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3.5.2. Microbial and enzyme activity analysis of strawberries

Previous results showed that US_LVA treatment effectively prevented firmness deterioration and weight loss in strawberries during storage while maintaining their appearance. This improvement in quality may be associated with the inhibitory effects of US_LVA treatment on microbial growth and enzymatic activities in strawberries. To explore this further, microbial and key enzyme analysis of strawberries during the storage were performed.

Fig. 8A-B shows the disinfection effects of various treatments on the natural microbial flora of strawberries. Compared with those in the control group, the microbial population immediately (day 0) decreased after treatment with ultrasound (US), LVA (1%), or their combination (US_LVA). Throughout the 8-day storage period, as expected, microbial counts gradually increased in all treated samples, with the US_LVA-treated samples showing the least growth. Compared with those in the control samples, the aerobic mesophilic counts in the samples treated with ultrasound, LVA (1%), and US_LVA decreased by 0.7 ± 0.1, 1.6 ± 0.2, and 2.1 ± 0.1 log CFU/g, respectively, at the end of the storage period. For the reduction of mold and yeast, the treatment groups achieved decreases of 1.5 ± 0.2 and 1.7 ± 0.3 log CFU/g, respectively, while the combined treatment maintained levels below the detection limit (< 2 log CFU/g) during storage. We believe that the microbial results demonstrate that the combined treatment effectively prevented excessive microbial infection during strawberry storage, thereby protecting the structural integrity of plant cells. These findings provide further evidence to explain how the combined treatment inhibited the

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595 decline in firmness and weight of strawberries.

596 On the basis of the above findings, ultrasound combined with LVA effectively
597 preserved strawberry quality by inhibiting microbial infection. Additionally, this
598 treatment may also induce plant defense responses. Therefore, further investigations
599 into the effects of ultrasound combined with LVA on key enzyme activities in
600 strawberries are essential to elucidate the underlying mechanisms for preserving fruit
601 quality during storage. This study demonstrated that ultrasonic and US_LVA
602 treatments significantly influenced the activity of various enzymes in strawberries,
603 thereby improving fruit storage stability and quality (Fig. 8C-F).

604 PPO and POD are key enzymes in the browning process of fruits and vegetables,
605 catalyzing the oxidation of polyphenols to brown pigments, which cause off-flavours
606 and negatively affect the sensory quality.[52] As shown in Fig. 8C-D, the ultrasound, LVA
607 (1%), and US_LVA treatments significantly reduced the PPO and POD activities in
608 strawberries. After 8 days of storage, the PPO activity decreased by 30.86%, 24.00%,
609 and 42.64%, whereas the POD activity decreased by 31.73%, 15.68%, and 47.78%
610 following the ultrasonication, LVA (1%), and US_LVA treatments, respectively,
611 compared with the control treatment. Ultrasound and US_LVA treatments notably
612 suppressed the activities of PPO and POD, possibly due to the deoxygenation effect of
613 LVA or the conversion of enzyme products into phenolic compounds, effectively
614 inhibiting enzymatic activity.[53] Additionally, the cavitation effect induced by
615 ultrasound might alter the microenvironment of the enzymes, and the shear forces
616 generated by bubble collapse cause structural changes, leading to enzyme denaturation

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and inactivation.[54] This inhibition of enzyme activity reduced oxidative reactions to some extent, contributing to the delay of strawberry quality deterioration.

The activity of PAL, the key rate-limiting enzyme in the phenylpropanoid pathway, initially increased during early cold storage but then decreased later due to senescence and browning (Fig. 8E). The ultrasound and LVA treatments significantly increased the activity of PAL ($p < 0.05$). This increase may be attributed to oxidative stress, as previous studies indicated that free radicals generated during ultrasonic water decomposition can activate PAL and promote the synthesis of phenolic defense compounds.[55, 56]

Changes in the MDA content are typically used as indicators of lipid peroxidation. Fig. 8F shows that the MDA content in strawberries from the different treatment groups continuously increased throughout the storage period, with the MDA content in the US_LVA-treated strawberries consistently remaining at the lowest level. After 8 days of storage, the MDA content in the US_LVA treatment group was 34.39% lower than that in the control group. The combined treatment inhibited the accumulation of MDA, thereby maintaining the integrity of strawberry cell structures and membranes.

In the present study, the combined treatment extended the shelf-life of strawberries and maintained their quality by exerting antimicrobial effects and enhancing the antioxidant defense system through induced oxidative stress, though certain aspects require further exploration. The non-targeted NMR-based metabolomic analysis provided valuable insights into metabolic changes. However, this approach may have limited sensitivity in detecting low-abundance metabolites and highly depends on the spectral database

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matching for compound identification, which can reduce accuracy when analyzing unknown or less-characterized compounds. To build upon these findings, future research should incorporate targeted metabolomic approaches to validate and quantify key metabolites identified through NMR. Including sensory evaluation and quantitative analysis of LVA residues would further strengthen the assessment of product quality, consumer acceptance, and regulatory compliance. In addition, evaluating economic feasibility and scalability is necessary to support the practical application of this combined treatment in industrial postharvest processing.

4. Conclusion

This study investigated the inactivation effects of ultrasound combined with LVA on various pathogens, the associated antibacterial mechanisms, and the preservation efficacy of LVA in strawberries. These results demonstrated that ultrasound combined with LVA has a significant inactivation effect on pathogens. An exploration of the inactivation mechanism of *L. monocytogenes* revealed that the combined treatment intensified oxidative damage, disrupted cell membrane integrity and permeability, and increased the leakage of intracellular components. This may have contributed to inhibited amino acid and protein synthesis, disrupted fatty acid metabolism, and the induction of oxidative stress responses and energy metabolism disorders, which might ultimately compromise membrane stability in bacteria. Additionally, the combined treatment effectively reduced the microbial loads on strawberry surfaces and improved their storage quality and antioxidant enzyme activities during storage at 4 °C. Overall,

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these findings demonstrate the fundamental potential of combining ultrasound with LVA to enhance microbial safety and extend the shelf-life of produce under controlled laboratory conditions. However, before translating this approach into commercial food processing, the feasibility of scale-up incorporating shorter cycles and process water, equipment compatibility with acidic LVA, economic considerations, sensory impacts, and post-wash residues remain to be systematically evaluated under industrially relevant conditions.

Author contributions

Zifei Liu: Writing – original draft, Writing – review & editing, Visualization, Software, Methodology, Investigation, Conceptualization. Lingdai Liu: Methodology, Writing – review & editing. Zejia Lin: Methodology, Writing – review & editing. Fion Chin Wei Lin: Methodology. Yuan Guo: Methodology. Dan Li: Conceptualization, Writing – review & editing, Supervision, Project administration.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting the findings of this research are available in the article.

Acknowledgment

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Figure Legends

Fig. 1. Changes in *L. monocytogenes* in broth with different treatments: control; ultrasound; LVA (1%); US_LVA. Different letters indicate statistically significant differences between the treatment groups ($p < 0.05$). The error bars represent the standard deviations (n = 3).

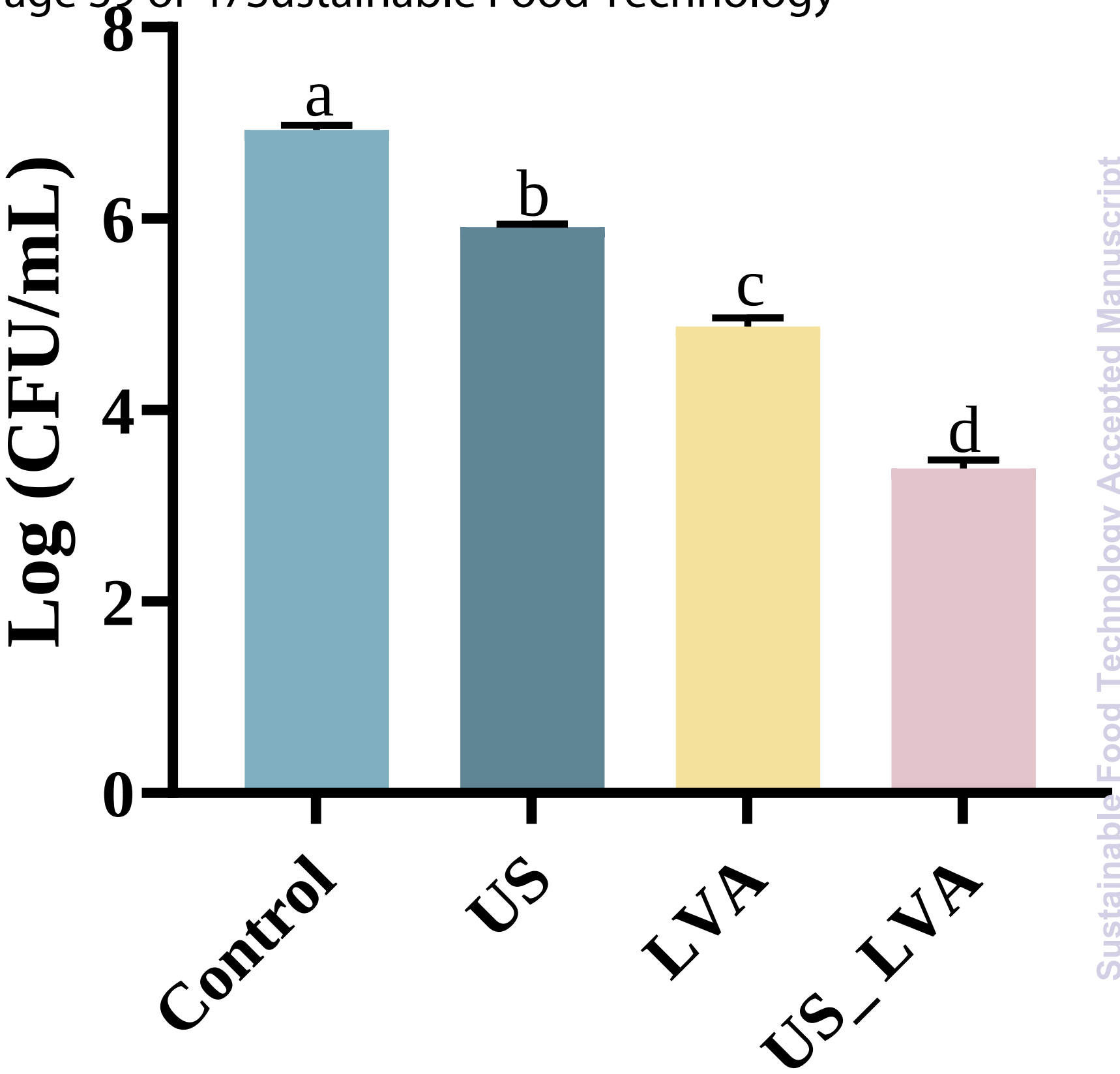
Fig. 2. Orthogonal partial least squares discriminant analysis (OPLS-DA) score plot and volcano plots comparing *L. monocytogenes* metabolite profiles between the control and treatment groups. Comparison between the control and US treatment groups (A1-A2), the control and LVA treatment groups (B1-B2), and the control and US_LVA treatment groups (C1-C2).

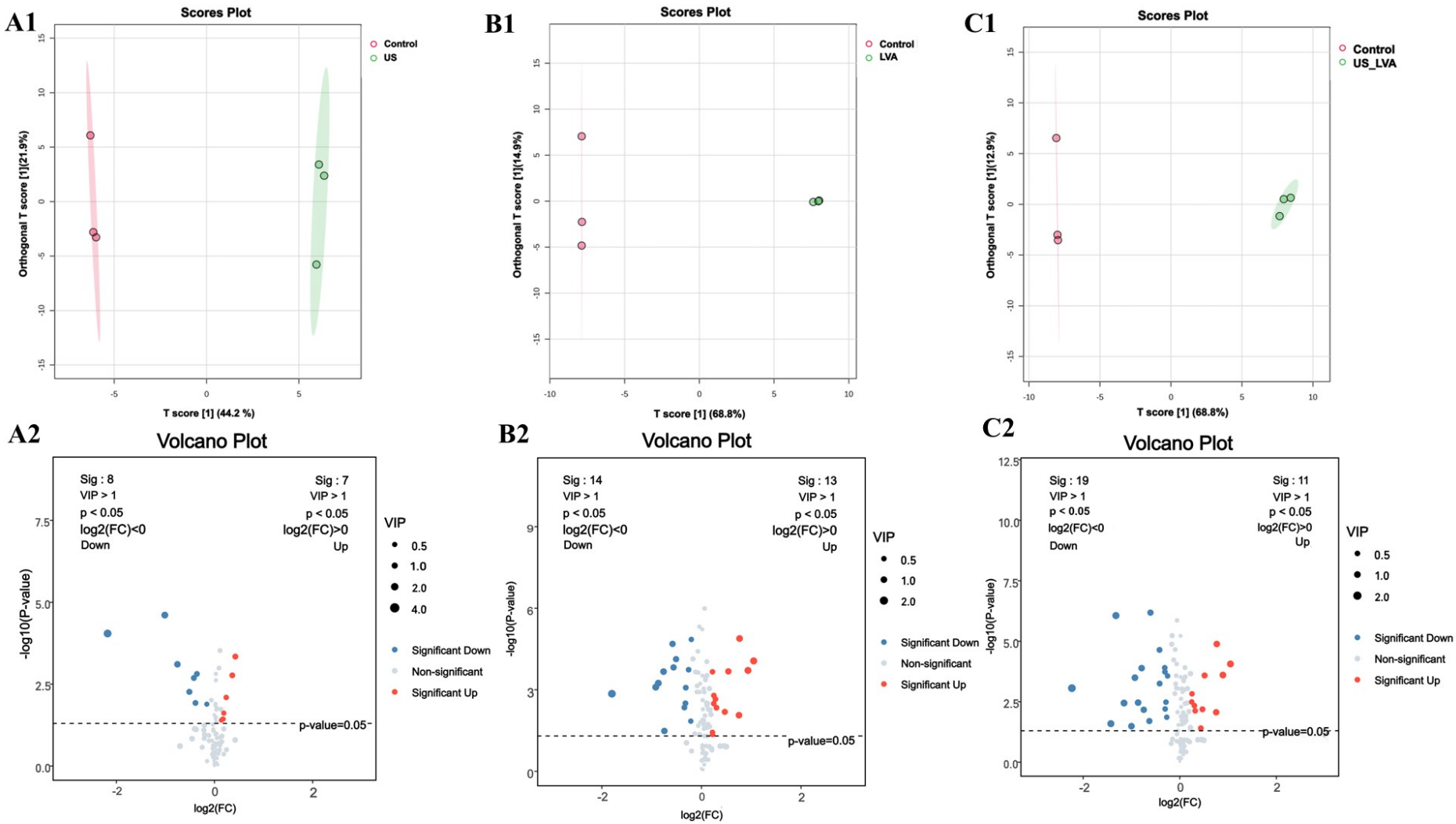
Fig. 3. Volcano plots of *L. monocytogenes* metabolites (VIP > 1) and pathway analysis overview. Comparison between the control and US treatment groups (A1-A2), control and LVA treatment groups (B1-B2), and control and US_LVA treatment groups (C1-C2).

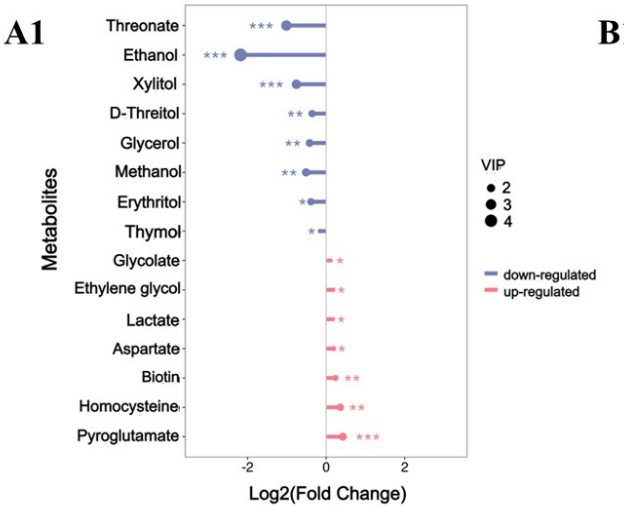
Fig. 4. Effects of different treatments on the intracellular ROS (A) of *L. monocytogenes*; protein release (B) and nucleic acid content (C) from *L. monocytogenes* cells under different treatments. Different letters indicate statistically significant differences between the treatment groups ($p < 0.05$). The error bars represent the standard deviations (n = 3).

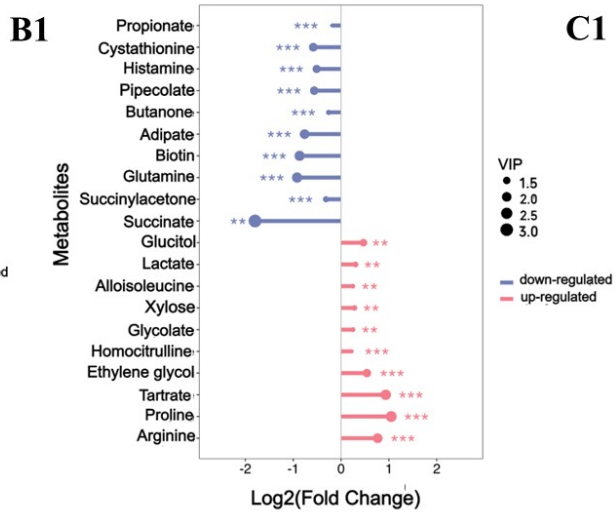
Fig. 5. Changes in *L. monocytogenes* in strawberry (A) and washing water (B) with different treatments: control; ultrasound; LVA (1%); US_LVA. Different letters

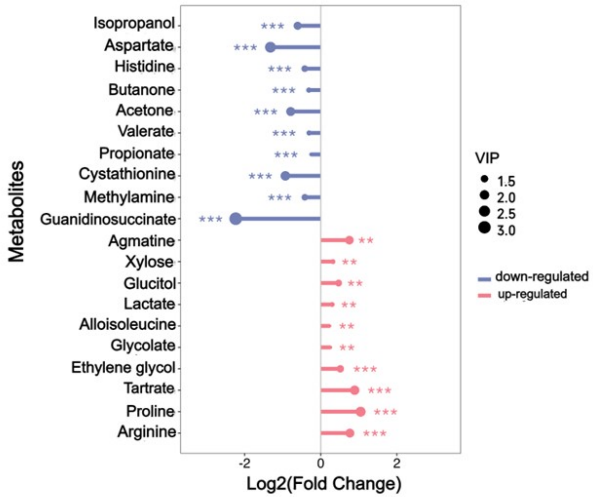
826 indicate statistically significant differences between the treatment groups ($p < 0.05$).
827 The error bars represent the standard deviations (n = 3).
828
829 Fig. 6. Changes in *E. coli* (ATCC 25922), *E. coli* O157:H7 (ATCC 35150), *S.*
830 Typhimurium (ATCC 14028), *S.* Enteritidis (ATCC 13076), and Tulane virus (TV),
831 MS2 bacteriophage (A-F) on the surfaces of strawberry plants after different treatments:
832 control, US, LVA, and US_LVA. Different letters indicate statistically significant
833 differences between the treatment groups ($p < 0.05$). The error bars represent the
834 standard deviations (n=3).
835
836 Fig. 7. Weight loss (A), firmness (B), and images of strawberries subjected to different
837 treatments (C) in different treatment groups: control, US, LVA, and US_LVA during
838 storage. Within the same storage period under different treatments, significant
839 differences are shown by different lowercase letters; for the same treatments at different
840 storage times, significant differences are shown by different capital letters ($p < 0.05$).
841 The error bars represent the standard deviations (n = 3).
842
843 Fig. 8. Aerobic mesophilic count (A), yeast and mould (B), PPO (C), POD (D), PAL
844 (E) and MDA (F) of strawberry in different treatment groups: control, US, LVA, and
845 US_LVA during storage. Within the same storage period under different treatments,
846 significant differences are shown by different lowercase letters; for the same treatments
847 at different storage times, significant differences are shown by different capital letters
848 ($p < 0.05$). The error bars represent the standard deviations (n = 3).
849

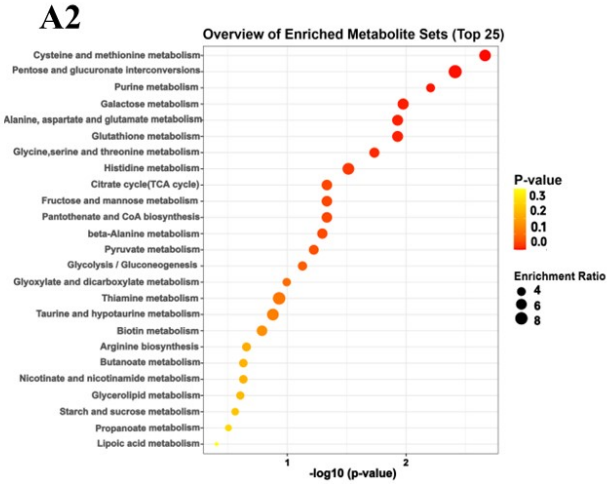


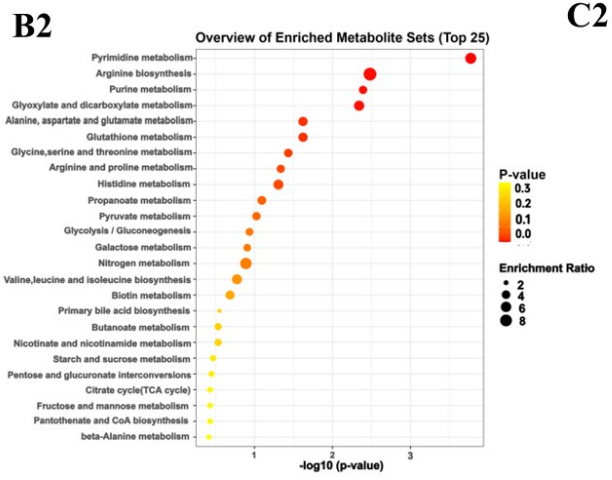


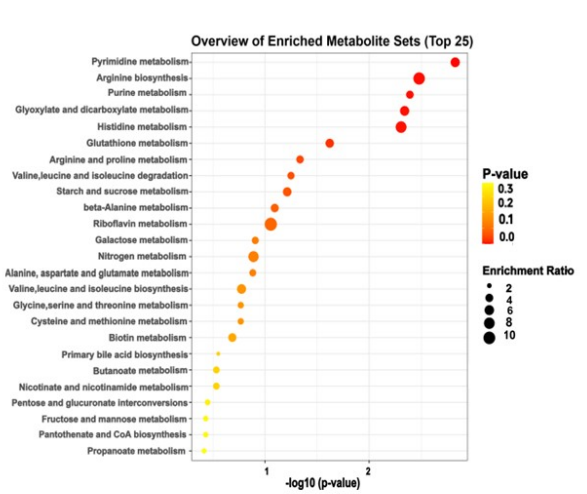


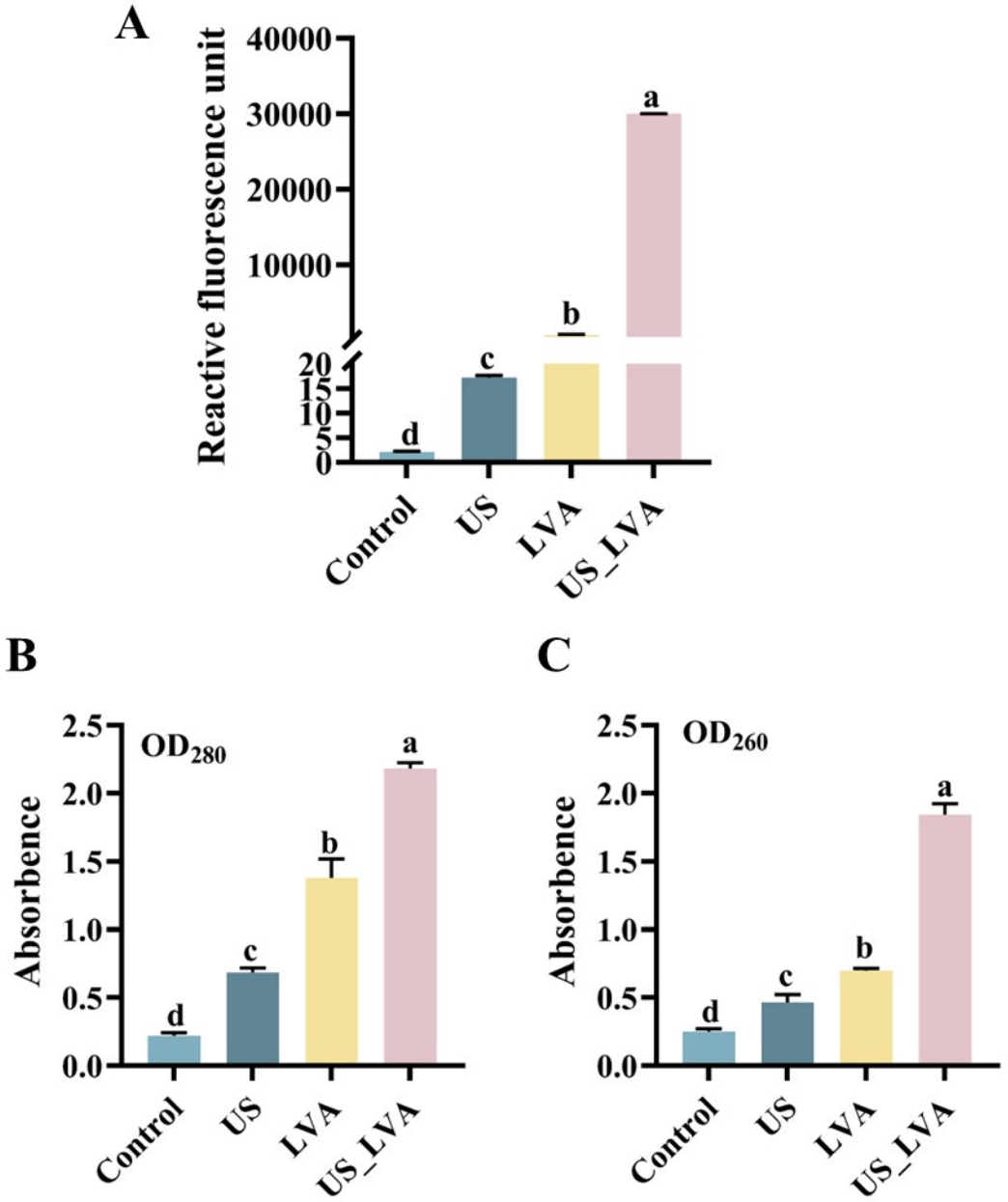


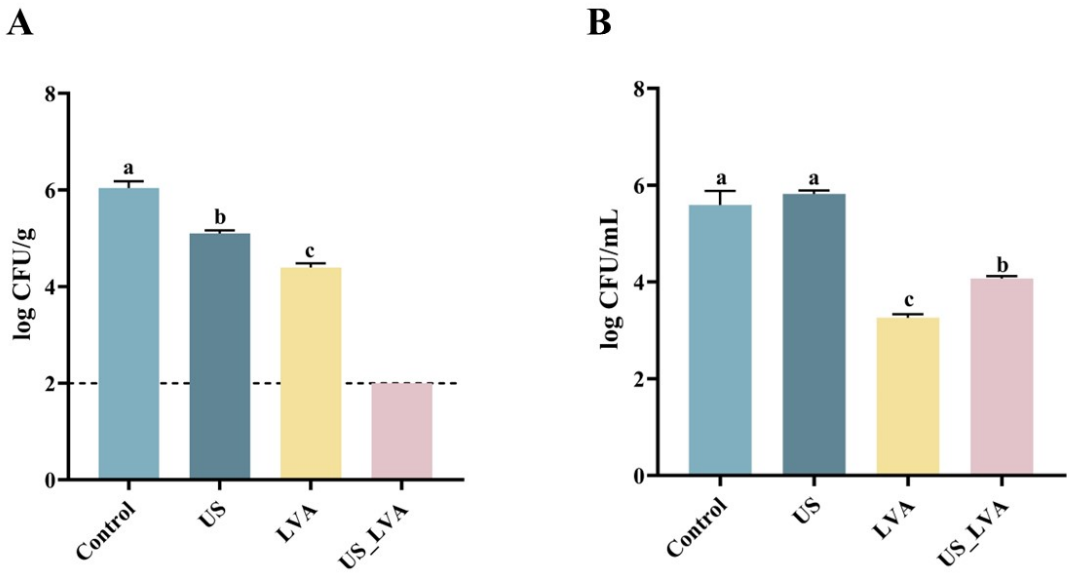


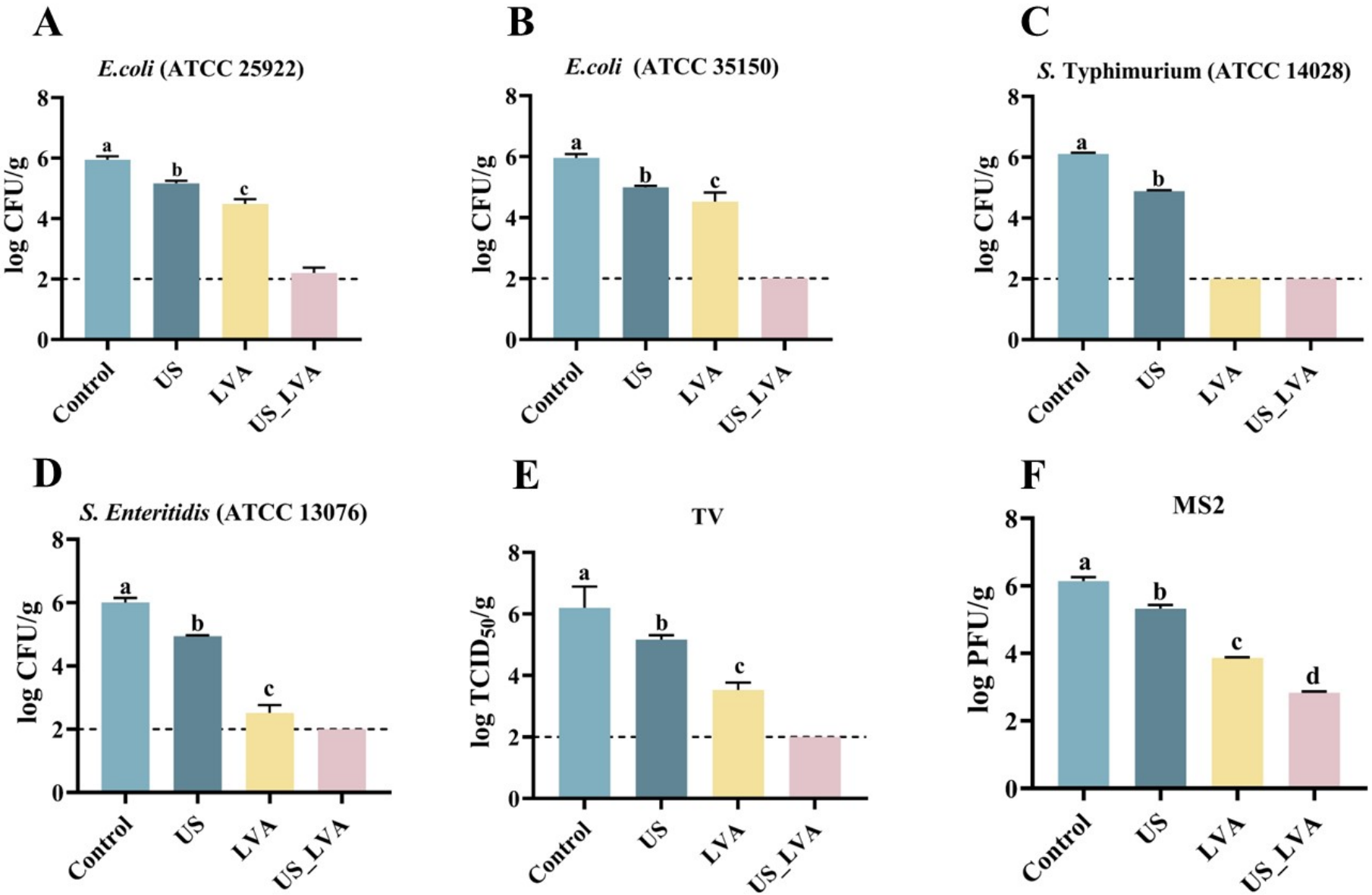


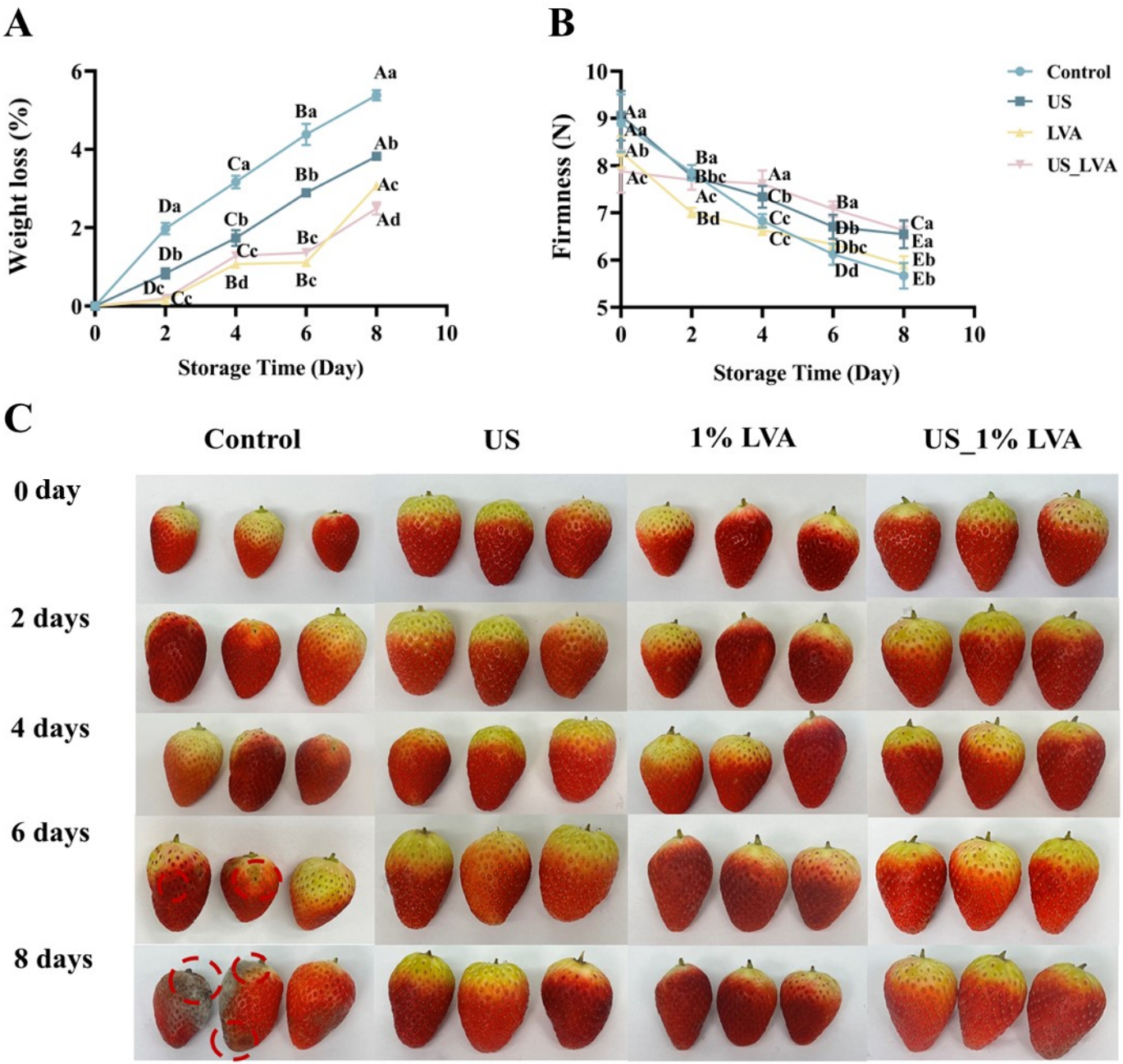


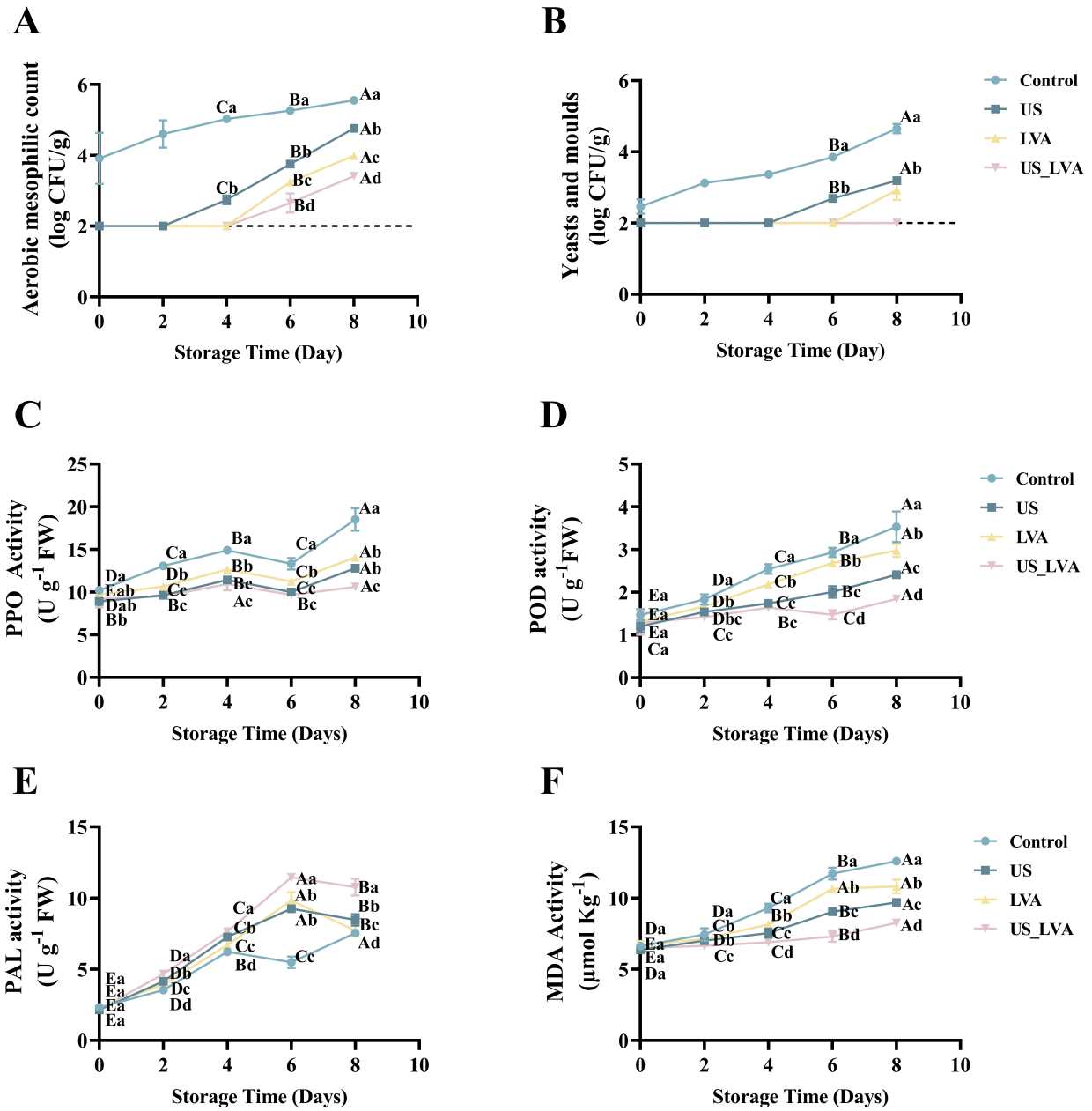












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Data availability

The data supporting the findings of this research are available in the article.