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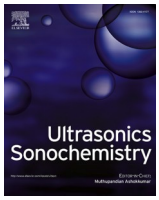

Ultrasound and lactic/malic acid treatment for mitten crab decontamination: Efficacy and mechanisms against *A. hydrophila*

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ABSTRACT

The Chinese mitten crab (*Eriocheir sinensis*), recognized as a high-value aquatic product, necessitates effective cleaning to ensure both safety and quality. Conventional cleaning methods frequently fail to eliminate biofilms and pathogenic bacteria, such as *Aeromonas hydrophila*, which pose significant health risks and contribute to spoilage. This study explores the bactericidal efficacy and underlying mechanisms of ultrasound treatment combined with a lactic acid and malic acid complex solution for decontaminating crabs and enhancing food safety. Employing a range of methodologies, including microscopic imaging, live/dead staining, RT-qPCR, and texture and microstructure analysis, the results indicate that the combined treatment significantly reduced *A. hydrophila* counts by 4.16 lg CFU/mL and induced substantial bacterial membrane damage, as evidenced by scanning electron microscopy (SEM). Gene expression analysis revealed a pronounced downregulation of biofilm-related genes. Notably, the treatment also preserved the texture and sensory properties of crab meat, thereby ensuring high product quality. These findings suggest that the application of ultrasound in conjunction with a lactic acid-malic acid solution represents a green and effective strategy for improving food safety and quality in the processing of aquatic products, offering a sustainable and eco-friendly alternative to traditional cleaning methods.

1. Introduction

Aeromonas hydrophila is a Gram-negative bacterium commonly found in aquatic environments [1]. It produces various toxins, including hemolysins and enterotoxins, which pose significant threats to both human health and aquatic animals [2]. This bacterium is a leading cause of contamination in aquatic products, particularly during handling and storage processes. Inadequate storage conditions facilitate the rapid proliferation of *A. hydrophila*, resulting in spoilage and diminished quality of aquatic products [3]. Rahman [4] demonstrated the bacterium's ability to form resilient biofilms, complicating its removal from the surfaces of aquatic products. Similarly, Dorick [5] highlighted that biofilm formation enhances the bacterium's resistance to cleaning agents, rendering traditional decontamination methods less effective. Traditional cleaning methods, such as manual washing, high-pressure water spraying, and ozone treatment, have been widely applied in seafood processing. However, these approaches often fail to completely remove biofilms and bacterial contaminants [6,7]. These findings underscore the significant risks posed by *A. hydrophila* in the processing of aquatic products and the urgent need for improved control strategies. Chen [8] noted that contamination of seafood by spoilage organisms like *A. hydrophila* can result in substantial economic losses, further emphasizing the necessity for effective cleaning solutions. Therefore, addressing this issue is crucial to ensuring the safety and quality of aquatic products.

The Chinese mitten crab *(Eriocheir sinensis)*, regarded as a high-value aquatic product, requires proper cleaning as a crucial step in its processing. Ineffective cleaning of crabs can allow harmful bacteria, such as *A. hydrophila*, to remain on their surfaces, potentially leading to foodborne illnesses or spoilage [9]. Panebianco [10] compared traditional cleaning methods, including manual washing and chemical treatments, and found that these approaches often fail to adequately remove biofilms, resulting in residual bacteria. Furthermore, the application of chemicals can leave harmful residues, posing risks to both consumers and the environment [11]. Alternatives such as high-pressure washing and ozone treatments have been investigated, as discussed by Gokmen [12]. Although these methods demonstrate improved cleaning efficacy, their high costs and the requirement for specialized equipment limit

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their broader application. These challenges underscore the need for more effective and practical cleaning methods to enhance the safety and quality of crabs while addressing the limitations of current techniques.

Ultrasound and organic acids are widely utilized for cleaning and microbial control in food processing. Ultrasound generates cavitation effects that involves the rapid formation, growth, and implosive collapse of microbubbles in liquid media [13]. This process generates localized high temperatures and pressures, leading to free radical production and bacterial membrane disruption, ultimately enhancing microbial inactivation, effectively reducing contamination on fresh produce and seafood [14–16]. Additionally, ultrasound enhances cleaning efficiency by improving the penetration of water or cleaning agents. However, its effectiveness is limited against resilient bacteria, such as biofilm-forming *A. hydrophila*, particularly under low-power conditions [17]. Organic acids, including acetic and lactic acid, inhibit bacterial growth by lowering pH and damaging cell membranes [18]. They are effective in reducing spoilage bacteria and extending the shelf life of seafood and meat [19–21]. Furthermore, organic acids help preserve food quality by slowing microbial growth. However, the use of high concentrations can adversely affect food flavor and texture [22]. While both ultrasound and organic acids are effective, each has limitations when used independently; ultrasound may leave residual bacteria, and high levels of organic acids may alter sensory properties. The combination of these two methods could maximize their benefits while mitigating individual drawbacks. However, there has been limited research on the combined effects of ultrasound and organic acids for cleaning crabs or controlling *A. hydrophila*. This study aims to explore the combined use of ultrasound and organic acids to clean crabs, enhance quality, and inhibit *A. hydrophila*. By addressing existing gaps in current methods, this work seeks to provide an effective and practical solution for the aquatic products processing industry.

2. Materials and methods

2.1. Bacterial strains, culture conditions and reagents

The *Aeromonas hydrophila* XT1 strain (GenBank accession number: PQ380179) was isolated from spoiled Chinese mitten crab (*Eriocheir sinensis*) meat. This bacterial strain was identified through 16S ribosomal typing, and the resulting 16S rRNA sequence was verified using the BLAST server at NCBI. The strain is stored at the National Key Laboratory of Food Science and Resources, Jiangnan University (Wuxi, Jiangsu, China). *A. hydrophila* was cultured in LB broth (1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, and 10 % (w/v) NaCl, pH 7.0, purchased from Sinopharm Chemical Reagent Co., Ltd., Beijing, China) at 37 °C with shaking at 200 rpm for 24 h. Subsequently, the culture was centrifuged at 6000 rpm for 5 min at 4 °C. The supernatant was discarded, and the pellet was washed with PBS before being resuspended and adjusted to an optical density of OD600nm = 0.5 (approximately 10^8 CFU/mL). Lactic acid (LA) and malic acid (MA) were sourced from Innochem (CAS: 6915–15-7, Beijing, China) and Sinopharm Chemical Reagent Co., Ltd. (CAS: 50–21-5, Shanghai, China), respectively, and were dissolved in sterile water. Plate count agar (PCA) medium was obtained from Hangkai Microorganisms (Guangzhou, China). The SYTO-9/propidium iodide (PI) Live/Dead Bacterial Double Stain Kit was acquired from Makino Biotech Co., Ltd. (Shanghai, China). Reactive oxygen species (ROS) and alkaline phosphatase (AKP) assay kits were obtained from Biyuntian Biotechnology Co., Ltd. (Shanghai, China). RNA extraction reagent RNAiso Plus (9109), cDNA reverse transcription reagent PrimeScript™ RT Master Mix (Perfect Real Time) (RR036A), and qPCR reagent TB Green® Premix Ex Taq™ II FAST qPCR (CN830A) were all purchased from Takara Biomedical Technology Co., Ltd. (Beijing, China). All other chemicals used in this study were analytical-grade reagents sourced from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Preparation of crab sample

In accordance with welfare guidelines, crabs are euthanized through rapid cooling. River crabs (*Eriocheir sinensis*) were procured from Xinghua, Jiangsu, China, with an average carapace length of 6.0 ± 0.2 cm and an average weight of 100 ± 0.4 g. The purchased crabs were transported to the laboratory with ice blocks, ensuring delivery within 24 h. No pre-treatment was conducted prior to cleaning. Fig. 1 summarizes the experimental procedures and potential mechanisms. Fresh river crabs were divided into five groups, each containing ten individuals. An ultrasonic bath (HK ultrasonic generator, Wuxi Huaneng Ultrasonic Electronics Co., Ltd.) was utilized, operating at a power of 300 W and sound frequencies of 28, 40, and 80 kHz. The organic acids employed included lactic acid, ascorbic acid, phytic acid, malic acid, and citric acid, each at a concentration of 0.5–2 % (w/v). Preliminary screening results are illustrated in Fig. S1a, while repeat screening results are presented in Fig. S1b. The optimal sterilization conditions were categorized into three groups: (a) US, where crabs were first soaked in ultrapure water for 30 min and then ultrasonically cleaned for 10 min; (b) 0.5 % MA-1 % LA, where crabs were soaked in a solution of 0.5 % malic acid and 1 % lactic acid for 30 min; (c) 1 % LA-0.5 % MA-US, where crabs were initially soaked in a compound solution of 1 % lactic acid and 0.5 % malic acid for 30 min, followed by ultrasonic cleaning for 10 min. Crabs soaked in ultrapure water for 30 min served as controls (CK). Ultrasound treatment was applied in a continuous mode for 10 min following a 30 min soaking period in an organic acid solution. These parameters were determined based on preliminary optimization experiments, which demonstrated that this combination achieved the highest bacterial reduction while minimizing adverse effects on crab quality. The entire river crab was immersed in the soaking solution, and the temperature of the ultrasonic cleaning solution was maintained at room temperature (approximately 25 °C). To prevent excessive heat accumulation during cavitation, the ultrasonic bath temperature was maintained at approximately 25 °C. This temperature control ensured that heat-induced microbial inactivation was negligible, and bacterial reduction was primarily attributed to mechanical and chemical effects rather than thermal influences. The treated river crabs were then subjected to microbiological and physicochemical analyses.

2.3. Ultrasound treatment combined with organic acid inhibits the growth of A. hydrophila in vitro

2.3.1. Ultrasound treatment combined with organic acid inhibits bacterial growth

To evaluate the bactericidal effect on *A. hydrophila*, the bacterial suspension (OD600nm = 1.0) cultured for 24 h was washed with 10 mM PBS, subjected to centrifugation at 6000 rpm for 5 min, and then resuspended in PBS. The bacterial suspension was adjusted to an OD600nm of 0.5 (approximately 10^8 CFU/mL). Subsequently, the bacterial suspensions were treated with four groups: CK, US, 0.5 % MA-1 % LA, and 1 % LA-0.5 % MA-US, respectively. These suspensions were then plated onto LB agar medium using a gradient dilution method and incubated at 37 °C for 48 h.

2.3.2. Cellular leakage measurement

The bacterial suspension underwent four distinct treatments: control (CK), ultrasound (US), a combination of 0.5 % malic acid (MA) and 1 % lactic acid (LA), and a combination of 1 % LA and 0.5 % MA with ultrasound (1 % LA-0.5 % MA-US). Following treatment, the samples were centrifuged at 10,000 rpm for 10 min, and the supernatant was collected for subsequent leakage analysis. Cellular leakage of nucleic acids and proteins was evaluated using ultraviolet (UV) absorbance measurements. The supernatant was analyzed at wavelengths of 260 nm and 280 nm. These wavelengths are characteristic of nucleic acids and proteins, and the absorbance values indicate the extent of leakage of intracellular nucleic acids and proteins resulting from the treatments.

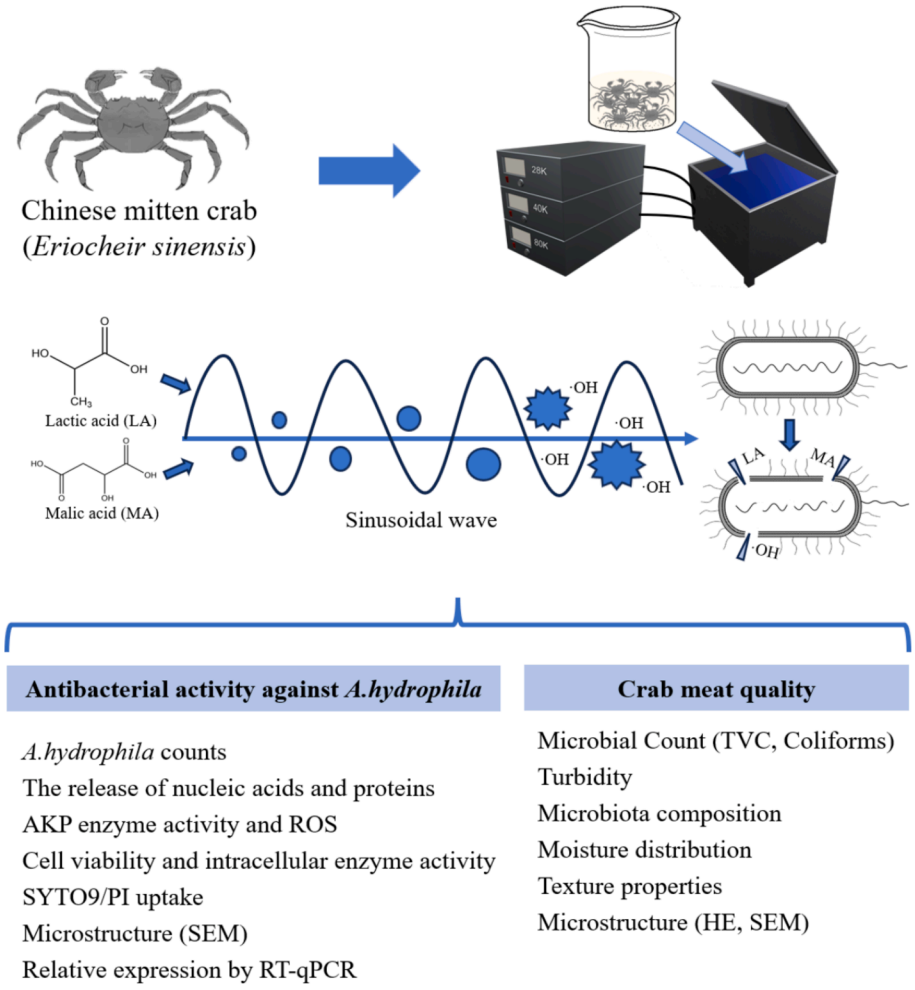

Fig. 1. Investigation pathways of ultrasonic and lactic acid and malic acid treatments on *A. hydrophila* and crabs.

2.3.3. Measurement of reactive oxygen species (ROS) and alkaline phosphatase (AKP) activity

The levels of reactive oxygen species (ROS) and alkaline phosphatase (AKP) enzyme activity were measured according to the instructions provided with the respective assay kits.

2.3.4. SYTO9/PI intake measurement

Bacterial cells were centrifuged at 6000 × g for 5 min at 4 °C, then resuspended in 0.85 % sterile saline. Subsequently, 3 μL of a mixed dye solution containing SYTO 9 (3.34 mM) and propidium iodide (PI) (20 mM) was added to the suspension and incubated in the dark for 15 min. For single staining, 1.5 μL of PI dye was added to the suspension and incubated in the dark for an additional 15 min. Subsequently, 5 μL of the stained bacterial suspension was placed on a glass slide, covered with a coverslip, and observed using an inverted confocal laser scanning microscope (LSM 880, Carl Zeiss Inc., Germany). The maximum excitation and emission wavelengths of the two dyes are 480/500 nm (SYTO 9) and 490/635 nm (PI), respectively. Image processing was performed using ZEN software.

2.3.5. Intracellular enzyme activity and cell membrane integrity assessment

Intracellular enzyme activity and cell membrane integrity were assessed using flow cytometry (BD FACS Aria III, BD Biosciences, USA) following the methodology outlined by Zhang [23], with minor modifications. Flow cytometric analysis was performed using single staining with propidium iodide (PI) or 5-carboxyfluorescein diacetate (5-cFDA), as well as dual staining with both 5-cFDA and PI. Cells stained with 5-cFDA exhibited green fluorescence at approximately 516 nm (FL1), whereas PI-stained cells displayed red fluorescence at around 647 nm (FL3). Data were collected by measuring forward scatter (FSC), side scatter (SSC), and fluorescence in channels FL1 (green) and FL3 (red) using an excitation wavelength of 488 nm. A total of 10,000 events were recorded per sample, with an event rate ranging from 400 to 600 events per second at a low flow rate. Flow cytometry data were subsequently analyzed using FlowJo v10 software.

2.3.6. Scanning electron microscopy (SEM) analysis

The morphological characteristics of *A. hydrophila* were investigated using scanning electron microscopy (SEM). Bacterial suspensions were subjected to various treatments, followed by centrifugation to obtain a pellet, which was then washed twice with PBS. The cells were fixed with 2.5 % glutaraldehyde (Sigma-Aldrich, Darmstadt, Germany) at 4 °C for 2 h. After fixation, the cells underwent additional washing with PBS and were subjected to a graded series of ethanol solutions (35 %, 50 %, 70 %, 80 %, 90 %, and 100 % (v/v)) for dehydration. Subsequently, the samples were gold-coated under vacuum and examined using a scanning electron microscope (JSM-7500F, JEOL, Japan) at magnifications of 10,000 × and 20,000 × . Untreated cells served as the control group for comparison.

2.3.7. Relative gene expression by RT-qPCR

Total RNA from *A. hydrophila* was extracted using the RNA-easy isolation reagent. Following the removal of genomic DNA with a DNA removal kit, the total RNA was reverse transcribed into complementary

DNA (cDNA). The purity of the cDNA was evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The RT-qPCR system was prepared with SYBR Green qPCR Premix, and quantitative PCR reactions were conducted on a PIKOREAL 96 instrument (Thermo Fisher Scientific, USA). The primers used for gene amplification are listed in Table 1. The relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.4. Physical and chemical properties of crabs

2.4.1. Microbial count

Microbiological parameters were determined before and after soaking in organic acids and sonication. Bacterial counts and coliform analysis were performed on crab shell and gill samples, as these surfaces are most susceptible to microbial contamination. For microbial community analysis, tissue samples from the shell and gill were collected. Total colonies in crabs were inoculated by the pour plate method using Plate Count agar (Hopebio, Qingdao, China) and incubated at 30 °C/72 h [24]. Coliforms were inoculated using lauryl sulfate tryptone (LST) broth (Hopebio) and brilliant green lactose bile salt (BGLB) broth (Hopebio) and incubated at 36 °C/48 h [25].

2.4.2. Bacterial community analysis

Crab samples of gill and shell were collected after soaking or sonication in different organic acids for bacterial community analysis. Each set of samples was measured in triplicate. After completing the genomic DNA extraction, 1 % agarose gel electrophoresis was used to detect the extracted genomic DNA. Illumina sequencing was performed by Shanghai Majorbio Biotechnology Company using 338F (ACTCCTACGGGAGGCAGCA) and 806R (GGACTACHVGGGTWTCTAAT) primers in the V3-4 region of 16S rRNA. Sequencing and bioinformatics analysis were performed using the Qiime platform (https://qiime.org/scripts/assign_taxonomy.html). Data were analyzed on the Majorbio cloud platform online platform (https://www.majorbio.com).

2.4.3. Turbidity

Turbidity measurements were conducted using the washing liquid obtained after treatment. And performed using a turbidity meter (TE-700Plus, Tianer, China), which was zeroed with distilled water before measurement.

2.4.4. Moisture distribution

The crab sample was cut into 10 mm × 10 mm × 40 mm rectangular parallelepipeds, placed in a 10 ml centrifuge tube, then placed in an NMR tube and placed in the magnet chamber. The LF-NMR parameters were based on the method of Liu et al. [26] with slight modifications. The experiment was measured three times in parallel.

Table 1
The primers used in the study.

Gene	Primer direction	Sequence of primers (5′→3′)
AerA	Forward	AAGACGGCCATCAAGGTCAG
	Reverse	CCAGTTGGTGGCAGTATCGT
exeA	Forward	AACTACGGGTTGCAGGATGG
	Reverse	AGATCTCGTTCGGTCAGGGA
fur	Forward	TGTTACCCGTCACCACTTCG
	Reverse	GTGGCCGTACAGATAGAGGC
hcp1	Forward	AAGACGAGATGCTGGTGCAA
	Reverse	TTCAGGGTCACTTTCGGCAG
TonB	Forward	ACTGGATCTGACCCTCTCCC
	Reverse	TTTTCTCCGTAGACCACCGC
cysE	Forward	TCCGGGAAGGGGTGATGATA
	Reverse	ACACTGATCCATGTCCAGCG
envZ	Forward	GAGGAGCGCAACCTGATGAT
	Reverse	CGGCCGAATGTAGTCGATGA
mshK	Forward	TGTCTGTTCAGTCTGCTGGC
	Reverse	ATAACTGCTGCCGTTGAGCA
ompR	Forward	TGACCCGTGAGAACTTCAGC
	Reverse	CAGGTAATCATCGGCACCCA
16S	Forward	CGATTAACGCTTGCACCCTC
	Reverse	TGCACAATGGGGAAACCTGA

2.4.5. Texture profile analysis (TPA)

TPA was conducted according to the protocol in previous study [27]. The TPA of crab leg muscle tissue samples with dimensions of 10 mm × 10 mm × 30 mm and crab hepatopancreas samples with dimensions of 20 mm × 20 mm × 20 mm was measured by the physical property measuring instrument (TMS-Pro, F.T.C., USA). The hardness, springiness, and chewiness of the properties were measured using a P/20 cylindrical probe with a measured speed of 1 mm/s and a measured deformation of 60 %.

2.4.6. Microstructure properties

The samples were cut into cubes (5 mm × 5 mm × 5 mm) and fixed with 4 % paraformaldehyde, and the resulting paraffin sections were dewaxed and stained with Hematoxylin and eosin (H&E). After dehydration, histological images were obtained for analysis by light microscopy (Eclipse E100, Nikon, Japan).

The microstructure of the samples was analyzed using SEM (SU8100, Hitachi, Tokyo, Japan) and observed at 300 × magnification as previously described by Zhu et al. [28].

2.5. Statistical analysis

Each experiment was repeated three times. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS 20 software, and average separation was performed by Tukey's multi-range test. $P < 0.05$ was considered significant difference. In addition, data were plotted using GraphPad Prism 5.0 software and the stacked bar chart was drawn using ChiPlot (https://www.chiplot.online/).

3. Results and discussion

This study investigates the effectiveness and mechanisms of various treatments, specifically the bactericidal effects of ultrasound (US) treatment combined with lactic acid and organic acid immersion (LA-MA), in controlling the growth of the specific spoilage bacterium *A. hydrophila* in crabs. Furthermore, the research explores the synergistic bactericidal effects of ultrasound and organic acids, along with their influence on the sensory quality of the crabs. The experimental approach is illustrated in Fig. 1.

3.1. Antibacterial activity of different treatments against A. hydrophila

Fig. 2A illustrates the inactivation effects of various treatments on *A. hydrophila* at 0 h and 24 h. Following treatment with ultrasound (US), lactic acid-malic acid (LA-MA), and the combined ultrasound and lactic acid-malic acid treatment (US-LA-MA), the counts of *A. hydrophila* cells decreased by 2.27, 3.77, and 4.16 log CFU/mL at 0 h, and by 0.72, 5.08, and 5.49 log CFU/mL at 24 h, respectively, compared to the control group. The reduction in *A. hydrophila* cell counts resulting from US treatment may be attributed to the physical effects of ultrasound, such as ultrasonic cavitation and mechanical vibration, which likely disrupted the cell membrane structure of *A. hydrophila*, leading to the leakage of cellular contents and cell death [29]. Additionally, ultrasonic treatment may have activated the stress response in *A. hydrophila*, thereby inhibiting its growth [30]. LA-MA treatment also exhibited antibacterial effects against *A. hydrophila*. Organic acids may enhance cell membrane permeability by lowering intracellular pH and disrupting metabolic pathways, resulting in the leakage of intracellular substances and consequently inhibiting the growth of *A. hydrophila* [31]. Compared to ultrasound treatment, organic acid treatment may have a more direct impact on the metabolic processes of *A. hydrophila*, as organic acids can penetrate the cell membrane and interfere with intracellular enzyme

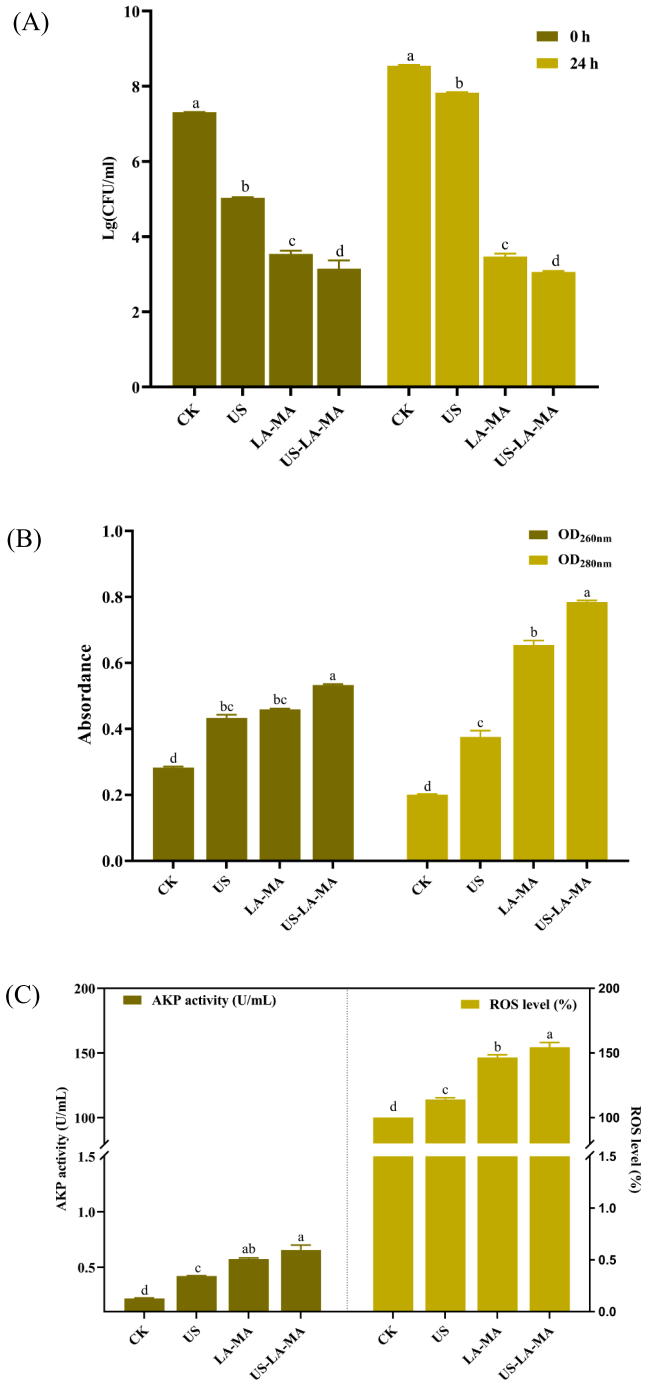

Fig. 2. Antibacterial activity of different treatments against *A. hydrophila*. A) The inactivation effects after different treatments on *A. hydrophila* at 0 h and 24 h; B) OD_{260} and OD_{280}; C) AKP activity and ROS level of *A. hydrophila* after different treatments. Letters (a, b, c, etc.) in the figure mean the statistic significant difference ($P < 0.05$).

activity [32,33]. The US-LA-MA group demonstrated the most significant antibacterial effect against *A. hydrophila*. This combined treatment likely produced a synergistic effect, wherein the physical disruption caused by ultrasound complemented the chemical interference of organic acids, leading to a greater degree of damage to the cell membrane. This synergistic effect may have resulted in a more pronounced reduction in the number of *A. hydrophila*, exhibiting more effective antibacterial activity compared to either treatment alone. Furthermore, ultrasound may have enhanced the susceptibility of bacteria to organic acids, as ultrasound pretreatment may weaken the barrier function of the cell membrane [34].

As shown in Fig. 2B, the release of nucleic acids and proteins from *A. hydrophila* cells exhibited variations following different treatments. The optical densities at 260 nm (OD_{260}) and 280 nm (OD_{280}) for untreated *A. hydrophila* were 0.23 and 0.17, respectively; these values increased to 0.43 and 0.33 after ultrasound (US) treatment. In contrast, after treatment with lactic acid and malic acid (LA-MA) and the combined treatment, the OD_{260} and OD_{280} for *A. hydrophila* rose to 0.53 and 0.55, and 0.52 and 0.59, respectively. These results indicate that the combination of US and LA-MA effectively enhances the release of nucleic acids and proteins from *A. hydrophila* cells. This finding aligns with the study by Sun [34], which demonstrated that ultrasound facilitates the entry of chlorogenic acid small molecules into bacterial cells, thereby contributing to a synergistic antibacterial effect. The combined ultrasound treatment may induce damage to the cell membrane, resulting in the leakage of cellular contents. It is believed that ultrasonic treatment disrupts the integrity of the cell membrane through physical mechanisms, such as cavitation [35]. Additionally, organic acids may exacerbate cell membrane damage by lowering intracellular pH or directly interfering with metabolic pathways [36]. This interplay of physical and chemical effects likely contributes to the substantial leakage of cellular contents. Furthermore, the CFU reduction results shown in Fig. 2A further corroborate these findings, confirming the synergistic antibacterial effects of the combined treatment. The synergistic application of ultrasound and organic acid effectively inhibits the growth and survival of *A. hydrophila* by compromising the cell membrane, leading to the leakage of intracellular proteins and nucleic acids.

The AKP enzyme activity, a marker of cell membrane damage, reflects the leakage of intracellular enzymes due to compromised membrane integrity [37]. As shown in Fig. 2C, the AKP enzyme activity in the supernatant of *A. hydrophila* cells was significantly increased in the US, LA-MA, and US-assisted LA-MA groups compared to the control group, with increases of 90.9 %, 159.1 %, and 200 %, respectively. These differences were statistically significant ($P < 0.05$). The combined treatment may have resulted in damage to the cell membrane of *A. hydrophila*, leading to increased membrane permeability and the leakage of AKP enzyme from the cells. Concurrently, the significant rise in ROS levels suggests that the synergistic effects of ultrasound and organic acids may induce ROS accumulation, triggering intracellular oxidative stress. This oxidative stress may damage cellular components, thereby inhibiting bacterial activity or resulting in cell death [29]. The slight increase in AKP activity and ROS levels in the US-LA-MA group compared to LA-MA indicates that ultrasound primarily enhances organic acid penetration rather than directly increasing ROS production. Although the difference in CFU reduction between LA-MA and US-LA-MA appears marginal, the combination treatment significantly enhances bacterial membrane disruption, as evidenced by increased AKP enzyme leakage and ROS level (Fig. 2C), suggesting a more profound bactericidal mechanism.

3.2. Cell viability and intracellular enzyme activity

Flow cytometry was employed to qualitatively and quantitatively assess the metabolic activity state, intermediate cell state, and death state of *A. hydrophila* cells. Non-fluorescent 5-cFDA, an esterified fluorescent substrate, has been extensively utilized to evaluate the esterase activity in bacteria. Its membrane permeability allows it to bind to nonspecific esterases within cells, subsequently hydrolyzing into fluorescent carboxyfluorescein, which emits green fluorescence. Propidium iodide (PI) can penetrate damaged cells and bind to nucleic acids, resulting in red fluorescence [38]. The results of the flow cytometer's PI/CFDA double staining are illustrated in Fig. 3. Cells located in the Q1 region indicate that both the cell membrane integrity and intracellular esterase activity have been compromised, categorizing them as dead

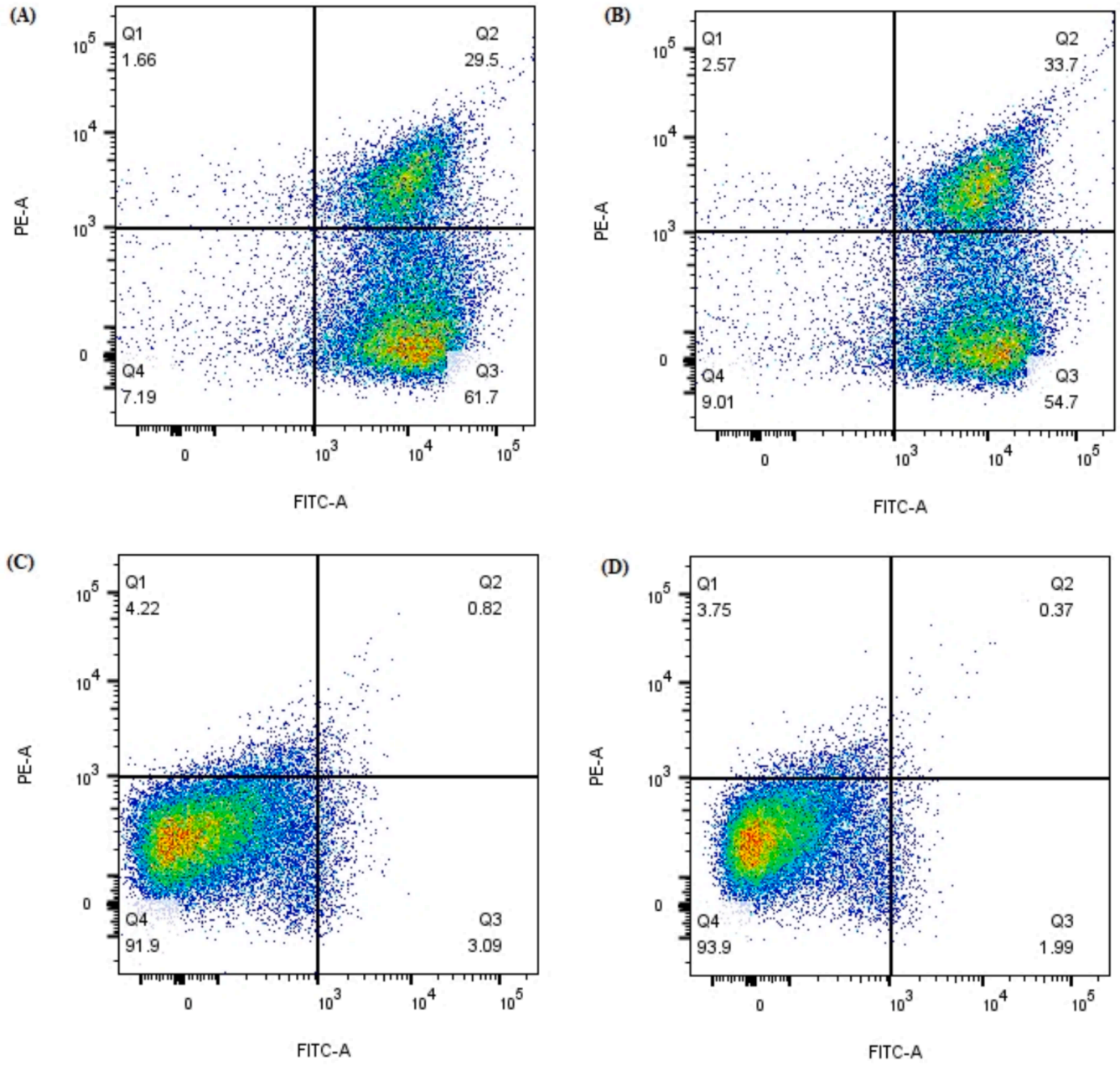

Fig. 3. Flow cytometry density plots of *A. hydrophila* cells after different treatments. (A) The control; (B) US treatment; (C) LA-MA treatment; (D) US-LA-MA treatment.

cells. The Q2 area signifies that while the cell membrane is damaged, intracellular enzyme activity remains high, indicating the presence of sublethal cells with metabolic activity. Cells in the Q3 area exhibit an intact cell membrane and high enzyme activity, identifying them as normal cells. Conversely, cells in the Q4 area have an intact membrane but lack enzyme activity, suggesting they are either dead cells or cell fragments [23]. Most cells in the untreated group are predominantly found in the Q2 and Q3 quadrants, indicating a substantial number of normal cells that maintain high activity and integrity. Following ultrasound (US) treatment, microorganisms gradually transitioned from Q3 to Q1, Q2, and Q4, with the proportions of Q1, Q2, and Q4 increasing from 1.66 %, 29.5 %, and 7.19 % to 2.57 %, 33.7 %, and 9.01 %, respectively. This result indicates that the US inactivation process preferentially targets the cell membrane structure of microorganisms, while leaving intracellular enzyme activity largely unaffected. Following LA-MA treatment, enzyme activity was nearly entirely inactivated, with the proportion of dead cells in the Q4 quadrant reaching 91.9 %. At this point, the intracellular enzymes of the microorganisms were fully inactivated, resulting in a complete loss of metabolic capacity. Concurrently, the organic acid treatment likely inflicted significant damage to the cell membrane, leading to the loss of viability in a substantial number of cells. After the combined treatment, the proportion of cells in the Q2 quadrant decreased to 0.37 %, while that in Q4 increased to 93.9 %. This distribution pattern further substantiates the synergistic destructive effect of the combined treatment of ultrasound and organic acid on the cell membrane, which may enhance membrane permeability, causing leakage of cellular contents and subsequent cell death. The mechanical action of ultrasound may disrupt the cell membrane through cavitation, while organic acids collectively target bacterial cells by lowering intracellular pH or directly interfering with metabolic pathways, ultimately inducing cell death [39].

3.3. SYTO9/PI uptake

PI (propidium iodide) is a hydrophilic red fluorescent probe commonly used to indicate membrane permeability, enabling the distinction between live and necrotic cells. This is due to its ability to traverse damaged cell membranes and bind to nucleic acids, resulting in a 30-fold increase in red fluorescence [40]. In contrast, the green fluorescent nucleic acid stain SYTO 9 labels both intact and damaged bacterial cells, with a significant enhancement of its fluorescence signal upon binding to nucleic acids [41]. When both dyes are present, PI exhibits a greater affinity for nucleic acids than SYTO 9, leading to the displacement of SYTO 9 by PI [42]. As illustrated in Fig. 4, untreated cells with intact bacterial membranes displayed green fluorescence due to the interaction of SYTO 9 with DNA, while PI did not fluoresce as it was unable to penetrate the cell interior. Following ultrasound (US) treatment, the proportion of green fluorescent cells decreased, and the percentage of red fluorescent cells slightly increased, suggesting that US induced minor membrane damage. After treatment with LA-MA, green fluorescent cells gradually diminished, with red fluorescent cells becoming predominant, indicating that organic acids compromised the integrity and membrane permeability of *A. hydrophila* cell membranes. Post combined treatment, nearly all observed fluorescence was red, with virtually no green fluorescence remaining, signifying a synergistic effect that enhanced bactericidal efficiency.

3.4. SEM analysis

The morphology of *A. hydrophila* cells following various treatments was examined using scanning electron microscopy (SEM) (Fig. 5). The surface of untreated *A. hydrophila* cells appeared smooth and intact (Fig. 5A and a). Ultrasound (US) treatment inflicted significant damage to the morphology of *A. hydrophila* (Fig. 5B and b), the cell surface is not as smooth as that of the untreated group cells and has obvious wrinkles. Following lactic acid-malic acid (LA-MA) treatment, more wrinkles and tiny holes emerged on the surface of *A. hydrophila* (Fig. 5C and c), indicating that organic acid treatment can alter the morphology of *A. hydrophila* and create pores, consistent with findings from other studies. For instance, Sun [34] reported that chlorogenic acid increased the surface roughness of Salmonella cells, causing them to appear wrinkled; treatment at ultrasound in combination with chlorogenic acid further increased the number of rough cells, which exhibited surface disintegration and lysis, resulting in the loss of their regular shape. Sun [43] noted that cells of *S. aureus* exhibited lysis and pore formation after treatment with 2 % CA and US. Additionally, the morphological characteristics of *A. hydrophila* cells were significantly altered after combined US and LA-MA treatment, evidenced by a reduction in intact cells and an increase in cell fragments (Fig. 5D and d). These observations align with the antibacterial efficacy results, indicating that the combined treatment had more pronounced deleterious effects on the bacterial surface structure compared to US or LA-MA treatment alone. The bacterial cell membrane plays an essential role as a protective barrier, serving as a fundamental target for many antimicrobial agents [44]. Its integrity is vital for maintaining the overall functionality and health of the bacterial cell. When this membrane is disrupted, it results in the leakage of important intracellular substances, which significantly inhibits bacterial growth and may ultimately lead to cell death [45]. This indicates that the bacterial cell membrane is a critical point of intervention in the fight against bacterial infections. In light of these mechanisms, it is proposed that a combined treatment approach may result in irreversible damage not only to the cell wall but also to the membrane of *A. hydrophila* cells, further enhancing the efficacy of antimicrobial strategies against this pathogen.

3.5. Relative expression by RT-qPCR

In Fig. 6, we observed the relative expression levels of multiple genes under different treatment conditions that are closely related to the virulence expression, flagella, and biofilm integrity of *A. hydrophila*. The figure illustrates the relative expression of each gene across four treatments (CK, US, LA-MA, and US-LA-MA) compared to the control group (CK). In the US-treated group, the expression levels of the *AerA* (encodes aerolysin, a pore-forming toxin linked to virulence [46]) and *hcp1* genes (affects the biological characteristics of *Aeromonas* such as motility, biofilm formation and cytotoxicity by regulating flagellar assembly [47]) were comparable to those in the control group, suggesting that these genes might not be significantly affected by US treatment;

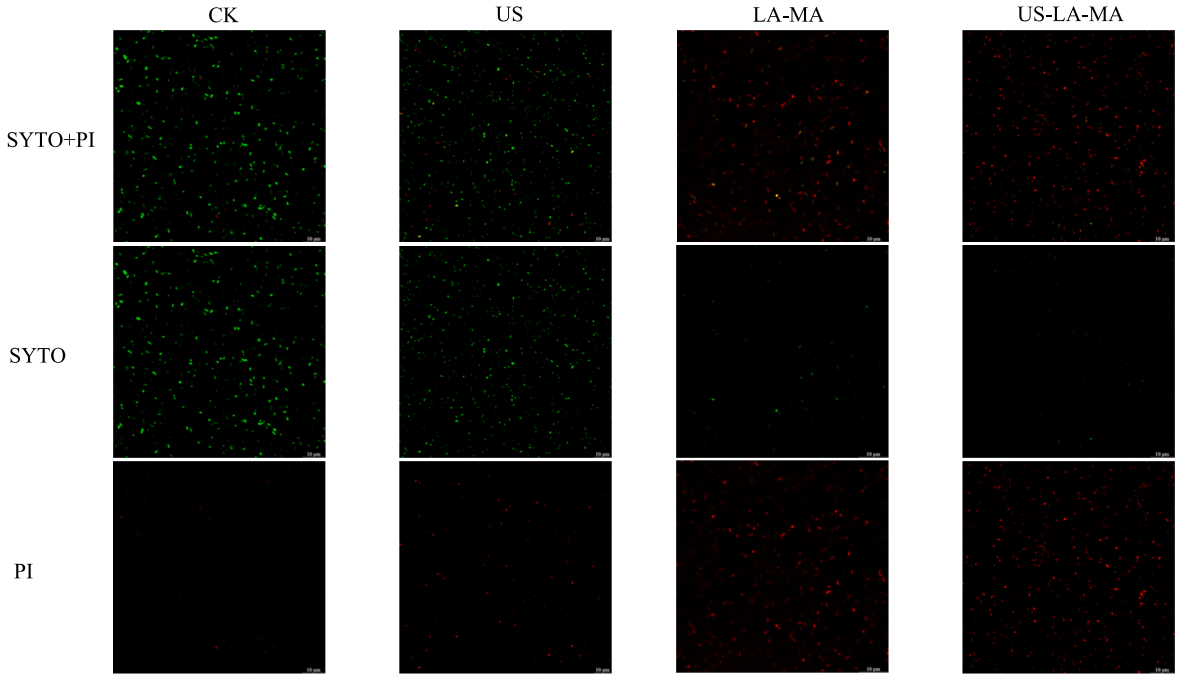

Fig. 4. Confocal laser scanning microscopy images of *A. hydrophila* after different treatments with SYTO 9 & PI double stained, with PI single stained, and SYTO 9 single stained.

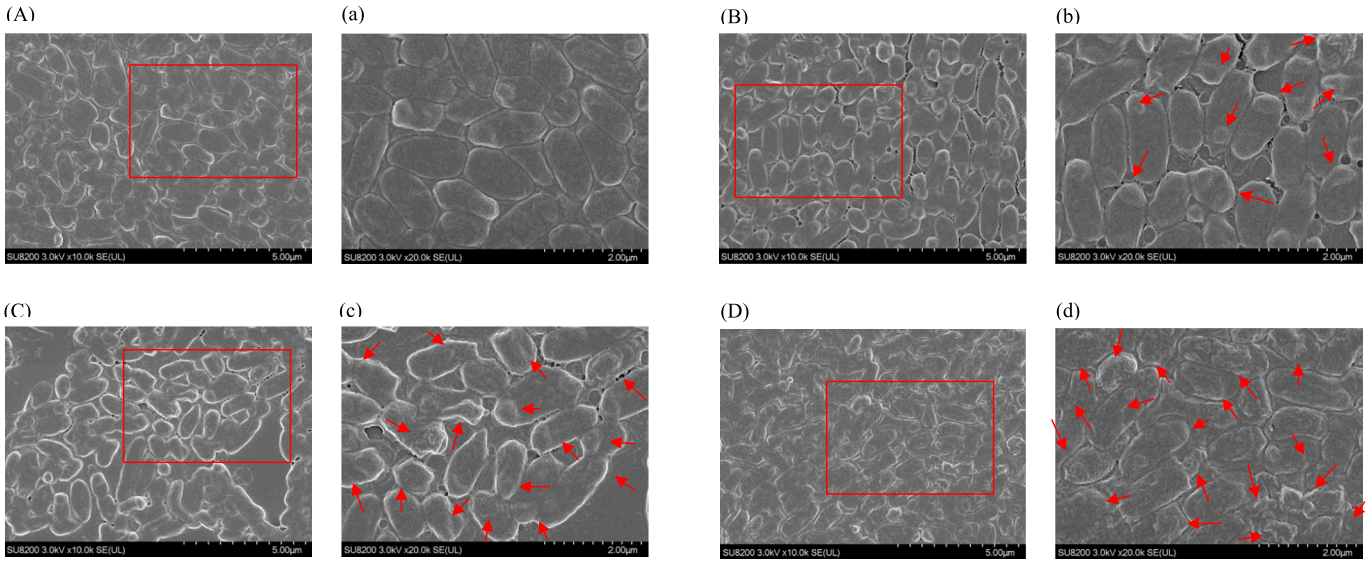

Fig. 5. The morphology of *A. hydrophila* cells observed by Scanning electron microscopy after different treatments. The red rectangle indicates the location of the 20,000x image in the 10,000x SEM image, and the arrows indicate pores or structural deformities. (A, a) The control; (B, b) US treatment; (C, c) LA-MA treatment; (D, d) US-LA-MA treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

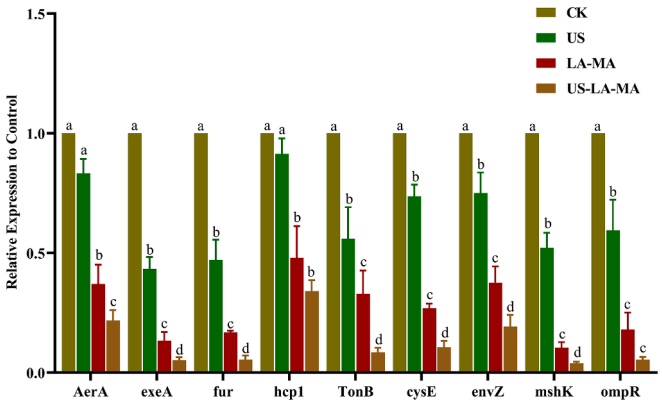

Fig. 6. Gene transcriptional level related to the virulence expression, flagella, and biofilm integrity of *A. hydrophila* after different treatments. Letters (a, b, c) in the figure mean the statistic significant difference ($P < 0.05$).

however, they were significantly influenced by LA-MA and the combined treatment ($P < 0.05$). The expression levels of the *exeA* (affects biological characteristics such as cell exotoxin secretion and biofilm formation by regulating T2SS assembly [48]) and *fur* (affects the pathogenicity of *Aeromonas* by regulating flagellar assembly and biofilm formation [49]) genes in the US group were significantly lower than those in the control group, indicating that ultrasound treatment may inhibit the expression of genes related to virulence, flagella, or iron metabolism [48,50]. Following LA-MA and the combined treatment, the expression levels were further reduced, implying that the combined treatment could synergistically enhance the expression of genes associated with virulence, flagella, or iron metabolism in *A. hydrophila*. Additionally, the expression levels of the *hcp1* and *TonB* (affects the motility of *Aeromonas* by controlling the secretion of flagellar proteins [51]) genes were significantly lower in the US-LA-MA group compared to the other groups, which may reflect the effects of ultrasound combined with lactic acid and malic acid treatment on bacterial protein synthesis, cell membrane function, and flagella [47,51]. The expression levels of the *cysE*, *envZ*, *mshK*, and *ompR* genes (plays a major role in drug resistance, stress resistance and virulence by regulating biofilm formation [52]) in the US group were significantly lower than those in the control group, and those in the LA-MA and US-LA-MA groups were also significantly lower than those in the US group, suggesting that lactic acid and malic acid treatment may impair the ability of *A. hydrophila* to form biofilms [53]. These changes in gene expression align with the previously reported effects of ultrasound and organic acid treatment on bacteria [52–55]. Specifically, ultrasound treatment may compromise the integrity of cell membranes through physical mechanisms, while organic acids can disrupt cellular metabolic pathways via chemical interactions. The combination of these treatments likely resulted in the leakage of cellular contents and subsequent cell death, as indicated by alterations in gene expression. Furthermore, the expression levels of multiple genes in the ultrasound combined with organic acid treatment group were significantly lower than those in the other groups, further validating the efficacy of this combined approach. This finding is corroborated by Chen [11], which demonstrated that the concurrent application of ultrasound and organic acid can substantially reduce bacterial counts and enhance antibacterial efficacy. The US-LA-MA treatment may induce cell membrane disruption and metabolic pathway interference by modulating the expression of key genes in *A. hydrophila*, thereby effectively inhibiting bacterial growth and survival.

3.6. The physicochemical properties of crab

3.6.1. Microbial count and turbidity

As shown in Fig. 7A–C, the TVC and coliform count of crabs were significantly reduced, and the turbidity was significantly increased ($P < 0.05$) after combined treatment with ultrasound and organic acids. Compared with untreated controls, US, LA-MA, and US-LA-MA group were observed to reduce the original bacterial load of crabs by 9.62 %, 37.36 %, and 42.03 %, respectively (Fig. 7A). The number of coliform bacteria was not detected in LA-MA, and US-LA-MA group (Fig. 7B). The turbidity value after US-LA-MA treatment was increased by 2.06 times compared with the control group (Fig. 7C). Ultrasonic waves form high-pressure shock waves through the cavitation effect (the rupture of tiny bubbles generated by high-frequency vibration), which can effectively remove mud and sand particles and biofilm in the crab shell and cracks. These detached materials are suspended in the cleaning liquid, causing the turbidity to increase significantly. Lactic acid and malic acid further loosen the adhesion of mud and sand to the crab shell by dissolving surface dirt and penetrating into the cracks, thereby enhancing the

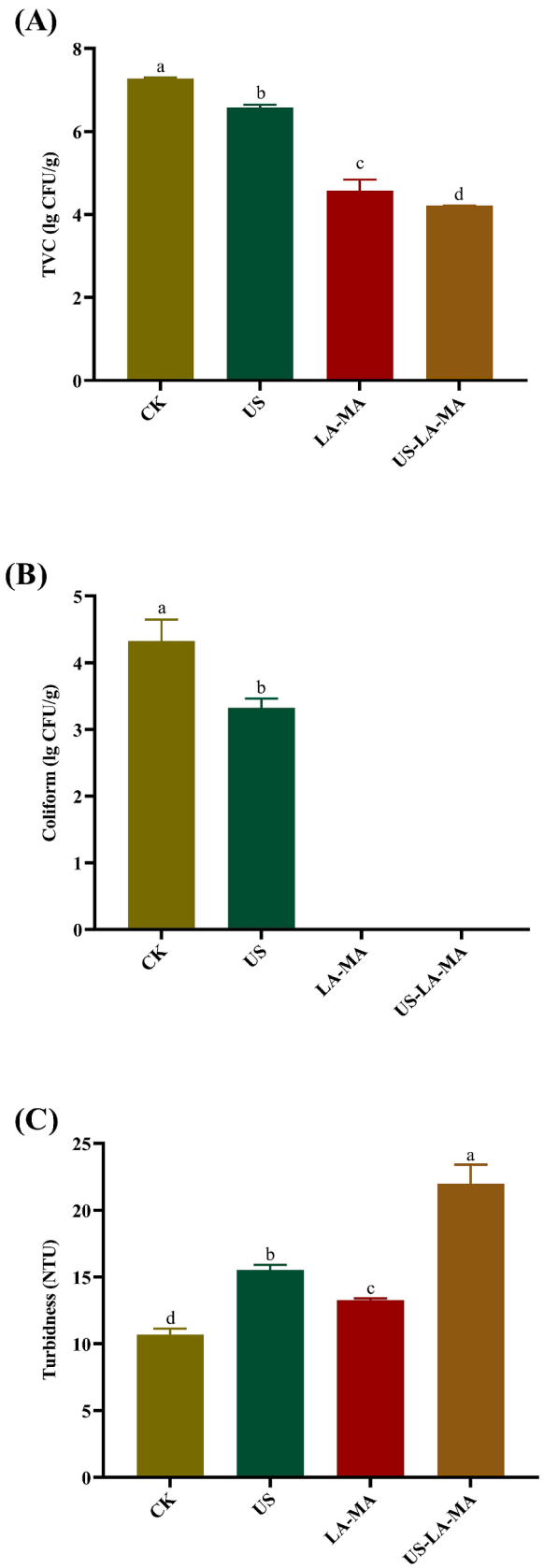

Fig. 7. Effect of different treatments on physicochemical properties of crab. A) Total cell population. B) Coliform population. C) Turbidness content. Letters (a, b, c) in the figure mean the statistic significant difference ($P < 0.05$).

efficiency of ultrasonic stripping of dirt. US has been reported to be able to effectively kill gram-positive bacteria by attacking the plasma membrane and internal structure of cells, while Gram-negative bacteria such as *Escherichia coli* are more difficult to kill than Gram-positive bacteria due to their bilayer structure of cell membrane [56]. Jeong and Ha [18] found that organic acids can achieve bacteriostasis by lowering the pH value of the bacterial environment. In addition, Yoon et al. [57] showed that non-dissociated organic acids enter bacterial cells through free diffusion as a complete molecule, and then dissociate into carboxyl ions and H^+ ions. Carboxyl ions have the function of inhibiting DNA replication, inhibiting protein synthesis and destroying bacterial cell membranes, thereby inhibiting bacteria from producing the next generation, while the dissociated H^+ reduces the pH value in bacterial cells. Bacteria pump excess H^+ out of the membrane through the Na-H^+-ATP (adenosine triphosphate) pump (active transport), which is an energy-consuming process. Therefore, bacteria expend energy to expel excess H^+ ions, a process that disrupts intracellular pH homeostasis, leading to metabolic inhibition and reduced bacterial viability [20]. The number of viable bacteria in crabs was the lowest after US was combined with compound organic acid, which confirmed that US-LA-MA had a synergistic antibacterial effect on crabs. This result shows that the cavitation effect of ultrasound may accelerate the dissociation of organic acids and cooperate with carboxyl ions and H^+ ions to enter the bacteria, leading to bacterial death and thus improving the antibacterial effect. This conclusion is consistent with the antibacterial effect on *A. hydrophila* mentioned above.

3.6.2. Microbiota composition

Most operational taxonomic units (OTUs) in crabs were identified within four bacterial phyla: *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Patescibacteria*. In comparison to the control group (72.48 %), the treatments with US, LA-MA, and US-LA-MA resulted in reduced levels of *Proteobacteria*, measuring 56.63 %, 43.71 %, and 45.01 %, respectively (Fig. 8A). *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Patescibacteria* are commonly reported in the microbiota of crabs and are also dominant in other food products and meat processing contexts [58–60]. As illustrated in Fig. 8B, an analysis of the composition and dynamics of bacterial communities at the genus level was conducted using common microbial histograms for untreated and combined-treatment samples. Following combined ultrasonic cleaning, the relative abundances of several bacterial genera, including *Acinetobacter*, *Flavobacterium*, and *Aeromonas*, decreased significantly; their values were recorded at 16.12 %, 9.55 %, and 0.87 %, respectively, which were lower than those of the control group (46.96 %, 12.37 %, and 3.44 %). Furthermore, the increased relative abundance of *Acinetobacter* and *Flavobacterium* in the US-LA-MA group compared with the LA-MA group may be due to selective microbial sensitivity and a decrease in the relative abundance of other bacteria. Ultrasound treatment may enhance the removal of more sensitive bacteria, allowing more resilient strains to persist and potentially increase in relative abundance. This phenomenon has been demonstrated in studies where the combination of ultrasound and antimicrobial agents significantly improved effectiveness against certain bacteria, while resistant strains such as Acinetobacter showed a survival advantage [61]. *Acinetobacter* is a well-known spoilage bacterium in aquatic products and meat, frequently studied in the literature [62]. *Flavobacterium* has been identified as an aquatic pathogen [63], while *Aeromonas* has also been documented in aquatic products and environments [1,64]. The bactericidal mechanism of ultrasound primarily operates through cavitation, whereby hydroxyl radicals penetrate and lyse bacterial cells. Pre-soaking with composite organic acids enhances the penetration of hydroxyl radicals and organic acids into the cells, thereby targeting components of the cytoplasmic membrane and intracellular enzymes, such as superoxide dismutase and catalase. The synergistic effect of these two treatments results in an enhanced bactericidal action.

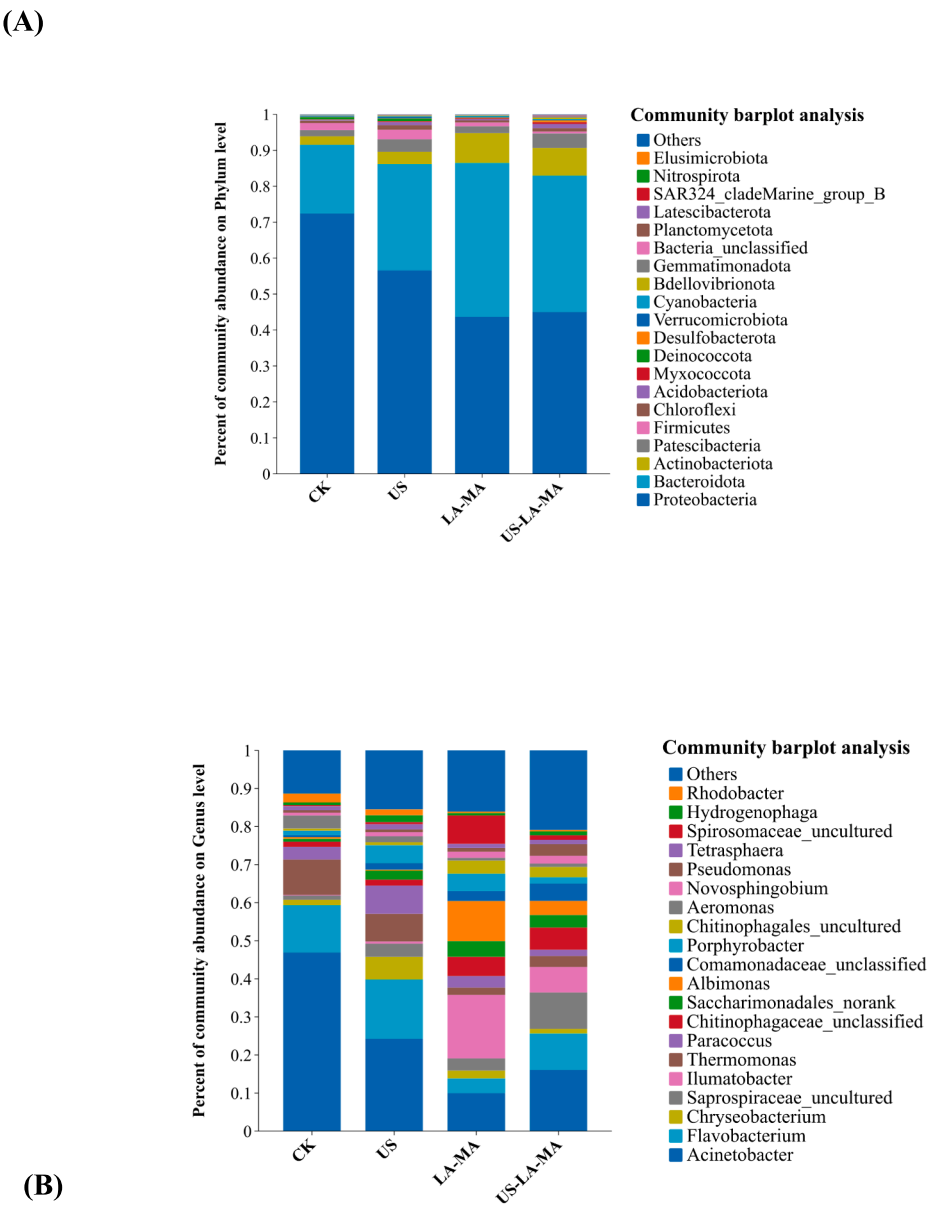

Fig. 8. Effect of different treatments on microbiota composition of crab. A) Composition and relative abundance at the Phylum level. B) Composition and relative abundance at the Genus level.

3.6.3. *Water distribution*

The moisture distribution of crab leg meat was elucidated using Low-Field Nuclear Magnetic Resonance (LF-NMR) technology. In the T_2 relaxation time, T_{21} (0.1–10 ms) is the bound water tightly bound to the polar groups of macromolecules. T_{22} (10–100 ms) is the immobilized water located within the muscle fiber network. T_{23} (100–1000 ms) is the free water in the myofibril lattice. As shown in Fig. 9, the CK group had the highest immobilized water content, and the US group had the lowest immobilized water content. In addition, the US group had the highest free water content, and the CK group had the lowest free water content. The cavitation effect of ultrasound will cause the structure of muscle fibers to be destroyed, and the water component in the cell lattice will be converted from immovable water to free water [28]. The addition of lactic and malic acids can mitigate ultrasound-induced protein damage, prevent bound water from converting to free water, and enhance the water-holding capacity of crab meat [22].

3.6.4. *Texture properties*

TPA is an important index of the quality and freshness of the aquatic products [65]. As shown in Fig. 10, the hardness, springiness, and chewiness of crab abdominal muscles was significantly decreased by 26.56 %, 0.07 %, 30.11 %, respectively, which was same in the crab hepatopancreas (40.42 %, 38.75 %, 60.81 %) after US treatment ($P < 0.05$). During the cavitation process, ultrasound destroys the integrity of myofibrils and induces the degradation of structural proteins, causing the structure of tissue cells to be destroyed, thereby reducing hardness [66]. Shi et al. [67] reported that the hardness of beef M. *semitendinosus* decreased with ultrasound treatment. However, the simultaneous use of ultrasound and LA combined with MA soaking will improve the hardness, springiness and chewiness. Li [22] reported that synergistic application of phytic acid and lactic acid can chelate transition state metal ions (Cu^{2+}) to maintain the textural characteristics of RTE shrimps. Therefore, it is speculated that LA and MA used in this study

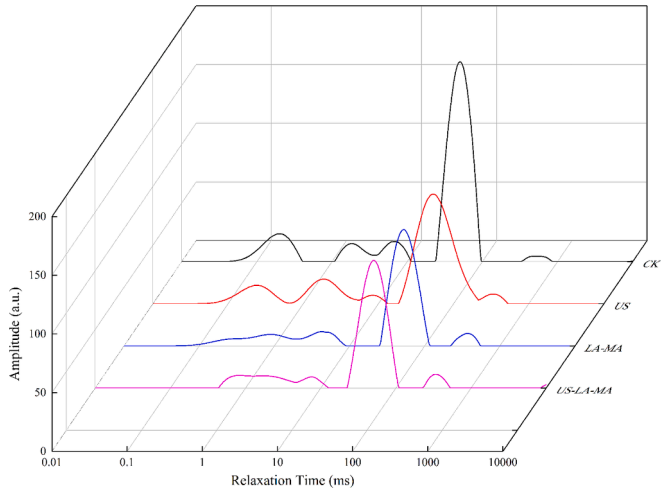

Fig. 9. Effect of different treatments on water distribution of crab.

may chelate transition metal ions (Cu^{2+}), which are present in crab tissues due to both endogenous processes (e.g., hemocyanin in muscle) and environmental exposure, and reduce the damage caused by ultrasound to the textural properties of crab muscles and hepatopancreas, thereby mitigating oxidative damage and preserving the quality of crab meat. However, these metal ions are not present in concentrations high enough to pose toxicity concerns in food applications [68].

3.6.5. *Microstructure*

Fig. 11 showed the microstructure images of crab tissues. The first column was the microstructure of the crab abdominal muscle (A-D), the second column was the microstructure of the hepatopancreas (a-d), and the third column was the SEM image of the abdominal muscle (i-iv). The muscle tissue fibers of the untreated crab were neatly arranged with complete microstructure. The SEM images showed smooth muscles without pores, complete shape of hepatopancreas, and clear internal structure. After US treatment, the muscle fibers of crabs were separated obviously, the fiber gap increased, the hepatopancreas were divided into segments, and the outer membrane was seriously damaged. SEM images showed that there were many pores in the muscle fibers, showing an uneven shape. Wang et al. [69] previously observed similar phenomena in mulberry cells using SEM and TEM. They explained that ultrasonic treatment would cause the contraction and expansion of mulberry cells, thus generating pore structure and damaging the microstructure of mulberry. In addition, the degree of muscle fiber separation and muscle fiber gap of crabs treated with LA combined MA were significantly smaller than those treated with US alone, and the degree of muscle fiber destruction of LA-MA-US was smaller, second only to the untreated group. These results indicated that LA-MA-US treatment can keep the muscle tissue of crabs intact. This finding is consistent with the results of the texture properties in Fig. 10.

4. Conclusion

This study systematically evaluates the synergistic effects and underlying mechanisms of ultrasound combined with a lactic acid-malic acid complex solution for the decontamination of Chinese mitten crabs. The combined treatment significantly enhances bactericidal activity by disrupting the membranes of *Aeromonas hydrophila* and downregulating key biofilm-associated genes, resulting in substantial reductions in bacterial counts. Furthermore, this approach preserves the structural integrity and textural properties of crab tissues, thereby maintaining their sensory quality. By integrating the physical effects of cavitation with chemical disruption, this method presents an eco-friendly and efficient solution for improving food safety and quality in

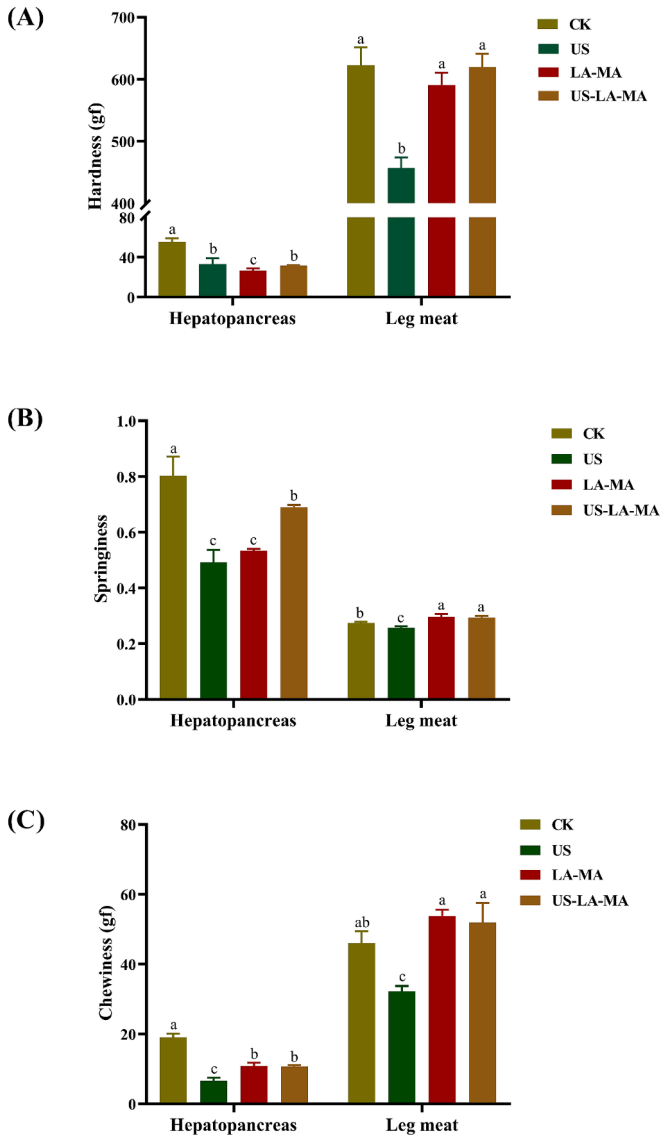

Fig. 10. Effect of different treatments on texture properties of crab. A) The hardness of crab hepatopancreas and leg meat. B) The springiness of crab hepatopancreas and leg meat. C) The chewiness of crab hepatopancreas and leg meat. Letters (a, b, c) in the figure mean the statistic significant difference ($P < 0.05$).

aquatic product processing. These findings underscore the potential for scaling this approach to industrial applications, offering a sustainable alternative to conventional methods while minimizing chemical residues and environmental impact. Future studies should concentrate on optimizing treatment parameters and investigating its applicability across diverse aquatic products to enhance its industrial viability.

5. Ethical guidelines

Ethics approval was not required for this research.

CRediT authorship contribution statement

Han Huang: Writing – review & editing, Writing – original draft, Methodology, Data curation. **Yanjun Tong:** Project administration, Funding acquisition. **Xiaomei Lyu:** Visualization, Methodology, Formal analysis. **Wei Zhao:** Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis. **Ruijin Yang:**

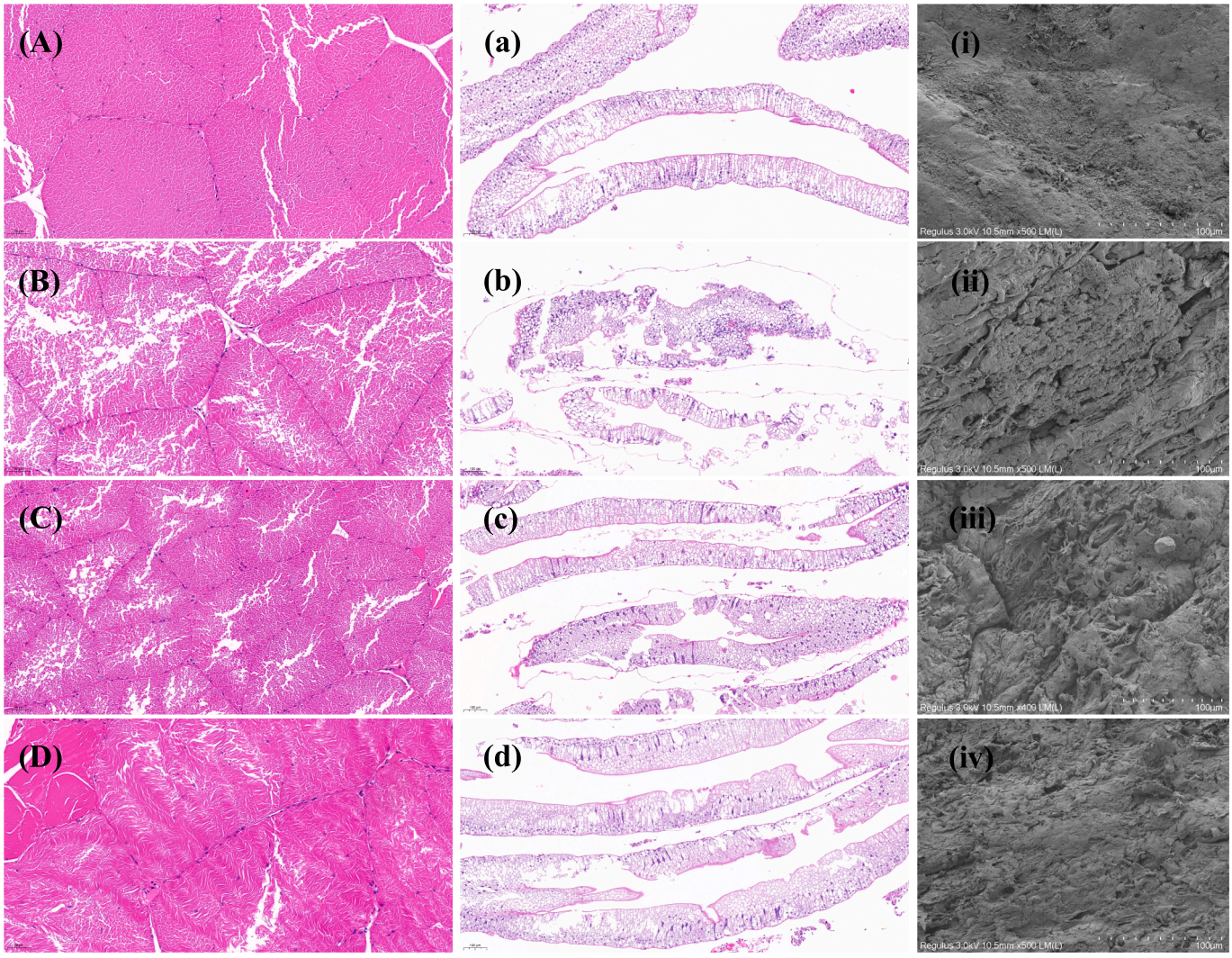

Fig. 11. Effect of different treatments on microstructure of crab. A) The microstructure of the crab abdominal muscle (A-D). B) The microstructure of the hepatopancreas (a-d). C) The SEM image of the abdominal muscle (i–iv). Labels correspond to untreated control (A, a, i), ultrasound treatment (B, b, ii), LA-MA treatment (C, c, iii), and US-LA-MA treatment (D, d, iv).

Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Funding acquisition.

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.ultsonch.2025.107294.

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