Effects of decontamination treatment combined with natural chemicals and/or ultra-high pressure on the quality and safety of ready-to-eat wine-pickled mud snails (*Bullacta exarata*)

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Abstract

Ready-to-eat wine-pickled mud snails (*Bullacta exarata*) typically host a large number of microorganisms and are frequently contaminated with pathogenic bacteria during processing, resulting in a higher risk for foodborne illness with consumption. In this study, the decontamination effects of different treatment methods, including the use of ultrasonic cleaning (USC), natural chemicals, and ultra-high pressure (UHP), on the quality and safety of pickled mud snails were investigated by assessing the total viable count (TVC), total volatile base nitrogen (TVB-N) content, thiobarbituric acid-reactive substance (TBARS), and pH value of the products after 12 months of storage at −20 °C. Treatment with 200 W USC for 5 min was the most effective approach for reducing TVC in raw mud snails, with a minimal change in food quality. Natural chemical treatment or UHP treatment significantly inhibited the increase in TVC, pH, and TBARS and TVB-N accumulation compared with the control group; however, their combined treatment had no synergistic effect. In contrast, the combined chemical treatment was more effective in inhibiting changes in the above indices in pickled mud snails than UHP treatment alone or combined chemicals + UHP treatment. In addition, the bacterial diversity of pickled mud snails before and after 12 months of storage at −20 °C was determined using Illumina MiSeq sequencing. Our results indicated that USC combined with natural chemicals can be utilized commercially to maintain the quality and safety of pickled mud snail during storage at −20 °C.

KEYWORDS

Ultrasonic cleaning; Illumina MiSeq; Bacterial diversity; Foodborne illness; Shelf-life
Introduction

The mud snail (*Bullacta exarata*), a shell mollusk, lives on inshore tidal land and is commonly used as a cooking material in the coastal regions of China (Wang *et al.*, 2016; Zhang *et al.*, 2013). Ready-to-eat wine-pickled mud snail is a type of processed aquatic products containing mud snails, salt, sugar, monosodium glutamate, and yellow wine to maintain the characteristic tastes, nutrients, and flavors of the raw materials (Liao *et al.*, 2020). It is usually eaten as a raw meat, without cooking or boiling. The mud snails filter enormous amounts of seawater and concentrate pathogens in its digestive glands, the mucous in its gills, and other tissues (Liao *et al.*, 2020). Thus, the pickled mud snail is highly susceptible to microbial spoilage, which can lead to foodborne illnesses when consumed (Lin *et al.*, 2020). Excessive microbial quantity leads to outbreaks of diarrheal illness associated with the consumption of raw seafood (Yang *et al.*, 2013). Thus, the main goal of pickled mud snail preservation is to reduce microbial spoilage and improve food safety by maintaining the original quality of the snail when harvested. To achieve this goal, several techniques have been examined, including ultrasonic cleaning (USC), ultra-high pressure (UHP) treatment, cold plasma, pulsed light, ultraviolet light, irradiation, ozone, and the application of natural chemicals (Alarcon-Rojoa *et al.*, 2019; Hygreeva and Pandey, 2016; Deng *et al.*, 2020a; Deng *et al.*, 2020b; Hassanzadeh *et al.*, 2017; Chung *et al.*, 2019).

Sodium hypochlorite (NaClO) is a widely used sanitizer; nevertheless, its limited efficacy and the toxicity of its chlorinated by-products that form in the presence of organic matter have prompted much concern. Therefore, the search for an effective and innocuous sanitization method is crucial (Dai *et al.*, 2012).
Ultrasound is a promising alternative to traditional methods of food preservation, and is regarded as a green emerging technology. USC is an eco-friendly, non-thermal method, widely applied to improve quality and inactivate microorganisms during food processing and preservation (Lee et al., 2014). USC at 20–100 kHz can produce cavitation phenomena in liquids. Implosion of cavitation bubbles generates rapid changes in localized pressure, which can cause microbial cell damage (Pedrós-Garrido et al., 2017). Many studies have reported that USC is beneficial for reducing microorganisms, retaining quality, and prolonging the shelf-life of fruits and vegetables (Vivek et al., 2016; Hashemi, 2018; Pinheiro et al., 2015). However, limited scientific information is available regarding the effects of USC on microbial reduction in mud snails.

Recently, the application of natural chemicals and UHP in food processing and preservation has attracted much attention. Several studies have reported that natural chemicals offer many benefits with regard to maintaining freshness and prolonging the shelf-life of aquatic products (Jia et al., 2019; Luo et al., 2018). These natural chemicals, such as chitosan (Soares et al., 2017), nisin (Schelegueda et al., 2015), ε-polylysine (Jia et al., 2019), tea polyphenol (Feng et al., 2017), and lysozyme (Wu et al., 2017), alone or in combination and at different concentrations, are effective in delaying microbial reproduction and/or lipid oxidation and extending the shelf-life of various seafoods. In addition, some researchers have indicated that UHP, a non-thermal technology, has minimal effect on the sensory and nutritional characteristics of food products and minimizes microbial loads while satisfying consumer demand for minimally processed products, improving food safety, and prolonging shelf-life (Mújica-Paz et al., 2011; Alba et al., 2019). However, to our knowledge, little
information is available on the effects of natural chemicals and UHP on the quality and safety of pickled mud snails during storage at −20 °C.

The objectives of the present work were to (a) compare the decontamination effects and select the optimal conditions of different treatment methods for decontamination treatment of mud snails, (b) evaluate the effects of natural chemicals and/or UHP on the quality and safety of pickled mud snails, and (c) determine the bacterial diversity of pickled mud snail before and after 12 months of storage at −20 °C based on a combination of treatments.

Materials and methods

Mud snail materials

Live mud snails were purchased from Ningbo Aquatic Product Wholesale Market, Ningbo, Zhejiang, China. The mud snails were kept on crushed ice and transported immediately (within 1 h) to the production workshop of Ningbo Nanlian Frozen Food Co., Ltd. Snails were selected based on uniform size (mean weight 5 ± 1 g) and an appearance free of visual defects. The snails were then placed in sterilized stainless steel containers with crushed ice for subsequent experimental use.

Chemicals and reagents

All chemicals (NaClO, hydrogen peroxide [H₂O₂], tea polyphenol, nisin, ε-polylysine, and lysozyme) used in the experiments were food-grade chemicals purchased from Sinopharm Chemical Reagent Co., Ltd. Ingredients, including salt, monosodium glutamate, sugar, and
yellow wine (alcohol content: 16%) were purchased from the metro supermarket in Ningbo City, Zhejiang Province, China.

**Preparation of decontamination solution**

Decontamination solutions were prepared by adding NaClO (effective chlorine concentration: 9%, final w/w) or H₂O₂ (30%, final w/w) liquid to sterilized tap water. Tap water meeting the Standards for Drinking Water Quality was used in the preparation of experimental chemical solutions. The solutions were cooled to 4 ± 1°C in a closed refrigerated chamber prior to their use in decontamination treatment.

**Sample preparation**

The mud snail material was placed in a NaClO solution (50, 100, 150, or 200 mg L⁻¹), H₂O₂ solution (0.5, 1.0, 1.5, or 2.0 g L⁻¹), or a KQ3200E ultrasonic cleaner (40kHz, Kunshan Ultrasonic Instrument Co., Ltd, Suzhou, Jiangsu, China) (150, 200, 250, or 300 W) for 10 min. The ratio of grams of mud snails to milliliters of solution was 1:10. Based on the results of the above mentioned experiments, solutions with 50 mg L⁻¹ NaClO or 1.5 g L⁻¹ H₂O₂ and 200 W USC were selected for further treatment time optimization experiments. The mud snail materials were dipped in 50 mg L⁻¹ NaClO or 1.5 g L⁻¹ H₂O₂ solution, or underwent ultrasound treatment at 200 W, for 2.5, 5, 7.5, or 10 min. The control samples were immersed in sterilized tap water (4 ± 1°C).

After the optimum decontamination treatment, the mud snails were mixed thoroughly with the other ingredients: 60 g kg⁻¹ salt, 10 g kg⁻¹ monosodium glutamate, 20 g kg⁻¹ sugar, and 50 g kg⁻¹ yellow wine. The pickled mud snails were then randomly divided into four
groups of 3 kg each. The first group of pickled mud snail was treated with combined chemicals (0.15 g kg\(^{-1}\) tea polyphenols + 0.05 g kg\(^{-1}\) nisin + 0.15 g kg\(^{-1}\) ε-polylysine + 0.1 g kg\(^{-1}\) lysozyme); the second group was treated with 300-MPa UHP at 25 °C for 15 min; the third group was treated with combined chemicals, followed by a 300-MPa UHP treatment at 25°C for 15 min; and the fourth group, used as the control, was not exposed to combined chemicals or 300-MPa UHP treatment. The optimum concentration of combined chemicals and the UHP treatment pressure were selected based on the results of pre-experiments (Table A1–4 and Figure A1 in Appendix 1).

UHP treatments were performed in a hydrostatic pressurization unit (UHP.L1-600/3, Tianjin Tyson Miao Biological Engineering Technology Co., Ltd., Tianjin, China) with a 5-L cylinder (diameter: 90 mm; height: 320 mm). Water was used as the pressure-transmitting fluid. The rate of pressure increase was approximately 200 MPa min\(^{-1}\), and the pressure release was immediate (<4 s) (Gómez-Guillén et al., 2005).

Following treatment, the pickled mud snail (300 ± 10 g) was packaged in glass bottles and sealed with a plastic screw lid (Figure 1). After sealing, ten bottles of pickled mud snail per treatment were stored at −20 °C for up to 12 months. All experiments were conducted at temperatures of less than 15°C and were replicated three times. The samples were taken at an interval of 3 months for further analysis.
Sensory and safety evaluation

Sensory evaluation was performed according to the method described by National Food Safety Standard - Processed aquatic products of animal origin (GB 10136-2015). Sensory characteristics (luster, taste, smell, state, and crispness) were screened by a panel consisting of ten experienced assessors (five women and five men aged 21-45 years). The total scores were 10 and the higher one represented the better sensory quality of pickled mud snails. The pickled mud snail samples were tasted by ten experienced assessors mentioned above. A glass of water was provided to restore the taste sensitivity. For safety evaluation, 50 g of pickled mud snails (all indices did not exceed the threshold levels recommended by GB 10136-2015) were eaten by each of the assessors mentioned above and observed for 72 h.

Microbial analysis

For the determination of coliforms and total viable count (TVC), pickled mud snail meat samples (25 g) were placed in aseptic bags with 225 mL of 0.85% sterile normal saline and homogenized for 3 min using a flapping homogenizer. The homogenate was diluted to appropriate concentrations according to the 10-fold dilution method. The coliforms were determined according to GB 4789.3-2016. The number of bacteria was determined by plating the homogenate on plate count agar and incubating at 37 °C for 48 h (Wang et al., 2016). The results are reported as \( \log_{10} \) colony-forming units, \( \log_{10} \) (CFU g\(^{-1}\)), for the mud snail samples. Each test was performed three times.
Total volatile base nitrogen content and thiobarbituric acid-reactive substance value analyses

Total volatile base nitrogen (TVB-N) content and thiobarbituric acid-reactive substance (TBARS) value were determined as described by Luo et al. (2018). Results are expressed in mg N per kg sample and mg MDA per kg sample, respectively.

Measurement of pH

Samples (10 g) were homogenized with 90 mL of distilled water in Stomacher bags for 120 s at 8 times per second. The samples were measured with a pHS-3C pH meter (Shanghai San-Xin Instrumentation, Inc., Shanghai, China).

Bacterial diversity analyses

Bacterial diversity analyses were performed by the Sangon Biotech (Shanghai) Co., Ltd. The DNA Extraction, polymerase chain reaction (PCR) amplification, bioinformatics and data analyses were performed as described by Luo et al. (2020). Alpha diversity analyses were conducted by Mothur 1.30.1 (http://www.mothur.org/). Cladistic analysis of the genus was performed using the ribosomal database project (RDP) classifier 2.12 (https://sourceforge.net/projects/rdp-classifier/) and RDP database (http://rdp.cme.msu.edu/misc/resources.jsp).
Statistical analysis

All measurements were carried out in triplicate. SPSS 19.0 software (IBM Corp., Armonk, NY, USA) was used to perform one-way analysis of variance (ANOVA) and least significant difference tests with $P = 0.05$ indicating significance. Excel 2016 software was used to produce graphs. Data are expressed as mean ± standard deviation (SD).

Results

Effect of pretreatment methods on TVC of mud snail

Figure 2 shows that the initial TVC in mud snail was $4.55 \pm 0.09 \log_{10} \text{CFU g}^{-1}$. All pretreatments significantly ($P < 0.05$) reduced the TVC compared with the control (Figure 2A–C). The greatest TVC reduction was achieved using 200 W USC, 50 mg L$^{-1}$ NaClO, or 2 g L$^{-1}$ H$_2$O$_2$ for 10 min. Although the antimicrobial effects of USC, NaClO, or H$_2$O$_2$ against TVC on mud snail materials improved with increasing power or concentration, treatments greater than 200 W USC, 50 mg L$^{-1}$ NaClO, or 1.5 g L$^{-1}$ H$_2$O$_2$ did not result in a substantial reduction in TVC. This indicates that the optimal conditions for pretreatment were 200 W USC, 50 mg L$^{-1}$ NaClO, or 1.5 g L$^{-1}$ H$_2$O$_2$. Therefore, these conditions were selected to further determine the treatment duration of mud snail.

Effect of treatment durations on TVC of mud snail

Figure 3A–C shows the antimicrobial effects of 200 W USC, 50 mg L$^{-1}$ NaClO, and 1.5 g L$^{-1}$ H$_2$O$_2$ at different treatment durations on mud snail materials, respectively. After treatment with 200 W USC, 50 mg L$^{-1}$ NaClO, or 1.5 g L$^{-1}$ H$_2$O$_2$, the TVC in mud snail material
was reduced significantly \((P < 0.05)\); the greatest TVC reduction was achieved after 10 min of treatment. However, no significant \((P > 0.05)\) differences in TVC reduction were found with an increase in contact (USC from 5 to 10 min, NaClO from 50 to 100 mg L\(^{-1}\)). Considering the bactericidal effect and edible safety, these results suggest that the optimal pretreatment method and condition for use in industry is 200 W USC treatment for 5 min. Thus, USC was selected for the subsequent production of pickled mud snail.

**Effects of combined chemicals and/or UHP on sensory and safety of pickled mud snail**

Table 1 shows the sensory scores significantly \((P < 0.05)\) decreased from 10.0±0.00 to 9.53±0.08, 9.51±0.05 when immediately determined after 300-MPa UHP or combined chemicals + 300-MPa UHP, respectively, while no significant \((P > 0.05)\) changes were found between the combined chemicals treated samples and the control. All four batches had no significant \((P > 0.05)\) influence on sensory characteristics and taste of pickled mud snails before and after 12 months of frozen storage at -20 °C (table 1). At the same time, foodborne illness was not observed in the 10 testers during a 72-h observation period.

**Effects of combined chemicals and/or UHP on coliforms, TVC, TVB-N, TBARS, and pH values of pickled mud snail**

The coliform colonies in all pickled mud snail samples, including the control, were below 10 CFU g\(^{-1}\) before and after storage at -20 °C. This result was further verified by Pony Testing International Group, Ningbo, China. Table 1 shows the TVC, TVB-N, TBARS, and pH values of pickled mud snail samples before and after 12 months of storage at -20 °C. UHP treatment resulted in the changes of these indices. The TVC, TVB-N, TBARS, and pH values
markedly decreased or increased from $3.58 \pm 0.03 \log_{10} \text{CFU g}^{-1}$, $95.9 \pm 0.7 \text{mg kg}^{-1}$, $1.47 \pm 0.07 \text{mg MDA kg}^{-1}$, and $7.35 \pm 0.04$ to $3.32 \pm 0.06 \log_{10} \text{CFU g}^{-1}$, $104.3 \pm 2.8 \text{mg kg}^{-1}$, $1.55 \pm 0.02 \text{mg MDA kg}^{-1}$, and $7.14 \pm 0.01$, respectively, when immediately determined after UHP treatment. Combined chemicals treatment has no significant ($P > 0.05$) influence in TVC and TVB-N contents of pickled mud snail, while it markedly decreased the TBARS and pH values. The TVC, TVB-N and TBARS values in all batches, including the control, remained below the maximum acceptable limit ($\text{TVC} \leq 4.7 \log_{10} \text{CFU g}^{-1}$, $\text{TVB-N} \leq 300 \text{mg kg}^{-1}$, $\text{TBARS} \leq 5 \text{mg MDA kg}^{-1}$) throughout storage. After 12 months of storage, the TVC, TVB-N, TBARS, and pH values in the control sample increased significantly ($P < 0.05$) to $4.06 \pm 0.07 \log_{10} \text{CFU g}^{-1}$, $127.4 \pm 4.2 \text{mg kg}^{-1}$, $3.45 \pm 0.07 \text{mg MDA kg}^{-1}$, and $7.51 \pm 0.02$, respectively. All treatments significantly ($P < 0.05$) inhibited the increase of these values compared with the control (Table 1). In contrast, the TVC values were lowered dramatically using combined chemicals, UHP, or combined chemicals + UHP treatments (Table 1). Among the three treatment groups, combined chemicals were more effective in inhibiting increases in these indices in pickled mud snails than were UHP and combined chemicals + UHP treatments.

Effect of combined chemicals and/or UHP on bacterial diversity of pickled mud snail

Alpha of pickled mud snail before and after 12 months of frozen storage at $-20 ^\circ \text{C}$

The bacterial diversity of pickled mud snails before and after 12 months of frozen storage at $-20 ^\circ \text{C}$ was determined by MiSeq sequencing of the 16S rDNA V3–V4 amplified regions. The sequence number and OTU number, ACE, Chao1, the Shannon and Simpson indices, and the sequencing coverage are shown in Table 2. The total number of species was
evaluated using the ACE and Chao1 index; higher values indicate higher community species richness. The higher estimated sample coverage values indicated a lower probability that sequences were unmeasured. The Shannon index was used to estimate the heterogeneity of microflora. The Simpson index was used to describe the diversity of microbes; larger Simpson values indicate lower bacterial diversity.

As shown in Table 2, the number of effective sequences in each group exceeded 30,000, and the percentage of effective sequences was more than 80%, indicating that the obtained effective sequences could meet the requirements of subsequent bacterial diversity analysis. The sequencing coverage of all samples was >93%, indicating that the probability of undetected sequences in samples was low. The number of OTU obtained by all groups ranged from 3535 to 5466. The microbial species of pickled mud snail samples in the test groups (Nos. 2–4) were relatively low compared with the control, indicating that the combined chemicals and/or UHP inhibited the growth and reproduction of microorganisms in pickled snails.

Table 2 also showed an increase in the Ace, Chao 1, and Simpson indices in all batches, including the control, whereas the Shannon index decreased compared with the initial values in the control. This indicated that microbial growth and reproduction in pickled mud snail samples continued to intensify, whereas the bacterial diversity in samples decreased after 12 months of storage at −20 °C.

Bacterial diversity of pickled mud snail before and after 12 months of frozen storage at −20 °C.
Changes in the bacterial diversity of pickled mud snails after 12 months of frozen storage at −20 °C are shown in Table 3. The dominant bacteria in the control at time 0 were identified as *Lactococcus* (9.63%), *Thauera* (6.33%), *Pseudomonas* (5.7%), *Bacteroides* (4.39%), *Brochothrix* (3.42%), *Legionella* (3.23%), *Comamonas* (2.59%), *Bifidobacterium* (2.31%), *Stenotrophomonas* (2.23%), *Mycoplasma* (2.13%), *Clostridium III* (1.98%), *Acinetobacter* (1.82%), *Barnesiella* (1.55%), *Lactobacillus* (1.38%), *Roseburia* (1.32%), *Faecalibacterium* (1.31%), *Methylophaga* (1.22%), *Trichococcus* (1.09%), *Bacillus* (1.06%), *Clostridium XIVa* (1.05%) and *Aestuariispira* (1.01%) (relative abundance >1%). After 12 months of storage at −20 °C, three dominant bacteria were identified in the control samples: *Pseudomonas* (65.52%), *Psychrobacter* (6.9%), and *Mycoplasma* (1.76%). The combined chemicals and/or UHP treatments significantly (*P* < 0.05) changed the bacterial diversity and the relative abundance. The major genera detected in the combined chemical-treated samples were *Pseudomonas* (76.62%), *Psychrobacter* (5.58%), and *Mycoplasma* (3.99%). The 300-MPa UHP treatment significantly increased (*P* < 0.05) the relative abundance of *Pseudomonas* (76.44%), *Psychrobacter* (8.63%), *Mycoplasma* (2.02%), and *Sphingomonas* (1.17%). The combined chemicals + 300-MPa UHP treatment increased the relative abundance of *Pseudomonas* (77.2%), *Psychrobacter* (7.87%), and *Mycoplasma* (1.26%) compared with the control.

**Discussion**

Mud snails carry a large number of microorganisms in the living environment. Microbial reproduction during catching, transportation, and processing result in higher initial TVC levels in pickled mud snail (Huang et al., 2011). Furthermore, pathogenic bacteria
contamination occurs frequently during processing due to poor conditions and manual operation, thereby increasing the risk of foodborne illnesses (Liao et al., 2020). Salt, yellow wine, and acetic acid have been used traditionally to reduce the initial TVC and inhibit microbial spoilage of pickled mud snail; however, adequate results have yet to be achieved (Sallam et al., 2007). In addition, slow microbial growth might occur during frozen storage, even stored at −20 °C, consequently negatively affecting safety and acceptability of frozen aquatic products. Soares et al. (2015) have reported that the TVC of salmon significantly increased from $2.73 \pm 0.03 \log_{10} \text{CFU g}^{-1}$ to $4.42 \pm 0.04 \log_{10} \text{CFU g}^{-1}$ after 250 days of storage at −22 °C. Also, Soares et al. (2017) have found an increase of TVC in salmon after 6 months of storage at −20 °C. Similarly, we found that the TVC in pickled mud snail significantly increased from $3.58 \pm 0.03 \log_{10} \text{CFU g}^{-1}$ to $4.06 \pm 0.07 \log_{10} \text{CFU g}^{-1}$ after 12 months of frozen storage at −20 °C.

Antibacterial treatment, as the less expensive processing technology, is largely used in fresh animal products to inhibit the deterioration of quality characteristics. Sarjit and Dykes (2015) found that a 60-mg L$^{-1}$ NaClO treatment for 10 min significantly reduced the numbers of Campylobacter and Salmonella (~ 0.2–1.5 log CFU cm$^{-2}$) in both duck and chicken meat products. Hou et al. (2016) reported that NaClO bacteria-reducing treatment significantly reduced the number of initial microorganisms in grass carp flank, with good sensory acceptance, and significantly prolonged the shelf life of refrigerated aerated grass carp flank.

Also, Lee et al. (2014) reported that ultrasound (37 kHz, 380 W) treatments for 5 min significantly reduced the bacterial populations in chicken skin. However, little research has been conducted to compare the effects of physical and chemical pretreatment methods in
reducing bacteria in aquatic products. Therefore, herein, we focused our attention on evaluating the effect of USC, NaClO, and H₂O₂ on TVC. All pretreatment methods tested in this study significantly reduced (P < 0.05) TVC in mud snail compared with the control (Figures 2 and 3). Compared with NaClO and H₂O₂ chemical decontamination agents, USC can better guarantee the edible safety of mud snail. Therefore, the mud snail was pretreated with 200 W USC for 5 min and studied further.

Natural chemicals and UHP are effective technologies to improve the quality of aquatic products and inhibit the growth and reproduction of microorganisms. Many studies have shown that the effect of a single natural chemical is limited; however, the combination of natural chemicals can improve the effects on preservation. Luo et al. (2018) found that adding 0.5 g L⁻¹ nisin + 5 g L⁻¹ chitosan + 0.2 g L⁻¹ phytic acid to ice-glazing was effective in maintaining freshness and prolonging the shelf-life of pacific Saury during frozen storage at −18 °C. According to Hughes et al. (2016) the refrigerated shelf life of abalone treated with 300-MPa UHP for 10 min was four times that of the control group. The combination of natural chemicals with UHP treatment has a synergistic effect on food preservation. Gómez-Guillén et al. (2005) using 15 % chitosan combined with 300-MPa UHP treatment of mackerel mincemeat, found that chitosan had no significant effect on the rheology or microstructural properties of the gel obtained under high pressure, but it did reduce lipid oxidation. In contrast, we found that combined chemical treatment + 300-MPa UHP treatment markedly promoted the accumulation of TVB-N and TBARS and the microbial reproduction of pickled mud snail during frozen (−20 °C) storage compared with the combined chemical treatment alone. Thus, UHP treatment likely caused membrane damage
and leakage of cell contents in pickled mud snail meat, resulting in an exacerbation of lipid oxidation and acceleration of microbial growth and reproduction.

Knowing the microbial community structure in marinated raw animal aquatic product is the key to predicting whether the product poses a microbiological hazard (Cho et al., 2016). The relative abundance of microorganisms can provide the targets for controlling the quality and safety of marinated raw animal aquatic product. In previous studies, some bacteria were isolated and identified in pickled mud snail using molecular biological principles (16S rDNA analysis), including Psychrobacter, Acinetobacter johnsonii, Acinetobacter junii, Burkholderia, Lactococcus garvieae, Pseudomonas, Sphingobacterium, Vibrio, etc (Huang et al., 2011; Zhang et al., 2011). Here, we investigated the bacterial diversity of pickled mud snail before and after 12 months of frozen storage at −20 °C using Illumina MiSeq sequencing. Our results showed 21 genera having a relative abundance above 1% in the control at time 0 (Figure A1 in Appendix 2). After 12 months of frozen storage at −20 °C, Pseudomonas, Psychrobacter, and Mycoplasma were the dominant bacteria in pickled mud snail, and their relative abundances were 65.52%, 6.9%, and 1.76%, respectively. The combined chemicals significantly (P < 0.05) reduced the relative abundance of the majority dominant genus, while increasing the relative abundance of Pseudomonas (76.62%), Mycoplasma (3.99%), and Lactobacillus (2.47%) (Table 3). UHP treatment increased the relative abundance of Pseudomonas (76.44%), Psychrobacter (8.63%), Mycoplasma (2.02%), and Sphingomonas (1.17%). Meanwhile, the combined chemicals + 300-MPa UHP increased the relative abundance of Pseudomonas (77.2%) and Psychrobacter (7.87%). These results suggest that the combined chemicals and/or UHP treatments are rather ineffective and inefficient for
inhibiting *Pseudomonas, Psychrobacter, and Mycoplasma* in pickled mud snail. It has been reported that *Pseudomonas, Psychrobacter, Mycoplasma, Lactobacillus*, and *Sphingomonas* are common in the marine environment and on the surfaces and in the intestinal contents of marine animals; in addition, the majority of the genera of these bacteria are non-pathogenic (Hou *et al*., 2016; Hughes *et al*., 2016; Cho *et al*., 2016). Nevertheless, the biological characteristics and pathogenicity of these bacteria require further study.

Moreover, sensory examination of the natural chemicals in pickled mud snail revealed that the combined treatment had no significant (*P* > 0.05) influence on sensory characteristics such as luster, taste, smell, state, or crispness. At the same time, foodborne illness was not observed in the 10 testers during a 72-h observation period. Given that nisin, tea polyphenols, ε-polylysine, and lysozyme are regulated as safe food additives by the National Health and Family Planning Commission of China (NHFPC) and have been widely used in the food industry worldwide, the application of these natural chemicals may offer an economical and practical approach on a commercial scale for maintaining the quality and safety of marinated raw animal aquatic products.

**Conclusions**

The findings from this study indicate that all pretreatment methods tested significantly reduced the TVC of mud snail materials. The optimal pretreatment condition was 200 W USC treatment for 5 min. Combined chemicals were more effective in inhibiting changes in the quality and safety of pickled mud snails than UHP treatment or treatment involving combined chemicals + UHP. The most effective combination was 0.15 g kg⁻¹ tea polyphenols...
+ 0.05 g kg\(^{-1}\) nisin + 0.15 g kg\(^{-1}\) ε-polylysine + 0.1 g kg\(^{-1}\) lysozyme. The combined chemicals and UHP treatment were rather ineffective in inhibiting Pseudomonas, Psychrobacter, and Mycoplasma in pickled mud snail during storage at -20\(^\circ\)C. Further research is needed to investigate how these bacteria can be effectively inhibited and eliminated from pickled mud snail.

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Table 1 Effect of combined chemicals and/or ultrahigh pressure (UHP) on sensory, TVC, total volatile base nitrogen (TVB-N), thiobarbituric acid-reactive substance (TBARS), and pH values of pickled mud snail before and after 12 months of frozen storage at −20 °C

| Indices       | Treatments                        | Storage time (months) |
|---------------|-----------------------------------|-----------------------|
|               |                                   | 0                     | 12                    |
| Sensory       | Control                           | 10.0±0.00Aa           | 9.89±0.07Aa           |
|               | Combined chemicals                 | 9.96±0.03Aa           | 9.91±0.06Aa           |
|               | 300-MPa UHP                        | 9.53±0.08Ab           | 9.49±0.04Ab           |
|               | Combined chemicals + 300-MPa UHP   | 9.51±0.05Ab           | 9.46±0.09Ab           |
| TVC           | Control                           | 3.58±0.03Ba           | 4.06±0.07Aa           |
| log_{10}(CFU g^{-1}) | Combined chemicals        | 3.56±0.07Aa           | 2.68±0.06Bb           |
|               | 300-MPa UHP                        | 3.32±0.06Ab           | 1.91±0.07Bc           |
|               | Combined chemicals + 300-MPa UHP   | 3.01±0.02Ac           | 1.98±0.02Bc           |
| TVB-N mg kg^{-1} | Control                        | 95.90±3.28Bc          | 127.4±4.2Aa           |
|               | Combined chemicals                 | 98.70±1.88Bc          | 116.2±1.4Ab           |
|               | 300-MPa UHP                        | 104.3±2.8Ab           | 99.40±3.8Ac           |
|               | Combined chemicals + 300-MPa UHP   | 107.1±2.6Aa           | 109.2±3.9Ab           |
| TBARS mg MDA kg^{-1} | Control                        | 1.47±0.07Ba           | 3.45±0.07Aa           |
|               | Combined chemicals                 | 1.03±0.01Bb           | 2.29±0.06Ad           |
|               | 300-MPa UHP                        | 1.55±0.02Ba           | 3.08±0.13Ab           |
|               | Combined chemicals + 300-MPa UHP   | 1.56±0.10Ba           | 2.83±0.10Ac           |
| pH value      | Control                           | 7.35±0.04Ba           | 7.51±0.02Aa           |
|               | Combined chemicals                 | 7.24±0.01Bb           | 7.36±0.01Ab           |
|               | 300-MPa UHP                        | 7.14±0.01Bc           | 7.32±0.01Ac           |
|               | Combined chemicals + 300-MPa UHP   | 7.23±0.03Ab           | 7.26±0.03Ad           |

Data are expressed as means±SD of triplicate assays. Mean values with different lowercase or capital letters in same column or row are significantly different (P<0.05), respectively.
Table 2  Alpha diversity of pickled mud snails before and after 12 months of frozen storage at −20 °C

| Samples                  | Control 0 month | Control 12 months | Combined chemicals 12 months | 300-MPa UHP 12 months | Combined chemicals + 300-MPa UHP 12 months |
|--------------------------|-----------------|-------------------|------------------------------|----------------------|-------------------------------------------|
| Sequence numbers         | 96402           | 49441             | 51184                        | 52352                | 55321                                     |
| OTU numbers              | 5466            | 3654              | 3593                         | 3581                 | 3535                                      |
| ACE                      | 22531.3         | 188383.01         | 328957.34                    | 211186.11            | 436151.94                                 |
| Chao 1                   | 15435.07        | 63032.92          | 87484.35                     | 55829.28             | 122154.29                                 |
| Shannon                  | 5.47            | 2.15              | 1.74                         | 1.71                 | 1.54                                      |
| Simpson                  | 0.02            | 0.45              | 0.59                         | 0.59                 | 0.6                                       |
| Coverage                 | 0.97            | 0.93              | 0.93                         | 0.94                 | 0.94                                      |
### Table 3 Effect of combined chemicals and/or UHP on bacterial diversity of pickled mud snail before and after 12 months of frozen storage at −20 °C

| Number | Bacterial genus  | Control 0 month | Control 12 months | Combined chemicals 12 months | 300-MPa UHP 12 months | Combined chemicals + 300-MPa UHP 12 months |
|--------|------------------|-----------------|-------------------|-------------------------------|-----------------------|---------------------------------------------|
| 1      | Lactococcus      | 9.63            | 0                 | 0                             | 0                     | 0                                           |
| 2      | Thauera          | 6.33            | 0.02              | 0                             | 0.04                  | 0                                           |
| 3      | Pseudomonas      | 5.7             | 65.52             | 76.62                         | 76.44                 | 77.2                                        |
| 4      | Bacteroides      | 4.39            | 0.07              | 0.11                          | 0.04                  | 0.02                                        |
| 5      | Brochothrix      | 3.42            | 0.01              | 0                             | 0                     | 0                                           |
| 6      | Legionella       | 3.23            | 0.04              | 0.02                          | 0.02                  | 0.02                                        |
| 7      | Comamonas        | 2.59            | 0                 | 0                             | 0                     | 0                                           |
| 8      | Bifidobacterium  | 2.31            | 0.01              | 0.02                          | 0.02                  | 0.01                                        |
| 9      | Stenotrophomonas | 2.23            | 0.02              | 0.01                          | 0.01                  | 0.01                                        |
| 10     | Mycoplasma       | 2.13            | 1.76              | 3.99                          | 2.02                  | 1.26                                        |
| 11     | Clostridium III  | 1.98            | 0.01              | 0.03                          | 0                     | 0.01                                        |
| 12     | Acinetobacter    | 1.82            | 0.04              | 0.01                          | 0.04                  | 0.04                                        |
| 13     | Barnesiella      | 1.55            | 0.2               | 0.11                          | 0.14                  | 0.07                                        |
| 14     | Lactobacillus    | 1.38            | 0.03              | 2.47                          | 0.03                  | 0.01                                        |
| 15     | Roseburia        | 1.32            | 0                 | 0.01                          | 0                     | 0                                           |
|   | Species               | 1   | 0.05 | 0.08 | 0.04 | 0.02 |
|---|----------------------|-----|------|------|------|------|
|16 | Faecalibacterium     | 1.31| 0.05 | 0.08 | 0.04 | 0.02 |
|17 | Methylophaga         | 1.22| 0.03 | 0    | 0    | 0    |
|18 | Trichococcus         | 1.09| 0    | 0    | 0    | 0    |
|19 | Bacillus             | 1.06| 0.01 | 0.02 | 0.14 | 0.01 |
|20 | Clostridium XIva     | 1.05| 0.02 | 0.01 | 0.02 | 0.01 |
|21 | Aestuariispira       | 1.01| 0.1  | 0.11 | 0.05 | 0.03 |
|22 | Psychrobacter        | 0.26| 6.9  | 5.58 | 8.63 | 7.87 |
|23 | Sphingomonas         | 0.06| 0.84 | 0.52 | 1.17 | 0.26 |
|24 | Unclassified         | 16.27| 18.92| 5.28 | 5.9  | 4.29 |
|25 | Other genera         | 26.66| 5.40 | 5.00 | 5.25 | 8.86 |
Fig. 1. Pickled mud snail products
Fig. 2. Effect of USC power (A) and NaClO (B) and H₂O₂ (C) concentrations on the total viable count (TVC) of mud snails. Vertical bars indicate the standard error of three replicates. Means with different letters for the same index are significantly different (P < 0.05).
Fig. 3. Effect of 200 W ultrasonic cleaning (USC) (A), 50 mg L\(^{-1}\) NaClO (B), and 1.5 g L\(^{-1}\) H\(_2\)O\(_2\) (C) on the TVC of mud snails. Vertical bars indicate the standard error of three replicates. Means with different letters for the same index are significantly different (\(P < 0.05\)).