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Investigating the microbial inactivation effect of low temperature high pressure carbon dioxide and its application in frozen prawn (*Penaeus vannamei*)

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ABSTRACT

During the pandemic of coronavirus disease 2019, the fact that frozen foods can carry the relevant virus raises concerns about the microbial safety of cold-chain foods. As a non-thermal processing technology, high pressure carbon dioxide (HPCD) is a potential method to reduce microbial load on cold-chain foods. In this study, we explored the microbial inactivation of low temperature (5-10 °C) HPCD (LT-HPCD) and evaluated its effect on the quality of prawn during freeze-chilled and frozen storage. LT-HPCD treatment at 6.5 MPa and 10 °C for 15 min could effectively inactivate *E. coli* (99.45%) and *S. aureus* (94.6%) suspended in 0.85% NaCl, SARS-CoV-2 Spike pseudovirus (>99%) and human coronavirus 229E (hCoV-229E) (>1-log virus tilter reduction) suspended in DMEM medium. The inactivation effect of LT-HPCD was weakened but still significant when the microorganisms were inoculated on the surface of food or package. LT-HPCD treatment at 6.5 MPa and 10 °C for 15 min achieved about 60% inactivation of total aerobic count while could maintain frozen state and quality of prawn. Moreover, LT-HPCD treated prawn exhibited significant slower microbial proliferation and no occurrence of melanosis compared with the untreated samples during chilled storage. A comprehensive quality investigation indicated that LT-HPCD treatment could maintain the color, texture and sensory of prawn during chilled or frozen storage. Consequently, LT-HPCD could improve the microbial safety of frozen prawn while maintaining its original quality, and could be a potential method for food industry to improve the microbial safety of cold-chain foods.

1. Introduction

The ongoing coronavirus disease 2019 (COVID-2019) has spread all over the world, causing serious loss and inconvenience. SARS-CoV-2 is a respiratory virus infecting people through mucous membranes. Apart from infection by breathing in respiratory droplets with viruses, contact can also be a possible transmission pathway (van Doremalen et al., 2020). That is, when objects with viruses on their surfaces contact mucous membranes such as the eyes, people may be infected (Colavita et al., 2020). Although there is no sufficient evidence to reach a consensus on whether SARS-CoV-2 transmitted through food can cause infection, people had raised concerns about the microbial safety of cold-chain foods (Anelich et al., 2020; Lu et al., 2021; Pang et al., 2020). For instance, HuNoV, a well-known enteric pathogen, can still be infectious after 10 days storage on refrigerated lettuce and turkey (Lamhoujeb et al., 2008), while frozen berry is an important vector for foodborne viruses, in particular HuNoV and HAV (Bozkurt et al., 2021). Except for the threat from virus, other microbes such as psychrophilic bacteria can also survive at low temperature and their growth can cause food spoilage or foodborne illness (Adam et al., 2010; Hampikyan et al., 2017). Hence, appropriate methods should be applied to eliminate the microbes of cold-chain foods to ensure their safety.

Non-thermal processing technologies are suitable for sterilizing cold-chain foods for their low temperature treatment to avoid causing deterioration effect on foods. At the same time, these physical sterilization technologies are easy to accept as they do not use chemical agents. In recent studies, non-thermal technologies are usually developed based on high hydrostatic pressure (HHP), ultraviolet irradiation, ultrasound, high pressure carbon dioxide (HPCD), high pressure homogenization, and microfiltration (Yu et al., 2020). Among them, HPCD has the

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capability of effectively inactivating bacteria (Balaban & Ferrentino, 2012; Ferrentino & Spilimbergo, 2011; Garcia-Gonzalez et al., 2007), but the efficiency of inactivating food viruses has rarely been studied (Perrut, 2012; Pexara & Govaris, 2020). Generally, HPCD creates a high pressure, high acidity and anaerobic environment using pressurized carbon dioxide as an effector in a sealed vessel at pressure of 5–50 MPa and temperature of 0-60 °C (Liao & Rao, 2020). This environment along with the explosive effect caused by depressurization contribute to the inactivation effect of enzymes and microbes, allowing for long-term storage of food products (Garcia-Gonzalez et al., 2007). The bactericidal mechanism of HPCD has been systematically outlined by Garcia-Gonzalez and colleagues (Garcia-Gonzalez et al., 2007), which includes (a) solubilization and dissociation of carbon dioxide in extracellular solution; (b) cell membrane modification; (c) intracellular pH reduction; (d) inactivation of key enzymes and inhibition of metabolism; (e) direct inhibition of metabolism by carbon dioxide and bicarbonate; (f) disruption of the intracellular electrolyte balance; and (g) loss of important constituents in cell or cell membrane components. Except for the prominent microbial inactivation effect, HPCD can also exhibit a freezing effect in the process of depressurization. For instance, after HPCD treatment at 5–10 MPa and 5-10 °C (the state of carbon dioxide is liquid), depressurization can turn 43% of CO_2 to dry ice and decrease the temperature of samples to −78.5 °C (Sui & Li, 2014). The heat taken away by the sublimation of dry ice accounts for about 84%, while gas carbon dioxide absorbs the remaining heat (Ciobanu et al., 1976). Thus, the bactericidal effect coupled with freezing effect of HPCD make it a potential sterilization technology for cold-chain foods.

In previous studies, HPCD sterilization is mainly applied to liquid foods, including various kinds of fruit and vegetable juices (Cappelletti et al., 2014; Li et al., 2012; Liao et al., 2010), milk (Liao et al., 2014) and apple cider (Gunes et al., 2006); while surface sterilization by HPCD is also possible for solid or semi-solid foods, such as meat (Huang et al., 2017; Galvanin et al., 2014; Ferrentino et al., 2013; Choi et al., 2009; Wei et al., 1991), seafood (Kustyawati et al., 2021; Ji et al., 2012; Meujo et al., 2010; Wei et al., 1991), Korean kimchi (Hong & Park, 1999), fresh fruit and vegetables (Bi et al., 2011; Galvanin et al., 2014; Matsufuji et al., 2009), shredded cheese (Sikin et al., 2016), ginseng powder (Dehghani et al., 2009) and etc. Most of the previous studies used HPCD conducted at room temperature or higher, at which the carbon dioxide were in the state of gas or supercritical fluid (Yu et al., 2020). Although HPCD at these conditions can reduce microbial load effectively, it may also cause the thawing of frozen foods and quality deterioration. To expand the scope of HPCD application for cold-chain foods, especially the frozen foods, it is necessary to explore the microbial inactivation effect of HPCD at low temperatures, as well as its effect on the quality of cold-chain foods.

In this study, we have preliminarily explored the microbial inactivation of low temperature (5-10 °C) HPCD (LT-HPCD). We tested the microbial inactivation effect of LT-HPCD on three different systems: (1) virus or bacterial suspensions, (2) virus or bacteria inoculated on food or package, and (3) total aerobic count on food, respectively. In addition, the effect of LT-HPCD on food quality was also investigated, including the immediate effect just after the treatment and the long-term effect during freeze-chilled storage or frozen storage.

2. Material and methods

2.1. Prawn samples

The prawns (*Penaeus vannamei*) were cultured in seawater in Zhangzhou, China and processed by brine freezing after catching. The whole prawns were stored at lower than −18 °C for about 3 months before being used in experiments.

2.2. Bacteria and virus cultures preparation and inoculation

For bacteria cultures, *Escherichia coli* 8099 and *Staphylococcus aureus* ATCC 6538 (China General Microbiological Culture Collection Center, CGMCC) were streaked on Luria-Bertani (LB) agar plates (LB broth: CM0996, OXOID, England; agar: BD, France) from frozen stock cultures and incubated at 37 °C for overnight. The single colony was taken to LB broth and incubate at 37 °C to $OD_{600} = 0.8$. After then, the cells were spun down by centrifugation (8000 *g*, 10 min, 4 °C) and washed with sterilized 0.85% NaCl (MACKLIN, China) solution twice. The cells were resuspended with 0.85% NaCl solution for LT-HPCD treatment and inoculation. The viable counts of bacteria were determined by plate counting.

SARS-CoV-2 Spike pseudovirus (PSV001, Sino Biological, China) and human Coronavirus 229E (hCoV-229E, ATCC VR-740) were chosen as representative viruses for evaluation of the virus inactivation effect. SARS-CoV-2 Spike pseudovirus was bought commercially. The hCoV-229E was prepared using Huh-7 cells (Chinese Academy of Medical Sciences), which was a permanent cell line established from male hepatoma tissue. Cells were inoculated with 100 μl hCoV-229E and incubated at 37 °C under 5% CO_2. After incubating for 1 h, DMEM medium (C11995500BT, GIBCO, US) containing 2% fetal bovine serum (FBS, 10270-044, GIBCO, US), 100 U/ml penicillin and 100 μg/ml streptomycin (Lukang, China) was added. Cytopathic effect (CPE) was observed using microscope every 24 h. The hCoV-229E could be used for the following experiment when the CPE reached more than 80%. The viruses were suspended in DMEM medium for LT-HPCD treatment or inoculation.

Cubed prawns (1 cm × 1 cm × 0.5 cm) and cubed polyethylene terephthalate (PET) package (1 cm × 1 cm) were sterilized by UV treatment for 30 min to inactivate surface microorganisms. The cubed materials were immersed in virus (SARS-CoV-2 Spike pseudovirus was 10^{10} virus copies/ml, hCoV-229E was $5 \times 10^{4.5}$ $TCID_{50}$/ml) or bacteria (10^8 CFU/ml) suspensions and mixed by vortex blending for 15 s to acquire inoculum. After inoculation, the cubed materials were taken out for next HPCD treatment (treated group) or kept in water bath at the same temperature for the same time (control group).

2.3. High pressure carbon dioxide (HPCD) treatment

The HPCD system (Haian, China) had a stainless steel pressure vessel with a volume of 3000 mL (*Φ*102 mm × 370 mm). The vessel is connected to a sterile clean bench to avoid contamination. The vessel temperature was maintained by a precision bath circulator (THD-0506, Tianheng, China). A temperature controller was placed at the middle wall of the vessel to monitor the processing temperature. A plunger pump (2TB-50/50, Huaan, China) was used to pressurize the vessel. A pressure transducer was fixed in the vessel to monitor the vessel pressure. All the data for temperature and pressure were displayed on a control panel. A vacuum pump (2XZ-2, Hongda, China) was connected to the vessel for evacuating the air in the vessel. Commercially-available CO_2 of 99.00% purity (Jingcheng, China) was passed through an active carbon filter before entering the pressure vessel. The HPCD system was sanitized with 75% ethanol (EtOH), and the removable shelves inside were autoclaved. The treated objects were placed into the vessel after the EtOH completely evaporated and the temperature reached to the set value. After sealing the vessel, carbon dioxide was aerated until the pressure reached the set value. The treatment time is the time during pressure keeping. When the treatment was finished, carbon dioxide inside the vessel was released and the objects can be taken out until the pressure reduced to atmospheric pressure. The suspensions and cubed materials were contained in small beakers covered with air permeable sealing film when treated in the HPCD system, while the whole prawns

were treated directly.

The combination of LT-HPCD and 1% (v/v) EtOH was achieved by mixing equal volume of $10^{5.5}$ $TCID_{50}$/ml hCoV-229E suspension and 2% EtOH in DMEM medium before LT-HPCD treatment. The EtOH was neutralized by the diluting effect of the added DMEM medium after LT-HPCD treatment. The blank group without hCoV-229E also showed that EtOH had no significant effect on CPE.

use. Viruses were ten-fold serially diluted with DMEM medium, which contained 2% FBS, double antibody (penicillin/streptomycin) and mycoplasma inhibitor (D103-01, Vazyme, China). The viruses were added to the cell culture plates in order of high dilution to low dilution with 6 replications, and then incubated for 120 h. Cytopathic effect was observed to determine virus titers by the Reed-Muench method (Reed & Muench, 1938):

$$\mathrm{Log}_{10}\,(\mathrm{TCID}_{50}\,/\,\mathrm{ml}) = |\mathrm{Log}_{10}\text{dilution above 50\%}| + \frac{(\%\text{ positive above 50\%}) - 50\%}{(\%\text{ positive above 50\%}) - (\%\text{ positive below 50\%})}$$

2.4. Plate counting and pH analysis

Viable count was performed by the spreading plate method for the bacterial suspensions and inoculated bacteria enumeration, while pouring plate method was used for the natural microflora enumeration of prawn. The inoculated bacteria on cubed material with or without LT-HPCD treatment were collected aseptically by vortex blending the material in sterile 0.85% NaCl solution for 15 s. Subsequently, the bacteria suspension was ten-fold serially diluted with 0.85% NaCl solution, and 0.1 ml of each dilution was spread on LB agar. After incubated at 37 °C for 12 h, the forming colonies were counted. Prawn sample (25 ± 1 g) were homogenized with 225 ml of 0.85% NaCl solution for 2 min in a stomacher bag using a stomacher (BagMixer400 VW, Interscience, France). The homogenate was used for pH analysis (FiveEasy Plus FE28, METTLER TOLEDO, Switzerland) and microbial enumeration after being serially diluted. One-milliliter of each dilution was poured in plate count agar (CM0325, OXOID, England) followed by incubation at 30 °C for 72 h, and the total bacteria were counted. For each treatment, three replications were performed.

2.5. Viral quantification

The inoculated viruses on cubed material with or without LT-HPCD treatment were collected by vortex blending the material in DMEM medium for 15 s.

SARS-CoV-2 Spike pseudovirus was quantified according to its instruction from Sino Biological and Kong et al. (2022). As the SARS-CoV-2 Spike pseudovirus had luciferase reporter gene, luciferase would express in 293T cell (OEC001, Sino Biological, China) after infection. 293T cells were seeded in cell culture plates (96-well) and cultured for 24 h at 37 °C. The culture medium was then replaced by DMEM with appropriate dilution of virus for a 2 h incubation. Following that, the inoculum was removed and fresh DMEM was added. The cells were continuously incubated for 48–72 h. After incubation, the culture media were discarded, and the cells were washed with phosphate buffer saline (SH30256.01, Hyclone, US) twice. Cell lysate solution was supplemented and the cell lysate was then transferred to an opaque 96-well white solid plate. Luminescence solution was filled into each well according to instructions of the Firefly Luciferase Reporter Gene Assay Kit (Beyotime, China). Immediately, the luciferase luminescence was determined at 578 nm by a microplate reader (TECAN SPARK 10M, Switzerland). The luciferase luminescence value of sample without LT-HPCD treatment was set as 100% for the control group. The luminescence value of treated sample was normalized according to the control group. According to these values, the survival rate was calculated and represented as percentage.

The quantification of hCoV-229E was based on Choi et al. (2022) with minor modification. Huh-7 cells were seeded in cell culture plates (96-well) and incubated for 18 h to acquire a single layer of cells. Cell culture plates were washed twice with phosphate buffer saline before

2.6. Texture and color analysis

The texture in terms of hardness and springiness were measured by using a Texture Analyzer (TA-XT plus, SEM, England). The first two abdominal somites of prawn were thawed in running water for about 20 min and tested immediately. A p/5 probe was used with a pre-test speed of 1 mm/s, a test speed of 1 mm/s and a post-test speed of 1 mm/s, a trigger point of 5 *g* and a target deformation of 30%. For each treatment, six replications were performed.

The color of prawn was measured in three different positions (cephalothorax, abdominal somite and telson) using a colorimeter (NR60CP, 3nh, China) and CIELAB system. In this color system, the L (lightness) variable represents the brightness, in which $L = 0$ for dark and $L = 100$ for white. The a (redness) scale ranges from negative values for green to positive values for red, and b (yellowness) scale ranges from negative values for blue to positive values for yellow. The colorimeter employed was firstly calibrated with a white standard followed by a black standard to obtain the final setting. For each treatment, six replications were performed.

2.7. Total volatile basic nitrogen (TVB-N) analysis

TVB-N was analyzed according to Zhuang et al. (2019) with minor modification. Briefly, 25 ml cooled ultrapure water was added to 2.5 g prawn muscle. After homogenization (F6/10, FLUKO, Germany) for 30 s on ice, the mixture was shaken on a shaker (ZWY-100H, Zhicheng, China) at 100 r/min for 30 min, then centrifuged at 3500 *g* for 3 min (CF16RXII, HITACHI, Japan). Five-milliliter of the resulting supernatant was well mixed with 5 ml MgO (Yuanye, China) suspension (10 g/l), and then the mixture was to distill completely through Kjeldahl Apparatus (KDY-9820, China). Meanwhile, 10 ml boric acid (Biorigin, China) solution (20 g/l) was used to absorb the distillate. After that, the boric acid solution was titrated with standard sulfuric acid solution (Haianhongmeng, China) to pH of 4.65. TVB-N value was determined and expressed as mg N/100 g flesh, according to the consumption of sulfuric acid. For each treatment, three replications were performed.

2.8. Salt-soluble protein

The determination of salt-soluble protein was based on Wu et al. (2014) with minor modification. One-gram prawn muscle was added with 15 ml cooled ultrapure water and then homogenized (F6/10, FLUKO, Germany) for 30 s on ice. The homogenate was extracted at 4 °C for 20 min and centrifuged at 10,000 *g* using a centrifuge at 4 °C for 20 min (CF16RXII, HITACHI, Japan). Supernatant was removed, and precipitate was added with 15 ml Tris-maleate buffer (0.6 M NaCl-20 mM Tris-maleate, pH 7.0) and then homogenized for 30 s. Then, the homogenate was kept at 4 °C for 60 min to extract salt-soluble protein and centrifuged at 10,000 *g* at 4 °C for 20 min. The obtained supernatant was diluted to 25 ml (0.6 M NaCl-20 mM Tris-maleate, pH 7.0), which was

myofibrillar protein solution for quantitative determination. Salt-soluble protein content was determined using a kit according to the Bradford method (Solarbio, China). For each treatment, three replications were performed.

2.9. Sensory analysis

Sensory analysis was performed to determine the effect of LT-HPCD processing and its long-term influence during storage. Frozen prawns were thawed in running water for about 20 min and then placed on crushed ice for sensory evaluation. Prawns in different groups were provided with randomized three-digit codes. Each group was consisted of three raw prawns of the same treatment. Original samples without any treatment were also provided as standards. Eleven evaluators scored the prawns on a scale of 0–10 in terms of color, flavor, muscular tissue and purity by observation, sniffing and touching. Besides, "Product acceptance of shucking effect" was only evaluated just after LT-HPCD treatment. Total score was the average of all attributes.

2.10. Storage study

The LT-HPCD group was obtained from the LT-HPCD treatment at 6.5 MPa, 10 °C, 15 min and the untreated prawns were the control group. The treated and control prawns were placed in sterile polyethylene bags in dark. Each bag contained a number of prawns. For the freeze-chilled storage study, three bags were set up as replication for each treatment and stored at 4 °C for 8 days, with a random sample taken every 2 days in aseptic condition. The samples were used to determine the pH, viable bacteria counts, texture, color, TVB-N and sensory score every 2 days and the salt-soluble protein content at the beginning and the end of the chilled storage was determined. In the frozen storage study, each group was also set up with three replicate bags of samples and frozen at −18 °C for 12 weeks. Samples were taken every 3 weeks to determine pH, texture and color, while sensory evaluation was carried out at the end of the frozen storage.

2.11. Statistical analysis

R 3.1.2 was used for statistical analysis. One-way analysis of variance (ANOVA) was performed on the data, and differences between group means were determined using Student-Newman-Keuls (SNK) at $p < 0.05$. Data results were expressed as mean ± standard error.

3. Results and discussions

3.1. Inactivation effect of LT-HPCD on microorganisms in suspensions

Since HPCD treatment at the temperature above 10 °C led to apparent melt and quality deterioration of frozen prawn (Fig. 1A), we mainly investigated the HPCD lower than 10 °C, which was termed low temperature HPCD (LT-HPCD). As shown in Fig. 1B, LT-HPCD effectively inactivated *E. coli* and *S. aureus* suspended in 0.85% NaCl solution and SARS-CoV-2 Spike pseudovirus suspended in DMEM medium, as 6.5 MPa treatment at 10 °C for 15 min respectively inactivated 99.45% *E. coli*, 94.6% *S. aureus* and >99.9% SARS-CoV-2 Spike pseudovirus. Prolonging the treatment time to 30 min did not significantly increase the inactivation effect (Fig. 1B). Notably, *S. aureus* exhibited stronger resistance to LT-HPCD than *E. coli*, which could be due to its thicker cell wall as a Gram-positive bacteria (Garcia-Gonzalez et al., 2007). As for the inactivation effect of LT-HPCD on human coronavirus 229E (hCoV-229E) suspended in DMEM medium, 6.5 MPa treatment at 10 °C for 15 min could reduce the virus tilter from $10^{4.8}$ $TCID_{50}$/ml to $10^{3.74}$ $TCID_{50}$/ml, and prolonged treatment time to 30 min at the same conditions also showed no significant increase to the virus inactivation (Fig. 1C). Hence, LT-HPCD treatment at 6.5 MPa and 10 °C for 15 min could effectively inactivate bacteria suspended in 0.85% NaCl solution or virus suspended in DMEM medium, while caused no apparent quality change to frozen prawn.

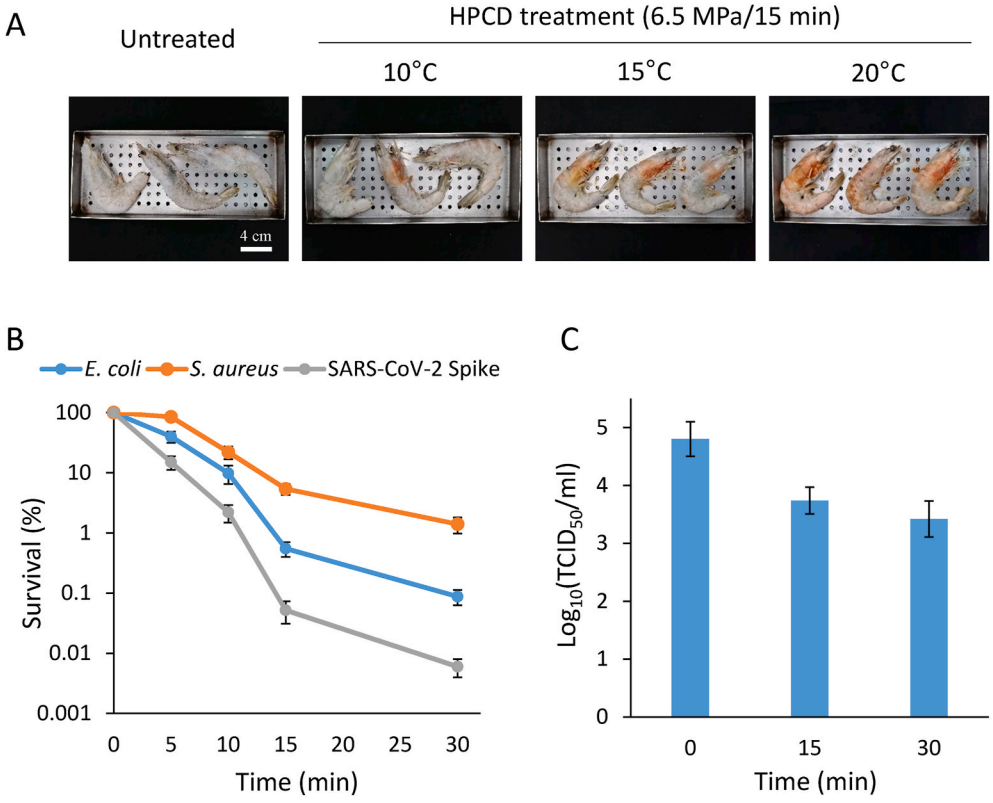

Fig. 1. Effect of HPCD treatment on frozen prawn (A) and inactivation effect of LT-HPCD on microbial suspensions (B–C). A: Frozen prawns were treated by HPCD at 6.5 MPa for 15 min at different temperature. B: Inactivation effect of LT-HPCD (6.5 MPa/10 °C/15 min) on *E. coli* and *S. aureus* suspended in 0.85% NaCl, SARS-CoV-2 Spike pseudovirus suspended in DMEM medium. C: Inactivation effect of LT-HPCD (6.5 MPa/10 °C/15 min) on hCoV-229E suspended in DMEM medium. Bars represented standard errors.

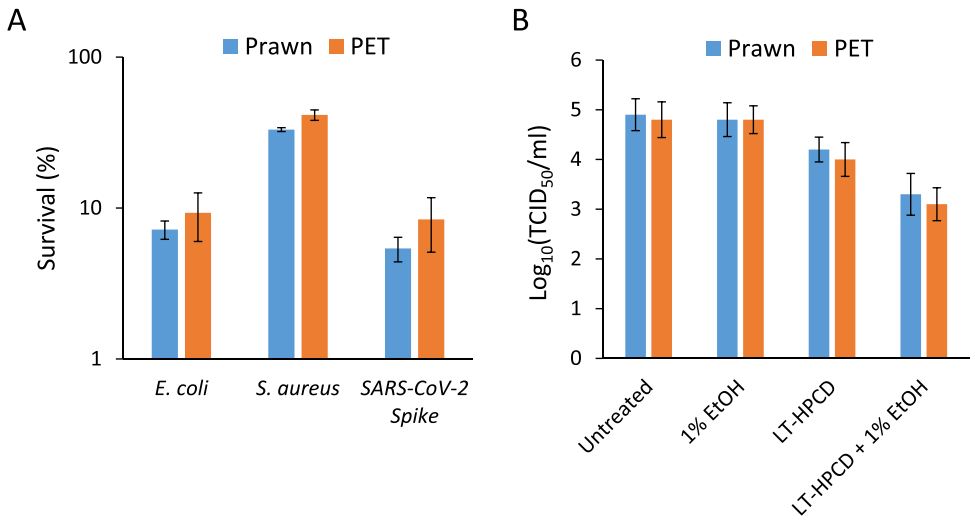

Fig. 2. Inactivation effect of LT-HPCD (6.5 MPa/10 °C/15 min) on microorganisms inoculated on the surfaces of prawn or PET. Bars represented standard errors.

3.2. Inactivation effect of LT-HPCD on microorganism inoculated on the surface of food or package

As LT-HPCD showed high inactivation effect on *E. coli*, *S. aureus*, SARS-CoV-2 Spike pseudovirus and hCoV-229E in suspension or medium, we wondered if it could inactivated these microorganisms inoculated on the surface of food or package. Since frozen prawn (*Penaeus vannamei*) was reported to carry the SARS-CoV-2, it was chosen as a representative food material to testify the microbial inactivation effect of LT-HPCD. Meanwhile, polyethylene terephthalate (PET) packaging material was used to test the microbial inactivation effect of LT-HPCD on food packages. *E. coli*, *S. aureus*, SARS-CoV-2 Spike pseudovirus and hCoV-229E were inoculated on the surface of prawn or PET followed by LT-HPCD treatment at 6.5 MPa, 10 °C for 15 min. As shown in Fig. 2A, LT-HPCD treatment could respectively inactivate 93%, 67% and 95% of *E. coli*, *S. aureus* and SARS-CoV-2 Spike on prawn, 91%, 59% and 92% of *E. coli*, *S. aureus* and SARS-CoV-2 Spike on PET. As for the inactivation effect of hCoV-229E, LT-HPCD achieved less than 1-log reduction of virus tilter on either prawn or PET (Fig. 2B). Compared with the high inactivation effectiveness in bacterial suspensions (Fig. 1B–C), the LT-HPCD showed much lower microbial inactivation effect on food or package surface. This could be due to (1) the protection effect from food components such as fat, protein and sugars (Sirisee et al., 1998), and (2) the low water content on food or package surfaces, which could weaken the acidification effect of carbon dioxide (Sirisee et al., 1998). Interestingly however, LT-HPCD combined with 1% EtOH achieved more than 1.5-log reduction of virus tilter for hCoV-229E, exhibiting a synergistic effect. Nevertheless, as there is no microbial limitation for frozen food in compulsory or recommended standard, we do not have to achieve very high level inactivation of the microorganisms on these food. A certain reduction of original bacterial count on frozen food can also be beneficial, since it will lower the capability of bacteria proliferation after thawing to cause food spoilage or foodborne illness (López-Caballero et al., 2007). Consequently, although LT-HPCD showed weakened microbial inactivation effect on food or package surface, it was still of great significance and had the capability to reduce the microflora on the frozen foods.

3.3. Inactivation effect of LT-HPCD on the natural microflora of prawn

To further investigated the microbial inactivation effect of LT-HPCD on real food systems, we tested the LT-HPCD inactivation effect on the natural microflora of prawn. At temperature of 5 °C, LT-HPCD treatment at 6.5 MPa or 10 MPa for 30 min showed no inactivation effect (Fig. 3A). When increasing the temperature to 10 °C, LT-HPCD could inactivate 60% of the natural microflora of prawn after 15 min treatment at 6.5

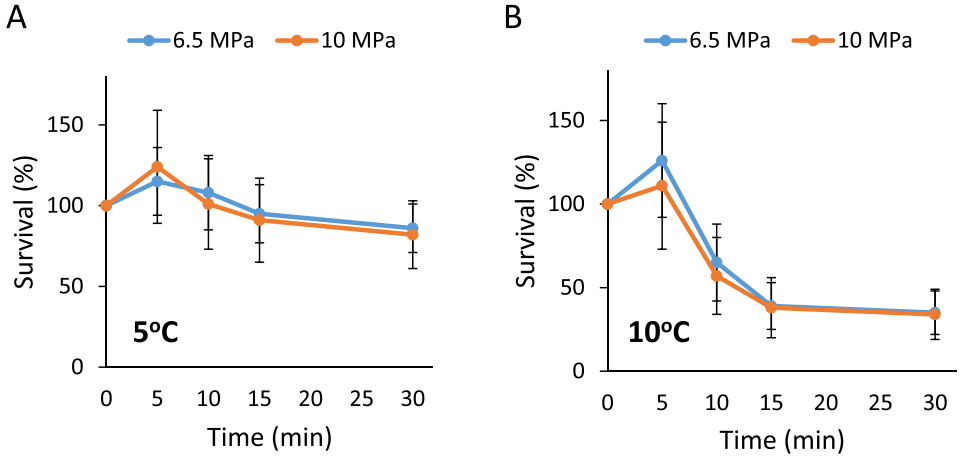

Fig. 3. Inactivation effect of LT-HPCD on the natural microflora of frozen prawn. A: LT-HPCD treatments were performed at 6.5 or 10 MPa and 5 °C for different times. B: LT-HPCD treatments were performed at 6.5 or 10 MPa and 10 °C for different times. Bars represented standard errors.

MPa or 10 MPa (Fig. 3B). The latter results also presented that (1) the inactivation effect of LT-HPCD treatment at 6.5 MPa or 10 MPa was similar, and (2) the enhancement of inactivation effect tended to slow down after 15 min of LT-HPCD treatment. As the pH of the carbon dioxide saturated solution drops to about 3 at 5 MPa and remains almost stable at even higher pressure (Bortoluzzi et al., 2011; Meyssami et al., 1992; Spilimbergo et al., 2005), the similar inactivation effect of LT-HPCD at 6.5 MPa and 10 MPa can be due to the comparable pH values at these pressures. As for the reduced inactivation effect with prolonged treatment time, this phenomenon was observed in many relevant studies (Furukawa et al., 2004; Kim et al., 2007; Liao et al., 2008, 2010), and might be attributed to the individual differences in microorganisms and protection from inactivated cells (Furukawa et al., 2002, 2006). Moreover, we also observed that the count of survival bacteria was unchanged in the first few minutes (Fig. 3B), which could be considered as the process of carbon dioxide penetration into bacteria (Liao et al., 2008). According to the inactivation effect as well as industrial practice, the optimal LT-HPCD treatment condition to inactivate microorganism on frozen prawn is 6.5 MPa and 10 °C for 15 min.

3.4. Effect of LT-HPCD on the quality of frozen prawn

To investigate the effect of LT-HPCD on frozen food quality, frozen prawn was treated at 6.5 MPa and 10 °C for 15 min and examined the frozen state, pH, texture, color and sensory score. As shown in Fig. 1A, LT-HPCD treatment at above mentioned conditions could maintain the frozen state of prawn, which was attributed to the frozen effect of carbon dioxide during depressurization. In fact, the state of carbon dioxide at 6.5 MPa and 10 °C is liquid, which has been extensively employed for quick-frozen foods such as carrot slices (Xu et al., 2014) and *Agaricus bisporus* (Tan et al., 2011). The changes of pH, texture, color and sensory scores after LT-HPCD treatments were presented in Table 1. LT-HPCD exhibited a carbon dioxide induced acidifying effect, converting the prawn from weakly alkaline to neutral. For texture, the hardness and springiness were still at their original level, showing no significant change ($p < 0.05$) after LT-HPCD treatment. Similarly, Kustyawati et al. (2021) have found no significant change of hardness when prawn was treated with HPCD at subcritical and supercritical states (6.2–7.6 MPa and >31 °C for 5–15 min). Liu et al. (2013) have also discovered no change in the hardness of the prawn after HPCD treatment at 15 MPa and 55 °C for 26 min, but observed a slight decrease in springiness, which can be attributed to its high treatment pressure and temperature.

Table 1
Quality attributes before and after LT-HPCD (6.5 MPa/10 °C/15 min) treatment.

Attributes			Control	LT-HPCD
pH			8.09 ± 0.04 [a]	7.21 ± 0.05 [b]
Texture	Hardness (*g*)		274.4 ± 18.98 [a]	268.1 ± 43.47 [a]
	Springiness		0.684 ± 0.021 [a]	0.722 ± 0.026 [a]
Color	*L*	Cephalothorax	51.79 ± 0.64 [a]	49.86 ± 0.83 [a]
		Abdominal somite	39.47 ± 1.14 [b]	48.03 ± 2.41 [a]
		Telson	44.83 ± 0.96 [a]	45.02 ± 2.01 [a]
	a	Cephalothorax	10.05 ± 0.88 [a]	10.31 ± 0.97 [a]
		Abdominal somite	−0.67 ± 0.25 [b]	1.70 ± 0.67 [a]
		Telson	2.55 ± 0.71 [a]	4.13 ± 1.06 [a]
	b	Cephalothorax	12.90 ± 0.81 [a]	15.74 ± 1.44 [a]
		Abdominal somite	−0.47 ± 0.48 [b]	3.78 ± 1.50 [a]
		Telson	5.46 ± 0.64 [a]	9.03 ± 1.13 [a]
Sensory	Color		8.70 ± 0.15 [a]	7.66 ± 0.17 [b]
	Flavor		7.60 ± 0.37 [a]	7.67 ± 0.44 [a]
	Muscular tissue		7.70 ± 0.37 [a]	7.56 ± 0.24 [a]
	Purity		8.70 ± 0.30 [a]	7.89 ± 0.31 [a]
	Product acceptance of shucking effect		–	8.33 ± 0.29
	Total score		8.15 ± 0.24 [a]	7.52 ± 0.17 [a]

Results were represented as mean ± standard error. Different letters in each row indicated the significant differences ($p < 0.05$) between different treatments. The control group did not have score of "Product acceptance of shucking effect".

Hence, LT-HPCD treatment could maintain the texture of prawn. On the aspect of color, there was no significant color change in cephalothorax and telson, whereas color change occurred in abdominal somite. The values of *L*, *a* and *b* in abdominal somite increased after LT-HPCD treatment, indicating that the abdominal somite turn brighter, redder and more yellow. However, this change was acceptable (see the following results of sensory evaluation) and was not apparent (Fig. 1A). So, the LT-HPCD treatment only brought light and acceptable appearance change to prawn.

Apart from instrumental measurements of food quality, sensory evaluation showed the direct feeling from people. Compared with the original samples, LT-HPCD treated prawn represented no significant differences in total sensory score. As for individual attributes, there was no significant difference in "Flavor", "Muscular tissue" and "Purity". The score of "Color" had a slight decrease, but it was acceptable (>6). These results indicated that prawn after LT-HPCD treatment retained their original sense characters. Collectively, LT-HPCD could inactivate a certain amount of microorganism of prawn, while maintaining their original quality.

3.5. The microbial and quality change of LT-HPCD treated prawn during freeze-chilled storage at 4 °C

Freeze-chilling is a combined technology comprising freezing and frozen storage, followed by thawing and chilled retail display of product (O'Leary et al., 2000). This technology can prolong the whole shelf life, expand market further and reduce waste (Fagan et al., 2003; O'Leary et al., 2000). In our study, the original samples were frozen prawn, and the freeze-chilled storage would be focused on the chilled storage (4 °C) after LT-HPCD treatment at 6.5 MPa and 10 °C for 15 min.

The quality change during freeze-chilled storage was shown in Fig. 4. Obviously, LT-HPCD treated prawn had better appearance (Fig. 4A). Melanosis occurred in control samples at the 4th day of storage, while LT-HPCD treated prawn exhibited nearly no melanosis during storage. Melanosis is caused by Polyphenoloxidase (PPO)-mediated oxidative enzymatic reactions followed by auto-oxidation and polymerization, manifesting the appearance of dark pigments at the joints and injured parts of crustacean (Gonçalves & de Oliveira, 2016; Rotllant et al., 2002). Illera et al. (2019) and Zhang et al. (2011) have proved that HPCD at supercritical or subcritical state can effectively inactivate the PPO in prawn and prohibit melanosis. Accordingly, our results suggested that LT-HPCD at liquid state could also inactivate PPO and thus exhibit a melanosis-inhibiting effect. Besides, HPCD treatment could reduce the oxygen in the prawn tissue, thus producing a melanosis-inhibiting effect similar to that of modified atmosphere packaging (MAP) (Qian et al., 2013; Thepnuan et al., 2008). Apart from inactivating enzyme, LT-HPCD treatment could also contribute to an effective bacteriostasis (Fig. 4B). The viable count microbes of LT-HPCD treated prawn only multiplied 10-fold after 8 days of storage, compared to a 10,000-fold increase for the control. The viable count difference between the two groups became significant ($p < 0.05$) after storage for 4 days, when the control showed a significant increase. However, the counts of LT-HPCD treated prawn showed no significant increase until the 8th day of storage. Ferrentino et al. (2013) have also discovered the bacteriostasis of HPCD. They found that the microbial load of HPCD treated (12 MPa, 50 °C for 5 min) cooked ham grew more slowly than control during the chilled storage of 30 days, and they thought that the dissolved carbon dioxide into the ham played a role in bacteriostasis. The dissolution of carbon dioxide could be proved by the change of pH (Fig. 4C). After LT-HPCD treatment, the pH of the prawn decreased significantly and was consistently lower than the control during the 8 days storage. The pH of the treated prawn was gradually increasing during the storage, and it was still much lower than the pH of control at the end of the storage. The pH increase of LT-HPCD treated prawn suggested that the carbon dioxide was gradually released from the prawn tissue to the air in the package, thus inhibiting aerobic bacteria.

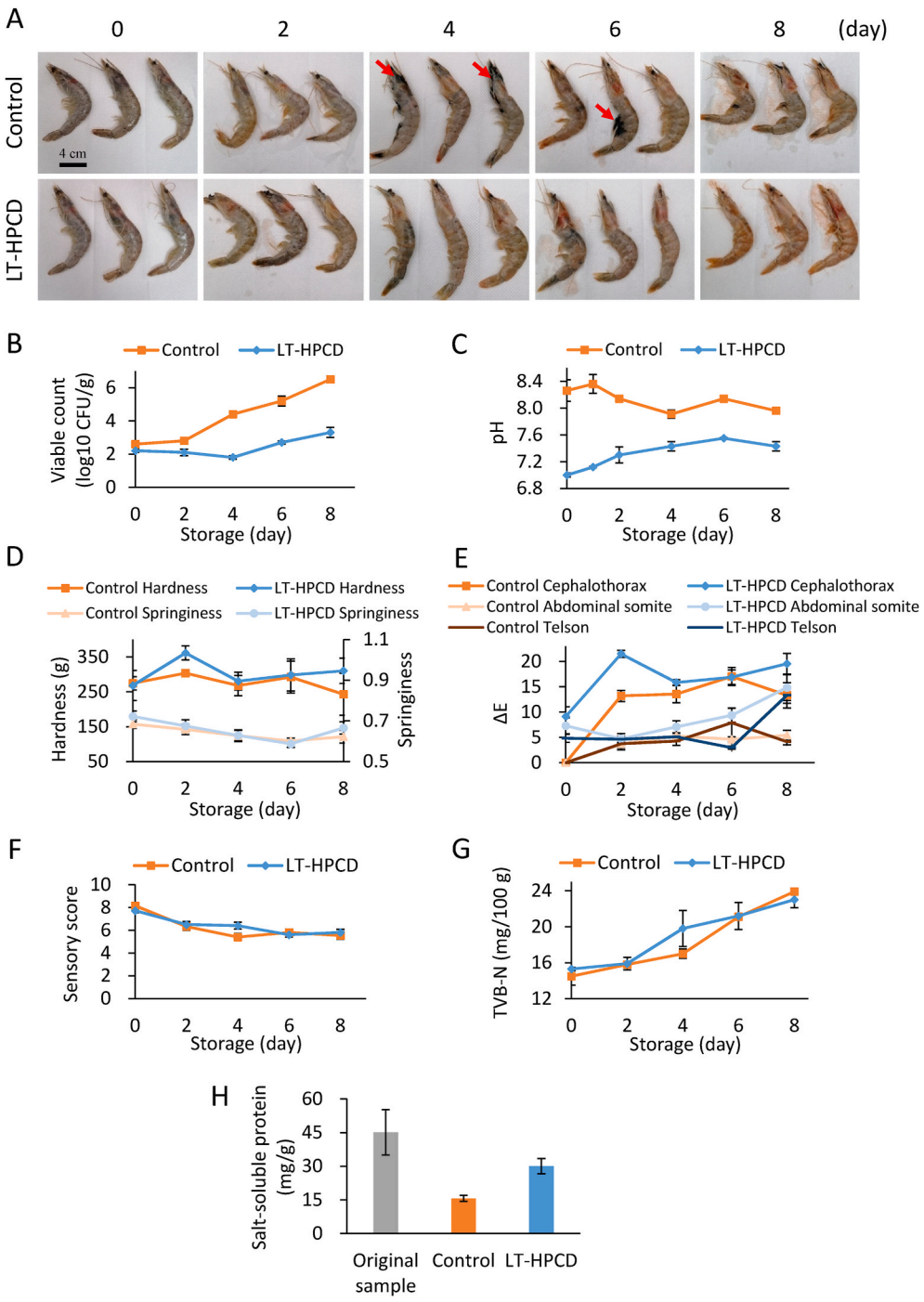

Fig. 4. The quality change of LT-HPCD (6.5 MPa/10 °C/15 min) treated prawn during storage at 4 °C. A: Changes of appearance. Melanoses were pointed out with red arrows. B: Changes of microbial viable count. C: Changes of pH. D: Changes of texture including hardness and springiness. E: Changes of color including the color of cephalothorax, abdominal somite and telson. Δ*E* was calculated by using the *Lab*-value of control at day 0 as the standard. F: Changes of total sensory score. G: Changes of total volatile basic nitrogen (TVB-N). H: Changes of salt-soluble protein at the end of the storage. The original sample was the frozen prawn without any treatment. The control was the prawn thawed and chilled for 8 days, and LT-HPCD was the prawn treated with LT-HPCD, thawed and chilled for 8 days. Bars represented standard errors.

Other attributions also reflected the influence of LT-HPCD treatment on prawn during freeze-chilled storage. For texture, no significant difference between the treated and control group was observed in hardness or springiness, and the values did not change significantly during the storage (Fig. 4D). Hence, the LT-HPCD treatment and continuous acidification brought no harmful effect to the texture of prawn during the chilled storage. The color change was shown in Fig. 4E. As for the cephalothorax, there was no significant difference between the two groups except for one outlier. Both the two groups had a color change in cephalothorax when transformed to chilling, but no further change occurred during the storage. The color change in abdominal somite and telson were similar. No significant difference between the two groups occurred until the 8th day of chilling, when the treated prawn showed larger color change. Thus, the prawn after LT-HPCD treatment had the same color change as the control during 6 days of chilled storage. The sensory scores echoed the above results. The differences in total scores between the two groups were not significant during the chilled storage (Fig. 4F). However, the treated group had maintained an acceptable sensory state (>6) for nearly 6 days, while the control maintained for less than 4 days. This was mainly caused by the score drop of "Color" (Supplementary Table 1). According to Fig. 4G, there was no significant difference in total volatile basic nitrogen (TVB-N) between the treated and control groups. TVB-N is derived from degradation of nitrogenous compounds like proteins, peptides, amino acids, and nucleotides during storage (Debevere & Boskou, 1996). Its steady rise during the chilled storage reflected the increase of spoilage level. The similar trends of

TVB-N change of the two groups might be due to the low microbial load and the variety of bacteria. Debevere and Boskou (1996) have found that MAP with 60% carbon dioxide was more effective to inhibit microbial growth than the production of TVB-N in cold preservation. They explained this as being related to growth of bacteria that were not related to spoilage but catalyzing the production of TVB-N. As for our results, it was possible that the microbial load of such TVB-N producing bacteria did not change significantly after LT-HPCD treatment. Fig. 4H showed the change of salt-soluble protein at the end of the storage, and the content of control had decreased significantly while the change of treated prawn was not significant. The reduction of salt-soluble protein indicates the protein denaturation (Matsumoto, 1980, pp. 95–124). This significant difference between the two groups indicated that LT-HPCD treatment could relieve protein denaturation. The positive impact of LT-HPCD treatment could be attributed to its enzyme and microbial inactivation effect, which relieve spoilage and quality deterioration.

3.6. The quality change of LT-HPCD treated prawn during frozen storage at −18 °C

The quality change during frozen storage was shown in Fig. 5. The two groups had nearly no change of appearance during storage (Fig. 5A). The pH of treated prawn had an obvious recovery in the first 3 weeks of storage, and then the difference between the two groups was not significant ($p < 0.05$) after 9 weeks (Fig. 5B). As the pH drop could quickly recover during the frozen storage, there was no need to concern the negative effect of acidification. For the texture, no significant difference had been formed between the two groups during the freezing (Fig. 5C). The hardness and springiness fluctuated in ranges without a clear trend of change in the storage. So, LT-HPCD treatment would not bring texture change in following frozen storage. The color changes between the two groups were not significantly different, and there was no apparent color change during the freezing (Fig. 5D). Fig. 5E showed the results of sensory evaluation at the end of the storage. There was no significant difference among the three groups in all attributes (Fig. 5E). Consequently, the LT-HPCD treatment would bring no negative effect to frozen prawn during the following frozen storage.

4. Conclusion

Low temperature HPCD (LT-HPCD) treatment at 6.5 MPa and 10 °C for 15 min showed an effective inactivation of bacterial suspensions and

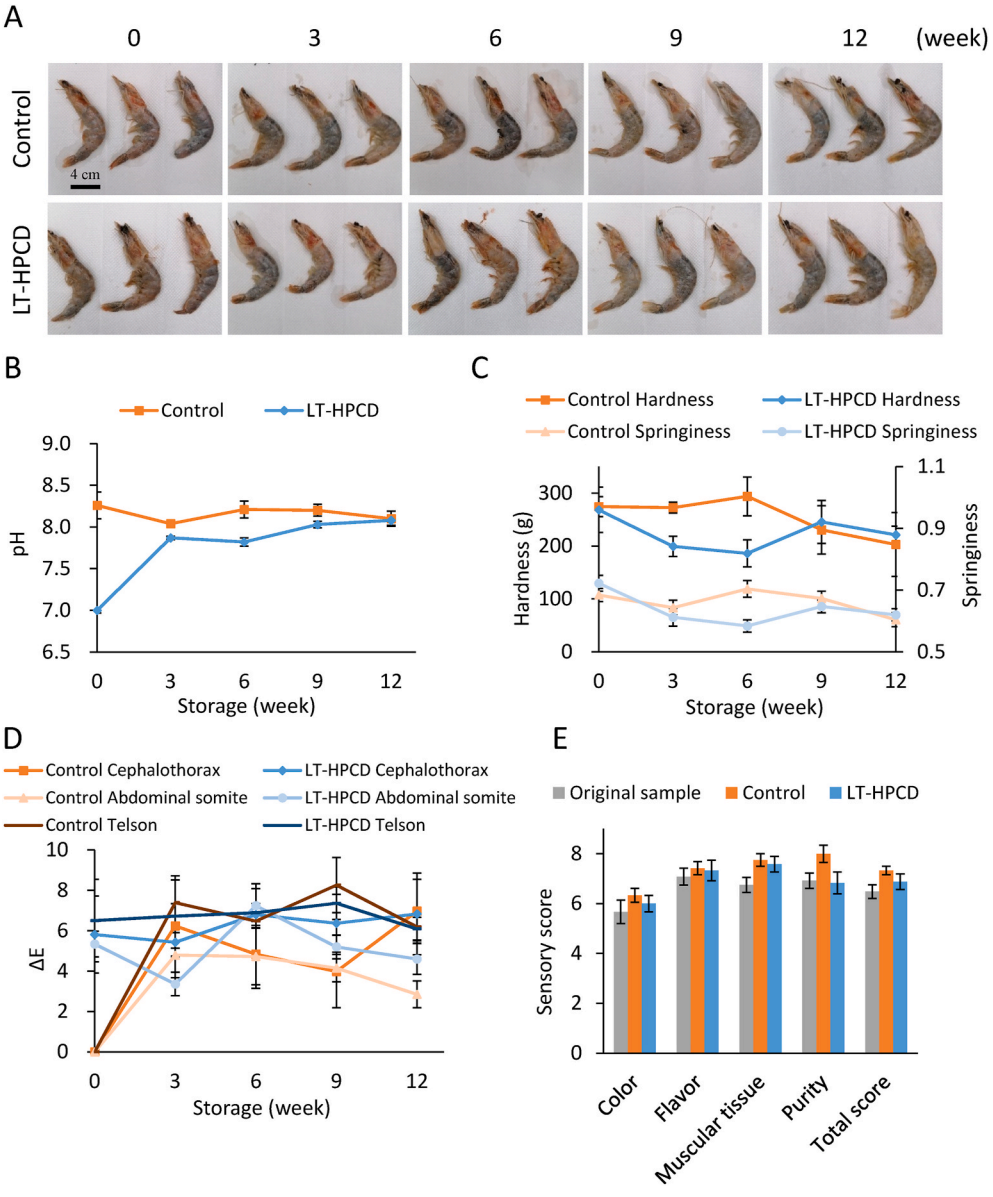

Fig. 5. The quality change of LT-HPCD (6.5 MPa/10 °C/15 min) treated prawn during frozen storage at −18 °C. A: Changes of appearance. B: Changes of pH. C: Changes of texture including hardness and springiness. D: Changes of color including the color of cephalothorax, abdominal somite and telson. Δ*E* was calculated using the *Lab*-value of control at day 0 as the standard. E: Changes of sensory score at the end of the storage. The original sample was the frozen prawn without any treatment. The control was the prawn frozen for 12 weeks, and LT-HPCD was the prawn treated with LT-HPCD and frozen for 12 weeks. Bars represented standard errors.

virus. It was also the optimal condition to treat frozen prawns, achieving a 60% reduction of natural microflora and maintain their original quality. There was only light pH change and acceptable color change in abdominal somite after treatment, while texture and sense were kept the same. The acidification effect of LT-HPCD was persistent during chilled storage, which could contribute to a long-term bacteriostasis. LT-HPCD was also beneficial to inhibit melanosis and protein denaturation during freeze-chilled storage. The use of high pressure carbon dioxide at low temperature achieved the combination of inactivation and freezing, making it more suitable for cold-chain food processing and quality maintenance. Frozen prawn could return to its original state after treatment without quality change in the following frozen storage. Hence, LT-HPCD was a suitable method for cold-chain food processing to improve microbial safety without changing original quality.

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CRediT authorship contribution statement

Zimeng Lian: Methodology, Investigation, Writing – original draft. **Dong Yang:** Methodology, Investigation. **Yongtao Wang:** Writing – review & editing. **Liang Zhao:** Writing – review & editing. **Lei Rao:** Conceptualization, Investigation, Supervision, Writing – review & editing. **Xiaojun Liao:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

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

Data availability

All data were showned in the article and supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2022.109401.

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