Original paper

Effects of Vacuum and Modified Atmosphere Packaging on Microbial Flora and Shelf-life of Pacific White Shrimp (*Litopenaeus vannamei*) during Controlled Freezing-point Storage at -0.8°C

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Effects of vacuum and modified atmosphere packaging (MAP) on microbial flora, pH, total volatile basic nitrogen (TVB-N), trimethylamine (TMA-N), the exudates and sensory attributes in Pacific white shrimp (*Litopenaeus vannamei*) during controlled freezing-point storage at -0.8°C for a period of 14 days were investigated. The results indicated vacuum packaging (VP), 40%CO₂/30%O₂/30%N₂ (MAP1) and 75%CO₂/10%O₂/15%N₂ (MAP2) were more effective for inhibiting growth of *Aeromonas* spp., *Pseudomonas* spp., H₂S-producing bacteria (including *Shewanella putrefaciens*) and *Enterobacteriaceae* and decreasing TVB-N and TMA-N contents as compared to air packaging (AP). pH values of VP and MAP2 samples were significantly ($P < 0.05$) lower than those of AP. Formation of exudates under AP, VP, MAP1 and MAP2 increased during the storage and the value was higher with the increase of CO₂ levels. Sensory evaluation indicated that the shelf-life of fresh Pacific white shrimp got extended and reached 7 days under VP, 9 days under MAP1 and 11–12 days for the optimum MA-packaged under MAP2 as compared to 4 days' shelf-life under aerobic storage.

Keywords: Pacific white shrimp, Vacuum packaging, Modified atmosphere packaging, Controlled freezing-point storage, Shelf-life

Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is one of the most important seafood traded worldwide, and also one of the most representative aquatic products for its positive culinary quality and high nutritional values (Oosterveer, 2006; Qian *et al.*, 2013). Since fresh shrimps have a short shelf life, which causes substantial practical problems for its transportation and distribution, and they are very sensitive to microbial spoilage and can be contaminated by

bacteria naturally present in the marine environment, such as lactic acid bacteria, *Vibrio* spp., *Enterobacteriaceae*, *Aeromonas* spp. and *Pseudomonas* spp. (Gopal *et al.*, 2005; Jeyasekaran *et al.*, 2006; Wang *et al.*, 2010; Behnam *et al.*, 2013; Binsi *et al.*, 2013; Carrascosa *et al.*, 2013), or in the digestive tract, which is not always removed directly after catch. Besides, flesh of shrimp after death is still active and biochemically alive, the organic decomposition or the change of shrimp composition may be triggered by various factors,

Abbreviations

MAP; Modified Atmosphere Packaging, VP; Vacuum Packaging, AP; Air Packaging, LAB; Lactic acid bacteria, TVB-N; Total Volatile Basic Nitrogen, TMA-N; Trimethylamine, TMAO; Trimethylamine Oxide, PPO; Polyphenoloxidase

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i.e. enzymes and microbiological activities, the need to develop methods for maintaining good postmortem quality of shrimps on their way to the market increases (Wang and Zeng, 2009; Cui *et al.*, 2013).

In order to extend the shelf-life of shrimp, many methods of packaging have been investigated to control the changes of sensory attributes and the proliferation of spoilage bacteria in raw shrimp, such as air packaging (AP), aerobic polyvinylchloride packaging (Chen and Xiong, 2008), vacuum packaging (VP), modified atmosphere packaging (Layrisse and Matches, 1984; Matches and Layrisse, 1985; Lu, 2009), especially modified atmosphere packaging (MAP), as one of the most effective applications for shelf-life extension of seafood, is becoming increasingly popular. The effectiveness of MAP to curtail bacterial growth and improve shelf-life stability of shrimp has also been evaluated in some studies (Bak *et al.*, 1999; Thepnuan *et al.*, 2008; Wang *et al.*, 2010).

More recently, an additional storage technique based on the Japanese concept of 'Hyo-on' has attracted considerable interest. 'Hyo-on', also called controlled freezing-point storage, employs a temperature range, in which foods remain in a non-frozen temperature-zone between the freezing point of water and that of an individual material (Yamane, 1982; Fukuma *et al.*, 1998). Storing food at controlled freezing-point temperatures can be advantageous in terms of maintaining food freshness and suppressing harmful microorganisms as compared to other storage temperature (Yamane, 1996; Wang and Zeng, 2009). In addition to these advantages, there is an additional effect that may keep food quality (Bohnert and Jensen, 1996; Guo *et al.*, 2008). However, to our knowledge, limited research has been conducted to assess the impact of MAP on the microbiological, physicochemical and sensory attributes of shrimps stored under chilled or refrigerated temperature. In particular, it is completely unknown how MAP with mixed CO₂, O₂ and N₂ gases would affect the quality attributes of raw shrimp during controlled freezing-point storage. The purpose of this study was to investigate the effects of air, vacuum and MAP on microbial flora and shelf-life of Pacific white shrimp (*Litopenaeus vannamei*) during controlled freezing-point storage at -0.8°C. The air packaging was used as control for comparison.

Materials and Methods

Samples preparation Fresh Pacific white shrimps (*Litopenaeus vannamei*), of the size of 55–65 shrimps/kg, were procured from local aquatic products market. The average length and weight of shrimp were ca. 14 cm and 17 g, respectively. The shrimps were placed in a large polyethylene bag with sufficient oxygen and transported to laboratory within 1 h. Upon the arrival, the shrimps were washed with clean water, in which 1368 lively and undamaged ones were used in two replications' experiments with repeated measures (n = 684 for each replication).

Packaging and storage of samples For all samples, an aliquot

of 12 ones were individually weighed and placed separately in a thermoplastic starch tray (TPS tray, 238 × 173 × 25, length × width × depth, in mm) (THP-34, Tianhe Environmental Technology Co., Ltd., Zhejiang). For each replication, a total of 56 trays, each containing 12 shrimps, were prepared. All trays allowed for 56 sub-treatments arranged with three types of packaging systems (AP, VP and MAP), which were packaged with pouches made of food grade nylon and polyethylene blending film (33.5 cm long × 27 cm wide; 160 µm in thickness; water vapor transmission rate of 1.30–1.52 g per m² at 37°C, 90% relative humidity, 24 h; oxygen transmission rate of 16.8–20.2 cm³ per m² at 23°C, 75% relative humidity, 24 h).

For AP, the pouches were sealed using an impulse heat-sealing machine (Model SF-200, P&S Co., Ltd., Shanghai, China). For VP, the ones were packed with a vacuum sealing machine (Model DZ 400/2S), supplied by Zhongjia Appliances Co., Ltd., Shandong, China. For MAP, the ones were filled with gas mixtures of 40%CO₂/30%O₂/30%N₂ (MAP1) and 75%CO₂/10%O₂/15%N₂ (MAP2) with 1.5 bar gas pressure, and packaged using a Modified Atmosphere Packing machine (Model DQB360, Qingpa Co., Ltd., Shanghai, China). These two particular MAP gas mixtures were used because it was reported by many researchers that MAP1 was markedly effective in extending the shelf-life of chilled fresh shrimp (López-Caballero *et al.*, 2002; Lu, 2009; Wang *et al.*, 2010) and MAP2 was the optimized result obtained from a simplex centroid mixture design with 3 components (partial pressures of CO₂, O₂ and N₂) and lattice 3 (9 runs + 3 replicates of centre point) (the conclusion from author's experiment). Immediately after packing, all the packs were kept in a precise refrigerator (Model SHP-2500, P&S Co., Ltd., Shanghai, China) (storage temperature as mentioned in the results of initial freezing point). At the end of each designated storage time within the same replication, one pack from each packaging system was removed for microbiological, pH, total volatile bases nitrogen (TVB-N), trimethylamine (TMA-N), exudates analysis and sensory evaluation, and for day 0, only one sub-treatment package was used.

DSC measurement Tests were conducted on a differential scanning calorimeter (DSC, 200PC, Netzsch Co., Ltd., Germany) with automatic data analysis software. Fresh shrimp samples were beheaded, peeled and ground to obtain uniformity in clean condition, and small samples (8–10 mg each) were hermetically closed in aluminum DSC pan and very precisely weighed. Prior to measurements, the DSC was calibrated for temperature and energy sensitivities using indium, Hg and C₆H₁₂. Samples were frozen in situ in the calorimeter with liquid nitrogen cooling and stabilized at -40°C and then heated from -40°C to 20°C at a rate of 2°C/min. An empty pan was used as a reference and the baseline was obtained from a scan realized with two empty pans and sapphire was used as a standard with a known specific heat value. Six replications were performed and specific heat capacity of these runs was calculated using the following equation and results were displayed as average curve with standard deviation in the figure.

Table 1. Standard references used in sensory evaluation of raw Pacific white shrimp

Category	Attribute	Definition	Reference/source
Odor	Old shrimp	Associated with old fish, from slight to strong	Shrimp powder
	Ocean/seawater	Associated with the ocean or seawater, from slight to strong	Clam juice (QianChuan Rubber Co., Ltd., Qingdao, China)
Shell Appearance	Darkness	The intensity of the shell color, from light to dark	White bond paper (L = 91.32, a = 0.03, b = 0.01) Black bond paper
	Stripe darkness	The darkness of the stripes on the shell, from light to dark	White bond paper Black bond paper
	Brown color	The brownness of the shell (from one section to six), from white to brown	White bond paper Light brown paper (L = 65.62, a = 8.12, b = 10.18) Chocolate syrup (Deli Foods Co., Ltd., Wuhu, China)
	Blotchiness	The amount of coverage of dark spots on the surface of the meat, from not blotchy to blotchy	None Fully covered
	Glossiness	The amount of light reflected from the shell, from dull to glossy	White bond paper Laminated card
	Tail iridescence/ Rainbow	The appearance of rainbow-like colors on the tail, from slight to extreme	Ribbon curl
	Plumpness	The appearance of being plump at the head, from flat to round	
	Brown color	The brownness of the meat near the head (cross-section at cut end), from white to brown	White bond paper Light brown paper Chocolate syrup

$$C_{app} = \frac{m_{std}}{m_s} \cdot \frac{DSC_s - DSC_b}{DSC_{std} - DSC_b} \cdot C_{p, std} \quad \dots\dots \text{Eq. 1}$$

Where C_{app} and $C_{p, std}$ are the specific heats of sample and sapphire, respectively (kJ/kg °C), m_s and m_{std} are the weights of sample and sapphire, respectively (kg), and DSC_s , DSC_{std} and DSC_b are the heat flow rates of sample, sapphire and baseline, respectively (mW).

Microbiological analysis A sample of approximately 25 g was taken aseptically from each packaging system within the same replication, transferred to a stomacher bag and 225 mL of 0.1% sterilized peptone (Bacto peptone, BD211667, NY, USA) water with salt (NaCl, 0.85%, wt/vol) were added, and the mixture was homogenized for 120 s in a Stomacher F-200 Laboratory Blender (Specimen and model factory, Shanghai, China). Then serial decimal dilutions of each homogenate were carried out with the same diluents. Samples (0.1 mL) of serial dilutions of shrimp homogenates were spread on the surface of the appropriate dry media in Petri dishes for determination of the mesophilic bacteria and psychrotrophic bacteria on Trypticase Soy agar (TSA, BD236950, NY, USA) and incubated at 37°C and 20°C for 2 days, respectively. *Pseudomonas* spp. and *Aeromonas* spp. were determined on Cefrimide Fusidin Cephaloridine agar (CFCA, Oxoid CM559 supplemented with selective supplement SR103, Oxoid, Basingstoke, UK) and *Aeromonas* Isolation agar (AIA,

Oxoid CM833 supplemented with selective supplement SR136) after incubation at 20°C for 2 – 3 days, respectively. For *Enterobacteriaceae* and H₂S-producing bacteria (including *Shewanella putrefaciens*), 1.0 mL was inoculated into 10 mL of molten (45°C) violet red bile glucose agar (VRBGA, Oxoid CM485) and iron agar (IA, Oxoid CM867), respectively. After setting, a 10-mL overlay of molten medium was added, and the former was incubated at 37°C for 24 h and the large colonies with purple haloes were counted while the incubation of IA plates was at 25°C and black colonies formed by the production of H₂S were enumerated after 3 days. Lactic acid bacteria (LAB) were enumerated on de Man Rogosa Sharpe agar (MRSA, pH6.2, Oxoid CM361) and incubated at 30°C for 5 days. Three replicates of at least three appropriate dilutions were enumerated. All plates were examined visually for typical colony types and morphological characteristics associated with each growth medium. In addition, the selectivity of each medium was checked routinely by Gram staining and microscopic examination of smears prepared from randomly selected colonies from all of the media. Microbiological data was transformed into logarithms of the number of colony-forming units (CFU/g).

Physicochemical analysis For pH determination, 5 g of shrimp meat was homogenized with 45 mL deionised water for 90 s and the homogenate was kept at room temperature for 5 min. The pH

Fig. 1. Experimental data of specific heat capacity versus temperature for fresh Pacific white shrimp samples

was measured using a pH meter (PHS-3C, Shanghai, China). Total volatile bases nitrogen (TVB-N) and trimethylamine (TMA-N) contents in shrimp meat were determined using the Conway micro-diffusion method (Conway and Byrne, 1936). Briefly, shrimp meat (2 g) was mixed with 8 mL of 4% TCA and homogenized at 6500 rpm using the Stomacher F-200 Laboratory Blender for 1 min. The homogenates were filtered through a filter paper (Whatman No. 41, Buckinghamshire, UK) and filtrates were used for analyses. Sample extract (1 mL) was placed in the outer ring, and then 1% boric acid containing the Conway indicator was pipetted into the inner ring. To initiate the reaction, saturated K_2CO_3 solution (1 mL) was mixed with sample extract. The Conway unit was closed and incubated at 37°C for 60 min. The inner ring solution was then titrated with 0.02 N HCl until the green color turned to pink. TMA-N content was determined in the same manner as TVB-N but 10% formaldehyde was added to the filtrates to fix ammonia present in the sample. The amounts of TVB-N or TMA-N were calculated and expressed as mg N/100 g shrimp meat. The exudates in the packages during storage were measured gravimetrically. The entire package (sample and film) was weighed. Then, the samples and any purge were removed from the package, and the shrimp and the entire package surface were wiped clean with a paper towel. Finally, the shrimp samples were placed back into its package and re-weighed. The mass of the exudates (g) was divided by the initial mass of the product (g) and reported as a g/100 g initial weight (Fernández *et al.*, 2009).

Sensory evaluation Sensory evaluation panel (12 persons) was recruited from a pool of panelists trained in descriptive analysis. Training consisted of preliminary trials and training of sensory evaluation standard. Eventually Standard references for shrimp odor and appearance were established based on Erickson and Wang's descriptive methods (Erickson *et al.*, 2007; Wang *et al.*, 2010) (Table 1). Briefly, raw shrimp was presented to a panel of trained panelists for sensory evaluation. Panelists were asked to rate samples as

acceptable or unacceptable on the basis of odor and appearance using a 10-point scale, where 10 corresponded to a product of highest quality and 0 corresponded to a poor quality of product. Scores of 6.0 and above were considered acceptable. Above of all, raw shrimps were placed into a TPS box (167mm length $\times$ 134mm width $\times$ 50mm depth, THH-09, Tianhe Environmental Technology Co., Ltd., Zhejiang) and held in a refrigerator (4°C) for less than 1 h before evaluations were conducted.

Statistical analysis Data from each replication was averaged, and means and standard deviations were calculated. The Origin 8.0 for Windows software (OriginLab Inc., Hampton, MA, USA) was used to explore the statistical significance of the results obtained, including multivariate contrasts and multiple comparisons by the Tukey's test. A confidence interval at the 95% level ($P < 0.05$) was considered in all cases.

Results and Discussion

Initial freezing point Experimental data of specific heat capacity versus temperature for fresh Pacific white shrimp samples is showed in Fig. 1. In all replicates, the mean value of specific heat and standard deviation gave a coefficient of variation lower than 10%, indicating that the variability in the measurements was not excessive.

Specific heat depends strongly on temperature. Just as illustrated in Fig. 1, specific heat capacity of the samples increased with temperature until the values peaked ($154.38 \pm 7.48 \text{ kJ/kg } ^\circ\text{C}$), and immediately dropped from peak values to $4.56 \pm 0.23 \text{ kJ/kg } ^\circ\text{C}$ due to phase change. Subsequently the value became flat no matter how the temperature rose in the non-frozen state. This trend indicated that specific heat capacity of samples in frozen status was lower than that in non-frozen ones. Obviously it was due to the fact that a major portion of latent heat was removed from the samples when the phase was changed from frozen form to non-frozen one (Ramaswamy and Tung, 1981; Tocci and Mascheron, 2008).

Therefore, the position of thawing peak ($-1.54 \pm 0.07^{\circ}\text{C}$) was defined as initial freezing point of fresh Pacific white shrimp samples in the heat capacity versus temperature plot.

Considering that it is important to prevent temperature fluctuations that result in the thawing and re-freezing of shrimp samples during controlled freezing-point storage, -0.8°C was chosen as the storage temperature of this work combined with the function and characteristics of equipment.

Microbiological analyses In present research, the monitoring of the following species of microorganisms during 14 days of storage was focused on: mesophilic bacteria, psychrotrophic bacteria, *Pseudomonas* spp., H_2S -producing bacteria (including *Shewanella putrefaciens*), *Aeromonas* spp., LAB and *Enterobacteriaceae*. The total mesophilic bacteria (day 0) in fresh shrimp (Fig. 2a) was 4.5 ± 0.3 log CFU/g, but under controlled freezing-point storage decreased by about one log in the first 3 days and later steadily increased to more than 6 log CFU/g at the end of storage. The psychrotrophic bacteria of fresh shrimp (3.2 ± 0.2 log CFU/g, Fig. 2b), which was ca. 1.3 log lower than the mesophilic bacteria, initially increased steadily on storage and also exceeded the value of 6 log CFU/g, which is considered as the upper acceptability limit for shrimp (ICMSF 2002). Length of controlled freezing-point storage at -0.8°C had a notable ($P < 0.05$) effect on both psychrotrophic bacteria and mesophilic bacteria which tended to increase as the storage time increased regardless of packaging conditions (Fig. 2a-b). However from day 3 on, psychrotrophic counts exceeded mesophilic counts in all samples. Similar observations were made earlier by Lakshmanan *et al.* (2002), Thepnuan *et al.* (2008) and Wang *et al.* (2010). This increase in counts can be due to the growth of psychrotrophic bacteria in Pacific white shrimp at -0.8°C .

It was clear that initial mesophilic counts and psychrotrophic counts for fresh Pacific white shrimp indicated acceptable quality. Given that for newly caught shrimp, initial mesophilic bacterial counts and psychrotrophic bacterial counts are between 2 and 6 log CFU/g, depending on environment, temperature and species. Similarly, initial mesophilic counts and psychrotrophic counts between 4 and 6 log CFU/g were reported for shrimp (*Penaeus semisulcatus*), freshly procured from the local fish market of India (Lakshmanan *et al.*, 2002). Of the packaging treatments (air, vacuum, MAP1 and MAP2) the use of VP and MAP was more effective for the inhibition of mesophilic counts and psychrotrophic counts as compared to AP ($P < 0.05$). This fact may be attributed to the inhibitory effect created either by the vacuum or by the presence of CO_2 (75% and 40%) on microbial growth. It is well established that under CO_2 , a bacteriostatic effect is exerted on aerobic flora growth and thus Gram-negative bacteria such as *Pseudomonas* spp. and H_2S -producing bacteria are inhibited.

It is shown from the available literatures that psychrotrophic bacteria are the major group of microorganism responsible for spoilage of fresh seafood, such as Mediterranean anchovies (*Engraulis encrasicolus*) (Pons-Sánchez-Cascado *et al.*, 2006),

Pacific white shrimp (*Litopenaeus vannamei*) (Thepnuan *et al.*, 2008; Wang *et al.*, 2010), rainbow trout (*Oncorhynchus mykiss*) (Behnam *et al.*, 2013). Furthermore it has been reported that mesophilic bacteria also plays an active role in decomposition of fish and crustaceans (Lakshmanan *et al.*, 2002; Manju *et al.*, 2007; Papadopoulou *et al.*, 2007; Wang *et al.*, 2010). Based on a microbiological acceptability limit of 6 log CFU/g for fresh shrimp and mesophilic counts and psychrotrophic counts of the present study, VP and MAP resulted in an extension of shelf-life of fresh Pacific white shrimp by ca. 3 days for VP, 5 days for MAP1 and 8 days for MAP2 as compared with AP samples, respectively. The initial load of microorganism in shrimp is dependent on the environment where it is farmed. Present results agreed with that reported by Thepnuan *et al.* (2008) and Wang *et al.* (2010) for Pacific white shrimp stored under VP and MAP, confirming that the two treatments were effective in extending the shelf-life of Pacific white shrimp. Moreover, Skandamis and Nychas (2002) reported that storage of fresh meat at increasing CO_2 concentrations caused a higher inhibition of psychrotrophic aerobes and an extended shelf life. In the present study, the total mesophilic bacteria were higher than psychrotrophic one in fresh shrimp in the first 2 days. But due to the effect of cold shock the psychrotrophic bacteria proliferated slowly and dominated the mesophilic bacterial load, which was in accordance with the findings of Lakshmanan *et al.* (2002), and exceeded the upper acceptability limit for shrimp on day 5 (AP), on day 8 (VP), on day 10 (MAP1) and on day 13 (MAP2).

Pseudomonas species are involved in the decadence process since these microorganisms are able to provoke the product deterioration developing odors and unpleasant tastes, caused by their strong biochemical activity. It has been reported that Gram-negative bacteria such as *Pseudomonas* spp. grow during aerobic storage on chilled fish and seafood products (Gram and Huss, 1996; Gram and Dalgaard, 2002). Initial population of *Pseudomonas* spp., Gram-negative contributor of natural flora of Pacific white shrimp (day 0), was 2.5 ± 0.4 log CFU/g, which was the most one of all the microorganisms enumerated (H_2S -producing bacteria, 2.1 ± 0.4 log CFU/g; *Aeromonas* spp., 1.7 ± 0.3 log CFU/g; LAB, 1.7 ± 0.4 log CFU/g; *Enterobacteriaceae*, 1.3 ± 0.2 log CFU/g), the results indicated that *Pseudomonas* spp. were the dominant microorganisms in fresh white shrimp, and on day 14, increased to reach final populations of 7.7 ± 0.3 log CFU/g (AP samples), 6.7 ± 0.3 log CFU/g (VP samples), 6.1 ± 0.3 log CFU/g (MAP1 samples) and 5.6 ± 0.4 log CFU/g (MAP2 samples) (Fig. 2c). In the same storage time, MAP and VP samples had a significantly lower ($P < 0.05$) *Pseudomonas* spp. count as compared to AP samples.

Of the packaging treatments examined in the present study, MAP2 was the most effective for the inhibition of *Pseudomonas* spp. in white shrimp. Between these bacterial groups, *Pseudomonas* spp. were dominant (counts of 2.5 ± 0.4 log CFU/g) on the initial storage period and the bacteria has been reported to be the major spoilage

Fig. 2. Effects of AP (■), VP (●), MAP1 (▲) and MAP2 (▼) on Mesophilic bacteria (a), Psychrotrophic bacteria (b), *Pseudomonas* spp. (c), *H₂S*-producing bacteria (including *Shewanella putrefaciens*) (d), *Aeromonas* spp. (e), LAB (f) and *Enterobacteriaceae* (g) of Pacific white shrimp during controlled freezing-point storage at -0.8°C . Each point is the mean $\pm$ SD of two replicate experiments with three samples analyzed per replicate ($n=2 \times 3$)

bacteria for seafood stored at refrigerated temperature in air (Gram and Melchiorson, 1996; Koutsoumanis and Nychas, 1999), and are inhibited or killed during storage in CO_2 (Lakshmanan *et al.*, 2002; Thepnuan *et al.*, 2008; Ravi Sankar *et al.*, 2008). This is probably attributed to the sensitivity of *Pseudomonas* spp. to CO_2 , especially the more CO_2 concentration; the more marked the inhibition (40% and 75% CO_2).

H_2S -producing bacteria (including *Shewanella putrefaciens*) are another important spoiled microbiology. Especially its initial counts of 2.1 ± 0.4 log CFU/g were second only to those of *Pseudomonas* spp. in all spoilers assayed, and as the storage time increased, H_2S -producing bacteria (including *Shewanella putrefaciens*) reached the final populations of 7.3 ± 0.4 log CFU/g, 6.6 ± 0.4 log CFU/g, 6.0 ± 0.2 log CFU/g and 5.1 ± 0.3 log CFU/g for AP, VP, MAP1 and MAP2, respectively. It is well documented that H_2S -producing bacteria (including *Shewanella putrefaciens*) can grow during chilled storage on seafood and is also considered as one of the strongest spoilers of seafood from cold and temperature water (Gram *et al.*, 1987). As expected in our experiment, H_2S -producing bacteria (including *Shewanella putrefaciens*) also dominated the spoiled microbiology in AP because of its higher O_2 and lower CO_2 content. But samples stored under different CO_2 concentration (MAP1 and MAP2) had significantly ($P < 0.05$) lower counts than other treatments, confirming the MAP, especially CO_2 in MAP, can effectively inhibit the growth of H_2S -producing bacteria (including *Shewanella putrefaciens*) in Pacific white shrimp. Likewise, the sensitivity of *Shewanella putrefaciens* – H_2S -producing bacteria – to CO_2 had already been described for shrimp (López-Caballero *et al.*, 2002).

Effects of AP, VP, MAP1 and MAP2 on *Aeromonas* spp. of Pacific white shrimp are depicted in Fig. 2e. *Aeromonas* spp. was identified as one of H_2S -producing bacteria and constituted 8–12% of H_2S -producing bacteria of Pacific white shrimp stored under AP and VP at the time of spoilage but their contribution gradually decreased with the increasing of CO_2 , especially for MAP2 (its contribution $< 1\%$). 75% CO_2 in MAP2 in conjunction with controlled freezing-point storage at -0.8°C affected the survival of *Aeromonas* spp. in Pacific white shrimp (Fig. 2e). The proliferation of *Aeromonas* spp. was noticeable in Pacific white shrimp stored under AP and VP from initial value of 1.7 ± 0.3 log CFU/g to ultimate 6.4 ± 0.4 log CFU/g and 5.6 ± 0.4 log CFU/g, respectively. The inhibition of CO_2 to *Aeromonas* spp. was especially pronounced, especially on the lag phase. Just as in the work, a lag phase of extension of 3–7 days was noticed for Pacific white shrimp stored under MAP2, and the psychrotropic character of *Aeromonas* spp. has been demonstrated in many aquatic foods such as cod and trout (Davies and Slade, 1995), pearlspot (Ravi Sankar *et al.*, 2008), etc.

LAB were also a dominant bacterial species in Pacific white shrimp spoilage (Fig. 2f). Initial lactic acid bacterial count was 1.7 ± 0.4 log CFU/g while the highest counts of 7.0 ± 0.4 log CFU/g were reached for VP samples on day 14, whereas respective counts

for the other samples did not reach this value throughout the entire storage period. After 14 days of storage, VP and MAP2 samples had a significantly higher ($P < 0.05$) count than all the rest. LAB, being facultative anaerobic bacterial species, were found to be members of the microbial flora of shrimp samples and the dominant species in VP and MAP2 samples. Obviously, the use of 75% CO_2 did not notably inhibit the growth of LAB due to their tolerance against the action of CO_2 , and on the contrary may inhibit growth of other bacteria because of the formation of lactic acid and bacteriocins and this fact may contribute to their selective growth during spoilage of seafood products. Likewise, Pournis *et al.* (2005) and Stamatis and Arkoudelos (2007) pointed out that there were high final counts of LAB in the microbial flora of refrigerated fish species, stored under various MAP conditions.

Initial *Enterobacteriaceae* counts of fresh shrimp were 1.3 ± 0.2 log CFU/g (Fig. 2g), which indicated a good hygiene of the marine environment from which the shrimp was caught, as well as good fishing practices and subsequent handling. At the end of storage, *Enterobacteriaceae* produced lower counts ($P < 0.05$) in VP (6.9 ± 0.5 log CFU/g), MAP1 (6.3 ± 0.5 log CFU/g) and MAP2 (5.7 ± 0.3 log CFU/g) samples as compared to the control (7.5 ± 0.5 log CFU/g for AP), obviously the growth of *Enterobacteriaceae* were partly inhibited under vacuum or in the presence of CO_2 . As one of the psychrotolerant species, *Enterobacteriaceae* are capable of growing at chilled storage but in this study did not well complete with other spoilers because of the formation of lactic acid under VP or the sensitivity of *Enterobacteriaceae* to CO_2 in MAP, similar conclusions were also found by López-Caballero *et al.* (2002), Kykkidou *et al.* (2009) and Wang *et al.* (2010).

Physicochemical analyses Effects of AP, VP, MAP1 and MAP2 on pH, TVB-N, TMA-N and exudates of Pacific white shrimp during controlled freezing-point storage are depicted in Fig. 3a-d. The initial value of pH (day 0) was found to be 7.3 ± 0.2 (Fig. 3a). In all shrimp samples, the values of pH decreased initially and then increased. On day 5, significant differences ($P < 0.05$) were observed in the pH values between AP (7.8 ± 0.3) and MAP2 (7.0 ± 0.2) samples, which may be attributed to the dissolution of CO_2 in the shrimp sample, acidifying it via the formation of carbonic acid (Banks *et al.*, 1980). During the storage period at -0.8°C , pH values of AP samples were significantly ($P < 0.05$) higher than those of all the rest, and the increase of pH was postulated to be due to the rapid spoilage of the product and the formation of alkaline compounds of autolysis and bacterial metabolites (Ruiz-Capillas and Moral, 2001). After 14 days of storage values of pH in VP and MAP2 samples were notably ($P < 0.05$) lower than those in the rest. However, values of pH for VP and MAP2 shrimp samples were no statistically significant differences ($P > 0.05$) between packaging treatments, which the formation of lactic acid maybe resulted in pH decrease. Similar observations were made by Alasalvar *et al.* (2001), Manju *et al.* (2007) and Fan *et al.* (2009).

Fig. 3b represents TVB-N contents of Pacific white shrimp

Fig. 3. Effects of AP (■), VP (●), MAP1 (▲) and MAP2 (▼) on pH values (a), TVB-N values (b), TMA-N values (c) and Exudates (d) of Pacific white shrimp during controlled freezing-point storage at -0.8°C . Each point is the mean $\pm$ SD of two replicate experiments with three samples analyzed per replicate ($n=2 \times 3$)

stored in air, vacuum and modified atmosphere. TVB-N values of Pacific white shrimp increased from an initial value of 8.5 ± 1.7 mg N/100 g shrimp meat to final values of 48.9 ± 2.2 mg N/100 g, 33.2 ± 1.4 mg N/100 g, 36.3 ± 2.7 mg N/100 g and 32.5 ± 2.2 mg N/100 g shrimp meat for AP, VP, MAP1 and MAP2 samples, respectively. As expected, on day 0 lower TVB-N contents in shrimp samples indicated that the shrimp was of good quality, in agreement with the relatively low mesophilic bacterial counts and psychrotrophic bacterial counts of 4.5 ± 0.3 log CFU/g and 3.2 ± 0.2 log CFU/g, respectively. Then its increase is related to the activity of spoilage bacteria and endogenous enzymes and lead to amount of ammonia and primary, secondary and tertiary amines. TVB-N values of MAP2 shrimp samples also increased slowly after 2 days of storage and finally produced lower ($P < 0.05$) values as compared to all the rest. The report of Thepnuan *et al.* (2008) in Pacific white shrimp (*Litopenaeus vannamei*) held at 4°C was similar to our observations. According to the shrimp sanitary standard, the TVB-N values of fresh shrimp should be less than 30 mg N/100 g (GB2741-94). TVB-N for shrimp samples stored under AP, VP, MAP1 and MAP2 exceeded the upper limit values of 30 mg N/100 g

shrimp meat after 5, 8, 10 and 13 days of storage, respectively. In agreement with results reported for raw Chinese shrimp in retail packages containing CO_2 -enriched atmospheres (Lu, 2009).

Although values of pH for VP and MAP1 shrimp samples were no statistically significant differences ($P > 0.05$) between packaging treatments as shown in Fig. 3a-b, it was more effective in controlling the increase of TVB-N value than the treatment of VP. The reason of this phenomenon might be explained by that formatted lactic acid dissolved in shrimp meat in VP storage would result in greater pH change and cause an ostensible rise in TVB-N value.

Changes in TMA-N content were similar to those observed for TVB-N content (Fig. 3b-c). Currently as in the case of TVB-N, 5 mg N/100 g shrimp meat was reported be to the acceptability limit for shrimp (Zeng *et al.*, 2005). In this study, there was no TMA-N detected up to day 1 of controlled freezing-point storage for all samples. TMA-N contents of 0.3 ± 0.1 mg N/100 g and 0.2 ± 0.1 mg N/100 g shrimp meat were found after 1 day of storage in the AP and VP, respectively. However, no TMA-N was found in MAP until day 4 (MAP1, 0.3 ± 0.1 mg N/100 g) and day 5 (MAP2, 0.3 ± 0.1 mg N/100 g) of storage. As described by Sivertsvik (2007), atmosphere

Fig. 4. Effects of AP (■), VP (●), MAP1 (▲) and MAP2 (▼) on Odor scores (a), Shell appearance scores (b), Meat appearance scores (c) and Overall acceptance scores (d) of Pacific white shrimp during controlled freezing-point storage at -0.8°C . Each point is the mean $\pm$ SD of two replicate experiments with three samples analyzed per replicate ($n=2 \times 3$)

with high CO_2 and O_2 content could inhibit the formation of TMA-N, and under controlled freezing-point condition, TMA-N is produced by the decomposition of trimethylamine oxide (TMAO) due to the action of intrinsic enzymes and microbial flora and recognized as the characteristic “shrimp” odor of spoiled shrimp. Throughout the entire storage, the TMA-N levels were maintained below 5 mg N/100g for shrimps in MAP1 and MAP2 until day 9 and day 12, respectively. Especially levels of above 10 mL O_2 /100 mL gas atmosphere have been shown to be inhibitory for the formation of TMA-N. As observed in the present study, combining O_2 with the inhibitory effect of CO_2 on spoilage organisms able to produce TMA-N, thus is a good way to maintain the characteristic seawater-like odor.

Levels of exudates of Pacific white shrimp stored in air, vacuum and modified atmosphere are shown in Fig. 3d. MAP treatment had practical implications on exudates values for the two MA-packaged samples, especially shrimp samples in MAP2 had higher ($P < 0.05$) exudates than those in MAP1 at the same storage time, while samples packed in air had less ($P < 0.05$) exudates than the rest

during the whole storage. Nevertheless as the storage time increased, exudates increased steeply after 4 days of storage for VP and MAP2 samples, reaching final values of 7.6 ± 0.7 g/100 g and 9.0 ± 0.8 g/100g, respectively. As indicated, elevated CO_2 concentrations in the packaging gas can increase exudates formation from MA packaged products even though not always observed (Fernández *et al.*, 2009). CO_2 are a weak acid and increased dissolving capacity under increased concentrations could alter the pH and water holding capacity of product accordingly. The results in our study support findings of increased exudates by increasing CO_2 levels and storage time (Fig. 3d), furthermore, due to the formation of lactic acid in VP samples, decrease in pH can presumably alter shrimp meat cell membrane function to affect nutrient uptake and absorption and increase exudates formation.

Sensory evaluation Fig. 4a-d presents the results of sensory evaluation (odor, shell & meat appearance and overall acceptance) of Pacific white shrimp under AP, VP, MAP1 and MAP2. On each sampling day, the spoilage patterns described by the panelists were similar in all packaging treatment and guaranteed no significant

differences ($P > 0.05$) among the evaluation by analysis of variance, and score 6.0 was considered to be lower limit of sensory acceptance. According to the test panel, sensory scores showed a significant decline ($P < 0.05$) in all the samples with increasing storage period. Until day 4 of storage all shrimp samples received mean odor scores in the range 6.7–8.3 (Fig. 4a). On day 5 of storage first off-odors developed in AP (score 5.7 ± 0.3) samples, which correlated rather well with microbiological data (6.1 ± 0.3 log CFU/g for psychrotrophic bacterial) but VP, MAP1 and MAP2 samples were still of acceptable quality. By day 8 of storage, VP samples became unacceptable (score 5.0 ± 0.3) with a loss of characteristic flavor indicating borderline quality, e.g. old-shrimp-like odor. But all MA packaged samples (MAP1 and MAP2) still received a score of 6.3 ± 0.3 and 7.0 ± 0.3 , respectively. The results indicated that high CO₂ concentration inhibited propagation of spoiled microorganisms, which was in agreement with the conclusions of Lu (2009). Data for shrimp meat appearance (Fig. 4c) generally agreed with that for odor. That was, MA packaged samples were the only ones receiving a score of more than 6.0 on day 9 of storage while AP and VP samples were unacceptable with a sign of leaked liquid, softening and inflexible meat. However, for shell appearance, due to the existence of polyphenoloxidase (PPO) the scores of MA packaged samples (MAP1 and MAP2) decreased to less than score 6.0 on day 10 and 12, respectively while VP samples have higher score of shell appearance compared to MAP1 samples at the same storage time (Fig. 4b). It was maybe attributed to the formation of lactic acid that inactivated the PPO in shrimp samples. Based on overall acceptance data, it was found that the rejection points for fresh Pacific white shrimp under various packaging conditions (AP, VP, MAP1 and MAP2) were ca. 4 days, 7 days, 9 days and 11–12 days, respectively (Fig. 4d).

Conclusion

In this research, the optimum MAP (75%CO₂/10%O₂/15%N₂), obtained from a simplex centroid mixture design and stored under controlled freezing-point storage at -0.8°C , including VP and MAP (40%CO₂/30%O₂/30%N₂) was studied through microbiological, physicochemical and sensory attributes of Pacific white shrimp, in which a detailed sensory evaluation has been conducted. Furthermore in our study, VP and MAP, especially 75%CO₂/10%O₂/15%N₂ atmosphere, markedly inhibited ($P < 0.05$) propagation of Gram-negative bacteria, such as *Pseudomonas* spp. H₂S-producing bacteria (including *Shewanella putrefaciens*), *Aeromonas* spp. and *Enterobacteriaceae*. and decreased ($P < 0.05$) the contents of TVB-N and TMA-N and based on sensory data, the shelf-life of fresh Pacific white shrimp got extended and reached 7 days (under vacuum), 9 days and 11–12 days (under modified atmosphere, 40%CO₂/30%O₂/30%N₂; 75%CO₂/10%O₂/15%N₂, respectively) as compared to 4 days' shelf-life under aerobic storage. However, considering the tolerance of lactic acid bacteria against the action of CO₂ and the existence of PPO, further studies are needed with regard to

preservation of Pacific white shrimp using both anti-browning and antimicrobial substances in combination with the optimum modified atmosphere packaging that will maximize shelf-life, while at the same time maintaining desirable sensorial characteristics. Additionally, survival of amine-forming bacteria and the changes of PPO in Pacific white shrimp under storage, not monitored in the present study, are crucial in view of the increasing consumption of white shrimp.

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