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Microbiological, physical and chemical characteristics of freshwater prawns (*Macrobrachium rosenbergii*) in modified-atmosphere packaging

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Summary This study evaluated the influence of packaging atmosphere (air versus 50% N_2/50% CO_2) on microbiological (mesophiles, psychrotrophs), physical (gas measurement) and chemical (pH, total volatile basic nitrogen [TVB-N], NH_3, H_2S and biogenic amines) parameters in freshwater prawns during storage at 0 ± 1 °C for 240 h. To select the most appropriate packaging, 21 batches of each treatment were analysed. Both the packaging permeability and the combination of gases affected the shelf life, but the modified-atmosphere packaging (MAP) was more efficient than air packaging, increasing the shelf life by 40 h. The parameters of pH and TVB-N showed no statistical difference between the two atmosphere conditions all along the storage period. The biogenic amine agmatine showed potential for use as a quality indicator due to the increased concentration during storage. In further studies, this amine can be applied as an indicator for public health issue.

Keywords Biogenic amines, food quality, *Macrobrachium rosenbergii*, modified-atmosphere packaging, shelf life.

Introduction

Farming of freshwater prawns has increased over the years and is still the main production method for these crustaceans (New *et al.*, 2012). Although widely marketed, prawns are highly perishable because of their high content of low-molecular-weight metabolites, such as free amino acids and nucleotides, resulting from hepatopancreas autolysis (Gokoglu & Yerlikaya, 2008; Nirmal & Benjakul, 2009). Because of the lack of established quality parameters for freshwater prawns, some researchers have used the same parameters as for marine shrimp. Non-nitrogenated substances and free amino acids are suitable substrates for the development of microorganisms, which can form volatile metabolites including ammonia, organic acids, hypoxanthine, acetate and volatile compounds of sulphur, resulting in off-flavours, and also nonvolatile compounds such as biogenic amines (Gram & Dalgaard, 2002; Silva *et al.*, 2011; Monteiro *et al.*, 2012; Carneiro *et al.*, 2013a,b; Santos *et al.*, 2013).

In stored products, biogenic amines (BA) are generated by the action of spoilage-bacteria decarboxylases (Ten Brink *et al.*, 1990; Lázaro *et al.*, 2013; Matejková *et al.*, 2013). Determination of the BA content in food is important because of the potential toxicity and significance for human health (Rodriguez *et al.*, 2014). Some researchers have examined the possibility of using these compounds as food-quality markers in nonfermented foods such as meat and fish during storage (Zhao *et al.*, 2007; Fadhlaoui-Zid *et al.*, 2012; Rodrigues *et al.*, 2013).

Modified-atmosphere packaging (MAP) has the potential to control chemical, enzymatic and microbiological reactions, minimising the major sources of the degradation that occurs during the storage period (Church & Parsons, 1995; Sivertsvik *et al.*, 2002). An appropriate combination of the gases O_2, N_2 and CO_2 in the package can inhibit spoilage microorganisms that grow under aerobic conditions, thus maintaining the sensory quality of the food matrix (Monteiro *et al.*, 2013).

The presence of oxygen in the package reduces the amount of exudates from fish during storage (Davis, 1995). This is important because drip loss could favour the growth of spoilage bacteria. Although some researchers have evaluated different fish-packaging materials (Pacquit *et al.*, 2007; Monteiro *et al.*, 2013),

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little information is available about the best packaging material for preserving freshwater prawns. The present study evaluated the influence of the permeability of multilayer and modified-atmosphere packaging on the shelf life of freshwater prawns (*Macrobrachium rosenbergii*) stored under controlled temperature (0 ± 1 °C), based on microbiological, physical and chemical parameters.

Materials and methods

Prawn sampling

Fifteen kilograms of freshwater prawns (*Macrobrachium rosenbergii*) was obtained during the autumn from Santa Helena Aquaculture, located in Silva Jardim, Rio de Janeiro, Brazil. Immediately after the harvest, the cephalothorax and exoskeleton were removed and the prawns were placed in isothermal expanded-polystyrene boxes containing ice, to maintain the temperature at 0 ± 1 °C (Fig. 1). The samples were immediately transferred to the laboratory for the analytical procedures. All the analyses were run in duplicate.

Quality of the raw prawns

The first stage consisted of an initial quality evaluation of bacteriological, physical and chemical parameters. Mesophilic, psychrotrophic, coagulase-positive *Staphylococcus*, *Salmonella* spp. and total and thermotolerant coliform bacteria were detected and quantified using methodology described by the American Public Health Association (APHA, 2001). Physical and chemical parameters including pH, TVB-N, ammonia and hydrogen sulphide were measured using methodology described by the Association of Official Analytical Chemists (AOAC, 2005). The contents of biogenic amines were determined according to Lázaro *et al.* (2013).

Packaging and gas composition measurements

Forty-two batches containing 10 prawns each were prepared. Twenty-one batches were placed on expanded-polystyrene trays and wrapped with polyvinyl chloride (PVC) film, 0.01–0.02 mm thick, and permeable to oxygen and carbon dioxide (T1). Another 21 samples were placed in Cryovac® BBL4 multilayer-barrier packaging (T2) and sealed with a Tecmaq® model AP 450, and the packages were filled with 100 g of prawns in 1L of 50% CO_2/50% N_2 (PBI Dansensor MAP MIX 9001 ME). The batches were prepared in one bag of 20 × 22 cm Cryovac® BB4L, with diffusion coefficients, according to the supplier, of 150 cm^3/m^2.24 h.bar to CO_2, 35 cm^3 m^{-2}.24 h.bar to O_2 and 1.4 cm^3 m^{-2}.24 h.bar to N_2 at 22 °C. The CO_2 and N_2 levels in the headspace of the packaged samples used for treatment 2 (MAP) were analysed with a Checkpoint® gas analyzer (PBI Dansensor A/S, Ringsted, Denmark).

Microbial and chemical analyses

To evaluate the shelf life of the prawns, all the samples were stored at (0 ± 1 °C) for 240 h. The time points were determined in previous studies where the bacterial growth behaviour was recognise in freshwater prawn to determine the most appropriate time to analyse the matrix. The microbiological assay was monitored by counting the mesophilic and psychrotrophic bacteria by pour plate method and stored at 35 °C during 48 h (APHA, 2001). In addition, pH was analysed using potentiometric method, total volatile basic nitrogen (TVB-N) according to Conway procedure (Siang & Kim, 1992), NH_3 using Nessler's colorimetric method and H_2S were determined by AOAC (2005) methods.

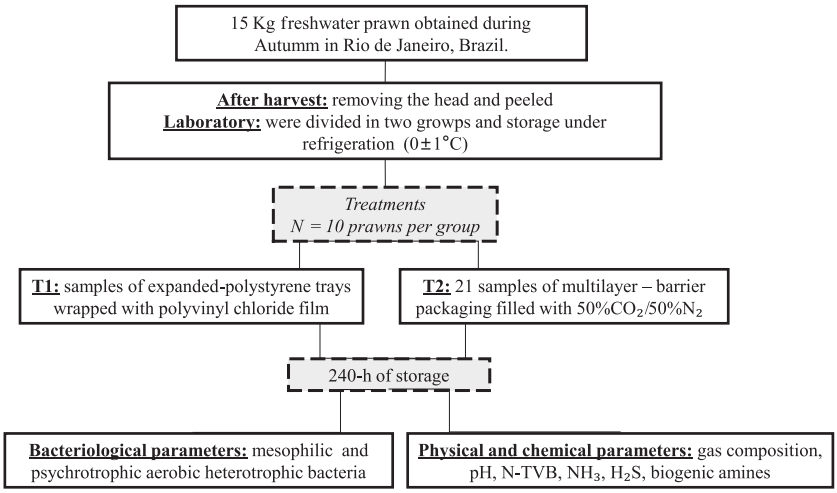

Figure 1 Experimental design.

Biogenic amines (tyramine, agmatine, cadaverine, tryptamine, histamine and espermine) were determined in triplicate by high-performance liquid chromatography (HPLC) Briefly, 5 mL of perchloric acid (5%) was added to 5 g of sample and stored under refrigeration (4 ± 2 °C) using periodic stirring for 1 h. In the next step, the solution was filtered (Whatman No. 1 filters), followed by the addition of 2 N sodium hydroxide that raised the pH to 6 or more. Subsequently, the solution was kept in an ice bath for 20 min and filtered (Whatman No. 1 filters) a second time. Next, 2 N sodium hydroxide was added until the pH raised to 12 or more. After this process, the solution was derivatised with 20 μL benzoyl chloride (99%), homogenised and left to stand at room temperature for 20 min. Then, 1 mL of diethyl ether (99%) was added, two phases in different polarity were formed, and then the supernatant was removed. The resulting solution was evaporated in a nitrogen stream using the sample concentration (Techne DB-3) and finally resuspended with the mobile phase (acetonitrile:H_2O; 42:58; v:v), as described by Lázaro *et al*. (2013).

Statistical analysis

Two-way ANOVA was performed to evaluate the differences obtained by comparison between the (T1) and (T2) prawn samples with respect to pH, TVB-N and biogenic amines (tiramine, agmatine, cadaverine, tryptamine, histamine and espermine) over the 240-h storage period. When a significant F was found, additional post hoc tests with Bonferroni's adjustment were performed. For data interpretation, analyses (from 0 to 240 h) were divided into three periods: Period 1 (P1) – analysis of the first 80 h of storage; Period 2 (P2) – analysis from 81 to 160 h; Period 3 (P3) – analysis from 161 to 240 h of storage. Statistical significance was set at the 0.05 level of confidence. All analyses were performed using a commercially available statistical package (GraphPad Prism version 6.00 for Windows, GraphPad Software, San Diego, CA, USA).

The bacterial growth curves were adjusted using the DMFit 2.0 (IFR, Norwich, UK) statistical program based on predictive microbiology and developed for an ideal data set by Baranyi & Roberts (1994).

Results and discussion

Quality of the raw prawns

Coagulase-positive *Staphylococcus* and *Salmonella* spp. were not present in the 25-g control sample, as specified by APHA (2001) for fish and fish products, although not specifically mentioned for freshwater prawns. The counts of mesophilic and psychrotrophic aerobic heterotrophic bacteria were 4 and 2 log CFU g^{-1}, respectively. These were below the levels established by the National Advisory Committee on Microbiological Criteria for Foods (NACMCF, 1990) and the International Commission on Microbiological Specification for Foods (ICMSF, 1988), which suggested a limit of 7 log CFU g^{-1}. In this case, the total coliform (TC) count was 110 MPN g^{-1}, and the thermotolerant coliform (TTC) count was 2.3 MPN g^{-1}. These results confirm that good manufacturing ractices (GMP) and asepsis were maintained during sample collection and handling.

Regarding chemical parameters in the raw material, the pH was 6.32 ± 0.02 and TVB-N 17.01 ± 0.44 mg 100 g^{-1}. Similar results were found by Chytiri *et al*. (2004) in ungutted rainbow trout, which could be compared with the prawns in this study, considering that both are freshwater animals and have similar characteristics. Tests for ammonia and hydrogen sulphide were negative, confirming that asepsis and the cold chain were maintained from harvest to handling in the laboratory.

Headspace gas composition in MAP samples

The concentrations of N_2 and CO_2 in the packages were measured during storage at each analysis time (Fig. 2). In the MAP-packaged samples, an initial decrease from 28.80 to 22.77% of the headspace CO_2 level and an increase from 62.51 to 66.10% of the N_2 level were observed in the first 40 h of storage, possibly due to dissolution of CO_2 in the water phase of the prawns (Devlieghere & Debevere, 2000; Sivertsvik *et al*., 2002). The concentrations of N_2 and CO_2 varied slightly until a significant decrease of CO_2 to 6.03% and an increase of N_2 to 70.61% occurred during further storage.

Shelf life

Bacteriological parameters

During storage, the mesophilic bacteria showed doubling times (generation times) of 4.43 and 4.60 h in T1 and T2, respectively. After 240 h of storage, the final counts for this bacterial group were 11.83 log CFU g^{-1} for the T1 group and 14.17 log CFU g^{-1} for the T2 group. For the psychrotrophic group, the initial count was 2.72 log CFU g^{-1} and the doubling times were 3.83 and 4.01 h for T1 and T2, respectively. At the end of the storage period, this bacterial group showed levels of 11.28 log CFU g^{-1} for T1 and 13.42 log CFU g^{-1} for T2.

Figure 3 shows the growth curves of mesophilic (A) and psychrotrophic (B) aerobic heterotrophic bacteria. The initial mesophilic bacteria count was higher than the psychrotrophic bacteria count for both treatments. However, after 40 h of storage, the numbers of

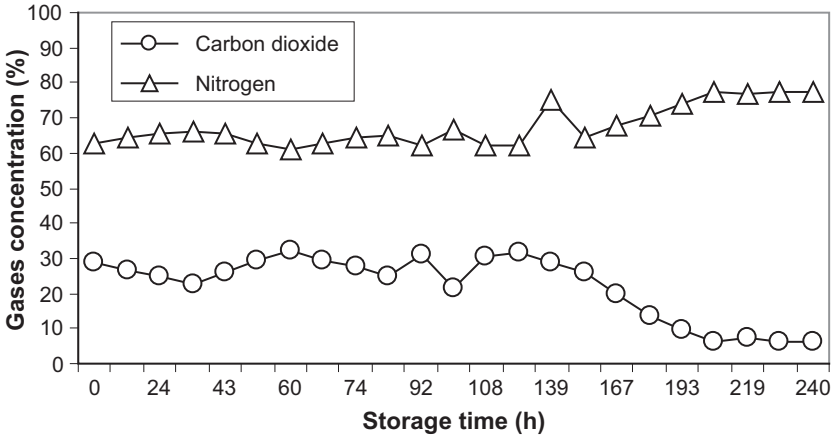

Figure 2 Concentration (%) of carbon dioxide (CO_2) and nitrogen (N_2) during 240-h storage under refrigeration (0 ± 1 °C) in samples of *Macrobrachium rosenbergii* stored in modified atmosphere packaging (50% CO_2/ 50% N_2).

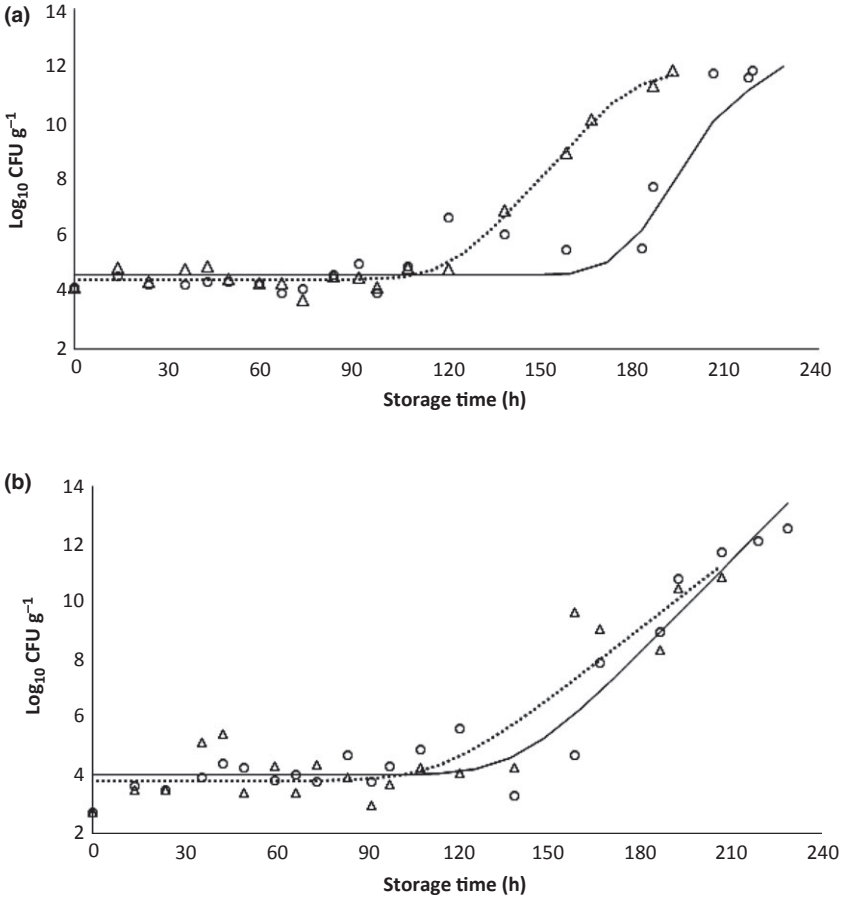

Figure 3 Linear regression of mesophilic (a) and psychrotrophic (b) aerobic heterotrophic bacteria growth curve showing bacterial behavior in T1 real values (Δ); T1 adjusted values by DMFit 2.0 (---); T2 real values (O) and T2 adjusted values by DMFit 2.0 (—).

psychrotrophic and mesophilic bacteria were similar, suggesting that the microbiota adapted to the refrigeration conditions of the matrix, as mentioned by Begum *et al*. (2012).

For determining prawn shelf life, the international guidelines established by the NACMCF (1990) and the ICMSF (1988) were adopted. These guidelines suggest a maximum value of 7 log CFU g^{-1} for mesophilic and psychrotrophic aerobic heterotrophic bacteria counts, as a microbiological criterion to ensure the quality level of freshwater prawns.

The analyses of bacterial growth suggested that the treatments affected the shelf life differently for both the mesophilic and psychrotrophic groups. The mesophilic

T1 group reached the threshold of 7 log CFU g^{-1} after around 140 h of storage, while the T2 group reached the same value after around 180 h. For psychrotrophic bacteria, the T1 group reached the threshold at around 155 h and the T2 group around 175 h. Although the mesophilic group was more influenced by MAP (which increased the lag phase), these results indicate that the combination of gases used here extended the shelf life of prawns.

Paranjpye *et al.* (2008), studying the marine shrimp *Pandalus jordani* packaged in oxygen-permeable and impermeable material, found no difference in the total bacterial count between these treatments. Begum *et al.* (2012) found a shelf life of 12 days for *M. rosenbergii* stored on ice. However, in their study, the total bacteria count stabilised at 10^7 CFU g^{-1} between the 6th and the 8th day, which suggests that the microorganisms had reached a stationary phase and that the shelf life was similar to that found in the present study.

The growth parameters (lag phase, log phase and number of bacterial colonies in the stationary phase) are shown in Table 1 for mesophilic and psychrotrophic bacteria in both treatments. The delay phase (lag phase) in the mesophilic and psychrotrophic counts was shorter in the T1 than in the T2 group, showing that the combination of gases effectively increased the lag phase, helping to extend the shelf life. Different factors can influence the lag phase, including the type of atmosphere used in the present study, suggesting that the more selective the atmosphere, the longer the lag phase (Monteiro *et al.*, 2013).

At the beginning, the bacteriological counts of all samples were similar; however, during the storage period, some changes in the microbiota were observed, which varied according to the packaging and the atmosphere used. It is well known that aerobic psychrotrophic genera such as *Pseudomonas* spp., *Alteromonas* sp. and *Aeromonas* sp. grow better in an O_2-rich atmosphere (Gram & Huss, 1996; Boulares *et al.*, 2012).

For both mesophilic and psychrotrophic bacteria, the doubling times were slightly higher in the T2 group, suggesting that MAP contributes to increase the log phase, another factor that contributes to extending the shelf life (Sivertsvik *et al.*, 2002). The cell number during the stationary phase was higher in the T2 group for both treatments, due to the oxygen and carbon dioxide (impermeable packaging), which can extend shelf life (Masniyom *et al.*, 2013).

Table 1 Growth parameters of mesophilic and psychrotrophic bacteria in samples of *Macrobrachium rosenbergii* stored in air and modified-atmosphere packaging (50% CO_2/50% N_2), kept at 0 ± 1 °C

Treatment	Parameter	Mesophilic	Psychrotrophic
T1	Lag	120.92	115.93
	Log	4.43	3.83
	NC	11.83	11.28
T2	Lag	175.67	140.66
	Log	4.60	4.01
	NC	14.17	13.42

Lag – Lag phase (hours).
Log – Log phase log (hours).
NC – Number of colonies in the stationary phase (log CFU g^{-1}).

Physical and chemical parameters

Regarding physical and chemical parameters, the pH did not increase significantly in T2 due to the impossibility of gas exchange with the environment, which causes carbon dioxide to accumulate and eventually dissolve in the water contained in the food, producing carbonic acid. Another possible explanation for the lack of an increase in pH is the production of lactic acid due to acidifying bacteria metabolism (Lerasle *et al.*, 2014).

As shown in Table 2, the pH did not increase during the entire storage period. Although pH is an indicative parameter of loss quality, in this study, it cannot be a conclusive parameter. The maximum value obtained during the storage period was 6.43 for T1 and 6.34 for T2 when the spoilage was evaluated by bacterial and some physical and chemical parameters. The pH obtained during P1 was not significantly different ($P = 0.98$) from the pH at the end of the storage period (P3), in both packaging types (T1 and T2). This reinforces the importance of using combined parameters to determine the quality of freshwater prawns.

Regarding TVB-N, the value obtained in the beginning was 16.70 mg N/100 g. Table 2 shows the fluctuation of TVB-N during 240 h of storage in air and modified-atmosphere packaging. For T1, there was no significant difference ($P < 0.01$) among periods, but in T2, the difference became apparent after 160 h of storage (P3). Monteiro *et al.* (2013) and Rodrigues *et al.* (2013) obtained low TVB-N values for freshwater fish compared with those for marine fish (Santos *et al.*, 2013) because of the high concentrations of TMAO found in marine fish (Tejada *et al.*, 2007).

The results for NH_3 production began to show some differences from 74 h of storage, when a positive test was obtained for T1. In this period, autolytic and microbial enzymes may have begun to affect the prawns, causing amino acid deamination and consequent NH_3 production (Pivarnik *et al.*, 2011). For T2, the modified atmosphere influenced the matrix: a positive result for NH3 was obtained only at 98 h.

Hydrogen sulphide (H_2S) was detected in the T1 and T2 groups after 92 and 121 h, respectively. Degradation of sulphur compounds occurs mainly from the action of mesophilic bacteria (Gram & Huss, 1996; Boulares *et al.*, 2012), and the presence of H_2S becomes more evident when bacteria grow and act on the sulphur-containing amino acids in the prawns.

Table 2 Range of pH, N-TVB (mgN 100 g^{-1}) and biogenic amines (mg kg^{-1}) of *Macrobrachium rosenbergii* in different periods of storage at a temperature of 0 ± 1 °C, in air and modified-atmosphere packaging (50%CO_2/50%N_2)

	T1			T2		
Parameters	**P1**	**P2**	**P3**	**P1**	**P2**	**P3**
pH	6.25 ± 0.10^{Aa}	6.26 ± 0.10^{Aa}	6.18 ± 0.10^{Aa}	6.20 ± 0.14^{Aa}	6.23 ± 0.04^{Aa}	6.12 ± 0.05^{Aa}
N-TVB	16.25 ± 1.10^{Aa}	15.86 ± 1.88^{Aa}	14.23 ± 2.33^{Aa}	14.75 ± 1.34^{Aa}	15.97 ± 1.45^{Aa}	18.15 ± 0.98^{Bb}
Tiramine	0.0030 ± 0.001^{Aa}	0.0031 ± 0.001^{Aa}	0.0027 ± 0.001^{Aa}	0.0027 ± 0.001^{Aa}	0.0030 ± 0.001^{Aa}	0.0043 ± 0.002^{Bb}
Agmatine	9.371 ± 0.71^{Aa}	12.472 ± 1.55^{Ab}	16.748 ± 0.89^{Ac}	14.436 ± 1.51^{Ba}	17.112 ± 2.55^{Bab}	19.641 ± 0.60^{Bb}
Cadaverine	0.029 ± 0.003^{Aa}	0.047 ± 0.006^{Ab}	0.048 ± 0.007^{Ab}	0.021 ± 0.003^{Aa}	0.033 ± 0.004^{Bb}	0.045 ± 0.008^{Ac}
Tryptamine	0.004 ± 0.001^{Aa}	0.005 ± 0.001^{Aa}	0.005 ± 0.001^{Aa}	0.004 ± 0.001^{Aa}	0.005 ± 0.001^{Aa}	0.005 ± 0.001^{Aa}
Histamine	0.005 ± 0.001^{Aa}	0.029 ± 0.001^{Ab}	0.059 ± 0.040^{Ac}	0.037 ± 0.004^{Ba}	0.028 ± 0.023^{Ab}	0.047 ± 0.001^{Bc}

^{A,B,C}Different upper-case letters in a row indicate significant differences ($P < 0.01$) among treatments (T1 and T2 groups) in the same storage period (ANOVA).

^{a,b,c}Different lower-case letters in a row indicate significant differences ($P < 0.01$) among storage periods (P1, P2 and P3) in the same treatment (ANOVA).

T1 refers to treatment in permeable packaging; T2 refers to treatment in modified-atmosphere packaging (50%CO_2/50%N_2).

P1 refers to 0–80 h of storage; P2 refers to 81–160 h of storage; P3 refers to 161–240 h of storage at 0 ± 1 °C.

During the storage period, the main biogenic amines (BA) for the prawns were identified and quantified by HPLC in both treatments (Table 2). In T1, after the end of 240 h storage, the concentrations of agmatine, tryptamine and histamine were 16.770 mg kg^{-1}, 0.005 mg kg^{-1} and 0.059 mg kg^{-1}, respectively. For the modified-atmosphere packaging (T2), the levels observed at the end of storage (240 h) were 19.642 mg kg^{-1}, 0.005 mg kg^{-1} and 0.047 mg kg^{-1} for agmatine, tryptamine and histamine, respectively. The concentration of agmatine was significantly higher than those of histamine and tryptamine because these amines originate, respectively, from the amino acids arginine, histidine and tryptophan, which are present in shrimp muscle, in decreasing order of concentration (Oujifard *et al*., 2012).

The T1 group showed no significant difference in agmatine concentrations during the first 160 h of storage (P1 and P2). However, in the final period (P3), the concentration of this amine increased significantly ($P < 0.001$). The samples from the T2 group also showed increased agmatine concentrations during storage, although the increase was significant ($P < 0.001$) only during the first 160 h (P1 and P2), and the level then remained steady until the end of storage (P3). The concentration of agmatine was significantly higher ($P < 0.001$) in the modified-atmosphere packaging (T2) than in T1 during the first 160 h of storage (P1 and P2). However, at the end of the storage period (P3), the concentrations of this amine showed no statistical difference between the types of packaging.

In view of the gradual increase of agmatine during the storage period, depending on the type of packaging, this amine could be considered a good-quality indicator (Galgano *et al*., 2009). The type of packaging affected the agmatine concentration mainly in the first 100 h of storage (P1 and P2). This initial increase may have resulted from the rapid log-phase growth of psychrotrophic bacteria, which then remained in the stationary phase until the end of storage.

Although little information is available about the physiological effects of the other amines, some of them can increase the potential biological effects of other amines. Putrescine and cadaverine increase histamine activity in the allergic process (Rodriguez *et al*., 2014). Some biogenic amines have been used as quality indicators for freshwater fish like tilapia (*Oreochromis niloticus*) for Cunha *et al*. (2013) and rainbow trout (*Oncorhynchus mykiss*) for Rodrigues *et al*. (2013). The present results showed that agmatine is a potential quality indicator for freshwater prawns.

The tryptamine concentration increased significantly ($P < 0.001$) in the first 80 h of storage and then increased only slightly in the final storage periods (P2 and P3), showing no significant differences among the periods. The increase in tryptamine concentration during 240 h of storage indicates that this amine could be used as a quality index for freshwater prawns, under similar conditions as this study. However, during the storage period, there was no significant difference ($P > 0.05$) between treatments (T1 and T2), showing that the type of packaging did not affect the production of this amine.

Conclusion

In this study, the shelf life of freshwater prawns stored under refrigeration (0 ± 1 °C) was 140 h for samples in permeable packaging and 180 h for samples in modified-atmosphere packaging, suggesting that the combination of gases used increased the storage period. The mesophilic aerobic heterotrophic bacteria count is the

main parameter for determining loss of quality. Biogenic amines, especially agmatine, show good potential as quality parameters, although further studies should be conducted to relate the predominant microorganisms to particular amines and to the risks for human health. These results encourage further studies, because extended shelf life and low-environmental-impact farming methods may increase the demand for prawns in both the domestic and international markets.

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