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Influence of high CO_2 modified atmosphere packaging on some quality characteristics of fresh farmed pufferfish (*Takifugu obscurus*) during refrigerated storage

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Abstract: The effect of modified atmosphere packaging (MAP) in combination with cold storage on physicochemical and microbiological changes of farmed pufferfish was studied during 14 days of storage. Results showed that samples with MAP3 (gas composition of 60% CO_2, 5% O_2, 35% N_2) treatment had the significantly lower total volatile basic nitrogen, *K* value, and total viable counts. The water holding capacity, low field NMR analysis and MRI indicated that MAP3 was more effective in maintaining the water content and water migration. Analyses of free amino acids (FAAs) also indicated that MAP3 treatment had a positive effect on preventing the degradation of proteins and retained better flavour quality.

Keywords: farmed pufferfish; high CO_2 MAP systems; shelf life; quality

Pufferfish (*Takifugu obscurus*) is famous for its extraordinarily palatable flavour, especially for its umami taste. Fresh pufferfish is extremely perishable because of its high protein and water activity. Controlling the growth of spoilage microorganisms is the key to extend the shelf life of pufferfish during storage. Cold storage and modified atmosphere packaging (MAP) are two common effective approaches to preserve fresh pufferfish. Carbon dioxide (CO_2) plays the most important role in MAP since it has bacteriostatic and fungistatic properties (Janjarassku & Suppakul 2018). Previous studies have shown that high CO_2 levels extend the shelf life of fish products due to the inhibition of microbial growth (Zhu et al. 2016; Sun et al. 2017). However, a high level of CO_2 leads to some negative sensory characteristics such as reduced texture quality, drip loss and colour change (Bono et al. 2012). Hence, it is vital to determine the amount of dissolved CO_2 in the MAP.

The objective of this research was to evaluate the potential applications of cold storage at 4 °C with different high-CO_2 MAP systems by monitoring the biochemical changes, and evaluating the organoleptic properties during storage.

MATERIAL AND METHODS

Preparation and treatment of pufferfish. A total of 96 live farmed pufferfishes with an average weight

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of 300 ± 10 g were purchased from Zhongyang Company (China). They were killed by trained professionals and randomly divided into six batches for air (AP), vacuum (VP), and MAP (MAP1: 40% CO_2, 5% O_2, 55% N_2; MAP2: 40% CO_2, 40% O_2, 20% N_2; MAP3: 60% CO_2, 5% O_2, 35% N_2; MAP4: 60% CO_2, 40% O_2) packages.

Then the packaged fish were kept at 4 ± 0.1 °C and the quality evaluation was performed at 2-day intervals until they were considered spoiled based on sensory evaluation.

Determination of water holding capacity (WHC). WHC was determined according to Wang et al. (2018) and the percentage of water retained by the sample after centrifugation was expressed as WHC.

Total volatile basic nitrogen (TVB-N). For TVB-N determinations, the distillation method was used and expressed as mg N (100 g)$^{-1}$ (Zhou et al. 2019).

Cooking loss (CL). CL was measured according to Li et al. (2018a) and the pufferfish were immersed in a water bath at 85 °C for 15 min. The weight of pufferfish was recorded before heating and after heating.

Electrical conductivity (EC) and pH value. 2.5 g of each sample were homogenized with 22.5 mL distilled water and centrifuged at 7 500 rpm for 10 min at 4 °C. EC and pH values of the supernatant were measured with an EC meter and a pH meter, respectively.

Texture profile analysis (TPA). TPA was conducted using the methods of Piedrahíta et al. (2019). The parameters of firmness, springiness, chewiness, and resilience were acquired using constant test speed of 1 m s^{-1} and sample deformation of 40%.

Evaluation of thiobarbituric acid (TBA) value. Lipid oxidation in pufferfish was monitored by the evaluation of thiobarbituric acid reactive substances (TBARS) according to the procedure of Milijasevic et al. (2018).

***K* value.** The ATP-related compounds were determined by a RP-HPLC procedure (Waters; USA). The *K* value was calculated as the ratio of the percentage amounts of inosine (HxR) and hypoxanthine (Hx) to the sum of adenosine-5'-triphosphate (ATP), adenosine-5'-diphosphate (ADP), adenosine-5'-monophosphate (AMP), inosine-5'-monophosphate (IMP), HxR and Hx were calculated as follows:

$$K \text{ value} = \frac{\text{HxR} + \text{Hx}}{\text{ATP} + \text{ADP} + \text{AMP} + \text{IMP} + \text{HxR} + \text{Hx}} \times 100 \ [\%]$$

where: HxR – inosine; Hx – hypoxanthine; ATP – adenosine-5'-triphosphate; ADP – adenosine-5'-diphosphate; AMP – adenosine-5'-monophosphate; IMP – inosine-5'-monophosphate.

Low field nuclear magnetic resonance analysis (LF-NMR). The proton relaxation experiments were performed as proposed by Li et al. (2018b).

Free amino acids (FAAs) analysis. FAAs were determined by the method of Tavakoli et al. (2018) using a Hitachi L-8800 amino acid analyser (Hitachi, Japan).

Microbiological analysis. Representative 10-g portions of pufferfish were blended with 90 mL of sterilized Ringer's solution (1/4 strength) and subjected to serial dilutions. Then the total viable count, *Pseudomonas* spp., and H_2S-producing bacteria were determined on nutrient agar medium, cetrimide agar and iron agar, respectively (Li et al. 2019).

Statistical analysis. The one-way ANOVA procedure followed by Duncan's test was used for multiple comparisons by the SPSS 22.0 software, and the results were expressed as means ± SD.

RESULTS AND DISCUSSION

Microbiological results. The initial mesophilic population was 2.84 log CFU g^{-1} and the microbial count increased during storage (Figure 1A). AP, VP, MAP2, MAP4 and MAP1 pufferfish had to be removed on day 8, 10, 10, 12 and 12, respectively, due to reaching the "shelf-life" limit of 7.0 log CFU g^{-1} for marine fish (Chen et al. 2019). The mesophile number of MAP3 samples was significantly lower than in other samples because of the remarkable inhibitory effect by 60% CO_2, 5% O_2, 35% N_2. The population of H_2S-producing bacteria and *Pseudomonas* spp. increased during storage in all the samples (Figure 1B and 1C). The initial count of H_2S-producing bacteria in pufferfish was approximately 2.41 log CFU g^{-1} and MAP3 sample was significantly lower ($P < 0.05$) than others at any of the storage time points (Figure 1B). *Pseudomonas* spp. growth was supported by storage in air and effectively inhibited by 60% CO_2 MAP (Kuuliala et al. 2018).

Changes in pH. There was a slight decrease in pH at the beginning for all samples (Figure 1D), which was due to the decomposition of glycogen, ATP and creatine phosphate (Wang et al. 2017). Then the pH of MAP treated samples was approximately 6.7–6.9 on day 14, which was a lower value than that of AP and VP samples. During storage, MAP3 showed lower average values and smaller pH changes, therefore it can be concluded that 60% CO_2, 5% O_2, 35% N_2 could have positive effects on the inhibition of bacterial spoilage.

EC. The EC value was determined as 604 μS cm^{-1} for fresh pufferfish and it increased in all samples

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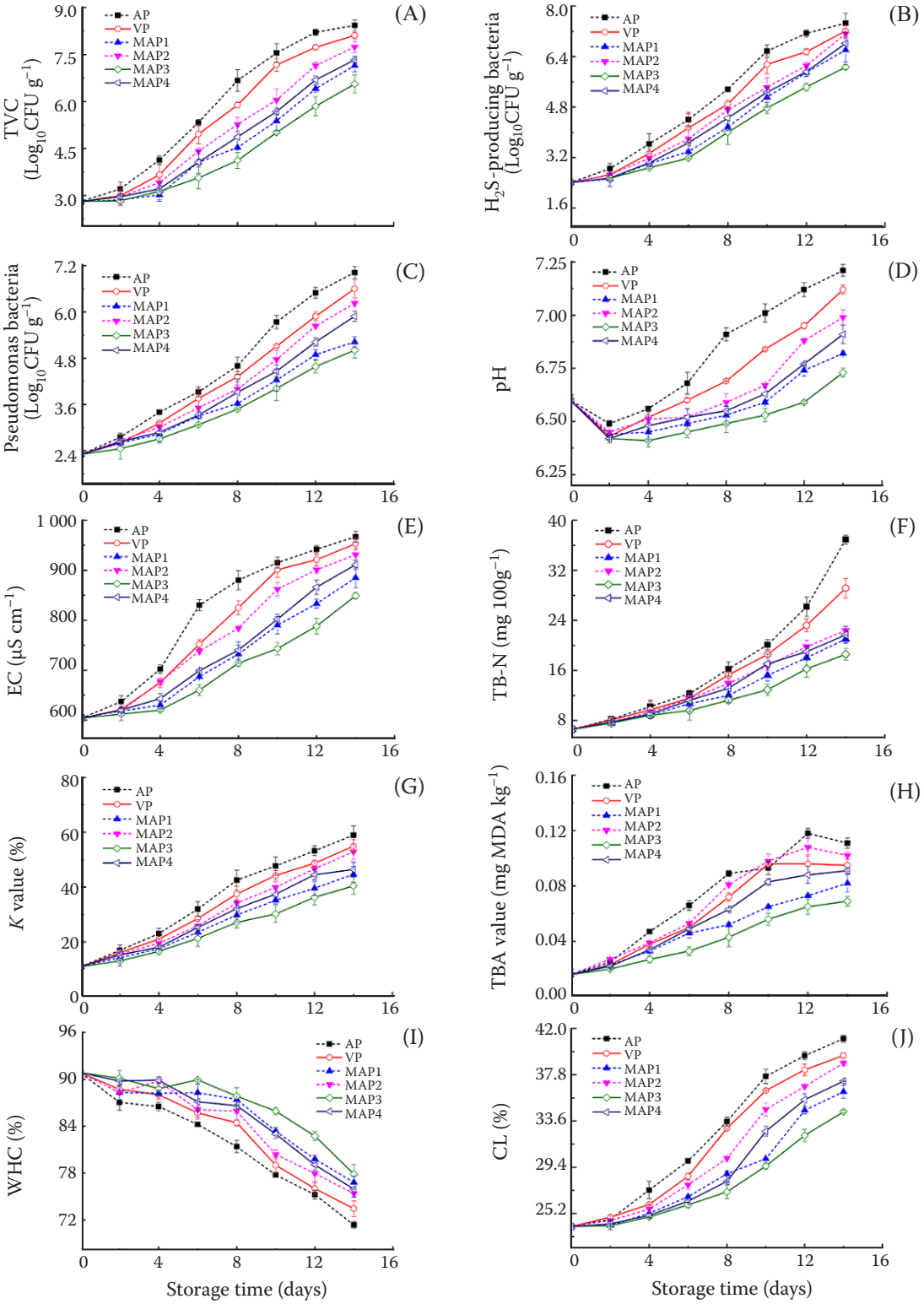

Figure 1. Changes in (A) total viable counts (TVC), (B) H_2S-producing bacteria, (C) *Pseudomonas* spp., (D) pH, (E) electrical conductivity (EC), (F) total volatile basic nitrogen (TVB-N), (G) *K* value, (H) thiobarbituric acid (TBA) value, (I) water holding capacity (WHC), (J) cooking loss (CL) of pufferfish samples under different MAP treatments during storage

AP – air packaging; VP – vacuum packaging; MAP – modified atmosphere packaging: MAP1 – 40% CO_2, 5% O_2, 55% N_2; MAP2 – 40% CO_2, 40% O_2, 20% N_2; MAP3 – 60% CO_2, 5% O_2, 35% N_2; MAP4 – 60% CO_2, 40% O_2

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during storage (Figure 1E). After 14 days of storage, the EC values of samples for AP and VP were 967 and 953 μS cm^{-1}. Corresponding reductions of 281, 327, 245, and 307 μS cm^{-1} were observed in comparison with AP for MAP1, MAP2, MAP3 and MAP4, respectively. The increase of EC value was mainly attributed to the ionic compounds caused by bacterial metabolism and biochemical reactions.

Changes in TVB-N. The TVB-N value of fresh pufferfish was only 6.59 mg N (100 g)$^{-1}$ (Figure 1F) and showed a progressive increase during storage. Samples stored in AP exhibited a higher increase reaching the value of 36.20 mg N (100 g)$^{-1}$ on day 14 that exceeded the maximum permissible level [35 mg N (100 g)$^{-1}$] in marine fish (Nasirifar et al. 2018). The use of MAP exerted an inhibitory effect on TVB-N of pufferfish ($P < 0.05$). On day 14, TVB-N values of 29.13, 22.39, 21.68, 21.01, and 18.57 mg N (100 g)$^{-1}$ were observed for VP, MAP2, MAP4, MAP1 and MAP3 pufferfish, respectively. The rate of TVB-N accumulation was 2.16 mg N (100 g)$^{-1}$ per day for AP samples compared to only 1.61, 1.20, 1.08, 1.03, and 0.86 mg N (100 g)$^{-1}$ per day for VP, MAP2, MAP4, MAP1 and MAP3 pufferfish, respectively.

***K* value.** The *K* value was 11.02% in fresh pufferfish (Figure 1G) and it increased in all samples during storage, however, MAP significantly delayed the *K* value increase, which had an analogous trend to TVB-N. After day 2, a difference in the *K* value between MAP and AP pufferfish was significant ($P < 0.05$). The final *K* values of AP, VP, MAP1, MAP2, MAP3 and MAP4 were 58.95, 54.91, 44.66, 52.86, 40.44 and 46.42%, respectively, which remained below this limit of acceptability. *K* values in MAP3 and MAP1 changed more slowly than in other samples ($P < 0.05$), which was attributed to the higher CO_2 concentration.

TBA. The initial TBA value of pufferfish was 0.016 mg MDA kg^{-1} (Figure 1H) and it showed upward trends during the first 12 days and then it decreased slightly at the end, which was mostly due to the reaction of malondialdehyde with aldehydes and ketones (da Silva et al. 2016). The highest TBA values of AP and VP were 0.118 and 0.096 mg MDA kg^{-1}, respectively, while the highest TBA values of MAP1, MAP2, MAP3 and MAP4 were 0.082, 0.102, 0.069, 0.630 and 0.091 mg MDA kg^{-1}, respectively. Compared with AP, TBA values in MAP3 and MAP1 pufferfish were relatively low ($P < 0.05$).

WHC and CL. WHC dropped from the initial value of 90.85% to about 73.45% for VP and 75.39–77.87% for MAP on day 14 (Figure 1I). The WHC decreased, reflecting a decrease in the water-protein interactions during cold storage. However, the influence of oxygen concentration in MAP on WHC is still unclear (Wang et al. 2019). MAP significantly inhibited CL of pufferfish during cold storage (Figure 1J). As a comparison with AP and VP samples in which the cooking loss increased from 24.02% on day 0 to almost 40% on day 14, the cooking loss of MAP samples remained below 40% throughout the storage. The lower cooking loss in MAP might be caused by the water holding capacity.

TPA. Pufferfish texture properties of all samples decreased during 14 days of storage (Figure 2), which indicates that the pufferfish lost its good quality and freshness. The average value of fresh pufferfish hardness was 2.8×10^4 g and a general decrease could be noticed in all samples during storage (1.37, 1.44, 1.72, 1.50, 1.88, and 1.63×10^4 g for AP, VP, MAP1, MAP2, MAP3 and MAP4 on day 14, respectively), in agreement with the findings of Feng et al. (2017) and Yadav et al. (2018). Springiness and chewiness showed similar behaviour to that of hardness values since a decrease in these two parameters was observed as the storage time increased, which indicated that pufferfish samples became softer during storage. AP showed significantly higher ($P < 0.05$) springiness and chewiness values compared to the other packages. The average value of fresh pufferfish springiness was 0.76, and it decreased significantly ($P < 0.05$) to 0.47, 0.49, 0.53, 0.50, 0.55, and 0.52 for AP, VP, MAP1, MAP2, MAP3 and MAP4, respectively, at the end of storage. The resilience of pufferfish showed a decreasing trend during the whole storage period, and the AP samples had a faster rate of decline.

Water distribution by LF NMR analysis. T_{21} representing the bound water ranged from 0.79% to 1.01% during storage (Figure 3), indicating that T_{21} could not be affected by treatment methods or storage time. T_{22} is considered as immobile water within the myofibril and *P* (T_{22}) diminished progressively during storage ($P < 0.05$). T_{23} represents free water and *P* (T_{23}) increased constantly, which indicated that the changes of free water were more obvious than those of bound and immobile water during storage.

In the present study, AP resulted in significantly lower immobilized water (from the initial value of 87.63% on day 0 to 69.29% on day 14) than the other types of packaging of other samples. MAP3 had the largest amounts of immobilized water on day 8 and day 14, probably owing to the conclusion that higher CO_2 concentration and lower O_2 concentration (gas composition of 60% CO_2, 5% O_2, 35% N_2) decreased the rate of diffusion of immobilized water into free water. Besides, this process of water migration was also well reflected in the following phenomenon that MAP treatments retarded the change rates of T_{22} and T_{23}.

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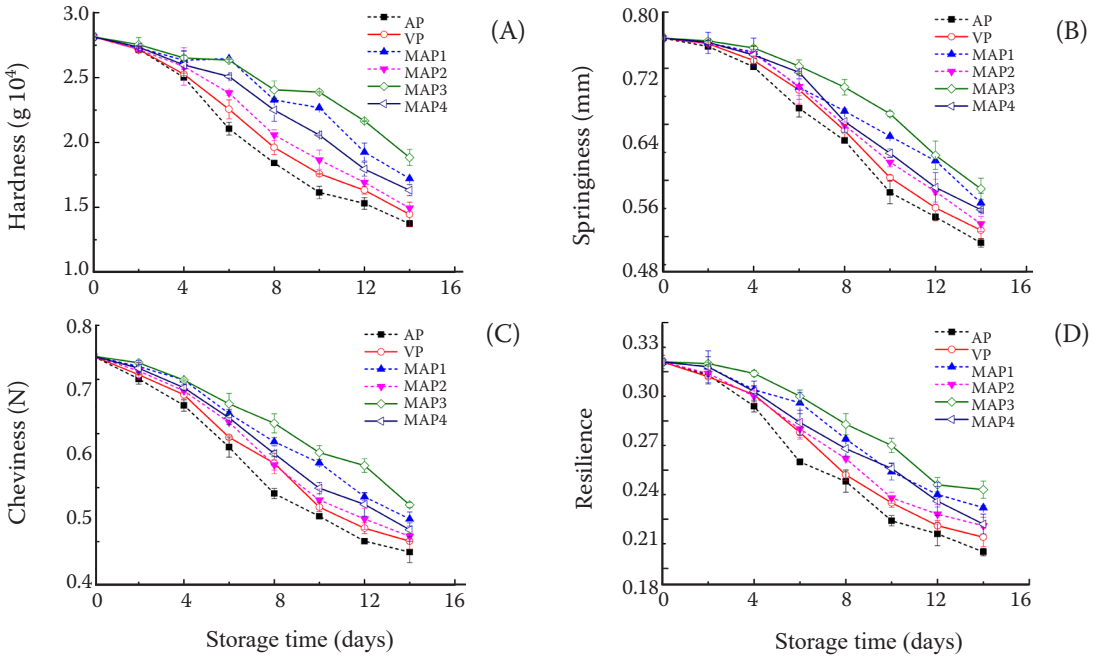

Figure 2. Results of (A) hardness, (B) springiness, (C) chewiness, and (D) resilience of pufferfish samples under different MAP treatments during cold storage

For abbrevaitions see Table 1

MRI (magnetic resonance imaging) provides visual information about turbot samples during storage. Red colour stands for high proton density and blue colour stands for low proton density in the pseudo-colour images. As shown in Figure 4, no significant difference was detected in the image brightness of pufferfish at the beginning and the brightness of pufferfish was darker and bluer with the increasing time. The co-

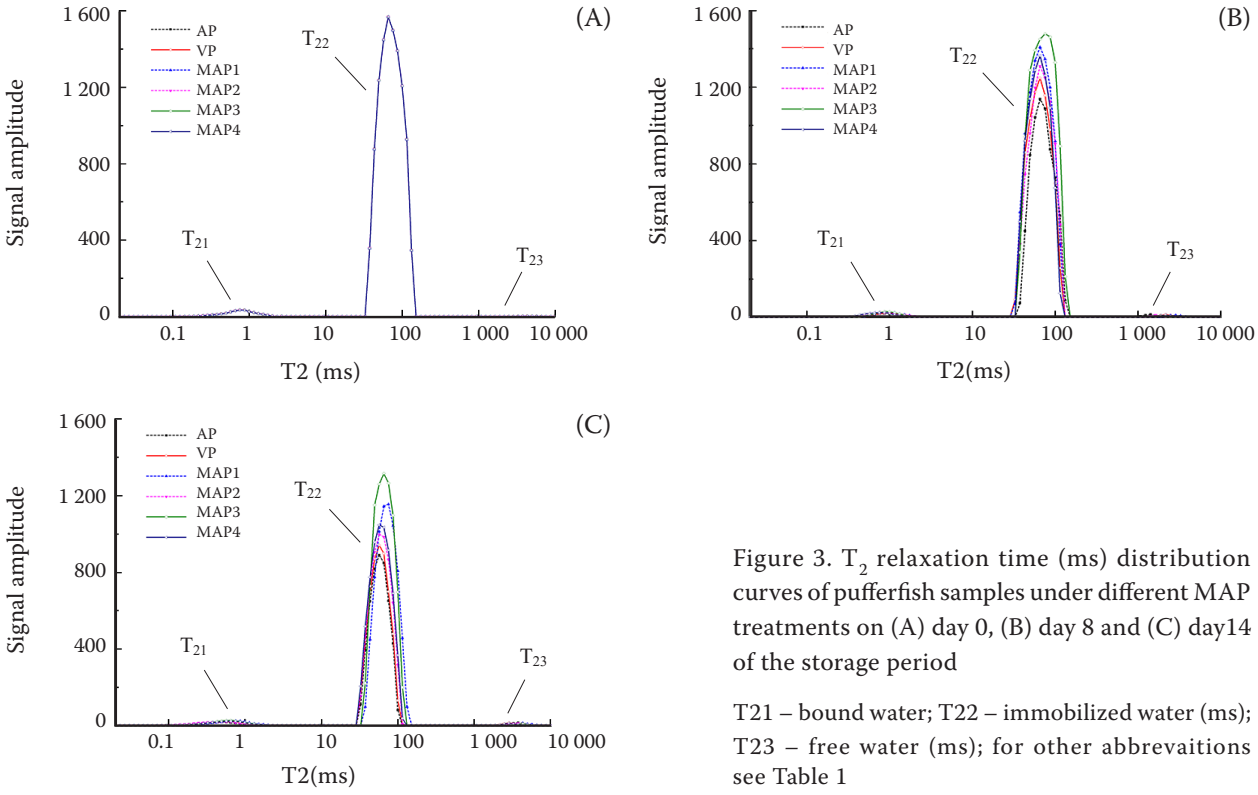

Figure 3. T_2 relaxation time (ms) distribution curves of pufferfish samples under different MAP treatments on (A) day 0, (B) day 8 and (C) day14 of the storage period

T21 – bound water; T22 – immobilized water (ms); T23 – free water (ms); for other abbrevaitions see Table 1

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lour of AP pufferfish on day 8 and 12 and VP samples on day 12 was bluer and darker than that of the other samples, demonstrating the myofibril degradation and microstructure destruction in AP pufferfish, while no significant difference ($P > 0.05$) was observed in MAP pufferfish. The brightness of MAP3 samples is lighter compared to other samples, which indicated that the MAP3 treatment (60% CO_2, 5% O_2, 35% N_2) is more suitable for the quality maintenance of pufferfish and the result was consistent with the variation of LF-NMR transverse relaxation.

Analysis of FAAs. In the present research, Asp, Ser, Glu, Ala, Val, Leu, Tyr, Lys, Arg and total FAAs showed upward trends at the beginning and afterwards they gradually decreased in all samples (Table 1). The MAP retarded changes in the levels of special FAAs, including Thr, Val, Met, Leu, Phe, Lys, His, and Arg, because MAP effectively controlled the microbial metabolism during storage. At the same time, Gly and Ala were the most abundant FAAs in pufferfish. His increased from the initial value of 3.64 mg (100 g)$^{-1}$ to 7.10, 6.61, 6.01, 6.43, 5.72 and 6.04 mg (100 g)$^{-1}$ for AP, VP, MAP1, MAP2, MAP3 and MAP4, respectively. Good acceptance of MAP3 pufferfish can be explained by its higher Gly and Ala concentration than in the other samples during storage and the two FAAs are responsible for stronger umami taste. Overall, MAP combined with cold storage could be effective in slowing down the process and keep the good edible value of pufferfish during storage.

Table 1. Changes in FAAs contents (mg 100g^{-1}) in differently treated pufferfish samples during refrigerated storage

Storage time	Samples	FAAs							
		Asp	**Thr**	**Ser**	**Glu**	**Gly**	**Ala**	**Val**	**Met**
Day 0		3.58 ± 0.05	6.71 ± 0.08	7.13 ± 0.03	3.17 ± 0.11	69.13 ± 0.52	58.22 ± 1.12	6.11 ± 0.10	1.34 ± 0.02
Day 8	AP	6.53 ± 0.03	9.07 ± 0.13	6.57 ± 0.01	3.90 ± 0.17	52.57 ± 1.03	62.87 ± 0.11	13.82 ± 0.27	1.51 ± 0.03
	VP	6.08 ± 0.15	8.65 ± 0.09	7.98 ± 0.16	4.40 ± 0.03	57.16 ± 0.79	64.69 ± 0.35	15.11 ± 0.16	1.88 ± 0.03
	MAP1	6.70 ± 0.05	7.47 ± 0.35	10.22 ± 0.07	8.99 ± 0.07	56.28 ± 0.71	72.01 ± 1.71	13.55 ± 0.04	1.48 ± 0.64
	MAP2	6.64 ± 0.03	8.58 ± 0.16	9.73 ± 0.11	10.07 ± 0.19	54.08 ± 2.21	66.17 ± 2.33	13.86 ± 0.46	1.05 ± 0.33
	MAP3	5.21 ± 0.12	7.19 ± 0.09	10.13 ± 0.24	9.50 ± 0.28	62.66 ± 0.91	75.45 ± 0.98	15.51 ± 0.23	1.39 ± 0.37
	MAP4	5.93 ± 0.08	7.61 ± 0.08	9.24 ± 0.07	8.06 ± 0.15	58.60 ± 1.22	67.49 ± 1.55	14.92 ± 0.17	1.75 ± 0.15
Day 14	AP	4.07 ± 0.11	13.62 ± 0.22	5.22 ± 0.21	4.81 ± 0.52	34.43 ± 1.75	46.41 ± 1.21	8.44 ± 0.15	1.40 ± 0.21
	VP	4.31 ± 0.13	12.56 ± 0.17	6.37 ± 0.13	6.36 ± 0.21	36.00 ± 0.97	49.89 ± 0.03	9.26 ± 0.27	1.65 ± 0.25
	MAP1	4.86 ± 0.06	9.89 ± 0.02	6.51 ± 0.06	8.88 ± 0.06	42.01 ± 1.31	62.53 ± 0.15	10.15 ± 0.09	1.79 ± 0.37
	MAP2	4.29 ± 0.05	11.67 ± 0.14	6.22 ± 0.08	7.41 ± 0.11	45.35 ± 1.44	52.57 ± 0.07	9.29 ± 0.13	2.07 ± 0.15
	MAP3	5.01 ± 0.10	9.29 ± 0.12	8.46 ± 0.16	9.83 ± 0.08	48.05 ± 0.85	64.81 ± 1.39	10.89 ± 0.18	2.55 ± 0.52
	MAP4	4.48 ± 0.03	11.78 ± 0.05	7.94 ± 0.11	9.81 ± 0.13	46.27 ± 2.36	55.95 ± 1.44	9.88 ± 0.54	2.18 ± 0.11
		Ile	**Leu**	**Tyr**	**Phe**	**Lys**	**His**	**Arg**	**Total**
Day 0		2.23 ± 0.17	3.28 ± 0.07	2.04 ± 0.11	3.03 ± 0.07	3.52 ± 0.21	3.64 ± 0.07	19.03 ± 1.36	192.16 ± 1.88
Day 8	AP	4.93 ± 0.21	6.93 ± 0.12	6.78 ± 0.17	8.81 ± 0.33	12.14 ± 0.13	6.19 ± 0.18	59.95 ± 1.87	262.57 ± 2.73
	VP	4.27 ± 0.19	6.18 ± 0.09	7.26 ± 0.16	8.16 ± 0.10	11.68 ± 0.20	5.70 ± 0.12	54.88 ± 0.68	264.08 ± 1.35
	MAP1	5.29 ± 0.22	5.87 ± 0.08	7.59 ± 0.07	6.64 ± 0.06	10.77 ± 0.24	4.58 ± 0.55	46.59 ± 0.94	264.03 ± 3.72
	MAP2	4.45 ± 0.09	6.53 ± 0.18	7.43 ± 0.11	7.82 ± 0.53	11.85 ± 0.13	5.48 ± 0.11	49.23 ± 1.16	262.97 ± 2.94
	MAP3	6.12 ± 0.14	5.64 ± 0.11	8.07 ± 0.34	6.09 ± 0.21	10.52 ± 0.55	3.95 ± 0.18	41.16 ± 2.15	268.59 ± 3.76
	MAP4	6.08 ± 0.19	6.01 ± 0.03	7.42 ± 0.22	7.33 ± 0.17	10.87 ± 0.42	4.82 ± 0.27	48.62 ± 1.71	264.75 ± 5.22
Day 14	AP	6.77 ± 0.35	3.62 ± 0.25	3.98 ± 0.25	11.34 ± 0.45	5.08 ± 0.22	7.10 ± 0.17	17.17 ± 0.82	173.46 ± 4.16
	VP	6.67 ± 0.17	4.41 ± 0.26	4.32 ± 0.21	11.18 ± 0.27	5.82 ± 0.27	6.61 ± 0.35	19.01 ± 1.22	184.42 ± 3.37
	MAP1	8.48 ± 0.27	3.91 ± 0.08	5.50 ± 0.09	10.56 ± 0.08	6.04 ± 0.17	6.01 ± 0.20	24.98 ± 0.45	212.10 ± 1.58
	MAP2	7.17 ± 0.33	3.87 ± 0.09	4.23 ± 0.17	9.88 ± 0.14	5.58 ± 0.43	6.43 ± 0.22	21.34 ± 2.78	197.37 ± 6.23
	MAP3	8.95 ± 0.14	4.05 ± 0.15	5.84 ± 0.15	9.15 ± 0.26	6.36 ± 0.26	5.72 ± 0.31	26.77 ± 2.06	225.73 ± 4.69
	MAP4	8.66 ± 0.22	3.88 ± 0.15	4.67 ± 0.41	9.71 ± 0.18	5.79 ± 0.33	6.04 ± 0.28	21.26 ± 1.66	208.30 ± 2.18

FAAs – free amino acids; for other abbrevaitions see Table 1

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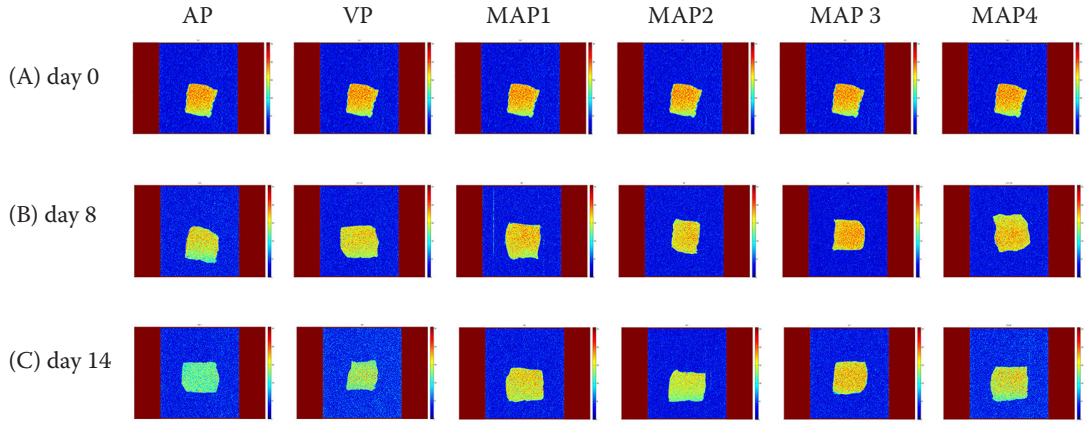

Figure 4. Magnetic resonance imaging (MRI) of pufferfish samples under different MAP treatments on (A) day 0, (B) day 8 and (C) day 14 of the storage period

For abbrevaitions see Table 1

CONCLUSION

The MAP in combination with cold (4 °C) treatment could slow down the rate of pufferfish spoilage during storage. The results of physicochemical and microbiological analyses indicated that the MAP3 (60% CO_2, 5% O_2, 35% N_2) pufferfish maintained the better flavour quality during cold storage, which was mainly due to the fact that high CO_2 concentration could effectively suppress the growth of spoilage microorganisms and extend the shelf life. Therefore, 60% CO_2, 5% O_2, 35% N_2 MAP combined with cold storage (4 °C) is suitable for maintaining the pufferfish freshness where an extended storage period may be necessitated.

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