Original Paper

Effect of CO_2-flushed Packaging on Quality of Pink Shrimp Contaminated with *Vibrio parahaemolyticus*

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CO_2-flushed packaging was effective for maintaining the quality of pink shrimp contaminated with *Vibrio parahaemolyticus*. Contaminated shrimp were sealed in Krehalon ML VS-20 film pouches flushed with air, N_2, or CO_2 and stored at 20°C. CO_2 concentration in the pouch was approximately 87% and was retained for at least 168 h. The numbers of *V. parahaemolyticus* in raw pink shrimp after 8 h (initially inoculated at 1–9 CFU·g^{-1}) or 12 h (initially inoculated at 100–999 CFU·g^{-1}) of storage and the maximum number of bacterial cells at steady state as a kinetic parameter of the Gompertz equation were smaller under the CO_2-flushed atmosphere than under the other atmospheres. The period before reaching the enteropathogenic concentration of 10^6 CFU·g^{-1} was prolonged by CO_2-flushed packaging. These results indicate that CO_2-flushed packaging is effective for suppressing *V. parahaemolyticus* growth. Shrimp blackening due to the activity of polyphenol oxidase (EC 1.14.18.1) was inhibited by CO_2-flushed packaging. Although CO_2 reduced the pH on the surface of shrimp, sensory scores were not affected. The results obtained in the present study indicate that CO_2-flushed packaging is effective for maintaining the appearance, taste, and hygiene of pink shrimp.

Keywords : Facultative anaerobe, Sea food, Storage, Inorganic gas, Blackening, quality, Gompertz model

1. Introduction

Pink shrimp (*Pandalus borealis* Krøyer, 1838) are an important seafood resource worldwide.[1)] Because shrimp can become contaminated with pathogens such as *Vibrio parahaemolyticus* (Fujino et al., 1951; Sakazaki et al., 1963 emend.; West et al., 1986), it is important that shrimp distributed to the market should be safe and hygienic for consumption.[2)]

It is well known that CO_2 inhibits the growth of aerobes.[3)] However, Kimura et al.[4)] reported that atmospheres containing 20–100% CO_2 did not significantly suppress the growth of *V. parahaemolyticus* purchased from the Institute for Fermentation, Osaka, Japan, which were inoculated on plate count agar and incubated at 30°C. Hirose et al.[5)] reported that 100% CO_2 atmosphere was not suppressed the growth of anaerobic bacteria though aerobic bacteria were suppressed at 10°C. Conversely, Makino et al.[6)]

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reported that a >99% CO_2 atmosphere significantly suppressed the growth of bacteria isolated from prawns [*Marsupenaeus japonicus*; (Bate, 1888)], which were inoculated on pink shrimp and incubated at 20°C. This research demonstrated the novel finding that CO_2 is effective in suppressing the growth of facultative anaerobes as well as aerobes. However, growth was measured in a gas-tight acrylic chamber. Moreover, the shrimp were sterilized by autoclaving for 15 min at 121°C to remove the initial microflora. Thus, these experimental conditions were very different from the practical conditions. Therefore, the suppression of *V. parahaemolyticus* growth by CO_2 required examination under practical conditions.

The appearance of shrimp degrades very rapidly after harvesting because of the enzymatic activity of polyphenol oxidase (PPO, EC 1.14.18.1), which results in the appearance of black spots on the shrimp surface.[7] Chen et al.[8] reported that high CO_2 pressure inhibited the activity of PPO, and Gonçalves et al.[7] suppressed the blackening of pink shrimp using a modified atmosphere with a high CO_2 concentration. These findings suggest that CO_2 is effective in maintaining both the appearance and hygiene of shrimp. However, Makino et al.[6] could not examine the effect of CO_2 on the suppression of shrimp blackening because PPO was inactivated by autoclaving, which was used as the sterilization method.

In the present study, the suppressive effect of CO_2-flushed packaging, as a practical method for the safe distribution of pink shrimp, on *V. parahaemolyticus* growth was investigated using the Gompertz equation, which is a kinetic model widely used for microbial growth analysis.[9,10] Simultaneously, the effects of CO_2 on shrimp blackening, pH, and sensory scores, which are considered as important factors determining quality, were examined. The experiments in the present study were conducted at 20°C, because this temperature was suitable value for investigating the atmospheric influence on *V. parahaemolyticus* growth as described in our previous report.[6]

2. Materials and Methods

2.1 Materials

Pink shrimp (17.5 ± 1.0 g per shrimp) harvested from the Tajima fishery harbor (Japan) were transported to the laboratory within a day of harvest. They were not treated with any chemical to suppress blackening.

V. parahaemolyticus isolated by the authors[6] was used in the present study. *V. parahaemolyticus*, stored by liquid drying,[11] were suspended in a small amount of sterilized Salt Polymyxin Broth "*Nissui*", and statically incubated in the same medium for 18 h at 30°C. The incubated broth containing 10^9 CFU·mL^{-1} of microbial cells was used for this study.

Laminated film pouches (Krehalon ML VS-20, Kureha Corporation, Tokyo, Japan, polyethylene terephthalate / nylon / ethylene vinyl alcohol / polyethylene) were used to store the shrimp. The properties were as follows: surface area, 0.2 m^2; film thickness, 40 μm; O_2 transmission rate, 25 mL·m^{-2}·d^{-1}·atm^{-1} [at 23°C, 80% relative humidity (R.H.)]; water vapor transmission rate, 20 g·m^{-2}·d^{-1} (at 40°C, 90% R.H.); standard plate

count (SPC), <4 CFU·m^{-2}.

2.2 Measurement of atmospheric changes in the laminated pouches

The ability of the laminated pouches to retain atmosphere was examined before the storage test for contaminated shrimp. N_2 (99.9995 vol%) or CO_2 (99.995 vol%) gas was flushed into a pouch through a sterilization filter (pore size, 0.1 μm) and immediately sealed using an impulse sealer. The preparations were stored for 168 h at 20°C. Changes in O_2, CO_2, and N_2 concentrations were measured using a GC-14A gas chromatograph (Shimadzu Co., Kyoto, Japan) equipped with a thermal conductivity detector, according to the method of Makino et al.[12] This experiment was repeated twice.

2.3 Measurement of *V. parahaemolyticus* growth in stored shrimp

SPC of the purchased shrimp was measured using Petrifilms™ (AC plates; Sumitomo 3M Ltd., Tokyo, Japan) before inoculation with *V. parahaemolyticus*. A suspension (0.1 mL) containing 10^2 or 10^4 CFU·mL^{-1} of *V. parahaemolyticus* was inoculated into the raw shrimp. This was equivalent to an initial inoculation at 10^0 (1–9) or 10^2 (100–999) CFU·g^{-1} of shrimp. Shrimp were also sterilized by autoclaving (121°C, 15 min) to confirm the effect of the initial SPC on *V. parahaemolyticus*growth. A suspension (0.1 mL) containing 10^2 CFU·mL^{-1} of *V. parahaemolyticus* was inoculated into the sterilized shrimp.

Shrimp were sealed in the laminated film pouches flushed with air, N_2, or CO_2. Pouches with air were simply sealed. N_2 and CO_2 gases were sealed in the pouches as described in the previous section. Atmospheres of air or high CO_2 concentration were used as the control and test, respectively. The atmosphere of high N_2 concentration (equivalent to air without O_2) was prepared to confirm the effect of O_2 on *V. parahaemolyticus* growth because the absence of O_2 in the high CO_2 atmosphere might affect *V. parahaemolyticus* growth, making it difficult to judge whether CO_2 suppressed the growth by simply comparing the growth at air and CO_2.

The preparations were stored for 48 h at 20°C. CFU of *V. parahaemolyticus* was measured every 4 h using thiosulfate citrate bile sucrose (TCBS) agar plates as the selection media for the bacterium, according to the method of Makino et al.[6] The contaminated shrimp before storage were used for the colony count at 0 h.

This experiment was repeated three times. Experimental data for the plate counts were statistically evaluated by analysis of variance (ANOVA) with Tukey–Kramer honestly significant difference (HSD) tests ($P < 0.05$) using JMP™ ver. 8.0.2 software (SAS Institute Inc., Cary, NC, USA).

Changes in plate counts over time were plotted on scatter graphs. Growth curves were generated from experimental data obtained in this study using the Gompertz equation (Eq. 1), which has been most often employed[9,10] in conjunction with SigmaPlot® ver. 9.01 (SPSS Inc., Chicago, IL, USA), a nonlinear least-squares fitting program that employs the Marquardt–Levenberg algorithm:

$$N_t = N_0 + Ce^{-e^{-B(t-M)}} \quad (1)$$

where N is the plate count (CFU·g^{-1}), C is the difference between the initial and maximum plate counts (CFU·g^{-1}), e is the Naperian base, t is the storage time (h), M is the time at which the absolute growth rate is maximum (h), B is the maximum relative growth rate (relative growth rate at M) (h^{-1}), and subscript 0 represents the start of storage.

The lag phase duration L (h) was estimated as shown in Eq. 2 from the Gompertz equation:[13]

$$L = M - \frac{1}{B} \quad (2)$$

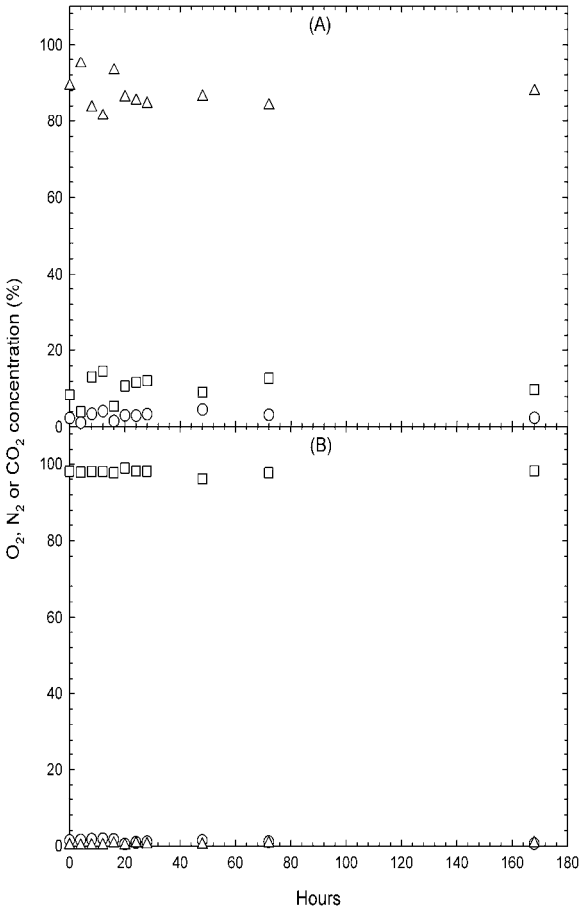

Fig. 1 Changes in O_2 (○), N_2 (□), and CO_2 (△) in the laminated film pouches (Krehalon ML VS-20) over time. (A) and (B): Initially flushed with CO_2 and N_2, respectively.

2.4 Observation of blackening of pink shrimp stored under various atmospheres

Blackening of pink shrimp initially contaminated with 10^0 CFU·g^{-1} of *V. parahaemolyticus* was recorded by taking images using a Coolpix L16 digital camera (Nikon Corporation, Tokyo, Japan) during the experiments mentioned in section 2.3.

2.5 Measurement of pH and sensory scores for pink shrimp stored under various atmospheres

Pink shrimp without inoculation were sealed in the laminated film pouches by the method described in section 2.3. The preparations were stored for 4 h at 20°C. After storage, the pH on the shrimp surface was measured using a YK-21 pH meter (Sato Shoji Corporation, Tokyo, Japan) equipped with a flat sensor for measuring pH on the surface of a sample.

Taste, flavor, and texture of the pink shrimp were evaluated using a four-star scale (1, bad; 2, poor; 3, fair; 4, good) by three trained panelists.

These data were statistically evaluated by ANOVA with the least significant difference (LSD) test ($P < 0.05$) using JMP™ ver. 8.0.2.

3. Results and Discussion

3.1 Gas barrier properties of the laminated pouches

Changes in the atmospheres in the laminated pouches over time are shown in Fig. 1.

After 168 h of storage, the atmosphere in the pouches initially flushed with CO_2 contained

approximately 87% CO_2, 2.8% O_2, and 10% N_2. The atmosphere in the pouches initially flushed with N_2 contained approximately 0.38% CO_2, 1.3% O_2, and 98% N_2. These barrier properties are suitable for gas-flushed packaging of pink shrimp contaminated with *V. parahaemolyticus* because this bacterium grows rapidly within 48 h.[6] Although 99.995% CO_2 and 99.9995% N_2 were flushed into the pouches, a certain amount of air may enter the pouch before sealing. Because air contains approximately 78% N_2, the N_2 concentration in the atmosphere shown in Fig. 1(B) may be higher than that shown in Fig. 1(A). The CO_2 concentration in Fig. 1(B) was expected to be approximately 0.03%, the usual CO_2 level in air. Because the CO_2 and N_2 concentrations in Fig. 1 were lower than those in the acrylic chamber in our previous report,[6] the influence of these gases on microbial growth in the present study may be smaller than that in the previous study.

3.2 Effects of various atmospheres on *V. parahaemolyticus* growth in stored shrimp

Changes in the plate counts of *V. parahaemolyticus* over time are shown in Fig. 2. Differences between the numbers of *V. parahaemolyticus* after the same storage period were compared by the Tukey–Kramer HSD test.

SPC of raw pink shrimp used in this study was log2.3 CFU·g^{-1}. *V. parahaemolyticus* grow on Petrifilms^{TM} (AC plates); however, the initial number of *V. parahaemolyticus* in the raw shrimp was below the limit of detection using TCBS agar plates. Therefore, microorganisms other than *V. parahaemolyticus* accounted for the initial microflora in the raw shrimp. Although the initial

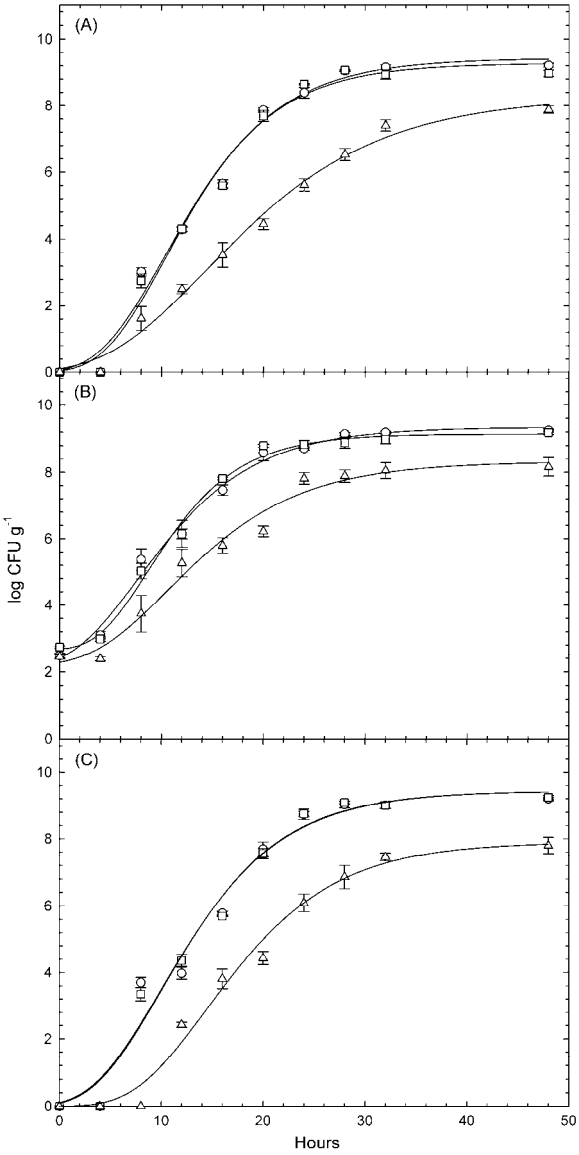

Fig. 2 Changes over time in plate counts of *V. parahaemolyticus* in pink shrimp stored in pouches flushed with air (○), N_2 (□), and CO_2 (△)at 20°C. (A): raw shrimp, 10^0 (1–9) CFU·g^{-1} initial plate count. (B): raw shrimp, 10^2 (100–999) CFU·g^{-1} initial plate count. (C): autoclaved shrimp, 10^0 (1–9) CFU·g^{-1} initial plate count. Means ± standard error of three observations are plotted.

microflora was unclear, effects of SPC on *V. parahaemolyticus* growth can be determined by comparing the results in Fig. 2(A) and 2(C). No significant differences were observed between these results. This indicates that the initial SPC in the shrimp did not affect the plate count data for *V. parahaemolyticus* in this study. Kimura[14] reported that the number of *V. parahaemolyticus* cells increased from 10 to 10^5 cells during 5 h at 30°C and that the growth rate was considerably higher than that of the bacteria growing on a standard plate. Therefore, rapid growth of inoculated *V. parahaemolyticus* may suppress the growth of other bacteria.

The number of bacteria after 8 h of storage under the CO_2-flushed atmosphere in Fig. 2(A) was lower than that under the other atmospheres. This suggests that the CO_2-flushed atmosphere is effective in suppressing *V. parahaemolyticus* growth. Although the CO_2 concentration in the present study was lower than that in the previous study,[6] according to the result in Fig. 1(A), bacterial growth was still suppressed. The number of bacteria after 12 h of storage under the CO_2-flushed atmosphere in Fig. 2(B) was also lower than that under the other atmospheres. The later suppressive effect of CO_2 in Fig. 2(B) compared with that in Fig. 2(A) may be due to the difference in the initial inoculation concentrations. However, the result at 12 h of storage in Fig. 2(B) is in agreement with the result reported by Makino et al.[6] in which shrimp were initially contaminated with 10^3 $CFU \cdot g^{-1}$ *V. parahaemolyticus*. This suggests that the suppression by CO_2 is delayed by an increase in the initial number of bacteria. Thus, reduction of the initial number of bacteria by chemicals, etc., is effective in delaying growth. The number of bacteria under the CO_2-flushed atmosphere in Fig. 2(A) was lower than that in Fig. 2(B) until 28 h. This suggests that the difference in the initial number of bacteria affected the number before reaching a steady state. O_2 in the air did not affect the growth of the bacteria because there were no significant differences between the numbers of bacteria in pouches flushed with air and N_2 in Fig. 2.

The growth kinetic parameters in the Gomperz equation are presented in Table 1. The maximum number calculated as $N_0 + C$ under the CO_2-flushed atmosphere was significantly lower than that under the other atmospheres, as indicated in Fig. 2. However, the maximum number was higher than that under the >99% CO_2 atmosphere reported by Makino et al.[6] This may be due to the difference in the CO_2 concentration. The lag phase duration, as the period just before the start of rapid growth, is important for evaluating storage methods and conditions. The duration for shrimp contaminated with 10^0 $CFU \cdot g^{-1}$ and stored under the CO_2-flushed atmosphere was slightly longer than that for shrimp under the other atmospheres. However, this tendency was unclear. In our previous study,[6] the lag period under the >99% CO_2 atmosphere was shorter than that under air. These results suggest that evaluation of the period using the Gompertz equation is difficult or that the effect of CO_2 on extending the lag period is small or non-existent.

Sakazaki et al.[15] reported that administration of approximately 10^6 viable cells of *V.*

parahaemolyticus caused gastroenteritis in a male subject. Therefore, the storage periods for the number of bacteria to reach 10^6 CFU·g^{-1} are presented in Table 1 as an index for evaluating enteropathogenicity per gram of shrimp. The storage periods under the CO_2-flushed atmosphere for shrimp initially contaminated with 10^0 and 10^2 CFU·g^{-1} bacterial cells were longer by 10 h and 5 h, respectively, than those under the other atmospheres. According to our previous study,[6] the maximum number at steady state under the >99% CO_2 atmosphere did not reach 10^6 CFU·g^{-1}. Thus, CO_2-flushed packaging is suggested to be effective in maintaining better shrimp hygiene, and the effect is higher with an increased CO_2 concentration.

3.3 Effects of atmospheres on the appearance of pink shrimp contaminated with *V. parahaemolyticus*

Blackening of stored raw shrimp initially contaminated with 10^0 CFU·g^{-1} was observed, as shown in Fig. 3.

The appearance of the cephala of shrimp stored under air and N_2-flushed atmospheres was blackened after 4 h and 16 h, respectively. This suggests O_2 effects blackening. Gonçalves et al.[7] reported that the enzymatic activity of PPO, which requires O_2, results in the appearance of black spots on pink shrimp. Therefore, reduction of O_2 in the storage atmosphere may be effective in suppressing shrimp blackening.

Table 1. Kinetic parameters in the Gompertz equation (Eq. 1), and the period of time for the number of bacteria to reach 10^6 CFU·g^{-1} under various atmospheres at 20°C.

Container	Auto-claving	Initial plate count, CFU·g^{-1}	Atmos-phere	Kinetic parameter					Period until 10^6 CFU·g^{-1}, h	Reference
				N_0	C	B	M	L		
Flexible pouch	No	10^0	Air	0	9.4	0.16	10	3.9	15	Present Study
			N_2	0	9.3	0.16	10	4.2	15	
			CO_2	0	8.3	0.10	14	4.6	25	
		10^2	Air	2.2	7.2	0.15	7.7	1.2	11	
			N_2	2.7	6.5	0.20	8.7	3.7	11	
			CO_2	2.2	6.1	0.14	11	3.2	16	
	Yes	10^0	Air	0	9.4	0.15	10	3.3	15	
			N_2	0	9.4	0.15	10	3.4	15	
			CO_2	0	7.9	0.14	15	7.4	24	
Acrylic gas-tight chamber	Yes	10^3	Air	3.4	6.2	0.17	13	7.6	14	Makino et al.[6]
			N_2	3.1	6.1	0.14	12	5.0	14	
			CO_2	3.2	2.5	0.22	10	5.9	∞	

N is the plate count (CFU·g^{-1}), C is the difference between the initial and maximum plate counts (CFU·g^{-1}), e is the Naperian base, t is the storage time (h), M is the time at which the absolute growth rate is maximal (h), B is the maximum relative growth rate (relative growth rate at M) (h^{-1}), and subscript 0 represents the start of storage, and L is the lag phase duration (h): $L = M - 1/B$.

Fig. 3 Appearance of pink shrimp initially contaminated with 10^0 CFU·g^{-1} *V. parahaemolyticus* stored in pouches flushed with air (A), N_2 (N), and CO_2 (C) at 20°C. The hyphenated numbers denote the storage period (h).

Table 2. pH and sensory scores for shrimp stored under various atmospheres.

Atmosphere	pH	Taste	Flavor	Texture
Air	7.3 ± 0.031^a	3.7 ± 0.35^a	4.0 ± 0.00^a	3.7 ± 0.35^a
N_2	7.3 ± 0.0067^a	4.0 ± 0.00^a	4.0 ± 0.00^a	4.0 ± 0.00^a
CO_2	6.9 ± 0.031^b	3.7 ± 0.35^a	4.0 ± 0.00^a	3.7 ± 0.35^a

Means ± standard error of three observations are presented. Within the same measurement, values followed by the same letter are not significantly different ($P > 0.05$; One-way ANOVA with the LSD test).

Conversely, the appearance of the shrimp stored under the CO_2-flushed atmosphere remained unchanged through all storage periods, although the images are shown only until 28 h. This result is in agreement with the research reported by Gonçalves et al.[7] that a modified atmosphere with high CO_2 concentration was effective in suppressing the blackening of pink shrimp. In the previous study by Makino et al.,[6] only the growth suppression of *V. parahaemolyticus* in high CO_2 concentration was reported because the stored shrimps were initially sterilized by autoclaving. This operation inactivated all enzymes, including PPO, and made it impossible to observe blackening of the samples. Therefore, the suppressive effects of CO_2 on *V. parahaemolyticus* and blackening are simultaneously confirmed in the present study for the first time.

3.4 Effects of atmospheres on pH and sensory scores for pink shrimp contaminated with *V. parahaemolyticus*

The pH values for shrimp stored under the three types of atmospheres for 4 h are presented in Table 2. pH under the CO_2-flushed atmosphere was significantly lower by 0.4 than that under the other atmospheres. This indicated that CO_2 affected the pH on the surface of the shrimp. However, *V. parahaemolyticus* can grow at a pH range of 5.6–9.6 (optimum pH, 7.6–8.0).[16] Therefore, the values in Table 2 alone did not have a suppressive effect on the growth, although pH under the CO_2-flushed atmosphere was further from the optimum pH than that under the other atmospheres, and this may have affected bacterial growth. Dixon and Kell[3] reported that a decrease in pH caused by an increase in CO_2 dissolved in the culture medium may not affect the growth of microorganisms. Therefore, inhibition of metabolic pathways by CO_2[3] may have suppressed bacterial growth.

The results of sensory tests are also presented in Table 2. In consideration of the safety of the panelists, the storage period of the samples for these tests was 4 h. The samples were tested after the pH measurements. No significant differences were observed between the storage atmospheres. Therefore, CO_2-flushed storage may not affect the eating quality of the shrimp.

The storage period for the measurement of pH and evaluation of sensory scores may have been much shorter than the average storage period of shrimp contaminated with bacteria. In future, we expect to measure pH after long-term storage. However, evaluation of sensory scores after long-term storage may be difficult.

In the present study, the possibility of CO_2-flushed packaging for maintaining the safety and quality of pink shrimp has been demonstrated. These novel results may contribute to improvements in distribution and storage methods for shrimp.

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