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Role of Sulfites and 4-Hexylresorcinol in Microbial Growth and Melanosis Prevention of Deepwater Pink Shrimp (*Parapenaeus longirostris*) Using a Controlled Atmosphere

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ABSTRACT

A controlled atmosphere containing 48% CO₂ and 7% O₂ was used in association with refrigeration for storage of deepwater pink shrimp (*Parapenaeus longirostris*). Shrimp were treated with two different concentrations of sodium metabisulfite or 4-hexylresorcinol and subjected to the controlled atmosphere immediately after capture onboard ship or on arrival in port. Total volatile basic nitrogen, total viable counts, enterobacteria, lactic acid bacteria, and luminescent bacteria were determined, and black spot progression was evaluated. The combined effect of controlled atmosphere and melanosis inhibitors was used to delay black spot development as compared to the shrimp stored in ice alone. Storage under the controlled atmosphere without ice limited microbiological quality, namely, total viable counts, but enterobacterial growth was lower.

The shelf life of fresh crustaceans is limited primarily by the formation, during cold storage, of large quantities of free amino acids and other substances that are ideal substrates for microbial growth, coupled with the onset of melanosis, produced by polyphenol oxidase (PPO). This enzyme is responsible for sclerotization (hardening) of the exoskeleton after molting (2), and indeed certain workers have observed a relationship between molt stage and black spot formation in shrimp (10, 24). The discoloration appears to be harmless to consumers, but it nonetheless greatly reduces market value and hence results in substantial economic loss.

Sulfites and sulfite derivatives have traditionally been used as melanosis inhibitors (11). However, their effectiveness is variable, they do not prevent black spot completely, and they may also sometimes pose a health risk (31, 32), mainly in the form of allergic reactions and severe asthma attacks in asthmatics. Benzoic acid, kojic acid, and 4-hexylresorcinol, or cumic acid, have proved to be partially effective black spot inhibitors (23). These chemical agents are capable of stopping black spot formation nearly completely. Nevertheless, microbial spoilage continues and becomes the factor limiting the shelf life of crustaceans kept under chilled storage (5).

There is therefore a need to find alternatives that will inhibit black spot formation, avoid health safety issues for consumers, and retard microbial growth. Sivertsvik and others (27) reported that use of a controlled atmosphere (CA) rich in CO₂ and poor in O₂ during cold storage helped delay melanosis formation while at the same time delaying microbial growth. Inhibition of the growth of microorganisms by controlled or modified atmospheres has been described in packaged shrimp (16, 17, 19). However, very little in-

formation is available on the shelf life of crustaceans treated with CA onboard ship or immediately after off-loading in port and then followed by cold storage.

An earlier work found that an atmosphere with approximately 55% CO₂, in combination with sulfiting, inhibited black spot entirely in iced shrimp for at least 2 weeks of storage. However, the texture of the product after cooking was deteriorated with significant losses in shrimp flavor; an effect ascribed to diffusion of the CO₂ into the flesh as the ice melted. Various researchers (3, 13) have also described adverse effects on fish color and texture in atmospheres overly enriched with CO₂, in large part caused by increased muscle acidity due to diffusion of the CO₂ (8, 17, 27).

The object of this study was to establish whether treatment using a controlled atmosphere (48% CO₂, 7% O₂) immediately after capture onboard ship or on arrival in port and application of 4-hexylresorcinol or sulfites combined to have a greater inhibitory effect on melanosis, thereby prolonging the shelf life of shrimp in cold storage.

MATERIALS AND METHODS

The experiment was performed using deepwater pink shrimp (*Parapenaeus longirostris*) caught off the coast of southern Spain (Cadiz) by trawlers in November 2002. Shrimp mean size and weight were 7 ± 1 cm and 5 ± 1.5 g, respectively. Onboard ship they were separated from the bycatch, washed with seawater, and placed in perforated polystyrene boxes (approximately 1 kg per box). Except for one treatment for which no inhibitors were used (treatment control), all the shrimp were dusted with either 4-hexylresorcinol plus sorbitol as excipient (0.25 g powder per 100 g shrimp) (treatment R) or a commercial sodium metabisulfite-based product (Freskor HA-5501, Hasenos, Spain) at a recommended concentration of 4% (treatment S4%), and also at 2% (treatment S2%), to test the possibility of lowering the sulfite levels used with the protective atmosphere. Inhibitor concentration

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has been expressed as grams of commercial product per 100 g of shrimp. All lots were chilled by covering with flaked ice to facilitate diffusion of the black spot inhibitors into the shrimp and to keep shrimp temperature down until landing. Two replications were performed for all treatments.

A portion of each of the treatments with the black spot inhibitors was placed in airtight stainless steel containers to be subjected to a controlled atmosphere onboard ship (CA_{board}) (treatments R-CA_{board}, S4%-CA_{board}, and S2%-CA_{board}). The same was done with a portion of the treatment without any inhibitor (treatment control-CA_{board}) for use as a control. The containers were immediately filled with a previously prepared gas mixture consisting of 7% O₂ and 48% CO₂, the remainder being N₂. The composition of the atmosphere was chosen according to preliminary trials.

On arrival in port 8 h after capture, the containers were opened to remove the ice from the boxes. They were then immediately resealed and placed in cold storage (2°C), and the controlled atmosphere gas mixture was replaced.

Another portion of the shrimp with added inhibitors was to be stored in the controlled atmosphere upon arrival in port (CA_{port}). Then, the ice was likewise removed and shrimp were placed in cold stored containers, which were filled with the same gas mixture (treatments R-CA_{port}, S4%-CA_{port}, S2%-CA_{port}, and control-CA_{port}). The lots that did not undergo CA treatment were kept in ice throughout the entire storage period (treatments R-ice, S4%-ice, S2%-ice, control-ice). The containers and the non-CA-treated lots were transported to Madrid (Spain) in a refrigerated truck that left the port within 12 h of landing. On arrival they were placed in cold storage at 2°C, and the CA was refreshed over the course of storage to ensure that the gas mixture remained stable.

A portion of each of the CA_{port}-treated lots was removed from the containers after 48 h and held in chilled storage covered with ice. The object was to be able to assess the possible residual effect of the CO₂ over the remainder of the storage period (treatments R-CA_{residual}, S4%-CA_{residual}, and S2%-CA_{residual}).

Over the 10-day storage period a group of trained panelists routinely evaluated shrimp appearance every 2 days (14 individuals per treatment per evaluation), scoring melanosis according to a scale of from 1 to 4, where 1 = complete absence of black spots; 2 = a few small spots on the carapace; 3 = considerable spotting on the carapace; 4 = substantial spotting over the entire shrimp. The assessors also examined shrimp discoloration and the commercial viability of each treatment. On day 2 of storage a taste test was performed for all treatments to detect possible shrimp aromas or flavors that might be related to the melanosis inhibitors or the CA employed. These aromas and flavors were rated as being either absent or present.

Total volatile basic nitrogen (TVBN) determinations were carried out in triplicate over the storage period using the method of Antonacopoulos and Vyncke (1). Exactly 10 g $\pm$ 0.1 g of the ground sample were weighed in a suitable container and homogenized for 2 min with 100 ml of 6% perchloric acid solution. After being filtered, the extract was alkalized with 20% sodium hydroxide solution and submitted to steam distillation. The volatile base components were absorbed by an acid receiver and determined by titration of the absorbed bases.

For microbiological analyses, duplicate shrimp samples were blended (1:9 dilution) with peptone water (Oxoid, Basingstoke, UK) containing 0.85% added NaCl in a Stomacher model 400 type BA 7021 Lab Blender (Seward Medical, Colworth London, UK) for 1 min. Total viable counts (TVC) and luminescent bacteria were determined by spread plating on Long and Hammer's agar with 1% NaCl (28), incubated at 15°C. Counts were per-

FIGURE 1. Melanosis scores in shrimp stored without black spot inhibitors (control) over the storage period. Control-ice = stored in ice; control-CA_{board} = onboard CA treatment; control-CA_{port} = in-port CA treatment. Melanosis index: 1 = complete absence of black spots; 2 = a few small spots on the carapace; 3 = considerable spotting on the carapace; 4 = substantial spotting over the entire shrimp.

formed after 3 days (in darkness for luminescent bacteria) and after 5 days (for TVC), Enterobacteria were determined on violet red bile glucose agar (30°C, 2 days), according to López-Caballero et al. (19). Lactic acid bacteria (LAB) were incubated in deMan Rogosa Sharpe agar at 30°C for 3 to 5 days, according to Man et al. (9).

Two-way analysis of variance was performed to evaluate the statistical significance of the differences between mean TVBN value pairs using a significance level setting of $P \leq 0.05$. The Statgraphics plus 2.1 computer program (STSC, Inc., Rockville, Md.) was used for statistical processing. Melanosis evaluation scores over the storage period were fitted to a curve by regressing the mean daily scores for all the panelists for each treatment.

RESULTS AND DISCUSSION

Effect of the CA on the shrimp stored without inhibitors. The use of a CO₂-enriched, O₂-poor CA (in both onboard and in-port treatments) did not prevent black spot development, showing higher values than those observed in shrimp stored in ice without any CA treatment (Fig. 1). This agreed with the findings of Lannelongue et al. (16), who attributed reduced intensity of black discoloration to washing of the shrimp by melting ice. This may also explain partially the TVBN values were higher than 30 mg/100 g after storage for 4 days in the CA-treated treatments (Table 1).

Initial microbial counts were generally high in all control treatments (Table 2), a finding doubtless ascribable to the absence of added preservatives. The TVC and luminescent bacterial counts were lower in the shrimp stored in ice after 2 days of storage. The melting ice could have washed away bacteria present on the surface of the shrimp, as has been suggested by Lannelongue et al. (15). Luminescent bacteria, chiefly the gram-negative organism *Photobacter-*

TABLE 1. Total volatile basic nitrogen (TVBN) (means and two-way analysis of variance) in shrimp stored without blackspot inhibitors (control) over the storage period

Lots ^a	Days of storage		
	2	4	7
Control-CA _{board}	19.08 AX ^b	34.89 BXY	
Control-CA _{port}	27.95 AX	38.78 AX	
Control-ice	18.26 AX	23.05 AY	21.69 A

^a Control-ice, stored in ice; Control-CA_{board}, onboard CA treatment; Control-CA_{port}, in-port CA treatment.

^b Different letters (A, B) in the same row indicate significant differences ($P \leq 0.05$) as a function of storage time; different letters (x, y) in the same column indicate significant differences ($P \leq 0.05$) as a function of treatment.

ium phosphoreum, have been regarded as one of the principal causes of spoilage in fish products stored in atmospheres with high CO₂ contents (6, 27), producing high levels of trimethylamine. In this study, luminescent bacterial counts were slightly higher in the CA_{port} treatment than in the CA_{board}, which may be ascribable to the residence time of the shrimp onboard the fishing vessel prior to CA treatment, around 8 h. So, 7% O₂ was not enough to inhibit the luminescence bacteria growth along storage.

LAB levels were slightly higher in the CA_{board} treatment. Partially anoxic conditions with higher acid levels due to the CO₂ encouraged LAB growth, and consequently the growth of the ordinary spoilage microflora was partially inhibited (5, 17, 27). The change in the balance in favor of the lactic acid microflora (17) is very important, given that these microorganisms do not produce indole (17) and trimethylamine (19) in significant amount. Gram-negative microorganisms, and *Enterobacteriaceae* in particular, show a significant production of indole levels, which have been used by the U.S. Food and Drug Administration to validate the sensory evaluation of shrimp decomposition (22).

Refrigeration at higher CO₂ concentrations has resulted in reported slow enterobacterial growth in shrimp (17). Lannelongue et al. (16) described gram-negative bacteria generally as being sensitive to CO₂ and to low temperatures. Nevertheless, after storage for 2 days the total counts of presumptive *Enterobacteriaceae* (26) were close to 10³ CFU/g. No putative washing effect by the melting ice was discernible in this case.

Effect of the CA on the sulfited shrimp. Dusting of commercial black spot inhibitor at a concentration of 4% plus storage in ice sufficed to halt melanosis development

FIGURE 2. Melanosis scores in shrimp treated by adding sulfites at concentrations of 4% (wt/wt) (S4%) and 2% (wt/wt) (S2%) during cold storage. S4%-ice, S2%-ice = stored in ice; S4%-CA_{board}, S2%-CA_{board} = onboard CA treatment; S4%-CA_{port}, S2%-CA_{port} = in-port CA treatment; S4%-CA_{residual}, S2%-CA_{residual} = stored in ice after in-port CA treatment for 48 h (residual effect). Melanosis index as in Figure 1.

in the shrimp and to keep shrimp appearance acceptable for at least 4 days without the need for any CA treatment (Fig. 2). The acceptability period was appreciably shorter when the black spot inhibitor was added at a concentration of 2%.

CA_{port} treatment of shrimp in combination with sulfiting at both concentrations, 4% and 2%, held black spot formation to lower levels over the storage period than with CA_{board} treatment. Thus, addition of a low concentration of

TABLE 2. Microbiological counts (log CFU/g) in shrimp (means plus or minus standard deviation) with no additives (control) after 2 days of cold storage

Lots ^a	TVC	Luminescent bacteria	Lactic acid bacteria	Enterobacteria
Control-CA _{board}	6.50 ± 0.02	3.7 ± 0.00	2.32 ± 0.04	<2
Control-CA _{port}	6.59 ± 0.03	4.24 ± 0.24	2.07 ± 0.03	<2
Control-ice	5.02 ± 0.03	3.00 ± 0.00	2.09 ± 0.01	2.24 ± 0.24

^a Control-ice, stored with ice; Control-CA_{board}, CA applied on board; Control-CA_{port}, CA applied in port.

TABLE 3. Total volatile basic nitrogen (TVBN) (means and analysis of variance) in shrimp treated by adding sulfites at concentrations of 4% (wt/wt) (S4%) and 2% (wt/wt) (S2%) during cold storage

Lots ^a	Days of storage		
	2	4	7
S4%-CA _{board}	26.06 AY ^b	35.07 BXYZ	69.28 CZ
S4%-CA _{port}	26.06 AY	40.14 BYZ	66.38 CZ
S4%-CA _{residual}	26.06 AY	31.47 AX	39.67 BY
S4%-ice	19.36 AX	18.99 AW	18.50 AX
S2%-CA _{board}	26.13 AY	35.76 BXYZ	62.02 CZ
S2%-CA _{port}	26.92 AY	41.65 BZ	62.98 CZ
S2%-CA _{residual}	26.92 AY	33.42 BXY	38.19 BY
S2%-ice	18.13 AX	19.52 AW	18.30 AX

^a S4%-ice, S2%-ice, stored in ice; S4%-CA_{board}, S2%-CA_{board}, on-board CA treatment; S4%-CA_{port}, S2%-CA_{port}, in-port CA treatment; S4%-CA_{residual}, S2%-CA_{residual}, stored in ice after in-port CA treatment for 48 h (residual effect).

^b Different letters (A, B, C) in the same row indicate significant differences ($P \leq 0.05$) as a function of storage time; different letters (w, x, y, z) in the same column indicate significant differences ($P \leq 0.05$) as a function of treatment.

sulfites in combination with CA treatment may be a suitable means of preventing melanosis.

In terms of melanosis development and the possible residual effect of the CO₂, CA treatment for 48 h followed by conventional storage in ice (CA_{residual}) did not yield better results than sulfiting alone, irrespective of the concentration employed. Surprisingly, not only did the CA treatments not yield any apparent benefit in terms of shrimp appearance, they proved detrimental to sensory quality.

The TVBN content in the CA-treated shrimp exceeded 30 mg/100 g of flesh from day 4 (Table 3), in spite of the beneficial effects of storing under CO₂ (17). In contrast, in the shrimp stored in ice from the outset, the TVBN level held steady at around 20 mg/100 g because of the melting ice effect, with no significant differences ($P \leq 0.05$) observed according to the sulfite concentration employed (Table 3). Furthermore, the values recorded were similar to those recorded for the shrimp stored without the use of inhibitors (Table 1). The shrimp treated with CA_{residual} exhibited intermediate TVBN levels ($P \leq 0.05$) at the end of storage. Thus, the washing effect of the melting ice would appear to be a more important factor for this index than the effect of either the CA treatment or sulfiting.

Neither the addition of sulfites, which certain workers have regarded as antimicrobial agents (4, 20) nor the high CO₂ levels prevented high total viable microbial counts after 2 days of storage (Table 4). However, the microflora could be dominated by gram-positive bacteria, which are much more resistant to CO₂ (17, 27). The lowest counts were observed in the shrimp stored in ice without CA treatment until day 7, after which the values were similar to those in the other treatments, in consonance with the results reported by López-Caballero et al. (19). Siverstsvik et al. (27) showed that CO₂ can inhibit the normal spoilage flora, increasing shelf life significantly. The absence of this effect

TABLE 4. Microbiological counts (log CFU/g) in shrimp (means plus or minus standard deviation) with added sulfites at 4% (S4%) and 2% (S2%) (wt/wt) during cold storage

Lots ^a	Total viable counts			Luminescent bacteria			Enterobacteria			Lactic acid bacteria		
	Day			Day			Day			Day		
	2	7	10	2	7	10	2	7	10	2	7	10
S4%-CA _{board}	6.46 ± 0.02	7.01 ± 0.03	7.19 ± 0.02	3.45 ± 0.15	<4	<4	<2	2.48 ± 0.00	2.95 ± 0.05	2.48 ± 0.02	3.85 ± 0.01	4.50 ± 0.02
S4%-CA _{port}	6.53 ± 0.03	7.43 ± 0.02	7.15 ± 0.02	4.00 ± 0.00	6.09 ± 0.09	<4	<2	3.06 ± 0.02	3.5 ± 0.2	2.66 ± 0.03	4.12 ± 0.01	4.20 ± 0.02
S4%-CA _{residual}	6.53 ± 0.03	7.11 ± 0.02	7.30 ± 0.08	4.00 ± 0.00	<4	5.00 ± 0.00	<2	2.91 ± 0.13	6.21 ± 0.04	2.66 ± 0.03	3.87 ± 0.03	4.58 ± 0.12
S4%-ice	5.26 ± 0.08	5.88 ± 0.03	7.30 ± 0.05	3.00 ± 0.00	3.45 ± 0.47	<4	2.22 ± 0.11	2.97 ± 0.07	5.8 ± 0.18	2.17 ± 0.03	2.75 ± 0.15	3.16 ± 0.21
S2%-CA _{board}	6.50 ± 0.11	7.44 ± 0.02	6.94 ± 0.02	3.15 ± 0.15	4.15 ± 0.15	<4	<2	3.08 ± 0.00	2.89 ± 0.11	2.47 ± 0.01	4.19 ± 0.04	3.31 ± 0.06
S2%-CA _{port}	6.64 ± 0.00	7.31 ± 0.05	6.94 ± 0.02	4.45 ± 0.15	5.15 ± 0.15	<4	<2	<2	<2	<2	3.45 ± 0.45	3.31 ± 0.06
S2%-CA _{residual}	6.64 ± 0.00	7.36 ± 0.01		4.45 ± 0.15	<4		<2	3.15 ± 0.00		<2	3.82 ± 0.11	
S2%-ice	5.04 ± 0.02	5.90 ± 0.01		3.89 ± 0.05	3.60 ± 0.30		<2	2.77 ± 0.17		2.14 ± 0.06	2.70 ± 0.00	

^a S4%-ice, S2%-ice, stored with ice; S4%-CA_{board}, S2%-CA_{board}, CA applied onboard; S4%-CA_{port}, S2%-CA_{port}, CA applied in port; S4%-CA_{residual}, S2%-CA_{residual}, with ice, after 48 h in CA applied in port (residual).

may be due to the lack of melting ice effect. In addition, unlike the findings observed by Stammen et al. (29), the residual effect of the CO₂ did not appear to have any beneficial influence on preservation.

As in the case of the shrimp without added inhibitor, luminescent bacterial counts were slightly lower in the CA_{board} treatments compared with those CA_{port} treatments, irrespective of the sulfite concentration used (Table 4). Sulfiting did not directly affect the growth of these microorganisms. As discussed by Dalgaard et al. (7), this slight inhibitory effect of the CA_{board} treatment can be attributed more to the lower O₂ levels than to the higher CO₂ levels, since *Photobacterium* is highly resistant to CO₂. Counts were somewhat higher than for the treatments stored in ice without any CA treatment.

CA treatment of sulfited shrimp was conducive to LAB growth over the storage period (Table 4), although growth was low because of the low temperature (18). The growth of LAB spp. in high CO₂ concentrations was reported by Stenström (30). The differences observed with respect to the lot that did not undergo CA treatment can be attributed to the washing effect of the melting ice and to the higher acidity levels caused by diffusion of the CO₂. This effect also appeared to be unrelated to the sulfite concentration used, and similarly no clear differences were discernible with respect to CA_{board} or CA_{port} treatment. Regarding the possible residual effect of the CO₂ on the shrimp, at the end of 10 days counts of LAB were similar to those in the CA treatments and significantly higher than those in the treatment stored in ice from the start. This indicates that there really was an appreciable residual effect on the growth of LAB, which was not counteracted by the washing effect of the melting ice.

The presumptive enterobacteria grew slightly during the storage period in CA (Table 4). Counts were similar in all treatments until day 7. However, by day 10 growth was considerably higher in the treatments stored in ice. It thus appears that under the conditions of this experiment the CO₂ exerted no residual influence and that CA treatment must be ongoing if it is to be able to limit the growth of these microorganisms. The high CO₂ content, combined with the presence of higher levels of LAB, may have been responsible for the lower growth recorded in the CA treatments. López-Caballero et al. (19) reported enterobacterial counts similar to those recorded here during CA storage of shrimp using 45% CO₂, 5% O₂, and 50% N₂ and even higher counts using 40% CO₂, 30% O₂, and 30% N₂. Here again, the concentration of the added sulfite likewise did not appear to exhibit any definite influence.

According to the sensory analysis performed 48 h after capture, the shrimp gave off a slightly acidic odor when the containers were opened, but this odor dissipated over time. On cooking a certain sour though not unpleasant taste was perceptible, possibly caused by diffusion of the CO₂ into the flesh (17). In any case, half of the panelists were unable to perceive this taste.

Effect of the CA on the shrimp treated with 4-hexylresorcinol. Based on preliminary studies into the use of

FIGURE 3. Melanosis scores in shrimp treated by adding 4-hexylresorcinol at a concentration of 0.25% (wt/wt) (R) during cold storage. R-ice = stored in ice; R-CA_{board} = onboard CA treatment; R-CA_{port} = in-port CA treatment; R-CA_{residual} = stored in ice after in-port CA treatment for 48 h (residual effect). Melanosis index as in Figure 1.

4-hexylresorcinol to stop melanosis in white shrimp (23), a concentration of 0.25 g of 4-hexylresorcinol per 100 g of shrimp was selected as the minimum dose required to prevent the appearance of black spot for at least 7 days in shrimp caught in autumn, when higher concentrations of melanosis inhibitors are needed to prevent black spot formation (23). This effective dose was appreciably higher than the concentrations reported by other researchers (12, 21, 25). These differences may be due to the fact that different species are involved, certain physiological states may be more susceptible than others, or different volumes of shrimp or application methods may have been used.

As shown in Figure 3, combining a CO₂-enriched atmosphere with 0.25% 4-hexylresorcinol resulted in the nearly complete prevention of melanosis over 9 days of the storage period. In any case, a certain greenish hue on the carapace was visible in all the treatments from day 4 onward, associated with the use of 4-hexylresorcinol in shrimp caught in autumn, as previously reported (23). The treatment stored in ice without CA treatment underwent a substantial increase in the level of melanosis after 7 days, an increase that appeared still sooner in the CA_{residual}-treated shrimp. The combination of CA treatment plus 4-hexylresorcinol changed the greenish hue on the carapace to a light gold.

The results for TVBN content revealed a trend very similar to that for the sulfited treatments, with slightly lower values at the end of storage (Table 5). TVBN levels increased significantly ($P \leq 0.05$) in the CA treatments, which were stored without ice. As in the other experimental cases already referred to above, values were higher than those recommended by Huss (14). In contrast, the treatment stored only in ice the whole time had constant TVBN levels that were less than 30 mg/100 g of flesh.

Viable aerobic counts (Table 6) at the start of storage were similar to those for the black spot inhibitor-free and

TABLE 5. Total volatile basic nitrogen (TVBN) (means and two-way analysis of variance) in shrimp treated by adding 4-hexylresorcinol at a concentration of 0.25% (wt/wt) (R) during cold shrimp

Lots ^a	Days of storage		
	2	4	7
R-CA _{board}	23.95 AX ^b	35.22 AY	48.86 CY
R-CA _{port}	29.25 AY	38.08 BY	64.45 CZ
R-CA _{residual}	29.25 AY	32.29 AY	44.65 BY
R-ice	19.76 AX	22.22 AX	19.69 AX

^a R-ice, stored in ice; R-CA_{board}, onboard CA treatment; R-CA_{port}, in-port CA treatment; R-CA_{residual}, stored in ice after in-port CA treatment for 48 h (residual effect).
^b Different letters (A, B, C) in the same row indicate significant differences ($P \leq 0.05$) as a function of storage time; different letters (X, Y, Z) in the same column indicate significant differences ($P \leq 0.05$) as a function of treatment.

sulfited treatments, except for the treatment kept on ice from the outset, which as previously mentioned had the lowest counts. After storage for 10 days, the TVCs in all the treatments treated with 4-hexylresorcinol were slightly lower than in the treatments dusted with 4% sulfites (commercial product). After 2 days counts were slightly lower in the CA_{board} treatment, but this effect was not prolonged until the end of storage, when there were no differences between the different CA treatments, including the treatment testing the residual effect of the CO₂ during storage. Consequently, from a microbiological standpoint, once again the washing effect of the melting ice seemed to outweigh the protective effect of the CA.

The use of a combination of CA and 4-hexylresorcinol did not afford any definite advantage in terms of the luminescent bacterial counts (Table 6), which in some cases were slightly higher than in the sulfited treatments, particularly at the end of storage.

LAB counts were low and similar in all the treatments until day 7 (Table 6). After storage for 10 days appreciably lower growth than in the sulfited treatments was observed (Table 4). It is possible that 4-hexylresorcinol could inhibit LAB, too. As in the two cases previously discussed above, the shrimp stored on ice without CA treatment had the lowest LAB counts.

After storage for 10 days, presumptive enterobacterial counts in the CA treatments were below the limit of 10³ CFU/g (Table 6) and were lower than in the corresponding sulfited treatments. This may be due to the presumable antimicrobial effect of 4-hexylresorcinol. Moreover, storage in ice from the time of capture or after 48 h under CA treatment (CA_{residual}) proved ineffective in delaying the growth of these microorganisms.

The combination of melanosis inhibitor plus CA treatment under refrigeration without ice was more effective in preventing black spot development than conventional cold storage in which the shrimp are treated with melanosis inhibitor and covered with ice. However, early CA treatment onboard ship did not yield any apparent improvement com-

TABLE 6. Microbiological counts (log CFU/g) in shrimp (means plus or minus standard deviation) with added 4-hexylresorcinol (R) at 0.25% (wt/wt) during cold storage

Lots ^a	Luminescent bacteria				Enterobacteria				Lactic acid bacteria			
	Total viable counts				Day				Day			
	2	7	10	2	7	10	2	7	10	2	7	10
R-CA _{board}	6.47 ± 0.06	6.82 ± 0.09	6.91 ± 0.02	<3	<4	4.35 ± 0.35	<2	<2	2.5 ± 0.2	<2	<2	3.16 ± 0.12
R-CA _{port}	6.77 ± 0.03	6.80 ± 0.03	6.97 ± 0.08	5.28 ± 0.10	<4	5.90 ± 0.05	2.31 ± 0.08	<2	<2	2.39 ± 0.09	2.72 ± 0.12	2.30 ± 0.30
R-CA _{residual}	6.77 ± 0.03	7.45 ± 0.03	6.90 ± 0.02	5.28 ± 0.10	4.00 ± 0.00	<4	2.31 ± 0.08	<2	5.44 ± 0.01	<2	<2	<2
R-ice	4.62 ± 0.09	6.36 ± 0.04	6.75 ± 0.03	<3	<3	4.00 ± 0.00	<2	<2	5.19 ± 0.03	<2	<2	<2

^a R-ice, stored with ice; R-CA_{board}, CA applied on board; R-CA_{port}, CA applied in port; R-CA_{residual}, with ice, after 48 h in CA applied in port (residual).

pared with treatment in port approximately 8 h later. Applying 0.25% 4-hexylresorcinol proved to be more efficient in inhibiting black spot formation than applying a 4% concentration of a commercial sodium metabisulfite-based product. Additionally, 4-hexylresorcinol was a more effective inhibitor of enterobacteria and lactic acid bacteria than sulfites, which, conversely, were more effective against luminescent bacteria.

Microbiological quality (TVBN content and TVC) of shrimp stored without ice under a controlled atmosphere consisting of 48% CO₂ and 7% O₂ was severely compromised as a consequence of the absence of melting ice effect. Luminescence bacteria and LAB counts were high because of the high levels of CO₂, and they could be the main spoilers. The presence of a high population of LAB, combined with the high CO₂ content, might inhibit presumptive enterobacteria, as compared to conventional treatment in ice.

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