Effects of storage in ozonised slurry ice on the sensory and microbial quality of sardine (*Sardina pilchardus*)

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Abstract

The use of slurry ice, both alone and in combination with ozone, as compared with traditional flake ice was investigated as a new refrigeration system for the storage of sardine (S*ardina pilchardus*). Microbiological, chemical and sensory analyses were carried out throughout a storage period of 22 days. According to sensory analyses, sardine specimens stored in ozonised slurry ice had a shelf life of 19 days, while counterpart batches stored in slurry ice or flake ice had shelf lives of 15 and 8 days, respectively. Storage in ozonised slurry ice led to significantly lower counts of aerobic mesophiles, psychrotrophic bacteria, anaerobes, coliforms, and both lipolytic and proteolytic microorganisms in sardine muscle, and of surface counts of mesophiles and psychrotrophic bacteria in sardine skin as compared with the slurry ice and the flake ice batches. In all cases, the slurry ice batch also exhibited significantly lower microbial counts, both in muscle and skin, than the flake ice batch. Chemical parameters revealed that the use of slurry ice slowed down the formation of TVB-N and TMA-N to a significant extent in comparison with storage in flake ice, the evolution of TMA-N being the most adequate parameter to detect quality losses. A combination of slurry ice with ozone also allowed a better control of pH and TMA-N formation as compared with slurry ice alone. *Proteus vulgaris* and *Staphylococcus sciuri* were identified as the main spoilers of sardine muscle. This work demonstrates that the combined use of slurry ice and ozone for the storage of sardine is can be recommended to improve the quality and extend the shelf life of this fish species.

Keywords: slurry ice, ozone, sardine, shelf life, chilled storage, microbial quality, sensory quality

1. Introduction

The leading role of microorganisms in marine fish spoilage is well known. Bacteria degrade fish constituents, particularly non-protein nitrogen compounds, thus inducing the development of off-odors and flavors typically associated with fish spoilage (Ababouch *et al.*, 1996). However, the rate of spoilage depends on several factors, the nature of fish species and the handling and storage conditions being of capital importance (Whittle *et al.*, 1990; Olafsdóttir *et al.*, 1997). Once the fish have been caught, refrigeration should be applied immediately in order to preserve the quality of the specimens. In this sense, currently different refrigeration systems are used: conventional flake ice (Nunes *et al.*, 1992), refrigerated seawater (Kraus, 1992), ozonised refrigerated water (Sugita *et al.*, 1992; Kötters *et al.*, 1997), and slurry ice (Huidobro *et al.*, 2001, 2002).

Slurry ice –also known as flow ice, fluid ice, slush ice or liquid ice– represents a relatively novel refrigeration system and consists of an ice-water suspension at a subzero temperature. Among its main advantages, two should be highlighted: (i) its faster chilling rate –due to a more rapid heat exchange–, and (ii) the reduced physical damage caused to seafood products by its spherical microscopic particles, as compared with conventional flake ice, which tend to be aciculate. Additionally, full coverage of the fish surface, thus avoiding the formation of air pockets, prevents direct contact of the fish material with oxygen, with the subsequent benefits derived from minimisation of oxidation and dehydration events. From a technical point of view, the slurry ice mixture can be pumped, thus allowing a more hygienic fish handling and process automatization. Another relevant feature of slurry ice is that it can be combined with other additives for different purposes; i.e., ozone- to achieve a better microbial control of the fish catch– or melanosis inhibitors, to minimise browning reactions in crustaceans (Huidobro *et al.*, 2002).

Despite these theoretical and practical advantages, few empirical data about the experimental use of slurry ice-based storage systems have been reported for commercial fish species. Thus, Chapman (1990) found that finfish stored in slurry ice on board had a better quality as compared with other chilling methods. Similar results were obtained for albacore (Price *et al.*, 1991) and shrimp (Huidobro *et al.*, 2002).

On the other hand, ozone has been traditionally used as a water-disinfecting agent. Its strong oxidizing nature makes it a useful tool for the inactivation of microorganisms. In this sense, ozone has been successfully employed as a disinfectant for fresh water aquaculture systems, and its applications in improving the sensory quality and shelf life of fish (Kötters *et al.*, 1997; Kim *et al.*, 1999; Kim *et al.*, 2000) have been reported recently. The bactericidal effect of ozone depends on several factors, such as temperature, relative humidity, pH and the presence of organic matter (Kim *et al.*, 1999). A few years ago, the FDA considered ozone a GRAS substance for use in different food applications, which has increased its worldwide.

Sardine (*Sardina pilchardus*) is a small pelagic fish. With the exception of the negligible amounts sold as retail, this species is mainly used as fresh or frozen raw material destined for further processing. One of the factors limiting its commercial use is the difficulty of its preservation at low temperature. Thus, the shelf life of sardines may be limited by rapid bacterial degradation and lipid oxidation mechanisms, which may cause off-flavors and flesh discoloration. The main goal of the present study was to evaluate the evolution of the sensory and microbial quality of sardines as affected by storage in slurry ice, either alone or combined with ozone. The identification of the main bacteria responsible for spoilage in this fish species is also described.

2. Materials and methods

2.1. Refrigeration systems

Slurry ice was prepared using a FLO-ICE prototype (Kinarca S.A.U., Vigo, Spain). The composition of the flow ice binary mixture was 40% ice and 60% water, prepared from filtered seawater (salinity: 3.3%). The temperature of the slurry ice mixture was -1.5ºC. The injection of ozone in the slurry ice mixture was accomplished with a prototype provided by Cosemar Ozono (Madrid, Spain), the redox potential being adjusted to 660 mV (0.17 mg ozone/l). In this batch, the ozone concentration was monitored by readings of the redox potential in the liquid phase. Flake ice was prepared with an Icematic F100 Compact device (Castelmac SPA, Castelfranco, Italy). The fish specimens were surrounded by either ozonised slurry ice, slurry ice, or flake ice at a fish:ice ratio of 1:1, and stored for up to 22 days in a refrigerated room at 2ºC. When required, the ice mixtures were renewed.

2.2. Fish material, processing and sampling

Sardine (*Sardina pilchardus*) specimens were caught in the day and kept in ice as they arrived at our laboratory. The fish specimens were neither headed nor gutted. Te length of the specimens was in the 16–21 cm range and average weight was 150 g. Three different batches, one for each refrigeration system, were used and studied separately along the whole experimental period. Samples were taken from each batch on days 0, 2, 5, 8, 12, 15, 19, and 22. All analyses were performed in triplicate.

2.3. Sensory analyses

Sensory analyses were conducted in whole fish by a panel consisting of five experienced judges, according to official guidelines (Table 1) concerning fresh and refrigerated fish (DOCE, 1989). Four categories were ranked: highest quality (E), good quality (A), fair quality (B), and unacceptable quality (C). Sensory assessment of the fish included the examination of the following parameters: skin, external odor, gills, consistency and flesh odor. The scores of the different panelists were averaged.

2.4. Microbiological analyses

Fish skin sections of 5 cm^2 were swabbed with sterile 0.1% peptone water (Oxoid Ltd., London, UK) and the microbial load was resuspended in 10 ml of 0.1% peptone water. Samples of 5 g of fish muscle were also dissected aseptically in parallel from skinned chilled specimens, mixed with 45 ml of 0.1% peptone water, and homogenized in a stomacher (Seward Medical, London, UK). In both cases, serial

dilutions from the skin or muscle microbial extracts were prepared in 0.1 % peptone water. Total aerobic and psychrotrophic bacteria were investigated in Plate Count Agar (PCA, Oxoid) after incubation at 30°C for 48 h or at 7-8°C for 10 days, respectively. Anaerobes were investigated as in the case of aerobic mesophiles, except that an anaerobic atmosphere kit (Oxoid) was placed together with the plates inside the anaerobiosis jar. Lactose-fermenting *Enterobacteriaceae* (coliforms) were investigated in Violet Red Bile Agar (VRBA medium, Merck, Darmstadt, Germany), following the manufacturer's instructions (Merck Microbiology Manual, 2002). The proteolytic phenotype was investigated in casein-agar medium (Phaff *et al.*, 1994), as previously described (Ben-Gigirey *et al.*, 2000). Microorganisms exhibiting a lipolytic phenotype were detected in tributyrine-agar medium (Ben-Gigirey *et al.*, 2000). In addition, H_2S-producing bacteria were investigated in triple sugar iron agar (TSI agar, Oxoid), while histamine-producing bacteria were detected in solid Niven medium (Niven *et al.*, 1981).

Routine microbiological tests included the investigation of colony morphology, cell morphology, Gram stain, and the production of cytochrome oxidase and catalase, as previously described (Rodriguez *et al.*, 2003). The identification of spoilage bacteria was carried out using API miniaturized biochemical tests (BioMèrieux, Marcy L'Etoile, France). The results of the identification tests were interpreted using the APILAB PLUS software (BioMèrieux).

2.5. Chemical analyses

The evolution of pH values in sardine muscle along the storage time was determined by means of a 6-mm diameter insertion electrode (Crison, Barcelona, Spain). Total volatile base-nitrogen (TVB-N) contents were measured as described elsewhere (Aubourg *et al.*, 1997). Briefly, fish muscle (10 g) was extracted with 6% perchloric acid and brought up to 50 ml. TVB-N contents were determined, after steam-distillation of the acid extracts rendered alkaline to pH 13 with 20% NaOH, by titration of the distillate with 10 mM HCl. The results were expressed as mg TVB-N/100 g muscle. Trimethylamine-nitrogen (TMA-N) values were determined by the picrate method, as previously described (Tozawa *et al.*, 1971). This involves the preparation of a 5% trichloroacetic acid extract of fish muscle (10 g /25 ml). Results were expressed as mg TMA-N/100 g muscle.

2.6. Statistical analyses

A multivariate analysis was performed to analyze the effect of each refrigeration system on the microbiological and chemical parameters. One-way analysis of variance (ANOVA) was also used to explore the significance of differences among microbiological and chemical parameters throughout storage for each refrigeration system. Multiple comparisons between parameters were carried out by the Tukey test. All tests were carried out using the SPSS software (SPSS Inc., Chicago, IL). A confidence interval at the 95% level ($p<0.05$) was considered in all cases.

3. Results and Discussion

3.1. Sensory analyses

The results from the sensory analyses are shown in Table 2. It can be seen that sardines refrigerated by the combined used of slurry ice and ozone retained a good quality (E and A categories) up to day 8 (Figure 1A) and were acceptable up to day 19. However, when slurry ice was used alone, the good quality was only retained up to day 5 and the product was acceptable up to day 15 (Figure 1B). The appearance of the gills and eyes were the first parameters that limited fish acceptability in both batches. In the case of sardines stored in flake ice, they only maintained good quality up to day 2 and were acceptable up to day 8 (Figure 1). This observation is in agreement with previously published information (El Marrakchi *et al.*, 1992; Ababouch *et al.*, 1996)

It should be stressed that the use of slurry ice –either alone or in combination with ozone– produced a significant increase in sardine shelf life, this trend being enhanced when ozone was present. In previous studies, it was demonstrated that the use of slurry ice extends the shelf life of non-fat fish species –such as farmed sea bream (Huidobro *et al.*, 2001), and European hake (Losada *et al.*, 2004) – and shrimp (Huidobro *et al.*, 2002). Moreover, the application of ozone has been reported to extend the shelf life of rockfish (*Sebastes* spp.) (Kötters *et al.*, 1997), and catfish (*Ictalurus punctatus*) fillets (Kim *et al.*, 2000), while other authors have reported benefits as regards the discoloration of minced horse mackerel as a result of washing with ozonised water for 10 to 20 min (Chen *et al.*, 1997). Recently, fresh scad (*Trachurus trachurus*) treated on-board with gaseous ozone also showed improved sensory scores as compared to non-treated samples (da Silva *et al.*, 1998).

3.2. Microbiological analyses

The evolution of microbial growth in sardine muscle along refrigerated storage in the slurry ice, ozonised-slurry ice and flake ice batches is shown in Figure 2 (panels A to F). As can be seen for all bacterial groups investigated, the use of flake ice allowed notable increases in the microbial populations, the counts of mesophiles, psychrotrophic bacteria, and of both proteolytic and lipolytic microorganisms reaching figures of approximately 10^6 CFU/g after 12 days of storage (Fig. 2, panels A, D, E, and F). By contrast, microbial growth was significantly slower in the slurry ice batch, the average differences in the counts of mesophiles, psychrotrophic bacteria, and both lipolytic and proteolytic microorganisms after 12 days of storage being in the range of 1.5-2.5 log units below those determined for the flake ice batch (Fig. 2, panels A, D, E, and F). By this time, according to sensory analysis the samples stored in flake ice were unacceptable, while slurry ice samples still had an acceptable quality (Table 2). According to sensory analyses, the less intense bacterial growth in sardine muscle when slurry ice was employed coincided with an extended shelf life of this batch. This trend is in agreement with the results of a previous study that reported significantly lower bacterial counts and an extended shelf of shrimp stored in slurry ice, as compared with conventional flake ice (Huidobro et al., 2002).

It is also well known that microbial proteolysis of muscle causes sensory spoilage (Kobatake *et al.*, 1992; Asakawa *et al.*, 1998; Gennari *et al.*, 1999), while the production of extracellular proteases by certain microorganisms affects the lipolytic breakdown of fat fish species such as albacore tuna (Ben-Gigirey *et al.*, 2000). In this sense, the lower counts determined for proteolytic and lipolytic bacteria in the slurry ice batch also seems to correlate with the results of the sensory analyses.

The combined use of ozone and slurry ice produced an additional reduction in the counts of the anaerobes, psychrotrophic bacteria, and of both proteolytic and lipolytic microorganisms along storage (Fig. 2, panels B, D, E, and F). A similar trend was observed for the evolution of mesophilic bacteria, but in this case the beneficial effect of ozone was only observed after 15 days of storage (Fig. 2, panel A). It should also be highlighted that the use of ozone combined with slurry ice induced a decline in the growth of mesophiles and lipolytic bacteria that was so important that their counts were similar to those determined at the beginning of the storage period (3.16 log CFU/g for mesophiles, and 2.17 CFU/g for lipolytic microorganisms).

The antibacterial activity of ozone in fish has been previously reported by other authors. Thus, treatment of the skin of gutted fish with a 3% NaCl solution containing 0.6 ppm of ozone for 30 to 60 minutes decreases viable counts by a factor of 2 to 3 log units and increases shelf life up to a factor of 60%. Chen *et al.* (1987) found that ozone, either in water or in a NaCl solution, was effective for the inactivation of microorganisms such as *Vibrio cholerae, Escherichia coli, Salmonella typhimurium, Vibrio parahaemolyticus*, and *Staphylococcus aureus.* Da Silva *et al.* (1998) found that gaseous ozone decreased both total viable counts and H_2S-producing bacteria by a factor of 1.0 log unit in fresh scad (*T. trachurus*).

It should also be mentioned that in our study coliform counts were below 10^4 CFU/g until the end of the storage period for the samples stored in flake ice, while the counts fell to below 10^2 CFU/g in the counterparts stored in slurry ice, either alone or combined with ozone (Fig. 2, panel C). Moreover, one-way ANOVA revealed that the combined use of slurry ice and ozone kept coliform counts comparable to the initial numbers at the beginning of storage. In any case, this bacterial group exhibited a limited development in sardine, confirming a previous report for jack mackerel (Figueroa *et al.*, 1990).

The evolution of surface microbial growth on sardine skin along refrigerated storage in the three batches is shown in Figure 3 (panels A and B). The mesophilic and psychrotrophic bacterial populations increased with storage time and reached counts of 10^6-10^7 CFU/cm^2 after 2 weeks of storage in flake ice. In contrast, mesophilic and psychrotrophic microorganisms only reached counts of 10^4-10^5 CFU/cm^2 in the slurry ice batch at that time. The surface washing of sardine skin caused by slurry ice could be a possible explanation for the lower surface counts determined in this batch. Interestingly, the combined use of ozone and slurry ice exerted an additional reduction of the microbial counts at 12 days of storage. Different possibilities have been reported to explain the ability of ozone to inactivate food surfaces. Thus, while some authors have suggested that ozone decreases the surface contaminants of fish during refrigerated storage (Dondo *et al.*, 1992; da Silva *et al.*, 1998), others defend the notion that ozone inactivates microorganisms less effectively when they are on food surfaces as compared to low ozone-demand liquid media (Kim *et al.*, 1999). The results obtained here clearly indicated a significant reduction of the microbial populations present on the fish surface due to ozone, this supporting the former statement.

The statistical significance of all the microbial results obtained is shown in Table 3. Thus, the use of slurry ice –either alone or in combination with ozone– resulted in significantly ($p<0.05$) lower counts of all microbial populations in sardine muscle and skin as compared to flake ice. It should also be stressed that the combined use of slurry ice and ozone significantly decreased the average populations of mesophiles, psychrotrophic bacteria, anaerobes, coliforms, proteolytic and lipolytic bacteria in sardine muscle, as well as of both surface mesophiles and psychrotrophic bacteria on sardine skin.

3.3. Chemical analyses

The evolution of chemical parameters in sardine muscle along storage is shown in Figure 4. In the case of sardines stored in flake ice, pH increased significantly with storage time, reaching a value of 7.0 by the end of storage (Fig. 4, panel A). This kind of behavior suggests a significant growth of alkalinizing bacteria in this batch, inducing the accumulation of ammonia compounds with the concomitant deleterious effects on sensory quality. Similar increases in pH have also been reported for sardine (Ababouch *et al.*, 1996) and for other fish species stored in flake ice (Nunes *et al.*, 1992; Ruiz-Capillas and Moral, 2001). A significantly different kind of behavior was observed in sardines stored in slurry ice: in this batch, the pH of the sardine muscle only underwent a slight increase: from an initial value of 5.90 to a final pH of 6.20 at the end of storage. When ozone was incorporated into the slurry ice mixture, the pH at the end of storage was 6.07, confirming a significant inhibition of alkalinizing microflora in this batch.

The evolution of TVB-N and TMA-N contents in the three batches is shown in Fig. 4 (panels B and C, respectively). No significant differences were observed among batches up to day 12, nor with respect to the initial counterparts at day 0. However, after that time a sharp increase in both parameters was observed in sardine muscle in the flake ice batch, a result that was not observed in either of the other two batches. El Marrakchi *et al.* (1990) studied quality changes in sardine stored in flake ice and suggested that contents of 5-10 mg TMA-N/100 g, and 25-35 mg TVB-N/100 g limited the acceptability of the specimens. In our work, the results obtained for TVB-N (Fig. 4, panel B) at the moment of sensory rejection –8 days for flake ice, 15 days for slurry ice and 19 days for ozonised slurry ice– matched the range of acceptability previously mentioned. However, the TMA-N contents determined in our study (Fig. 4, panel C) were below the proposed limits of acceptability, this probably indicating a very limited

growth of TMA-producing bacteria such as *Shewanella putrefaciens* and *Photobacterium phosphoreum,* as will be discussed below.

In global terms, multivariate analysis revealed that the storage of sardine in flake ice elicited significantly higher values for pH, TVB-N, and TMA-N as compared with storage in slurry ice (Table 4). Moreover, the addition of ozone to slurry ice significantly decreased the pH and TMA-N parameters as compared to the slurry ice batch (Table 4).

In order to establish the accuracy of the chemical parameters studied as potential indices of the quality changes taking place along storage, their correlations with storage time were investigated. High Spearman correlation coefficient values were obtained for the three parameters ($r^2 = 0.92$, for TVB-N, $r^2 = 0.96$ for TMA-N, and $r^2 = 0.95$ for pH) in the flake ice batch. In contrast, the correlation values decreased for the samples stored in slurry ice ($r^2 = 0.71$ for TVB-N; $r^2 = 0.99$ for TMA-N; and $r^2 = 0.77$ for pH). Conversely, for ozonised-slurry ice samples, the only parameter that showed a satisfactory correlation value was TMA-N ($r^2 = 0.99$). According to these results, the evolution of TMA-N in sardine muscle proved to be the most adequate for revealing losses in sardine quality. It is remarkable that(Strikingly,) this parameter also proved to be the best one for determining quality losses in other fish species preserved in flake ice, such as horse mackerel (Aubourg, 2001).

In sum, the refrigeration and storage of sardines in slurry ice significantly slowed down the formation of TVB-N and TMA-N and prevented significant increases in pH as compared with storage in flake ice. The combination of slurry ice with ozone resulted in a better control of both alkalinizing and TMA-producing bacteria.

3.4. Isolation and identification of spoilage bacteria from sardines stored in slurry ice

El Marrakchi *et al.* (1992) studied the microflora of sardines stored at room temperature, finding that Gram-positive bacteria –such as *Staphylococcus* spp. and *Micrococcus*– predominated over Gram-negative microorganisms such as *E. coli, Vibrio fluvialis, Aeromonas hydrophila, Moraxella* spp., and *Flavobacterium*. By contrast, storage in ice promoted the selection of Gram-negative bacteria due to their ability to grow and multiply at low temperature, *Proteus mirabilis* being isolated as the predominant bacterium in the spoiled product. Gennari *et al.* (1999) found that *S. putrefaciens* and *Pseudomonas fluorescens* were the main spoilers of sardines stored in ice. With respect to histamine-producing bacteria, *Morganella morganii* –a well known and prolific histamine-producer (Kim *et al.*, 2003a; Kim *et al.*, 2003b) – was the only bacterial species reported (Gennari *et al.*, 1999).

There is no information available concerning the identification of spoilage microflora in sardines stored in slurry ice. From the 14 bacterial strains isolated from sardine muscle stored in flake ice, slurry ice or ozonised slurry ice for 22 days and then subjected to room temperature (25°C), nine were selected for identification. These strains were chosen because they exhibited: (i) different phenotypes in the preliminary microbiological study, (ii) a proteolytic, lipolytic, H_2S-producing and/or histamine-producing phenotype, and (iii) relative abundance as compared with the whole microbial population. As can be seen in Table 5, nearly all the bacterial isolates identified exhibited proteolytic activity, most of them also exhibiting a histamine-producing and/or lipolytic activity. In addition, a few isolates were able to produce H_2S. *Proteus vulgaris* and *Staphylococcus sciuri* proved to be the predominant spoilers in sardine muscle. These two bacterial species have been isolated previously from other fish species. Thus, while Daczkiwsaka-Kozon *et al.* (2001) reported that *S. sciuri* represented 5.2 % of all the staphylococci isolated from marine fish, *S. sciuri* and

Serratia spp. have recently been isolated from a variety of farmed fish species (Melka *et al.*, 2003).

The ability to produce histamine by some of the bacterial isolates identified in this work was expected, since certain *Proteus* spp. –such as *P vulgaris*, *P. mirabilis* and *P. morganii*, now re-named as *M. morganii*– are well-known histamine producers (Kimata *et al.*, 1960; Middlebrooks *et al.*, 1988; Flick *et al.*, 2001; Kim *et al.*, 2003b). Moreover, Ababouch *et al.* (1991) reported that certain *Proteus* spp. isolated from sardine produced histamine concentrations between 100 and 2,000 ppm. Although sardine is not the most common fish associated with histamine poisoning, this fish species has occasionally been involved in outbreaks (Flick *et al.*, 2001; Kim *et al.*, 2003b).

4. Conclusions

Storage of sardine in slurry ice –alone or in combination with ozone– improves the sensory, microbiological and biochemical quality of this fish species as compared with storage in conventional flake ice, a result that implies a significant extension of the shelf life of this fish species. Of special relevance are the significant reductions of the psychrotrophic bacteria –both in muscle and– as well as of proteolytic and lipolytic bacteria. *Proteus vulgaris* and *Staphylococcus sciuri* were identified as the main spoilers isolated from sardine muscle. On the basis of the results obtained, the use of slurry ice –either alone or combined with ozone– for the refrigerated storage of sardine is advisable.

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Legends to the Figures

Figure 1. External aspect of sardines stored in ozonised slurry ice, slurry ice, or flake ice at days 8 (A) and 15 (B).

Figure 2. Evolution of microbial growth in sardine muscle along refrigerated storage using flake ice (o), slurry ice (ڤ) and ozonised slurry ice (Δ). Panel A: mesophiles; panel B: anaerobes; panel C: coliforms; panel D: psychrotrophic bacteria; panel E: lipolytic bacteria; panel F: proteolytic bacteria.

Figure 3. Evolution of surface microbial growth on sardine skin along refrigerated storage using flake ice (o), slurry ice (ڤ) and ozonised slurry ice (Δ). Panel A: mesophiles; panel B: psychrotrophic bacteria.

Figure 4. Evolution of chemical parameters in sardine muscle along refrigerated storage using flake ice (o), slurry ice (ڤ) and ozonised slurry ice (Δ). Panel A: pH; panel B: total volatile base nitrogen (TVB-N): panel C: trimethylamine nitrogen (TMA-N)

FIGURE 2

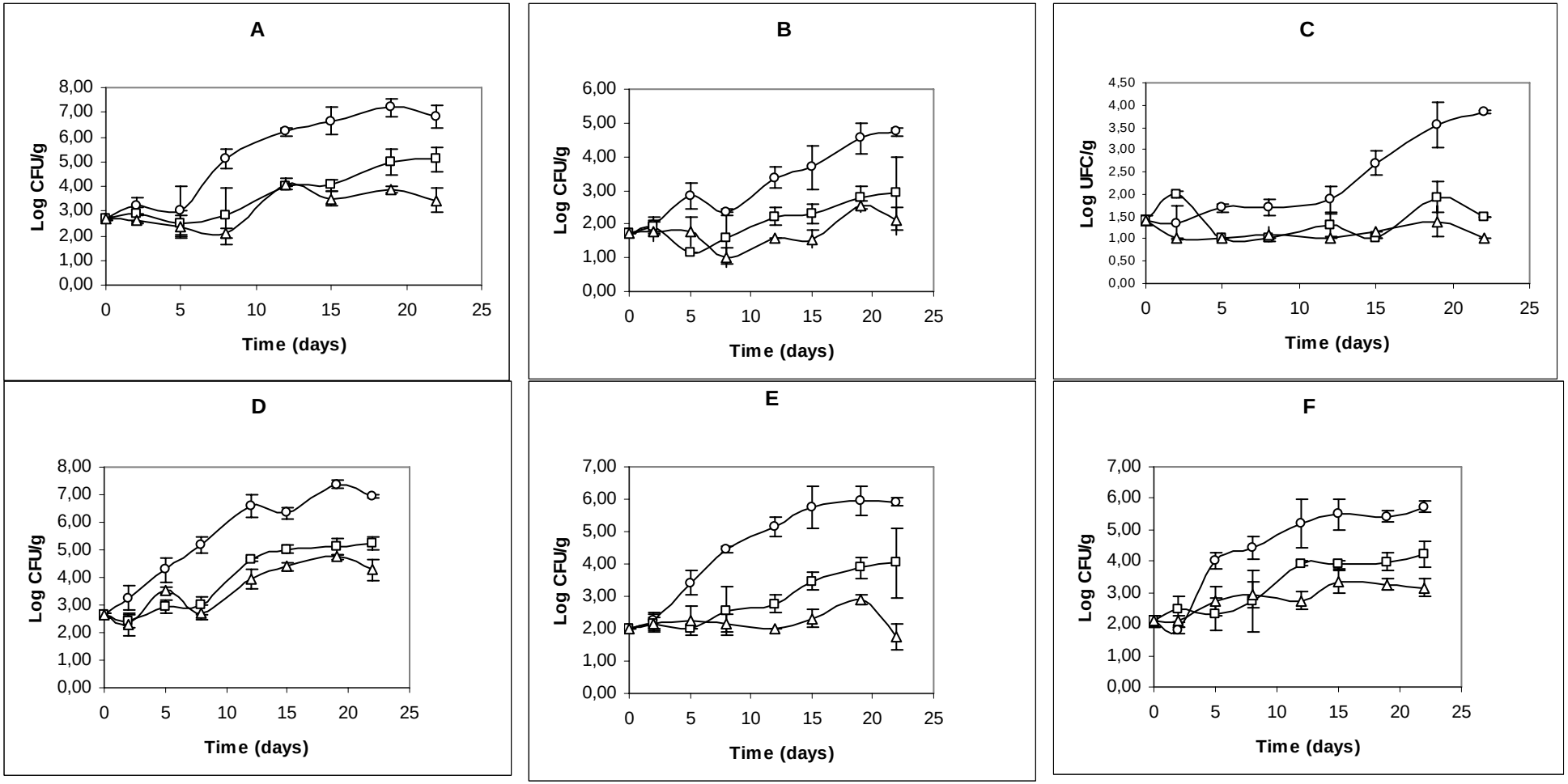
A
Log CFU/g
8,00
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
0
5
10
15
20
25
Time (days)
B
Log CFU/g
6,00
5,00
4,00
3,00
2,00
1,00
0,00
Time (days)
C
Log UFC/g
4,50
4,00
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
Time (days)
D
Log CFU/g
Time (days)
E
Log CFU/g
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
Time (days)
F
Log CFU/g
Time (days)

FIGURE 3

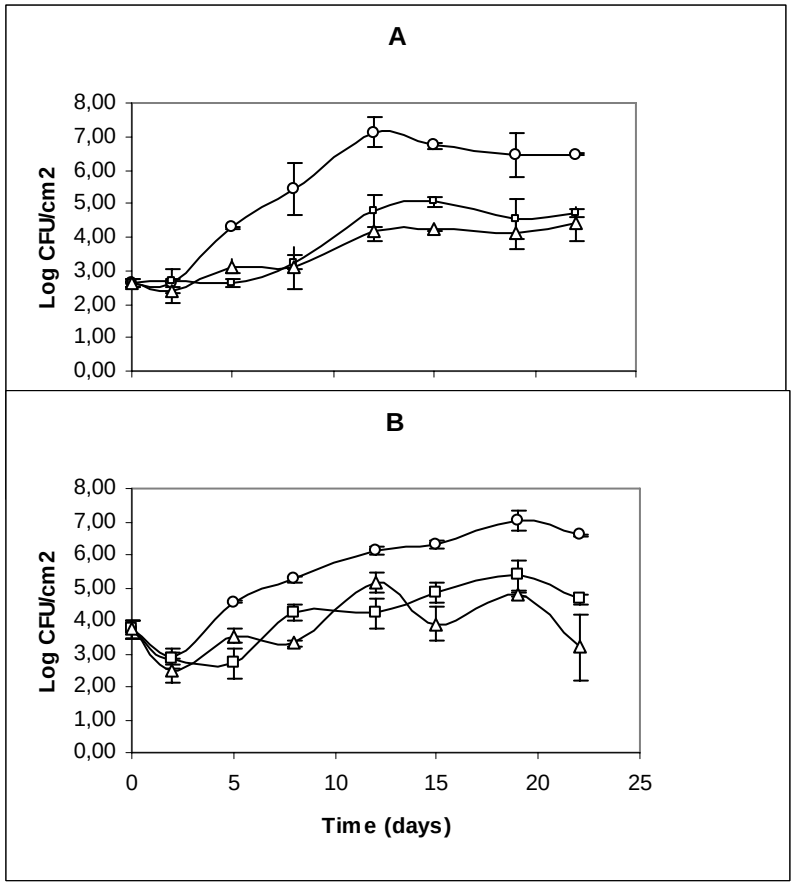

FIGURE 4

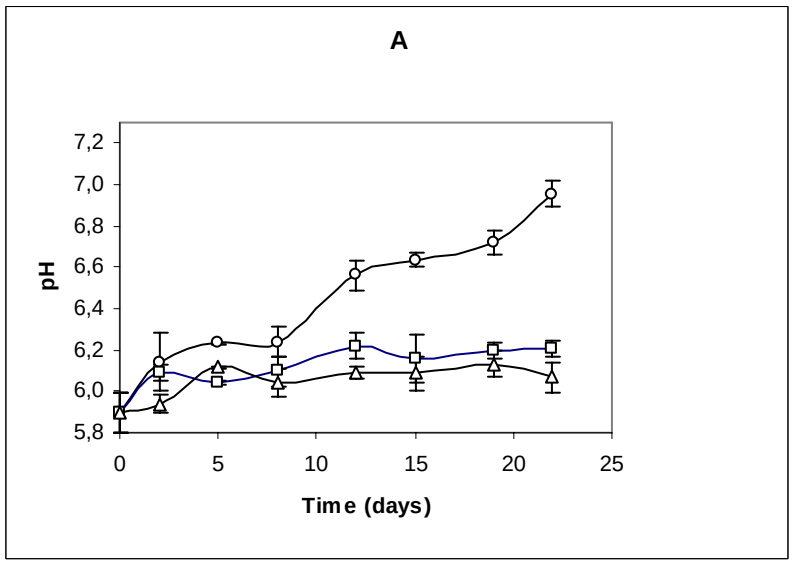

Table 1. Scale employed for evaluating the sensory quality of sardine batches.

Attribute	Highest quality (E)	Good quality (A)	Fair quality (B)	Unacceptable (C)
Skin	Very intense pigmentation; transparent mucus	Milky mucus; insignificant pigmentation losses	Slightly greyish mucus; pigmentation without shine	Widely opaque mucus; important pigmentation losses
External odor	Sharply seaweedy and shellfish	Weakly seaweedy and shellfish	Incipiently sour and putrid	Sour and putrid
Gills	Brightly red; without odor; lamina perfectly separated	Rose colored; without odor; lamina adhered in groups	Slightly pale; incipient fishy odor; lamina adhered in groups	Grey-yellowish color; intense ammonia odor; lamina totally adhered
Consistency	Presence or partial disappearance of rigor mortis symptoms	Firm and elastic; pressure signs disappear immediately and completely	Presence of mechanical signs; elasticity notably reduced	Important shape changes due to mechanical factors
Flesh odor	Sharply seaweedy and shellfish	Weakly seaweedy and shellfish	Incipiently sour and putrid	Sour and putrid

Table 2. Comparative sensory acceptability of sardine batches.

	Ozonised slurry ice (days of storage)								Slurry ice (days of storage)							Flake ice (days of storage)						
	0	2	5	8	12	15	19	22	2	5	8	12	15	19	22	2	5	8	12	15	19	22
Skin aspect	E	E	E	A	B	B	B	B	E	E	A	B	B	B	B	A	A	B	C	C	C	C
External odor	E	E	A	A	B	B	B	C	E	A	A	B	B	C	C	A	A	C	C	C	C	C
Gills	E	E	A	A	A	B	C	C	E	A	A	B	C	C	C	E	A	B	B	C	C	C
Eyes	E	E	A	A	B	B	C	C	E	A	B	B	C	C	C	A	B	C	C	C	C	C
Consistency	E	E	E	A	A	A	B	C	E	E	A	A	B	B	C	E	A	B	B	C	C	C
Flesh odor	E	E	A	A	B	B	B	C	E	A	A	B	B	C	C	A	A	C	C	C	C	C

Table 3. Effects of the type of storage system used on Mean microbial counts in muscle (log CFU/g) and skin* (log CFU/cm^2).

	Storage system		
	Ozonised slurry ice	Slurry ice	Flake ice
Aerobic mesophiles	3.16[a]	3.95[b]	5.00[c]
Anaerobes	1.81[a]	2.30[b]	3.11[c]
Coliforms	1.13[a]	1.44[b]	2.22[c]
Proteolytic bacteria	2.79[a]	3.48[b]	4.20[c]
Lipolytic bacteria	2.17[a]	3.10[b]	4.27[c]
Psychrotrophic bacteria	3.61[a]	4.21[b]	5.29[c]
Psychrotrophic* bacteria	3.92 [a]	4.34[b]	5.23[c]
Aerobic mesophiles*	3.56[a]	4.08[b]	5.13[c]

*Surface counts determined on skin. For each parameter, all mean values followed by different superscripts are significantly different at the $p< 0.05$ level.

Table 4. Effects of the type of storage system used on mean values of the chemical parameters studied.

	Storage system		
	Ozonised slurry ice	Slurry ice	Flake ice
pH	3.16[a]	3.95[b]	5.00[c]
TMA-N	1.02[a]	1.45[b]	2.80[c]
TVB-N	32.22[a]	32.80[a]	43.13[b]

For each parameter, all mean values followed by different superscripts are significantly different ($p < 0.05$).

Table 5. Identification of spoilage microorganisms isolated from sardine muscle stored in slurry ice.

Strain	Storage conditions	Spoilage activity	Bacterial species
1	Ozonised slurry ice*	P, L, H_2S, His	*Proteus vulgaris*
2	Ozonised slurry ice*	L, H_2S, His	*Proteus vulgaris*
3	Ozonised slurry ice*	P, L, H_2S, His	*Proteus penneri*
4	Slurry ice*	P, His	*Proteus vulgaris*
5	Slurry ice*	P, His	*Staphylococcus sciuri*
6	Slurry ice*	P, His	*Staphylococcus sciuri*
7	Flake ice*	P, L, His	*Serratia liquefaciens*
8	Flake ice*	P, L	*Staphylococcus haemolyticus*
9	Flake ice*	P, His	*Staphylococcus sciuri*

*All isolates were recovered from sardine stored for 22 days and then subjected to 25°C for 3 days. P: proteolytic activity; L: lipolytic activity; H_2S: production of H_2S; His: histamine production.

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