Bibiana García-Soto[1], Santiago P. Aubourg[3], Calo-Mata, P., Jorge Barros-Velázquez[2]/

Extension of the shelf life of chilled hake (*Merluccius merluccius*) by a novel icing medium containing natural organic acids

Running Title: Chilled hake and citric and lactic acids
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1. INTRODUCTION

Marine foods deteriorate rapidly post-mortem as a consequence of a variety of biochemical and microbial breakdown mechanisms, although the rate of quality loss depends directly on the nature of the fish species in question and on the handling and storage conditions used (Whittle et al., 1990; Ashie et al., 1996). To retard fish damage as long as possible, and accordingly extend shelf life, flake ice chilling has been the most employed method. However, and according to the actual need for high-quality fresh products, flake ice has been combined to other preservative strategies such as previous chemical (Manju et al., 2007; Özogul et al., 2011) or physical (González-Fandos, E., Villarino-Rodríguez, A., García-Linares, M. C., García-Arias, M. T., & García-Fernández, M. C.,2005; Briones et al., 2010) treatment, employment of preservative packaging (Ozen and Floros, 2001; Sivertsvik et al. 2002) and presence in the icing medium of preservative compounds (Oral et al., 2008) or plant extracts (Quitral et al., 2009; Özyurt et al., 2012) in the ice.

In this context, natural low molecular weight organic acids and their sodium salts represent a relevant choice because of their easy availability, low commercial cost and wide range of permitted concentrations for their use. Thus, citric acid (CA) is widely known for its role as chelator and acidulant in biological systems; its presence has led to a profitable effect on fish fillets (Badii and Howell 2002; Kilinc et al., 2009) and whole fish (Aubourg and others 2004) quality. Further, lactic acid (LA) has been reported to be effective in preserving and extending shelf-life in fish fillets (Kim and others 1995; Metin and others 2001), coated fish (Gogus and others 2006) and fish slices (Sallam 2007). Finally, the presence of both acids in the icing medium employed as refrigeration system has led to a damage inhibition in fatty fish species such as mackerel (Sanjuás-Rey et al., 2012) and horse mackerel (Sanjuás-Rey et al., 2011).

Gadiforms are a large group of fish species (cod, hake, pollack, haddock, whiting, saithe, etc.) that represent an important percentage of the overall fish catching and consumption in most European countries. Thus, some members of the genus *Merluccius* merit considerable appreciation as freshly consumable products because of their excellent sensory features. Among them, *Merluccius merluccius*, commonly known as European hake, is the most appreciated species. According to its easy deterioration, previous research accounts for different attempts to enlarge its shelf life time under refrigerated conditions such as ice slurries (Rodríguez et al., 2004), ozonized ice slurry (Campos et al., 2005), controlled and modified atmospheres (Ruiz-Capillas et al., 2001), active packaging (Pereira de Abreu et al., 2011) and high hydrostatic pressure (Vidacek et al., 2009).

The present research focuses on the quality retention of European hake during chilling storage. With the aim of enhancing its quality, aqueous solutions including CA

and LA were employed as icing media. Their effects on microbial activity inhibition, lipid oxidation stability and sensory acceptance were monitored up to 13 d of chilled storage.

2. MATERIALS AND METHODS

2.1. Icing systems

Three aqueous solutions including the following concentration (w/v) values of CA and LA, respectively, were prepared: 0.075% and 0.050% (condition C-75), 0.125% and 0.050% (condition C-125) and 0.175% and 0050% (condition C-175). All solutions were packed in polythene bags and kept frozen at –20ºC until use. Traditional ice was prepared starting only from water (condition C-0; control) that was packed and kept frozen in the same way as the ones including both organic acids. Before addition to individual fishes, the different ices were ground to obtain common flakes. Organic acids encountered in the present research are regarded as safe (GRAS) for use in foods according to European and American administrations (Madrid et al., 1994; Giese, 1996).

Preliminary trials were carried out in order to establish a convenient concentration of CA and LA to be used to prepare the ice. Thus, solutions combining the two organic acids in the 0.005-0.250% concentration range were preliminary tested. Possible fish appearance modifications as a result of the acids presence in the ice were evaluated. According to the results obtained, the above-mentioned combinations were chosen as being the most appropriated to be employed for further investigation.

2.2. Fish material, processing and sampling

Fresh European hake (*Merluccius merluccius*) (153 individuals) were caught near the Galician Atlantic coast (north-western Spain) and transported on ice to the laboratory ten hours after catching. The length and weight of the fish specimens were included in the following ranges: 32 to 35 cm and 180 to 210 g, respectively.

Upon arrival in the laboratory, nine individual fishes were separated and analysed as starting raw fish (day 0); for it, three different groups (three individuals per group) were analysed independently in order to achieve the statistical analysis (n = 3). The remaining fish were divided into four batches (36 individuals in each batch), placed in boxes and directly surrounded, respectively, by the four kinds of ices previously mentioned (conditions C-0, C-75, C-125 and C-175); a 1:1 fish-to-ice ratio was employed. All batches were placed in a refrigerated room (4ºC). Boxes employed allowed draining and ice was renewed when required. Fish samples from all batches were taken for analysis on days 2, 6, 9 and 13. At each sampling time, nine individuals of each batch were taken for analysis, being considered into three groups (three individuals in each group) that were studied independently (n=3).

Sensory analysis was carried out on the whole fish, while microbiological and chemical analyses were carried out on the white muscle.

2.3. Sensory analyses

Sensory analysis was conducted by a sensory panel consisting of five experienced judges, according to traditional guidelines concerning fresh and refrigerated fish (Council Regulation, 1990). Sensory assessment of the fish included the following attributes: skin and mucus development, eye, external odor, gills appearance and color, consistency, flesh odor (raw and cooked) and flesh taste (cooked). Each attribute was ranked according to the following four categories: highest quality (E), good quality (A), fair quality (B), and unacceptable quality (C).

2.4. Microbiological analyses

Samples of 10 g of fish muscle were dissected aseptically from chilled fish specimens, mixed with 90 ml of 0.1% peptone water (Merck, Darmstadt, Germany), and homogenized in sterilized stomacher bags (AES, Combourg, France) as previously described (Ben-Gigirey and others 1998; Ben-Gigirey and others 1999). In all cases, serial dilutions from the microbial extracts were prepared in 0.1% peptone water.

Total aerobes were investigated by surface inoculation on plate count agar (PCA, Oxoid Ltd., London, UK), after incubation at 30ºC for 48 h. The anaerobe counts were also determined in PCA at 30ºC, except that an anaerobic atmosphere kit (Oxoid) was placed together with the plates inside the anaerobiosis jar. Psychrotrophes were also investigated in PCA but incubation was carried out at 7-8ºC for 7 days. Enterobacteriaceae were investigated by pour plating using Violet Red Bile Agar (VRBA) (Merck, Darmstadt, Germany) after incubation at 37ºC for 24 h. Microorganisms exhibiting a proteolytic phenotype were investigated in casein-agar medium after incubation at 30ºC for 48 h, as previously described by Ben-Gigirey and others (2000). In all cases, bacterial counts were transformed into log CFU/g muscle before undergoing statistical analysis. All analyses were done by triplicate.

2.5. Chemical analyses

The evolution of pH values in hake muscle along storage time was determined by means of a 6-mm diameter insertion electrode (Crison, Barcelona, Spain).

Trimethylamine-nitrogen (TMA-N) values were determined by the picrate method, as previously described by Tozawa and others (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.

Lipids were extracted from the fish muscle by the Bligh and Dyer (1959) method, by employing a single-phase solubilization of the lipids using a chloroform-methanol (1:1) mixture. Quantification results were expressed as g lipid/100 g muscle.

Free fatty acid (FFA) content was determined in the lipid extract of the fish muscle by the Lowry and Tinsley (1976) method based on complex formation with cupric acetate-pyridine followed by spectrophotometric (715 nm) assessment. Results were expressed as g FFA/100g lipids.

The peroxide value (PV) was determined spectrophotometrically (Beckman Coulter, DU 640, London, UK) in the lipid extract by previous peroxide reduction with ferric thiocyanate, according to the Chapman and McKay (1949) method. Results were expressed as meq active oxygen/kg lipids.

The thiobarbituric acid index (TBA-i) was determined according to Vyncke (1970). This method is based on the reaction between a trichloracetic acid extract of the fish muscle and thiobarbituric acid. Content of thiobarbituric acid reactive substances (TBARS) was spectrophotometrically measured at 532 nm and calculated from a standard curve using 1,1,3,3-tetraethoxy-propane (TEP); results were expressed as mg malondialdehyde/kg muscle.

Formation of fluorescent compounds (Fluorimeter LS 45; Perkin Elmer España, Tres Cantos, Madrid, Spain) was determined by measurements at 393/463 nm and 327/415 nm as described by Aubourg (1999). The relative fluorescence (RF) was calculated as follows: $RF = F/F_{st}$, where F is the fluorescence measured at each excitation/emission maximum, and F_{st} is the fluorescence intensity of a quinine sulfate solution (1 µg/ml in 0.05 M H_2SO_4) at the corresponding wavelength. The fluorescence ratio (FR) was calculated as the ratio between the two RF values: $FR = RF_{393/463\ nm}$ /

$RF_{327/415\ nm}$. The FR value was determined in the aqueous phase resulting from the lipid extraction of the fish muscle (Bligh and Dyer, 1959).

2.6. Statistical analysis

Data obtained from the different microbial and chemical analyses were subjected to the ANOVA method to explore differences by two different ways: icing conditions effect and chilling time effect. For it, the PASW Statistics 18 software for Windows (SPSS Inc., Chicago, IL, USA) was employed. The comparison of means was performed using the least-squares difference (LSD) method. Differences between batches and among icing times were considered significant for a confidence interval at the 95% level ($p<0.05$) in all cases.

3. RESULTS AND DISCUSSION

3.1. Microbiological analyses

The development of aerobic mesophiles in hake muscle throughout storage is shown in Figure 1. The results revealed statistically-significant ($p<0.05$) differences between the control batch (C-0) and the batches corresponding to the icing systems including CA and LA. Special protection of fish microbial quality was exerted by the C-175 batch, which, unlike the other batches, did not exhibit counts above 7 log CFU/g even after 13 d of storage. It should also be remarked that the differences between the microbial counts of C-175 and C-0 batches were above 2 log CFU/g on sampling days 6 and 9, this revealing a significantly high inhibition of the growth of this microbial group in the batch including the highest concentration of organic acids.

The anaerobes counts also revealed a statistically-significant ($p<0.05$) slowing down in the batches corresponding to the organic acids icing system, as compared with the C-0 batch (Figure 2). As in the case of the aerobic mesophiles, these differences were higher when comparing C-0 and C-175 batches, such differences being as high as 2.01 and 2.70 log CFU/g after 6 and 13 d of storage, respectively. Thus, and as in the case of the aerobes assessment, and even to a higher extent, the storage of hake in the organic acid icing system implied a better protection of the microbial quality of hake.

With respect to the psychrotrophes, the results of this study also indicated some benefitial effects derived from the use of the organic acid icing system (Figure 3). However, stastitically-significant ($p<0.05$) differences were only observed when comparing C-0 and C-175 batches at moderately advanced storage times (6 and 9 d). Thus, average differences between both batches rose up to 1.21 and 1.54 log CFU/g after 6 and 9 d of storage, respectively. C-125 and C-150 batches also provided lower microbial counts as compared to the C-0 batch, although the differences among them were not statistically-significant ($p>0.05$) in any case.

The investigation of Enterobacteriaceae revealed low numbers for all batches, all counts being below 2.65 log CFU/g even after 13 d of storage (Table 1). However, the batches corresponding to the organic acid icing system provided lower counts for this microbial group, as compared to the C-0 bath. Moreover, the differences between C-0 and C-175 batches proved to be statistically-significant ($p<0.05$) at advanced storage times, with differences of 0.79 and 0.89 log CFU/g between batches at days 6 and 9, respectively. Remarkably, and unlike the other batches, the counts of Enterobacteriaceae were always below 2 log CFU/g in the C-175 batch, even after 13 d of chilled storage.

Finally, the study also included the investigation of proteolytic bacteria, a microbial group whose role in the spoilage of fish muscle has been reported (Rodríguez, Barros-Velazquez, Ojea, Pineiro, & Aubourg, 2003; Rodríguez, Losada, Aubourg &

Barros-Velazquez, 2004; Campos et al., 2005). Thus, the inhibition of proteolytic bacteria in hake muscle by the organic acids included in the icing system would be a remarkable result in terms of fish quality. The results evidenced that the presence of CA and LA in the icing system inhibited the growth of this microbial group, with statistically-significant ($p<0.05$) differences being determined between C-0 batch and both C-150 and C-175 batches (Table 1). Remarkably, the highest differences between batches were observed when comparing C-0 and C-175 batches, such differences rising up to 1.07 and 0.90 log CFU/g after 9 and 13 days of storage, respectively.

The results of this study revealed that the C-175 batch, including ice crystals formed with a 0.175% solution of CA and a 0.050% of LA, provided a significantly ($p<0.05$) reduction of the growth of all microbial groups considered in this study (Table 1, Figures 1-3), and led to a better maintenance of microbial quality of hake even after 13 d of chilled storage.

Previous reports on the enhanced preservation of hake and other marine species under advanced refrigeration systems, such as ice slurries prepared from marine water, proved that the melting of the ice crystals during chilled storage exerted a washing effect of the salts solution on the fish surface, this reducing both the surface contamination of fish and bacterial diffusion towards the muscle (Losada et al., 2004; Rodríguez, Losada, Aubourg, & Barros-Velázquez, 2004; Campos et al., 2005). In the present study, the melting of the ice crystals containing the natural organic acid solution, especially in the C-175 batch, might work in the same way and exert a similar washing effect, which the subsequent reduction of the surface microbial load and its diffusion to the muscle.

This study also supports a previous work on novel preservation methods for chilled hake, developed at our laboratory. Such work included the evaluation of a commercial icing system prepared with an aqueous formula including CA, LA and ascorbic acid (AA) (Sanjuás-Rey, García-Soto, Fuertes-Gamundi, Aubourg & Barros-Velázquez, 2012lwt); as a result, an inhibitory effect on aerobic mesophiles formation could be observed in hake muscle as well as in megrim (*Lepidorhombus whiffiagonis*) and angler (*Lophius piscatorius*) flesh. Likewise, other studies have also reported the usefulness of including preservative compounds in the icing system for the inhibition of microbial development. These account for an organic acid mixture (CA, LA and AA) during chilled storage of mackerel (*Scomber scombrus*) (Sanjuás-Rey et al., 2012jfs) and a wild-thyme hydrosol extract in chilled Transcaucasian barb (*Capoeta capoeta capoeta*) (Oral et al., 2008).

3.2. Chemical analyses

Table 2 shows the results obtained for the pH assessment. Treated fish (C-75, C-125 and C-175 conditions) did not show a definite tendency with time. However, control samples showed a progressive increase with time ($p<0.05$), in agreement with the increased microbial activity development revealed from the microbiological parameters (Table 1 and Figures 1-3). Differences among icing treatments were scarce and a definite effect of the acid presence in the ice on the pH value of the hake muscle can not be concluded ($p>0.05$).

It is widely accepted that increases in pH in fish muscle denote the accumulation of undesirable alkaline compounds, such as ammonia and trimethylamine (TMA), which are mostly derived from microbial action. In this sense, it has been suggested that pH values above 7 may limit the consumption of certain lean fish species such as hake (Ruiz-C and Moral, 2001, FRI 34, 441-447); in the present research, pH values measured throughout the chilled storage did not attain such score.

In agreement with the present results, the employment of a commercial formula including CA, LA and AA in the icing system did not provide any changes in the pH value throughout the chilled storage of hake muscle (Sanjuás-Rey et al., 2012). However, other studies have reported a pH decrease as a result of including preservative compounds in ice during the chilled storage of other marine species. These account for oregano and rosemary extracts during the chilled storage of Chilean jack mackerel (*Trachurus murphyi*) (Quitral et al., 2009), a rosemary extract applied to sardine (*Sardionella aurita*) (Özyurt et al., 2012) and a wild-thyme hydrosol extract employed in the chilled storage of Transcaucasian barb (*Capoeta capoeta capoeta*) (Oral et al., 2008).

TMA formation was found very low in all kinds of samples during the 0-9-day period (Table 2). After this time, a marked increase ($p<0.05$) could be observed in all cases, so that fair quadratic correlation values with chilling time were obtained for the different kinds of samples ($r = 0.86$-0.89). However, none of the different kinds of conditions analyzed led to overpassed values according to the legal limits established for this species (5 mg TMA-N/100g muscle; Baixas-Nogueras et al., 2003). Icing treatment showed an inhibitory effect ($p<0.05$) on TMA-N content, this increasing with the CA presence in the ice. Volatile amine compounds such as TMA have been reported to be produced partially by means of endogenous enzyme activity, but mostly as a result of microbial development (Whittle et al., 1990; Ashie et al., 1996). In the present case, the organic-acid icing effect on TMA formation is in agreement with the inhibitory effect found in the different microbial measurements (aerobes, anaerobes, psychrotrophes, proteolytics and Enterobacteriaceae; Figures 1-3 and Table 2).

Previous research shows the inhibitory effect on TMA content as a result of including preservative components in the icing system. Thus, the presence of CA, LA and AA led to a lower TMA formation in chilled mackerel (Sanjuás-Rey et al., 2012jfs). When the same organic acids were included in a commercial formula and applied in the icing system, an inhibitory effect on TMA content was produced in hake and angler during the chilled storage (Sanjuás et al., 2012).

A progressive FFA formation ($p<0.05$) could be observed throughout storage for all kinds of samples (Table 2); thus, fair linear correlation values with chilling time were obtained for the different kinds of samples ($r = 0.83$-0.93). Differences as a result of the icing condition could only be observed for the 9-13-day period; however, a definite effect of the acid presence and concentration on the FFA content in hake muscle could not ($p>0.05$) be concluded.

FFA formation during fish chilling storage has been reported to be produced as a result of endogenous enzyme activity and microbial activity (Whittle et al., 1990; Ashie et al., 1996). Before the end of the microbial lag phase (up to 6-9 days, depending on several factors), FFA formation should be produced mostly as a result of endogenous enzyme (namely, lipases and phospholipases) activity. Later on, microbial activity should gain importance as a result of bacterial catabolic processes. The effectiveness lack found in the present study for the organic-acid icing system in preventing the lipid hydrolysis development could be explained on the basis of a predominance of the FFA formation by means of the endogenous enzyme pathway.

According to the actual results, the presence of CA, LA and AA in the icing system did not lead to an inhibitory effect on FFA formation during the chilled storage of horse mackerel (*Trachurus trachurus*) (Sanjuás-Rey et al., 2011); the same conclusion was obtained by Özyurt et al. (2012) by employing a rosemary extract during the chilling storage of sardine. Contrary, an inhibitory effect on fish FFA

formation could be observed by applying a rosemary or oregano extract in chilled Chilean jack mackerel (Quitral et al., 2009).

No formation of peroxides was evident in the 0-6-day period, showing a general marked increase ($p<0.05$) at day 9 (Table 3) and followed by no changes at the end of the experiment. Values can be considered as not specially high since all values remained below the 9.0 score. Concerning the icing effect, higher mean values were obtained throughout the whole experiment for samples corresponding to the C-125 condition; however, scarce significant differences could be outlined, so that a definite effect of the ice employed could not be assessed ($p>0.05$) on the primary oxidation compounds level.

Formation of TBARS was low throughout the chilling storage, being all values included in the 0.18-0.60 range (Table 3). It is concluded that an important development of secondary lipid oxidation compounds did not occur, according to values obtained for the primary oxidation development. A definite effect of icing condition on TBARS formation could also not be concluded ($p>0.05$). Thus, a TBARS formation inhibition was observed at day 2 as a result of the acid presence in the ice; however, at the end of the storage a higher formation of secondary oxidation compounds was observed as a result of applying the highest CA concentration.

Fluorescent compound formation was low in the 0-9-day period, so that FR scores remained in the 0.30-0.63 range in all kinds of samples (Table 3). After that time, a marked general increase ($p<0.05$) was evident. An inhibitory effect ($p<0.05$) of the acid presence in ice could be concluded at the end of the experiment on the fluorescent compound formation, according to the following decreasing sequence: C-0 > C-75 > C-125, C-175.

Lipid oxidation has been recognised as a complex process where different kinds of molecules are produced, most of them unstable, susceptible to breakdown and originate lower weight compounds, or react with other molecules present in the fish muscle; as a result, determination of each kind of compound cannot always provide an accurate method for the quality loss assessment in fish.

In the present research, a low primary and secondary lipid oxidation compound formation could be assessed, according to the common knowledge that lipid oxidation development is not a markedly important damage pathway during the chilled storage of a lean (lipid content range in the present experiment: 0.55-0.63 g/100g muscle) fish species (Whittle *et al*., 1990; Losada *et al*., 2004). The electrophilic character of such lipid oxidation compounds has led them to interact with food constituents possessing nucleophilic functions (Howell, 1995; Aubourg, 1999), so that FR evaluation (tertiary lipid oxidation compounds) has afforded in the present research the most accurate assessment of lipid oxidation development. Additionally, a fair linear correlation was observed between FR and TMA-N values ($r = 0.83$-0.93), so that a good agreement can be inferred between microbial activity development and lipid oxidation evolution.

Previous research accounts for an inhibitory effect on fish lipid oxidation (PV and TBA-i assessments) as a result of including preservative compounds in the icing system. This concerns Chilean jack mackerel as a result of including an oregano or rosemary extract in the icing system (Quitral et al., 2009), horse mackerel by applying an ice including an organic acid mixture (CA, LA and AA) (Sanjuás-Rey et al., 2011) and sardine by including a rosemary extract during chilled stored of sardine (Özyurt et al., 2012).

3.3. Sensory analyses

Sensory evaluation of the different kinds of samples is expressed in Table 4. In agreement to the above-mentioned results on microbial activity development (microbial

counts and TMA formation), a general decreasing appreciation with chilling time was evident for the different attributes under study. Previous research on chilled European hake (Ruiz-Capillas and Moral, 2001; Baixas-Nogueras et al., 2002) observed longer shelf life times (20-25 days) than in the present work; this can be explained by the relative smaller size of the hake specimens examined in the actual research (Losada et al., 2004; Rodríguez et al., 2004).

A preservative effect of the acid mixture presence in the ice system could be proved. Thus, fish samples corresponding to the C-75 and C-0 conditions were not found acceptable by the panel at day 13; however, fish belonging to the C-125 and C-175 batches were still acceptable at that time. External odor, flesh odor (raw and cooked) and flesh taste were found as the limiting factors. A slight inhibitory effect was also concluded for the C-75 batch, since better scores were appreciated for eye (day 9), external odor (day 6) and gills (day 6) evaluation. Finally, some differences between samples corresponding to the two CA-highest content conditions were observed; thus, a better score was obtained for C-125 fish in eye (day 9) and flesh taste (day 2). Such differences, which are contrary to the results obtained for chemical and microbiological analyses, could be explained by means of a negative effect of a higher presence of CA in the icing medium.

An increased shelf life time has also previously observed as a result of including preservative compounds in the icing system. This is the case of chilled megrim, hake and angler by applying a commercial formula including CA, LA and AA in the ice (Sanjuás te al., 2012), a rosemary extract during chilled storage of sardine (Özyurt et al., 2013), oregano and rosemary extracts during the chilled storage of Chilean jack mackerel (Quitral et al., 2009), an acid mixture (CA, LA and AA) during the chilled storage of horse mackerel (Sanjuás-Rey et al., 2011) and wild-thyme hydrosol in chilled Transcaucasian barb (Oral et al., 2008).

CONCLUSIONS

Previous research accounts for positive effects on quality retention in chilled lean fish by including preservative compounds in the icing system. Such studies consist basically on commercial formulae and plant extracts. However, in the present case two organic natural acids are employed, widely known for their easy availability and low commercial cost. Results described here allow us to conclude that employment of a CA-LA-icing system can provide a profitable and practical strategy and a relevant choice in order to obtain higher quality and safer products, according to the actual demand for fresh products.

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FIGURE LEGENDS

Figure 1

Aerobic mesophile count (log CFU/g muscle) assessment* in chilled hake stored under different icing conditions**

* Mean values of three (n=3) replicates; standard deviations are indicated by bars. For each chilling time, values accompanied by different letters (A, B, C) denote significant differences ($p<0.05$) as a result of the icing condition. No indication is provided when no significant differences are found ($p>0.05$). Starting raw fish value: 2.46±0.03.

** Abbreviations of icing conditions: C-0 (Control; no acid presence in ice), C-75 (0.075% citric acid and 0.050% lactic acid), C-125 (0.125% citric acid and 0.050% lactic acid) and C-175 (0.175% citric acid and 0.050% lactic acid).

Figure 2

Anaerobe count (log CFU/g muscle) assessment* in chilled hake stored under different icing conditions**

* Mean values of three (n=3) replicates; standard deviations are indicated by bars. For each chilling time, values accompanied by different letters (A, B, C) denote significant differences ($p<0.05$) as a result of the icing condition. No indication is provided when no significant differences are found ($p>0.05$). Starting raw fish value: 1.98±0.28.

** Abbreviations of icing conditions as expressed in Figure 1.

Figure 3

Psychrotrophe count (log CFU/g muscle) assessment* in chilled hake stored under different icing conditions**

* Mean values of three (n=3) replicates; standard deviations are indicated by bars. For each chilling time, values accompanied by different letters (A, B) denote significant differences ($p<0.05$) as a result of the icing condition. No indication is provided when no significant differences are found ($p>0.05$). Starting raw fish value: 0.99±0.00.

** Abbreviations of icing conditions as expressed in Figure 1.

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TABLE 1

Evolution of Enterobacteriaceae and proteolytic bacteria (log CFU/g)* in chilled hake stored under different icing conditions**

Chilling time (days)	Enterobacteriaceae				Proteolytic bacteria			
	C-0	C-75	C-125	C-175	C-0	C-75	C-125	C-175
0	0.99 a (0.00)	0.99 a (0.00)	0.99 a (0.00)	0.99 a (0.00)	2.10 a (0.20)	2.10 a (0.20)	2.10 a (0.20)	2.10 a (0.20)
2	0.99 a (0.00)	0.99 a (0.00)	0.99 a (0.00)	0.99 a (0.00)	2.10 a (0.20)	1.99 a (0.00)	2.10 a (0.20)	1.99 a (0.00)
6	1.89 ab (0.81)	1.97 b (0.43)	1.42 ab (0.42)	1.33 ab (0.37)	3.49 Bb (0.07)	3.53 Bb (0.07)	3.02 Ab (0.24)	2.69 Aab (0.70)
9	2.61 Bb (0.30)	1.85 Ab (0.23)	2.15 Ac (0.15)	1.82 Ab (0.22)	4.56 Bc (0.19)	4.26 Bc (0.23)	3.65 Ab (0.39)	3.49 Ab (0.41)
13	2.25 Bb (0.60)	2.26 Bb (0.31)	1.64 Ab (0.20)	1.36 Aab (0.36)	5.36 Bd (0.55)	4.78ABd (0.12)	4.47ABc (0.23)	4.46 Ac (0.09)

* Mean values of three replicates (n = 3); standard deviations are indicated in brackets. For each parameter and for each chilling time, mean values followed by different capital letters (A-B) indicate significant ($p<0.05$) differences as a result of the icing condition. For each parameter and for each icing condition, values followed by different low-case letters (a-d) denote significant ($p<0.05$) differences as a result of the chilling time. No letters are indicated when significant differences are not found ($p>0.05$).

** Abbreviations of icing conditions: C-0 (Control; no acid presence in ice), C-75 (0.075% citric acid and 0.050% lactic acid), C-125 (0.125% citric acid and 0.050% lactic acid) and C-175 (0.175% citric acid and 0.050% lactic acid).

TABLE 2

Chemical quality parameters assessment* in chilled hake stored under different icing conditions**

Chilling time (days)	pH				Trimethylamine (TMA)				Free fatty acids (FFA)			
	C-0	C-75	C-125	C-175	C-0	C-75	C-125	C-175	C-0	C-75	C-125	C-175
0	6.50 a (0.15)	6.50 ab (0.15)	6.50 (0.15)	6.50 ab (0.15)	0.00 a (0.00)	0.00 a (0.00)	0.00 a (0.00)	0.00 a (0.00)	0.85 a (0.11)	0.85 a (0.11)	0.85 a (0.11)	0.85 a (0.11)
2	6.60 Aa (0.04)	6.69 Ab (0.05)	6.56 B (0.03)	6.63 ABb (0.06)	0.01 a (0.00)	0.00 a (0.00)	0.01 a (0.01)	0.01 a (0.02)	1.26 ab (0.38)	1.91 bc (0.39)	1.46 b (0.31)	1.58 bc (0.39)
6	6.61 a (0.03)	6.53 a (0.07)	6.62 (0.08)	6.57 ab (0.07)	0.02 a (0.01)	0.02 b (0.01)	0.02 ab (0.02)	0.00 a (0.01)	1.43 b (0.31)	1.68 b (0.08)	1.81 b (0.23)	1.35 b (0.34)
9	6.79 ab (0.18)	6.62 ab (0.09)	6.56 (0.05)	6.50 ab (0.15)	0.19 Bb (0.07)	0.07 Ac (0.03)	0.05 Ab (0.01)	0.08 Ab (0.02)	2.53 Bc (0.41)	2.44ABbc (0.81)	2.58 Bc (0.41)	1.98 Ac (0.05)
13	6.84 Bb (0.08)	6.56 Bab (0.19)	6.58 AB (0.14)	6.37 Aa (0.18)	1.80 Cc (0.35)	0.95 Bd (0.21)	1.14 Bc (0.24)	0.50 Ac (0.19)	3.12 ABc (0.42)	2.46 Ac (0.54)	3.67 Bd (0.41)	3.02 ABc (1.18)

* Mean values of three replicates (n = 3); standard deviations are indicated in brackets. For each parameter and for each chilling time, mean values followed by different capital letters (A-C) indicate significant ($p<0.05$) differences as a result of the icing condition. For each parameter and for each icing condition, values followed by different low-case letters (a-d) denote significant ($p<0.05$) differences as a result of the chilling time. No letters are indicated when significant differences are not found ($p>0.05$).

** Abbreviations of icing conditions as expressed in Table 1.

TABLE 3

Lipid oxidation assessment* in chilled hake stored under different icing conditions**

Chilling time (days)	Peroxide value (PV)				Thiobarbituric acid index (TBA-i)				Fluorescence ratio (FR)			
	C-0	C-75	C-125	C-175	C-0	C-75	C-125	C-175	C-0	C-75	C-125	C-175
0	3.95 a (0.21)	3.95 a (0.21)	3.95 a (0.21)	3.95 b (0.21)	0.18 a (0.03)	0.18 a (0.03)	0.18 a (0.03)	0.18 a (0.03)	0.51 a (0.07)	0.51 a (0.07)	0.51 ab (0.07)	0.51 a (0.07)
2	3.24 a (0.95)	3.66 a (0.71)	4.28 a (1.16)	3.60 ab (0.65)	0.33 Bb (0.10)	0.16 Aa (0.04)	0.14 Aa (0.01)	0.14 Aa (0.08)	0.52 ABa (0.16)	0.48 ABa (0.07)	0.63 Bab (0.11)	0.44 Aa (0.06)
6	3.29 ABa (0.86)	3.22 ABa (0.97)	4.75 Ba (1.22)	2.11 Aa (0.34)	0.27 ab (0.08)	0.25 ab (0.05)	0.28 b (0.04)	0.27 a (0.07)	0.66 Ba (0.25)	0.31 ABa (0.19)	0.52ABab (0.11)	0.35 Aa (0.04)
9	6.72 ABb (2.05)	6.39 Ab (0.87)	8.72 Bb (0.53)	7.94 ABc (1.19)	0.30 b (0.05)	0.29 ab (0.13)	0.39 b (0.08)	0.44 b (0.08)	0.50 a (0.23)	0.30 a (0.16)	0.45 a (0.09)	0.33 a (0.12)
13	5.04 Aab (0.91)	5.66 ABb (1.23)	7.56 Bb (1.14)	7.12 ABc (1.37)	0.29 Aab (0.07)	0.28 Ab (0.03)	0.34 Ab (0.08)	0.59 Bb (0.16)	1.77 Cb (0.49)	0.96 Bb (0.11)	0.74 Ab (0.17)	0.76 Ab (0.07)

* Mean values of three replicates (n = 3); standard deviations are indicated in brackets. For each parameter and for each chilling time, mean values followed by different capital letters (A-C) indicate significant ($p<0.05$) differences as a result of the icing condition. For each parameter and for each icing condition, values followed by different low-case letters (a-c) denote significant ($p<0.05$) differences as a result of the chilling time. No letters are indicated when significant differences are not found ($p>0.05$).

** Abbreviations of icing conditions as expressed in Table 1.

TABLE 4

Assessment of sensory acceptance* in chilled hake stored under different icing conditions**

Sensory attribute	Chilling time (days)	Icing condition			
		C-0	C-75	C-125	C-175
Skin	2	A			
	6	A			
	9	A			
	13	B			
Eye	2	A			
	6	A			
	9	B	A		B
	13	B			
External odour	2	A			
	6	B	A		
	9	B		A	
	13	C		B	
Gills	2	A			
	6	B	A		
	9	B			
	13	B			
Consistency	2	A			
	6	A			
	9	B			
	13	B			
Flesh odour (raw)	2	A			
	6	A			
	9	B			
	13	C		B	
Flesh odour (cooked)	2	A			
	6	A			
	9	B			
	13	C		B	
Flesh taste (cooked)	2	A			B
	6	A			
	9	B			
	13	C		B	

* Quality categories: E (excellent), A (good), B (fair) and C (unacceptable). Starting fish (day 0) was category E in all attributes.

** Abbreviations of icing conditions as expressed in Table 1.