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4            Microbial activity inhibition in chilled mackerel  
5            (*Scomber scombrus*) by employment of an organic  
6                                    acid-icing system

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28 **ABSTRACT**

29

30 The present work concerns Atlantic mackerel (*Scomber scombrus*) traded as a  
31 chilled product. The study was aimed to investigate the effect of including a mixture of  
32 organic acids (citric, ascorbic and lactic) in the icing medium employed during the fish  
33 chilled storage. To this end and according to preliminary trials results, an aqueous  
34 solution including 0.050 % (w/v) of each acid was employed as icing medium; its effect  
35 on the microbial activity development in mackerel muscle was monitored for up to 13  
36 days of chilled storage and compared to a counterpart-fish batch kept under traditional  
37 water ice considered as control. Results indicated a lower bacterial growth in mackerel  
38 muscle subjected to storage in the organic acid-icing system by comparison with control  
39 fish. Thus, statistically-significant ( $p < 0.05$ ) differences between both batches for all six  
40 microbial groups investigated (aerobes, anaerobes, psychrotrophes, Enterobacteriaceae,  
41 lipolytics and proteolytics) and for two chemical indices related to microbial activity  
42 development (total volatile bases and trimethylamine) were obtained. The surface wash  
43 caused by the melting of the ice during storage and the subsequent antimicrobial effect  
44 of such acids on skin microflora of the fish can be invoked as the main reasons for the  
45 limited bacterial growth found in the corresponding mackerel muscle.

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48 **Running Head:** Microbial inhibition in chilled mackerel.

49 **Keywords:** *Scomber scombrus*, ascorbic, citric, lactic, chilling, microbial activity.

50

51 **PRACTICAL APPLICATION**

52

53 Among natural antioxidants, citric, ascorbic and lactic acids are low molecular  
54 weight organic compounds that represent a relevant choice because of their easy  
55 availability, low commercial cost and wide range of permitted concentrations for their  
56 use in foods. Present results obtained by their inclusion in a novel icing system have led  
57 to a lower microbial development in chilled mackerel when compared to its counterpart  
58 fish kept under traditional icing conditions. Such a finding indicated that inclusion of  
59 this acid mixture in the icing medium can lead to a marked quality and safety  
60 enhancement as well as to profitable commercial value increases.

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## INTRODUCTION

63

64

65           Maintain good quality and shelf life extension of fresh fish are nowadays  
66 mandatory. Flake ice has been the most employed method to cool and store fish  
67 products and partially inhibit detrimental effects on the commercial value. However,  
68 significant deterioration of sensory quality and nutritional value has been detected in  
69 chilled fish as a result of microbial and biochemical degradation mechanisms (Whittle  
70 and others 1990). To retard fish spoilage as long as possible, a wide number of  
71 preservative strategies to be combined to flake ice chilling have been tested  
72 satisfactorily such as chemical (washing or dipping by means of an aqueous solution  
73 including preservative compounds) and physical (hydrostatic high pressure, low-dose  
74 irradiation, etc.) treatments (Ashie and others 1996; Richards and others 1998) and  
75 employment of preservative packaging (Ozen and Floros 2001).

76           Among previous chemical treatments to chilling storage, natural low molecular  
77 weight organic acids and their sodium salts have shown to represent a relevant choice  
78 because of their easy availability, low commercial cost and wide range of permitted  
79 concentrations for their use. Thus, ascorbic and citric acids (AA and CA, respectively)  
80 are widely known for their role as chelators, acidulants in biological systems and  
81 synergists of primary antioxidants, so that a profitable effect on fish fillets (Badii and  
82 Howell 2002; Pourashouri and others 2009) and whole fish (Aubourg and others 2004)  
83 has been observed. Further, lactic acid (LA) has been reported to be effective in  
84 suppressing Gram-negative bacteria, which are known to be the most important fish  
85 spoiler group; thus, LA pre-treatment has shown to be effective in preserving and  
86 extending shelf-life in fish fillets (Kim and others 1995; Metin and others 2001), coated  
87 fish (Gogus and others 2006) and fish slices (Sallam 2007).

88           Small pelagic fatty fish species can constitute food products of great economic  
89 importance in many European countries (FAO 2007a). Some of these fish species are  
90 captured in high proportions when their demand is relatively low, so that a large portion  
91 of their catches is underutilized and transformed into fish meals for animals. Thus, great  
92 attention is being accorded by manufacturers in the search of appropriate technological  
93 treatments that may increase their shelf-life and accordingly, their trading value. One  
94 such abundant species at both North Atlantic coasts is Atlantic mackerel (*Scomber*  
95 *scombrus*) belonging to the *Scombridae* family (FAO 2007b). Although it is recognized  
96 as a healthy food, it remains underutilized because of its short chilled shelf life (up to 9-  
97 10 days). Most research has been focused on the assesment of lipid hydrolysis and  
98 oxidation as these are the most relevant mechanisms of quality loss during mackerel  
99 chilling storage (Decker and Hultin, 1990; Saeed and Howell, 2001). In this sense,  
100 different technologies have been checked to partially inhibit them (Hwang and  
101 Regenstein, 1995; Richards and others 1998). However, chilling storage of mackerel  
102 has shown an important microbial activity (Jhaveri and others 1982; Bennour and others  
103 1991), so that great efforts should also be directed to the inhibition of this damage  
104 pathway.

105           The present work concerns mackerel traded as a chilled product. The study was  
106 aimed to investigate the effect of including a mixture of organic acids in the icing  
107 medium employed. To this end, an aqueous solution including CA, AA and LA was  
108 employed as icing medium; its effect on microbial activity development was monitored  
109 in mackerel muscle for up to 13 days of chilled storage.

110

## **MATERIAL AND METHODS**

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### **Icing systems**

113 An aqueous solution containing 0.050% (w/v) of each natural organic acid (CA,  
114 AA and LA) was prepared, packed in polythene bags and kept frozen at  $-20^{\circ}\text{C}$  until use.  
115 Traditional ice was prepared starting only from water that was packed and kept frozen  
116 in the same way as the one including the organic acid mixture. Before addition to  
117 individual fishes, the different ices were ground to obtain common flakes. Organic acids  
118 encountered in the present research are regarded as safe (GRAS) for use in foods  
119 according to European and American administrations (Madrid and others 1994; Giese  
120 1996).

121 Preliminary trials were carried out in order to assess a convenient concentration  
122 of the organic acid mixture used to prepare the ice. Thus, a solution combining the three  
123 organic acids in the 0.005 % to 0.250 % concentration range was preliminary evaluated.  
124 According to results obtained on the visual analysis of individual fishes, the 0.050%  
125 concentration was chosen as the most suitable for further investigation.

126

### **Raw fish, processing and sampling**

128 Fresh mackerel (81 individuals) were caught near the Galician Atlantic coast  
129 (north-western Spain) in Autumn 2010 and transported on ice to the laboratory. The  
130 length and weight of the fish specimens were included in the following ranges: 21-25  
131 cm and 175-230 g, respectively.

132 Upon arrival in the laboratory, nine individual fishes were separated and  
133 analyzed as starting raw fish (day 0); for it, three different groups (three individuals per  
134 group) were analyzed independently in order to achieve the statistical analysis ( $n = 3$ ).  
135 The remaining fish were divided into two batches (36 individuals in each batch). The

136 first batch (preserved fish; P batch) was placed in boxes and directly surrounded by ice  
137 prepared with the organic acid-mixture above mentioned. Fish corresponding to the  
138 second batch (control batch; C batch) was placed in boxes and surrounded by traditional  
139 ice prepared with water.

140 In both batches, a 1:1 fish-to-ice ratio was employed. Both batches were placed  
141 in a refrigerated room (4 °C). Boxes employed allowed draining and ice was renewed  
142 when required. Fish samples from the two different batches were taken for analysis on  
143 days 3, 6, 10 and 13. At each sampling time, nine individuals of each batch were taken  
144 for analysis, being considered into three groups (three individuals in each group) that  
145 were studied independently in order to achieve the statistical analysis (n=3).

146

#### 147 **Microbial analysis**

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

153 Total aerobes were investigated by surface inoculation on plate count agar  
154 (PCA, Oxoid Ltd., London, UK), after incubation at 30°C for 48 h. Psychrotrophes were  
155 also investigated in PCA but incubation was carried out at 7-8 °C for 7 days.  
156 Enterobacteriaceae were investigated by pour plating using Violet Red Bile Agar  
157 (VRBA) (Merck, Darmstadt, Germany) after incubation at 37 °C for 24 h.  
158 Microorganisms exhibiting a proteolytic or lipolytic phenotype were investigated in  
159 casein-agar medium or tributyrin-agar, respectively, after incubation at 30 °C for 48 h,  
160 as previously described by Ben-Gigirey and others (2000).

161 In all cases, bacterial counts were transformed into log CFU/ g muscle before  
162 undergoing statistical analysis. All analyses were done by triplicate.

163

#### 164 **Chemical analysis of microbial activity**

165 Total volatile base-nitrogen (TVB-N) values were measured as previously  
166 reported (Aubourg and others 1997). Briefly, fish muscle (10 g) was extracted with 6%  
167 perchloric acid (30 ml) and brought up to 50 ml. An aliquot of the acid extracts was  
168 rendered alkaline to pH 13 with 20% NaOH and then steam-distilled. Finally, the  
169 TVB-N content was determined by titration of the distillate with 10 mM HCl. Results  
170 were expressed as mg TVB-N/ 100 g muscle.

171 Trimethylamine-nitrogen (TMA-N) values were determined by the picrate  
172 method, as previously described by Tozawa and others (1971). This involves the  
173 preparation of a 5% trichloroacetic acid extract of fish muscle (10 g / 25 ml). Results  
174 were expressed as mg TMA-N/ 100 g muscle.

175

#### 176 **Statistical analysis**

177 Data obtained from the different microbial and chemical analyses were subjected  
178 to the ANOVA method ( $p < 0.05$ ) to explore differences by two different ways: icing  
179 conditions effect and chilling time effect. For it, the PASW Statistics 18 software for  
180 Windows (SPSS Inc., Chicago, IL, USA) was employed. The comparison of means was  
181 performed using the least-squares difference (LSD) method. Correlation analysis among  
182 parameters (chilling time, microbial indices and chemical values) was carried out by  
183 means of the Pearson correlation coefficient (r value).

184



## **RESULTS AND DISCUSSION**

### **Microbial count analysis**

The development of aerobic bacteria was significantly ( $p < 0.05$ ) slowed down in the batch submitted to the organic acid-icing system, as compared to the control batch (Table 1). Thus, the differences in the microbial numbers reached its maximum, more than two log units, at day 6 of storage, while the average difference for aerobic mesophiles throughout storage was 1.18 log units. Remarkably, the control batch stored in traditional ice for 13 days reached levels above  $10^7$  CFU/ g. Bennour and others (1991) and Gram and Huss (1996) reported aerobe count to be the breakpoint for relevant microbial spoilage (Gram and Huss 1996). On contrast, the aerobe counts in the batch preserved with organic acids were round  $10^6$  CFU/ g at that time (Table 1). Aerobe counts as high as  $10^7$  CFU/ g have been reported in the skin of horse mackerel stored for 7 days at  $4^\circ\text{C}$  (Kuda and others 1996). This result is quite in agreement with our results and that underscores the microbial inhibition exerted by the organic acid-icing system. In the present research, both fish batches showed fair correlation values between aerobe counts and chilling time ( $r = 0.93$  and  $0.84$  for C- and P-fish, respectively; Table 2).

With respect to the development of anaerobic bacteria, significant ( $p < 0.05$ ) advantages derived from the use of the organic acid-icing system were also observed (Table 1). Thus, the average differences between batches C and P in the anaerobic counts throughout storage were higher than 2 log units, which means that the slowing down of anaerobic bacterial growth was over 99%. Furthermore, final counts after 13 days of storage differed in more than 3.60 log units, which clearly indicates a remarkably better control of anaerobes growth as a result of the storage in the organic

209 acid-icing system. Correlation value between aerobe counts and chilling time was only  
210 good in the case of the C batch ( $r = 0.93$ ; Table 2).

211 The comparative analysis of psychrotrophe counts indicated significant ( $p < 0.05$ )  
212 differences among batches after 10 days of storage (Table 1). The development of this  
213 microbial group was quite limited in both batches on early storage periods, with  
214 microbial numbers below 4.85 log CFU/ g units until day six, although the organic acid  
215 icing system provided a remarkable protective effect. Differences between batches  
216 increased on day 10, with the psychrotrophes higher than 7 log units on the control  
217 batch while the organic acid icing system exhibited values below 6 log units. Even a  
218 more significant effect was observed on day 13, although both batches were above the 6  
219 log CFU/ g units breakpoint of microbial spoilage. The average differences between  
220 batches during the whole storage period rose to 1.17 log units, this indicating a  
221 remarkable slowing down of the growth of this bacterial group in the organic acid-icing  
222 system. Moreover, as storage progressed higher differences in the psychrotrophe counts  
223 were achieved, these reaching its maximum at day 13; at this time (day 13), a difference  
224 of 1.50 log units between batches was observed. As in the case of the aerobes, the  
225 psychrotrophes did not reach  $10^7$  CFU/ g in the muscle of the fish specimens stored in  
226 the organic acid-icing system, while this value was clearly surpassed in the control  
227 batch. Both fish batches showed good correlation values between psychrotrophe counts  
228 and chilling time ( $r = 0.94$  and  $0.93$  for C- and P-fish, respectively; Table 2), according  
229 to previous research on chilled mackerel (Bennour and others 1991).

230 With respect to Enterobacteriaceae, the counts were below 1.5 log units for both  
231 batches until day 10 (Table 3). These results confirm the very good initial quality of the  
232 mackerel specimens employed and the limited growth of enteric bacteria during chilled  
233 storage, regardless of the ice system considered. Low numbers of Enterobacteriaceae

234 had also been reported before by other authors for related fish species such as horse  
235 mackerel (Rodríguez and others 2005) or jack mackerel (Figuroa and others 1990),  
236 subjected to storage on flake ice and slurry ice conditions. However, in our study, the  
237 counts for this bacterial group were similar in the organic acid-icing system than in the  
238 control batch except for advanced storage times (day 13), where a difference of 1.95 log  
239 units between batches was determined. However, the fact that the counts for this  
240 bacterial group were quite low up to day 10 (below 1.30 log CFU/ g) and as a result of  
241 the ample standard deviation values obtained, no conclusion could be depicted related to  
242 any beneficial effect of the organic acid system up to day 10. The results also indicate a  
243 good control of this microbial group up to day 10 in both batches, this being probably  
244 related with the good hygienic practices and the rapid chilling of fish. However, on day  
245 10 the microbial quality of the fish was at its limit, this leading to a rapid increase in the  
246 numbers of Enterobacteriaceae at day 13. According to this Enterobacteriaceae count  
247 distribution, fair correlation values with chilling time were only obtained for fish  
248 corresponding to the P batch ( $r = 0.87$ ; Table 2).

249         This work was also aimed at evaluating the effect of the organic acid-mixture on  
250 microbial groups exhibiting specific spoilage phenotypes. Thus, the development of  
251 bacteria able to produce extracellular lipolytic enzymes has been described to negatively  
252 affect the preservation of medium-fat fish species (Rodríguez and others 2005).  
253 Likewise, the inhibition of proteolytic bacteria, able to synthesize and secrete  
254 proteolytic enzymes, has also been linked to a better preservation of fish species, due to  
255 the limitation of the physical damage of the muscle structure also reducing the  
256 formation of alkaline compounds such as ammonia and related metabolites (Venugopal  
257 1990; Odagami and others 1994; Makarios-Laham and Lee 1993; Rodríguez and others  
258 2003). Accordingly, the inhibition of bacteria exhibiting lipolytic and/or proteolytic

259 phenotypes may limit the negative effects of lipases and proteases on the quality of fish  
260 species, since these enzymes have always been characterized for retaining activity  
261 during long storage periods even under refrigeration temperatures (Alford and Pierce  
262 1961).

263         With respect to the lipolytic bacteria, and as in the cases of other microbial  
264 groups studied before, significant ( $p < 0.05$ ) differences were determined between  
265 batches (Table 3). Thus, the average difference during storage was 1.41 log units, and  
266 reached its maximum at advanced stages of storage. Remarkably, differences of 1.25  
267 and 2.73 log units were determined at 10 and 13 days of storage, respectively.  
268 According to these results, the growth of bacteria potentially involved in the lipolytic  
269 breakdown of mackerel was slowed down as a consequence of storage in the organic  
270 acid-icing system. Similar inhibition of lipolytic microorganisms was observed with  
271 other preservation methods such as ice slurries for other medium-fat and fat fish species  
272 like horse mackerel (Rodríguez and others 2005) and sardine (Campos and others  
273 2005), respectively. Good correlation values between lipolytic counts and chilling time  
274 were only obtained for fish corresponding to the C batch ( $r = 0.93$ ; Table 2).

275         With respect to the proteolytic bacteria, a similar type of behavior was observed  
276 than in the case of lipolytic ones (Table 3). Thus, the counts of proteolytic bacteria in  
277 mackerel muscle stored in the organic acid-icing system did not reach levels of  $10^4$   
278 CFU/ g at any storage time, while the control batch exhibited values higher than  $10^5$   
279 CFU/ g after 10 days of storage. The average difference in the counts between batches  
280 throughout storage was 1.19 log units, and the growth inhibition was more intense as  
281 storage progressed, a result that is in agreement with those observed for the majority of  
282 the microbial groups studied before. As with lipolytic counts, proteolytic ones only

283 provided fair correlation values with chilling time in the case of the C batch ( $r = 0.89$ ;  
284 Table 2).

285 A marked inhibitory effect of the novel icing system on several microbial groups  
286 investigated was observed, this being especially remarkable in the cases of the aerobes,  
287 anaerobes and lipolytic bacteria. The surface wash caused by the melting of the ice  
288 during storage and the subsequent antimicrobial effect of the organic acids on skin  
289 microflora of the fish can be invoked as the main reasons for the limited bacterial  
290 growth found in the corresponding mackerel muscle. Similar results had been reported  
291 before for other preservation methods such as ice slurries, where ice melting led to the  
292 release of salt that exerted a bacteriostatic effect on fish microflora (Rodríguez and  
293 others 2003; Rodríguez and others 2005; Campos and others 2005). Moreover, previous  
294 studies on horse mackerel have confirmed the presence of *Proteus penneri* and  
295 *Staphylococcus xylosum* strains exhibiting remarkable lipolytic and proteolytic  
296 phenotype, and, to a lesser extent, *Proteus vulgaris*. The fact that the organic acid-icing  
297 system evaluated in this study significantly ( $p < 0.05$ ) slowed down the growth of  
298 proteolytic and lipolytic bacteria in mackerel muscle, stresses the benefits that such  
299 storage system may have on the maintenance of the quality of this fish species.

300

### 301 **Chemical analysis of microbial activity**

302 Microbial activity development was also measured by chemical indices. The  
303 TVB-N content showed a relatively high value ( $30.21 \pm 1.48$ ) for starting fish (Table 4);  
304 this value is found quite similar to the one obtained by Civera and others (1993) (31.9  
305 mg/ 100 g muscle) but markedly higher than the values reported by Bennour and others  
306 (1991) and Fagan and others (2003). In this study (Table 4), a higher ( $p < 0.05$ ) TVB-N  
307 content in fish samples corresponding to the control batch could be observed when

308 compared to preserved fish in the 6-13-day period. Volatile amine content showed a  
309 marked increase in the 10-13-day period for C-fish, this being in agreement with a  
310 previous report on chilled mackerel (Bennour and others 1991); in the case of the P-  
311 batch, a marked increase on volatile amine formation was only attained at the end of the  
312 experiment. As for aerobic count assessment, TVB-N values showed a mean value  
313 decrease in the P-fish batch in the 0-6-day period; this result can be explained as a result  
314 of the preservative effect of the organic acid presence in the ice and by a relatively low  
315 microbial activity at that period. Good correlation values between TVB-N content and  
316 chilling time were obtained for fish corresponding to the C batch ( $r = 0.91$ ; Table 2),  
317 according to the strong relationship between TVB-N content and freshness loss (Whittle  
318 and others 1990; Bennour and others 1991). Volatile amine compounds have been  
319 reported to be produced partially by means of endogenous enzyme activity, but mostly  
320 as a result of microbial development (Whittle and others 1990).

321 Initial fish showed low TMA-N values ( $0.07 \pm 0.04$ ; Table 4), this indicating the  
322 high freshness of the raw material employed. All fish specimens showed a progressive  
323 TMA-N content increase ( $p < 0.05$ ) with time regardless the icing system employed; such  
324 increase was specially marked at the end of storage, this also being in agreement with  
325 previous reports on chilled mackerel (Jhaveri and others 1982; Bennour and others  
326 1991). As a result of this, good correlation values between TMA-N values and chilling  
327 time were observed ( $r = 0.93$  and  $0.95$ , for C- and P-fish, respectively; Table 2). Such  
328 results are in agreement with previous research where TMA-N assessment showed to be  
329 an accurate index for assessing quality loss during mackerel chilling storage (Bennour  
330 and others 1991; Civera and others 1993), according to the strong relationship between  
331 TMA-N content and freshness loss (Whittle and other 1990). As for TVB-N value,  
332 trimethylamine has been reported to be produced mostly as a result of microbial

333 development. Comparison between both kinds of icing conditions (Table 4) showed  
334 lower TMA-N mean values throughout the whole experiment as a result of employing  
335 the organic acid-mixture in the icing medium; such differences were found significant at  
336 the end of the experiment.

337 Relationship between chemical and microbial parameters was also analyzed in  
338 the present research (Table 5). Related to the control batch, better correlation values  
339 with microbial count assessments were obtained for TMA-N values ( $r = 0.84-0.92$ ) than  
340 for TVB-N scores ( $r = 0.78-0.89$ ). In this sense, previous research (Bennour and others  
341 1991) showed that TMA-N content was profitable in order to classify the freshness  
342 degree of chilled mackerel (first, second and third grade would correspond to 0-1, 1-3  
343 and 3-6 mg TMA-N/ 100 g muscle, respectively). In addition, a previous correlation  
344 study between TVB-N and TMA-N values and sensory acceptance in chilled mackerel  
345 concluded that this species was not suitable for consumption when both parameters  
346 were above 40 and 4 mg/ 100 g muscle, respectively (Civera and others 1993). In the  
347 present research, a higher TVB-N value was attained in the case of the control fish at  
348 the end of the experiment (Table 4).

349 Related to the preserved batch, correlation values were again better for TMA-N  
350 value than for TVB-N scores. For both chemical parameters, fair correlation values  
351 were obtained with aerobes, psychrotrophes and Enterobacteriaceae.

352

353

### **CONCLUSIONS**

354 The results obtained in this study indicated significant ( $p < 0.05$ ) differences  
355 between batches for all six microbial groups investigated, as well as in both chemical  
356 indices related to microbial activity development. These findings clearly indicated a  
357 significantly ( $p < 0.05$ ) lower bacterial growth in mackerel muscle subjected to storage in

358 the organic acid-icing system as compared with traditional flake ice. The novel icing  
359 system evaluated in this work contains three organic acids, all of them previously  
360 reported as possessing antimicrobial and antioxidant activities. In addition, such natural  
361 organic acids are known to represent a relevant choice because of their easy availability,  
362 low commercial cost and wide range of permitted concentration for their use in foods.

363 As being a highly fatty fish species, previous research on quality loss of chilled  
364 mackerel has mostly focused the lipid changes, these concerning specially lipid  
365 oxidation and hydrolysis. In the present investigation, an extensive study on microbial  
366 activity development during the chilled storage of this pelagic fish species has been  
367 undertaken; as a result, progress of different bacteria groups has been described, as well  
368 as its relationship with chemical parameters related to microbial activity development.

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370

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**TABLE 1**

**Aerobe, anaerobe and psychrotrophe count (log CFU/ g muscle) assessment\* in chilled mackerel kept under different icing conditions\*\***

Chilling Time (days)	Aerobes		Anaerobes		Psychrotrophes	
	C	P	C	P	C	P
Raw fish	3.31 C (0.08)	3.31 B (0.08)	1.97 D (0.85)	1.97 AB (0.85)	3.26 C (0.37)	3.26 B (0.37)
3	3.41 BC (0.65)	3.12 B (0.19)	3.33 C (0.60)	2.79 A (0.70)	4.02 BC (0.61)	3.16 B (1.02)
6	4.21 aB (0.37)	2.20 bC (0.17)	3.50 aC (0.41)	1.99 bB (0.05)	4.85 B (0.30)	3.77 B (1.06)
10	6.44 aA (0.45)	5.34 bA (0.52)	4.57 aB (0.43)	1.99 bB (0.05)	7.06 aA (0.37)	5.74 bA (0.45)
13	7.28 aA (0.32)	6.05 bA (0.77)	5.68 aA (0.44)	1.99 bB (0.05)	7.93 aA (0.50)	6.43 bA (0.51)

\* 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 low-case 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 capital 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: P (ice including the organic acid-mixture) and C (ice prepared only from water).

**TABLE 2**

**Correlation coefficient (r value)\* between the chilled storage time and the different parameters analyzed in chilled mackerel kept under different icing conditions\*\***

Quality parameter	r value	
	C	P
Aerobes	0.93 <sup>b</sup>	0.84 <sup>b</sup>
Anaerobes	0.93 <sup>a</sup>	-0.39 <sup>b</sup>
Psychrotrophes	0.94 <sup>a</sup>	0.93 <sup>b</sup>
Enterobacteriaceae	0.79 <sup>b</sup>	0.87 <sup>b</sup>
Lipolytics	0.93 <sup>b</sup>	0.80 <sup>b</sup>
Proteolytics	0.89 <sup>a</sup>	0.69 <sup>c</sup>
Total volatile base-nitrogen	0.91 <sup>b</sup>	0.79 <sup>b</sup>
Trimethylamine-nitrogen	0.93 <sup>b</sup>	0.95 <sup>b</sup>

\* For each index, linear<sup>a</sup>, quadratic<sup>b</sup> and logarithmic<sup>c</sup> fittings were studied. In each case, the best correlation coefficient value is expressed.

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

**TABLE 3**

**Enterobacteriaceae, lipolytic and proteolytic count (log CFU/ g muscle) assessment\* in chilled mackerel kept under different icing conditions\*\***

Chilling Time (days)	Enterobacteriaceae		Lipolytics		Proteolytics	
	C	P	C	P	C	P
Raw fish	0.99 B (0.01)	0.99 B (0.01)	2.16 D (0.28)	2.16 AB (0.28)	2.09 C (0.18)	2.09 B (0.18)
3	0.99 B (0.05)	0.99 B (0.05)	3.32 C (0.56)	2.85 A (0.41)	3.51 B (0.11)	3.16 AB (1.01)
6	0.99 B (0.05)	1.23 AB (0.41)	3.30 aC (0.52)	2.10 bB (0.18)	3.10 B (0.35)	2.76 AB (0.69)
10	0.99 B (0.05)	1.29 AB (0.53)	4.81 aB (0.07)	3.56 bAB (1.15)	5.30 aA (0.29)	3.78 bA (1.09)
13	4.42 aA (0.44)	2.47 bA (0.93)	6.40 aA (0.20)	3.67 bAB (1.85)	5.51 aA (0.52)	2.96 bAB (1.48)

\* 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 low-case 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 capital 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 4**

**Evolution of total volatile base-nitrogen (TVB-N) and trimethylamine-nitrogen (TMA-N) values\* in chilled mackerel kept under different icing conditions\*\***

Chilling Time (days)	TVB-N (mg/ 100 g muscle)		TMA-N (mg/ 100 g muscle)	
	C	P	C	P
Raw fish	30.21 C (1.48)	30.21 B (1.48)	0.07 D (0.04)	0.07 D (0.04)
3	30.74 C (2.00)	28.69 BC (1.70)	0.29 CD (0.20)	0.16 D (0.04)
6	30.15 aC (1.57)	27.44 bC (0.62)	0.54 C (0.27)	0.34 C (0.06)
10	35.12 aB (2.08)	30.47 bB (1.57)	1.04 B (0.12)	0.85 B (0.14)
13	44.61 aA (1.76)	35.21 bA (2.53)	2.32 aA (0.25)	1.47 bA (0.23)

\* 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 low-case letters 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 capital 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 5**

**Linear correlation coefficient values between microbial and chemical parameters analyzed in chilled mackerel kept under different icing conditions\***

Microbial parameter	Total volatile base-nitrogen		Trimethylamine-nitrogen	
	C	P	C	P
Aerobes	0.86	0.84	0.88	0.84
Anaerobes	0.84	-0.30	0.84	-0.37
Psychrotrophes	0.84	0.75	0.88	0.92
Enterobacteriaceae	0.89	0.84	0.87	0.88
Lipolytics	0.89	0.68	0.92	0.80
Proteolytics	0.78	0.07	0.88	0.42

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