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1 **Assessment of the bioprotective potential of lactic acid bacteria against *Listeria***  
2 ***monocytogenes* on vacuum-packed cold-smoked salmon stored at 8 °C.**

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10 **ABSTRACT**

11 Smoked salmon is a highly appreciated delicatessen product. Nevertheless, this ready-  
12 to-eat (RTE) product is considered at risk for *Listeria monocytogenes*, due to both the  
13 prevalence and growth potential of this bacteria on the product. Biopreservation may  
14 be considered a mild and natural effective strategy for minimizing this risk. In this study,  
15 we evaluated the following three potential bioprotective lactic acid bacterial strains  
16 against *L. monocytogenes* in three smoked salmon types with different  
17 physicochemical characteristics, primarily fat, moisture, phenol and acid acetic content:  
18 two bacteriocin-like producers that were isolated from smoked salmon and identified as  
19 *Lactobacillus curvatus* and *Carnobacterium maltaromaticum* and a recognized  
20 bioprotective bacteriocin producer from meat origin, *Lactobacillus sakei* CTC494. *L.*  
21 *sakei* CTC494 inhibited the growth of *L. monocytogenes* after 21 days of storage at 8  
22 °C in all the products tested, whereas *L. curvatus* CTC1742 only limited the growth of  
23 the pathogen (< 2 log increase). The effectiveness of *C. maltaromaticum* CTC1741  
24 was dependent on the product type; this strain limited the growth of the pathogen in  
25 only one smoked salmon type.

26 These results suggest that the meat-borne starter culture, *L. sakei* CTC494, may  
27 potentially be used as a bioprotective culture to improve the food safety of cold-smoked  
28 salmon.

29 **Keywords:** Food-borne pathogens; fish products; *Lactobacillus sakei* CTC494;  
30 listeristatic.

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## 33 1. Introduction

34 The consumption of ready-to-eat (RTE) foods has increased considerably during the  
35 last decades, which is likely related to the modern lifestyle (Cabedo et al., 2008). Cold-  
36 smoked salmon is normally made from salmon fillets with low levels of salt (< 6% in the  
37 water phase) that are subjected either to traditional wood smoking for prolonged  
38 periods (not exceeding 25 °C - 30 °C during the process) or to the application of  
39 artificial smoke flavouring (liquefied smoke preparations formulated from the  
40 condensation of wood smoke and either water, oil, or emulsifiers). In Spain, the  
41 production and consumption of cold-smoked salmon has been increasing in the last  
42 decade; indeed, Spain represents the sixth highest European country in terms of  
43 consumption of smoked salmon (IRI, 2015).

44 The latest European zoonoses summary report showed that *Listeria monocytogenes*  
45 continues to be a concern for RTE fishery products (EFSA-ECDC, 2018). The  
46 prevalence of *L. monocytogenes* varies depending on the type of fish matrix, the  
47 characteristics of the product, and the packaging but also on the manufacturing  
48 environment; there are differences between processing plants or fish slaughterhouses  
49 (Dauphin et al., 2001; Hoffman et al., 2003; Rotariu et al., 2014b; Thimothe et al.,  
50 2004). The risk of contamination of this RTE product has been described (Dauphin et  
51 al., 2001; Jami et al., 2014), and some authors linked a high prevalence of *L.*  
52 *monocytogenes* in processing plants with the ubiquitous contamination of the industry  
53 environment and final product (Gudmundsdottir et al., 2005; Nakari et al., 2014; Vogel  
54 et al., 2001; Vongkamjan et al., 2013). Moreover, the product may be a suitable  
55 environment for *L. monocytogenes* growth (Mejlholm and Dalgaard, 2007b, 2009).

56 Biopreservation strategies are methods for preserving food using non-pathogenic safe  
57 microorganisms (protective cultures) that are selected to prevent the development of  
58 other undesirable microorganisms. Such strategies are considered natural and  
59 effective means to control food-borne pathogens (Katla et al., 2003; Pilet and Leroi,

2011; Rotariu et al., 2014a). Among the biopreservation strategies, lactic acid bacteria (LAB) are considered good candidates because they produce natural antimicrobials, they are part of the common microbiota of different products, including smoked salmon, and they are recognized as non-hazardous to human health, classified as Generally Recognized As Safe (GRAS) or under the criteria of Qualified Presumption of Safety (QPS) (EFSA, 2018; FDA, 2012). Diverse studies have highlighted the bioprotective role of endogenous LAB (*Lactobacillus*, *Carnobacterium* and *Enterococcus*) in cold-smoked salmon (Brillet et al., 2004; Duffes et al., 1999a; Ghanbari et al., 2013; Leroi et al., 2015; Leroi et al., 1998; Nilsson et al., 1997; Richard et al., 2004; Weiss and Hammes, 2006; Tomé et al., 2008, Concha-Meyer et al., 2011; Rotariu et al., 2014).

The aim of this study was to evaluate the effectiveness of a meat-borne strain, *L. sakei* CTC494, in comparison with *in vitro*-selected LAB strains isolated from cold-smoked salmon against *L. monocytogenes* that was artificially inoculated on different cold-smoked salmons, vacuum-packaged and stored at 8 °C for 21 days. *L. sakei* CTC494 is a recognized bacteriocinogenic (sakacin K) starter and bioprotective meat culture (Aymerich et al., 2000; Hugas et al., 1995; Hugas, 1998; Ortiz et al., 2014; Ravyts et al., 2008). Recently it has been assayed as a bioprotective culture in fresh-filleted fish (Costa et al., 2019). This challenge test strategy is intended to provide scientific information to the industry, supporting the implementation of biopreservation strategies aiming to minimize the growth and associated risk of *L. monocytogenes* in RTE fish products.

## 2. Materials and methods

### 2.1. Identification of isolates and screening of antilisterial activity

A set of 80 isolates from de Man, Rogosa and Sharpe agar (MRS, Merck, Darmstadt, Germany) (n = 40) and CTSI (Cresol red thallium acetate sucrose inulin) (Wasney et al., 2001) (n = 40) were obtained from 8 different types of cold-smoked salmon, 7 different brands with 2 products from the same brand that differed in the fresh salmon

87 origin (Scottish and Norwegian). The isolates were assayed for their antimicrobial  
88 activity against *L. monocytogenes* CTC1500, the indicator strain. Previous assays  
89 showed that this strain is one of the fastest growing strains from a set of 4 different *L.*  
90 *monocytogenes* strains tested, including INIA G1 (serotype 1/2b) and INIA G15  
91 (serotype 1/2a) (both isolated from environmental samples of the smoked salmon  
92 industry and kindly provided by M. Medina, INIA, Madrid, Spain), CTC1500 (serotype  
93 1/2a, ST18) and CTC1680 (serotype 1/2c, ST155), which were isolated from smoked  
94 salmon and belong to the IRTA-Food Safety Program collection (unpublished results).  
95 The ability of this strain to grow at 8 °C in cold-smoked salmon was previously  
96 confirmed in samples of 6 different brands (including 4 brands used for LAB isolations  
97 plus 2 additional brands). The meat-borne *L. sakei* CTC494 strain, from our own  
98 collection, is currently marketed by THT s.a.(Gembloux, Belgium) as an antilisteria  
99 starter culture for fermented meat products; this strain was used as the antimicrobial  
100 positive control. Isolates were stored at - 80 °C in their respective growth media with  
101 20% glycerol.

102 To identify the isolates, DNA was isolated from overnight cultures using the DNeasy  
103 tissue kit (Qiagen, Hilden, Germany). Molecular identification was performed by the  
104 partial sequencing of the 16S rRNA gene with universal primers (1061R-,  
105 CACGRCACGAGCTGACGAC and 8F-AGAGTTTGATYMTGGCTCAG) and  
106 phenylalanyl-tRNA synthase (*pheS*) (*pheS*-21-F-CAYCCNGCHCGYGAYATGC and  
107 *pheS*-23R-GGRTGRACCATVCCNGCHCC) (Naser et al., 2007). Species assignment  
108 was performed through online homology alignment using the BLAST+ software and the  
109 NCBI-GenBank (USA), EMBL (EU) and DDBJ (Japan) databases.

110 To assess the antimicrobial bacteriocin-like activity of these strains, the cultures were  
111 grown in MRS (LAB) or CTSI (*Carnobacterium*) at 30 °C for 18 to 20 h until the culture  
112 reached ca. 1 x 10<sup>8</sup> CFU/mL. Partial purification of the culture supernatant was  
113 performed. Cells were removed by centrifugation at 5,000 rpm for 10 min at 4 °C. The

114 supernatant fluid was collected, and the potential antimicrobial compound was  
115 precipitated by the addition of 0.4 g/mL ammonium sulphate (Aymerich et al., 1996).  
116 After 45 min at 0 °C, the protein precipitate was pelleted by centrifugation at 10,000  
117 rpm for 30 min. The pellet was dissolved in 10 mM sodium phosphate buffer, pH 6.0,  
118 and heat-treated by pasteurization for 10 min at 80 °C.

119 LAB antimicrobial activity was examined using the agar spot test (Tagg et al., 1976).  
120 Serial two-fold dilutions were made from the pasteurized semi-purified extract. Then,  
121 10 µL of each dilution was placed on the surface of semisolid TSAYE overlay (Tryptone  
122 Soya agar with 0.6% yeast extract and 7.5 g/L agar) seeded with 50 µL of an overnight  
123 culture of *L. monocytogenes* CTC1500 in TSBYE (Tryptone Soya broth with 0.6%  
124 yeast extract) and incubated overnight at 30 °C 24 h. One arbitrary unit (AU/mL) was  
125 defined from the 10 µL of the highest dilution of bacteriocin-like solution that caused a  
126 definite zone of inhibition on the lawn of the indicator strain.

## 127 *2.2. Challenge test in different types of cold-smoked salmon*

128 Vacuum-packed cold-smoked Atlantic salmon (*Salmo salar* L.) from different producers  
129 was purchased at local retailers upon arrival (i.e. within few days after production) and  
130 transported (refrigerated) to the laboratory for further analysis. Only samples within  
131 their initial shelf life were selected in order to maximize, with limited variation, the  
132 remaining shelf life. Three different cold-smoked salmon types were considered as  
133 follows: salmon A and C were from fresh fish originating from Norway and  
134 manufactured by 2 different brands, and salmon B originated from Scotland and was  
135 elaborated by the same company that produced salmon A.

136 To perform the challenge tests, all samples were aseptically cut into 4 x 4 cm<sup>2</sup> portions  
137 (16 cm<sup>2</sup>), which weighed 4 g, and frozen overnight. Then, the samples were subjected  
138 to the freeze-thaw method before the surface inoculation with the pathogen to facilitate  
139 *L. monocytogenes* growth and test for the worst-case scenario, as reported by Kang et  
140 al. (2012). The appropriate dilution of a - 80 °C *L. monocytogenes* CTC1500 culture (to

141 simulate osmotically stressed cells in the dry environment of the food industry) (Hereu  
142 et al., 2014; Wesche et al., 2009) was inoculated on the surface of the product (1%  
143 v/w) and spread with a sterile spreader to reach ca. 2.6 log CFU/g. The samples were  
144 maintained in the safety cabinet for 10 min until the *L. monocytogenes* culture was  
145 completely absorbed. Afterward, the LAB cultures were independently spread over the  
146 previously inoculated samples (1% v/w) to a final concentration of ca. 4.6 log CFU/g,  
147 reabsorption was allowed, and then the samples were vacuum-packed using individual  
148 bags (Sacoliva S.L., Castellar del Vallés, Barcelona, Spain) and stored at 8 °C for 21  
149 days.

150 Different lots were prepared to test three LAB cultures according to the experimental  
151 design depicted in Figure 1. Two independent trials were performed. A minimum of 3  
152 smoked-salmon fillets were used per each whole trial. Cut samples were randomly  
153 distributed among the different lots. Samples were analysed in triplicate for each lot  
154 and type at time 0 (after inoculation) and after 21 days of storage at 8 °C. The storage  
155 temperature was controlled with the Evisense® system from Labguard (AES,  
156 BioMérieux, France).

### 157 2.2.1. Microbial analysis

158 Samples were weighed and ten-fold diluted in peptone physiological saline solution (1  
159 g/L peptone and 8.5 g/L sodium chloride). The suspension was mixed with the  
160 Smasher® blender (AES, BioMérieux) for 1 min at room temperature. Next, the  
161 appropriate dilutions were spread on selective agar plates for microbial counts, as  
162 follows: *Enterobacteriaceae* in Violet Red Bile Glucose agar (VRBG; Merck); LAB on  
163 de Man Rogosa and Sharpe Agar (MRS, Merck); *Carnobacterium* sp. on CTSI  
164 (Wasney et al., 2001); and *L. monocytogenes* on supplemented Chromogenic Listeria  
165 Agar (Oxoid Ltd, Basingstoke, UK). The quantification limit was set at 4 CFU/g for *L.*  
166 *monocytogenes*, 10 CFU/g for *Enterobacteriaceae*, and 100 CFU/g for LAB and  
167 *Carnobacterium*.



168 A representative portion of each product was collected before the inoculation to  
169 evaluate the initial hygienic status of the cold-smoked salmon (initial microbial load). To  
170 assess the growth potential ( $\Delta \log$ ) of *L. monocytogenes*, the difference between the  
171 average count (log CFU/g) at the end of the shelf life and the average count (log  
172 CFU/g) at the beginning of the assay was calculated.

### 173 2.2.2. *Physicochemical analysis*

174 Physicochemical characteristics of each smoked salmon type were determined from  
175  $n = 4$  samples from a representative sample of 200 g. The pH (Crison puncture  
176 electrode pH 5053, pHmetre 25, Crison Instruments S.S., Barcelona, Spain) and water  
177 activity ( $a_w$ ) (Aqualab®, Ferrer Lab, Spain) of the fish samples were analysed in  
178 triplicate. The moisture, fat and protein contents were determined by FoodScan®  
179 (Foss, Hilleroed, Denmark). The NaCl content was measured by analysing the chloride  
180 content using the ISO 1841-2:1996 method in a potentiometric titrator 785 DMP Titrino  
181 (Metrohm AG, Herisau, Switzerland). The total phenol content (mg/Kg) was quantified  
182 according to Cardinal et al. (2004). For organic acids, neutralized 10% perchloric acid  
183 extracts (Hansen et al., 1995) were analysed by high-performance liquid  
184 chromatography with an Aminex® HPX-87H column (Bio-Rad laboratories SA, Spain).

### 185 2.3. *Statistical analysis*

186 Data were statistically analysed by one-way analysis of variance (ANOVA) using the  
187 least significance difference (LSD) test to assess the potential effect of  
188 physicochemical parameters, type of smoked salmon and bioprotective culture. Means  
189 were compared by Tukey-Kramer and Dunnett's tests ( $p \leq 0.05$ ). To assess the growth  
190 potential, means were compared by paired Student's T-test within each bacterial group.  
191 The JMP 8.0.1 statistic software from SAS Institute Inc. (Cary, NC, United States) was  
192 used.

193 **3. Results**

194 **3.1. Identification and antimicrobial activity of isolates**

195 The 40 MRS isolates originating from the 8 different cold-smoked salmon types, were  
196 identified as *Lactobacillus sakei* (25%) and *Lactobacillus curvatus* (75%). All the CTSI  
197 isolates (n=40) were identified as *Carnobacterium maltaromaticum* (100%).

198 Considering all 80 isolates, *in vitro* antilisterial activity was observed in 12.5% of the  
199 isolates belonging to the genera *Lactobacillus* and 45% of those belonging to  
200 *Carnobacterium*. Antimicrobial activity ranged from 25,600 - 102,400 (AU/mL) and 200  
201 - 400 AU/mL, respectively. All the antilisterial isolates of *Lactobacillus* belonged to the  
202 same type of smoked salmon and were identified as *L. curvatus*. None of the *L. sakei*  
203 isolates exhibited antilisterial activity. Concerning *Carnobacterium*, 18 isolates from five  
204 different cold-smoked salmon types exhibited antimicrobial activity against *L.*  
205 *monocytogenes* CTC1500.

206 The isolates, *C. maltaromaticum* CTC1741 and *L. curvatus* CTC1742, with an *in vitro*  
207 antilisterial activity of 400 AU/mL and 102,400 AU/mL, respectively, were selected as  
208 potential bioprotective cultures to be tested in different types of commercial sliced cold-  
209 smoked salmon stored at refrigeration temperature (challenge test as described in  
210 section 2.2). The control strain, *L. sakei* CTC494, exhibited the highest *in vitro*  
211 antilisterial activity (153,600 AU/mL) when compared to *L. curvatus* CTC1742 and *C.*  
212 *maltaromaticum* CTC1741.

213 **3.2. Microbial and physicochemical characteristics of cold-smoked samples**

214 The microbiological quality of the initial samples (non-inoculated) demonstrated a good  
215 hygiene level of the types of smoked salmon used, with levels of *Enterobacteriaceae*  
216 under 1 log CFU/g in salmon A and B and  $1.52 \pm 0.81$  CFU/g in salmon C. *L.*  
217 *monocytogenes* levels were under the detection limit ( $< 0.60$  log CFU/g). LAB counts  
218 were under 2 log CFU/g in salmon B and C, and  $2.21 \pm 1.77$  log CFU/g in salmon A.

219 *Carnobacterium* levels were under 2 log CFU/g in salmon A, and  $2.15 \pm 0.22$  and  $2.81$   
220  $\pm 1.15$  log CFU/g in salmon B and C, respectively.

221 The physicochemical parameters of the three types of smoked salmon were analysed,  
222 and all three types exhibited a similar pH, water activity ( $a_w$ ) and NaCl content.  
223 Significant differences ( $p < 0.05$ ) were observed in the fat, protein, moisture, phenol,  
224 and acetic acid content (Table 1). Smoked salmon A and B, which were produced and  
225 sold by the same trademark but elaborated with fresh salmon from different origins  
226 (Norway and Scotland) had similar physicochemical characteristics. Salmon C (from  
227 Norwegian fresh salmon but elaborated and sold by a different trademark) had a higher  
228 fat content, which is likely associated with fresh salmon production systems. Salmon C  
229 also had a lower phenol content and higher acetic acid content, which are likely  
230 associated with the elaboration technology used (Table 1).

### 231 3.3. *L. monocytogenes* growth potential after storage

232 No immediate bactericidal effect on the food-borne pathogen was observed in any of  
233 the lots. *L. monocytogenes* achieved an average count of  $5.73 \pm 1.35$  log CFU/g after 21  
234 days of vacuum storage at 8 °C, and there were no significant differences in *L.*  
235 *monocytogenes* growth ( $p \geq 0.05$ ) among the three types of cold-smoked salmon  
236 (Table 2). The average growth potential of *L. monocytogenes* in the control samples  
237 was  $2.77 \pm 1.66$  log units (Figure 2).

238 No differences ( $p \geq 0.05$ ) could be attributed to the different smoked salmon types. No  
239 interaction between lot and type was observed when the growth potential of *L.*  
240 *monocytogenes* was analysed through a complete statistical model, taking into account  
241 the effect of the three selected bioprotective cultures and the three different types of  
242 salmon (Table 2). Nevertheless, a significant effect ( $p \leq 0.05$ ) of product type was  
243 observed concerning the antilisterial effect of *C. maltaromaticum* CTC1741 when  
244 partial models considering the *L. monocytogenes* growth capacity after 21 days of  
245 refrigerated storage were separately built for each bioprotective culture. In this case, *C.*

246 *maltaromaticum* CTC1741 demonstrated an antilisterial effect in salmon C (Figure 2),  
247 and no significant growth of *L. monocytogenes* was observed after 21 days of storage  
248 at 8 °C (Table 2).

249 The growth potential of *L. monocytogenes* was significantly affected by the type of  
250 bioprotective culture applied ( $p < 0.05$ ) (Figure 2). In the *L. sakei* CTC494 lot after 21  
251 days at 8 °C, *L. monocytogenes* achieved 2.25 log lower counts compared with the  
252 control samples, with average final counts of  $2.30 \pm 0.83$  log CFU/g (Table 2). Indeed,  
253 *L. sakei* CTC494 resulted in *L. monocytogenes* growth inhibition ( $\delta < 0.5$  log) (Figure  
254 2). In the *L. curvatus* CTC1742 lot, *L. monocytogenes* achieved an average log  
255 increase of  $0.80 \pm 0.68$  log CFU/g, while in the *C. maltaromaticum* CTC1741 lot, *L.*  
256 *monocytogenes* achieved an average log increase of  $1.81 \pm 1.06$  log CFU/g (almost  
257 greater than a 2 log increase) (Figure 2), with average counts of  $4.45 \pm 1.06$  log CFU/g  
258 at the end of the refrigerated storage period.

259 Thus, *L. sakei* CTC494, with bacteriostatic activity, demonstrated the best antilisterial  
260 results ( $p < 0.05$ ), followed by *L. curvatus* CTC1742 ( $p < 0.05$ ), as a limiting growth  
261 factor. The results of *C. maltaromaticum* CTC1741 lot were similar to those of the  
262 control lot (Figure 2).

263 The growth of *Lactobacillus* was similar on the inoculated lots, *L. sakei* CTC494 and *L.*  
264 *curvatus* CTC1742 in any of the different salmon types (A, B and C), after refrigerated  
265 storage for 21 days at 8 °C (Table 2); *Lactobacillus* counts averaged  $8.70 \pm 0.29$  log  
266 CFU/g. All the samples showed a satisfactory appearance concerning colour and  
267 odour. In the non-*Lactobacillus* inoculated lots, MRS counts were significantly lower,  
268 and no significant differences were observed between the non-inoculated *Lactobacillus*  
269 lots (Table 2), although highly variable counts were observed ( $2.63 \pm 2.26$  log CFU/g).

270 *C. maltaromaticum* CTC1741 showed significantly lower counts after 21 days of  
271 refrigerated storage in salmon C (Table 2). Whereas in salmon A and B, the counts  
272 increased more than 3 log units (Table 2), achieving average counts of  $7.21 \pm 1.05$  log

273 CFU/g, it did not grow (Table 2) in salmon type C; initial numbers were maintained, with  
274 average final counts of  $4.65 \pm 1.13$  log CFU/g. All the samples showed a satisfactory  
275 appearance concerning colour and odour.

276 No growth of endogenous *Enterobacteriaceae* populations, except on control C  
277 samples, were observed in any type of cold-smoked salmon or bioprotective culture lot.  
278 This finding demonstrates that proper hygiene standards were maintained until the end  
279 of the storage period (Table 2).

#### 280 **4. Discussion**

281 It is known that the growth potential of *L. monocytogenes* can vary depending on the  
282 type of matrix and the intrinsic properties of it, as well as the direct or indirect  
283 competition between natural or added strains against pathogenic bacteria (Mejlholm  
284 and Dalgaard, 2007a). Certain strains of psychotropic *Lactobacillus* spp. and  
285 *Carnobacterium* spp. from cold-smoked salmon, which exert an antilisterial effect  
286 through the production of organic acids and other antimicrobials, such as bacteriocins,  
287 have been previously identified (Ghanbari et al., 2013). Bioprotective strategies are  
288 considered relevant to microbiological food safety primarily in products that allow for  
289 the growth of the pathogens according to the results observed in control samples.  
290 Indeed, Vermeulen et al. (2011) reported that smoked salmon enabled the growth of *L.*  
291 *monocytogenes* after refrigerated storage for 8 days 2 °C, 10 days 4 °C and 13 days at  
292 8 °C, with a 1.3 to 2.8 log increase at the end of the shelf life. Concha-Meyer et al.  
293 (2011) also reported a 2.4 log increase of *L. monocytogenes* after 28 days of storage of  
294 smoked salmon at 4 °C. Katla et al. (2001) reported an even higher growth potential,  
295 with an increase of 4.5 logs of *L. monocytogenes* after 14 days in vacuum-packed  
296 samples. Notably, the cold-smoked salmon in that study had been previously irradiated  
297 to reduce natural microbiota; thus, there was no competitive microbiota.

298 In this study, we reported the efficacy of *L. sakei* CTC494, which inhibited the growth of  
299 *L. monocytogenes* in all the three smoked salmon types tested with different  
300 representative physicochemical characteristics, including fat, protein, moisture, phenol  
301 and acetic acid content, after 8 °C refrigerated storage for 21 days in the presence of  
302 endogenous microbiota. Indeed, *L. sakei* CTC494 has been previously recognized as a  
303 starter and bioprotective culture for fermented sausages and raw and cooked meat  
304 products (Hugas et al., 1998; Ravyts et al., 2008). More recently, it has been tested on  
305 fresh fish (Costa et al, 2019). Moreover, *L. sakei* CTC494 has been reported to reduce  
306 the adhesion of *L. monocytogenes* to the intestinal cell line HT29 (Garriga et al., 2015),  
307 suggesting its potential probiotic properties. Uyttendaele et al. (2009) reported that only  
308 when the pH was lowered to 5.5 - 6.0 and the  $a_w$  was lowered to 0.93 - 0.94, three  
309 different inoculated LAB strains of smoked fish stored at 4 °C during 3 - 4 weeks  
310 exerted an antilisterial effect. The pathogen was able to grow on 48% of the smoked  
311 fish samples with a higher pH and  $a_w$ . In contrast, in the present study, *L. sakei*  
312 CTC494 inhibited *L. monocytogenes* growth even in products with a non-acidic pH and  
313 a higher water activity (pH slightly over 6.0 and  $a_w$  of 0.96). Katla et al. (2001) also  
314 reported a bacteriostatic effect when two *L. sakei* strains, one bacteriocin sakacin P  
315 producer (*L. sakei* Lb790 (pMLS114)) and its isogenic strain were used as potential  
316 bioprotective cultures on vacuum-packed smoked salmon at 10 °C for 28 days.  
317 However, the authors previously irradiated the product to eliminate the natural  
318 background microbiota. Weiss and Hammes (2008) also reported the potential of *L.*  
319 *sakei* strains, LTH4122 and LTH5754, fish isolates, to improve the safety of cold-  
320 smoked salmon stored at 4 °C without changing sensorial properties.

321 In our study, the selected *Carnobacterium* strain exhibited antilisterial activity in the *in*  
322 *vitro* assays but did not exert a significant antilisterial effect on the product except for  
323 smoked salmon type C, a product which higher concentration of acetic acid than the  
324 other type of cold-smoked salmon and where the bioprotective strain was not able to

325 grow. It has been described that growth of *Carnobacterium* could be affected by the  
326 presence of acetate (Wasney et al, 2001). Moreover, acetate has also been described  
327 as an inducer for the production of A9b bacteriocin on *Carnobacterium piscicola*  
328 (Nilsson et al., 2002). It is known that food components can affect bacteriocin  
329 production and activity (Aasen et al., 2003). Two strains of *C. piscicola* were previously  
330 reported to strongly suppress the growth of *L. monocytogenes* inoculated in cold-  
331 smoked salmon with background microbiota when stored at 5 °C for 32 days (Nilsson  
332 et al., 1999). Duffes et al. (1999b) also reported that certain strains of *Carnobacterium*  
333 ssp. and *L. sakei* are bacteriocin-like producers that can inhibit the growth of *L.*  
334 *monocytogenes* in a cold-smoked salmon model. Concha-Meyer et al. (2011) also  
335 reported a bacteriostatic effect of two *Carnobacterium* strains, one endogenous and  
336 one from meat, when they were trapped in alginate films to be applied on smoked  
337 salmon at 4 °C. Indeed, the government of Canada has included *Carnobacterium*  
338 *divergens* M35 in the list of permitted food preservative to be added as bioprotective  
339 culture in cold-smoked salmon and trout (item n°C.1A) together with other additives,  
340 such as sodium diacetate up to 0.25% as a processing aid (Health Canada, 2019).  
341 However, some authors have suggested that several strains of *C. divergens* and *C.*  
342 *piscicola* are promising as protective cultures in products with approximately 4%  
343 moderate NaCl water phase content. Different microorganisms that are more resistant  
344 to NaCl and smoke may be needed for long-storage products (Brillet et al., 2005;  
345 Himelbloom et al., 2001; Nilsson et al., 1999). Thus, further research on alternative  
346 bioprotective cultures, such as the cultures used in the present study, with average  
347 values of 4.7 - 5.5% NaCl in the water phase, are warranted.

348 In this study, all the products except the lot with *L. sakei* CTC494 enabled the growth of  
349 *L. monocytogenes* (> 0.5 logs). Thus, from a practical point of view and considering  
350 current EU legislation, *L. sakei* CTC494 was the only bioprotective culture that enabled  
351 the product to be changed from category 1.2 (RTE food able to support the growth of *L.*

352 *monocytogenes*) to category 1.3 (RTE food not able to support the growth of *L.*  
353 *monocytogenes*) (European Commission, 2005), thus categorizing it at a lower risk.  
354 Nevertheless, if we consider that *L. monocytogenes* post-processing contamination is  
355 generally low (1 log CFU/g or even less), and the three-level RTE-product  
356 categorization of Health Canada policies (Health Canada, 2011, 2012) introduces the  
357 potential of growth as a useful tool to assess risk for consumers, *L. curvatus* CTC1742  
358 may also be considered an effective bioprotective culture.

359 In this context, while control samples and *C. maltaromaticum* CTC1741 lots should be  
360 classified at the higher risk Category 1 (products that could support the growth of *L.*  
361 *monocytogenes*), *L. curvatus* CTC1742 may be moved to Category 2A (products which  
362 enable limited growth of *L. monocytogenes* to levels not higher than 100 CFU/g  
363 throughout the stated shelf life). In addition, cold-smoked salmon with *L. sakei* CTC494  
364 may be classified as Category 2B (RTE food products in which the growth of *L.*  
365 *monocytogenes* cannot occur throughout the expected shelf life of that food), which is a  
366 less risky category, not only benefiting consumer and public health but also the food  
367 enterprise, with low levels of monitoring priority and legislation constraints.

368 Moreover, considering the USDA *Listeria* zero policy approach (FSIS, 2014), the  
369 bacteriostatic effect of *L. sakei* CTC494, and the capacity of *L. curvatus* CTC1742 to  
370 limit the growth of *L. monocytogenes*, these strains could potentially be classified as  
371 antimicrobial agents (AMAs). In addition, the total suppression of *L. monocytogenes*  
372 growth exerted by *L. sakei* CTC494 would make the product eligible for a labelling  
373 claim regarding enhanced protection on the RTE cold-smoked salmon.

374 The results of the present study extend knowledge and open the field for the potential  
375 application of *L. sakei* CTC494 as a suitable antilisterial bioprotective culture on RTE-  
376 cold-smoked salmon.

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609 **Table 1:** Physicochemical characteristics of the different types of cold-smoked salmon  
 610 used for the challenge tests.

Physicochemical parameters	Smoked salmon type		
	A	B	C
	Mean ± SD	Mean ± SD	Mean ± SD
Fat (%)	7.06 <sup>a</sup> ± 1.37	7.21 <sup>a</sup> ± 1.99	15.44 <sup>b</sup> ± 2.24
Protein (%)	20.48 <sup>a</sup> ± 0.85	22.50 <sup>b</sup> ± 1.00	19.99 <sup>a</sup> ± 1.17
pH	6.03 ± 0.03	6.07 ± 0.06	6.10 ± 0.10
a <sub>w</sub>	0.96 ± 0.00	0.96 ± 0.00	0.96 ± 0.00
Moisture (%)	67.42 <sup>b</sup> ± 0.67	64.47 <sup>b</sup> ± 0.15	58.57 <sup>a</sup> ± 0.31
NaCl (%)	3.90 ± 0.80	3.15 ± 0.86	3.32 ± 0.80
Total phenol content (mg/Kg)	37.80 <sup>b</sup> ± 15.77	42.59 <sup>b</sup> ± 11.52	12.35 <sup>a</sup> ± 2.85
Lactic acid (mg/Kg)	5267 ± 153	5551 ± 239	5277 ± 578
Acetic acid (mg/Kg)	667 <sup>a</sup> ± 104	652 <sup>a</sup> ± 242	1818 <sup>b</sup> ± 341

611  
 612 <sup>a,b:</sup> Tukey-Kramer significant differences between physicochemical parameters among  
 613 smoked salmon types ( $p < 0.05$ ) are indicated by different small letters (in rows).

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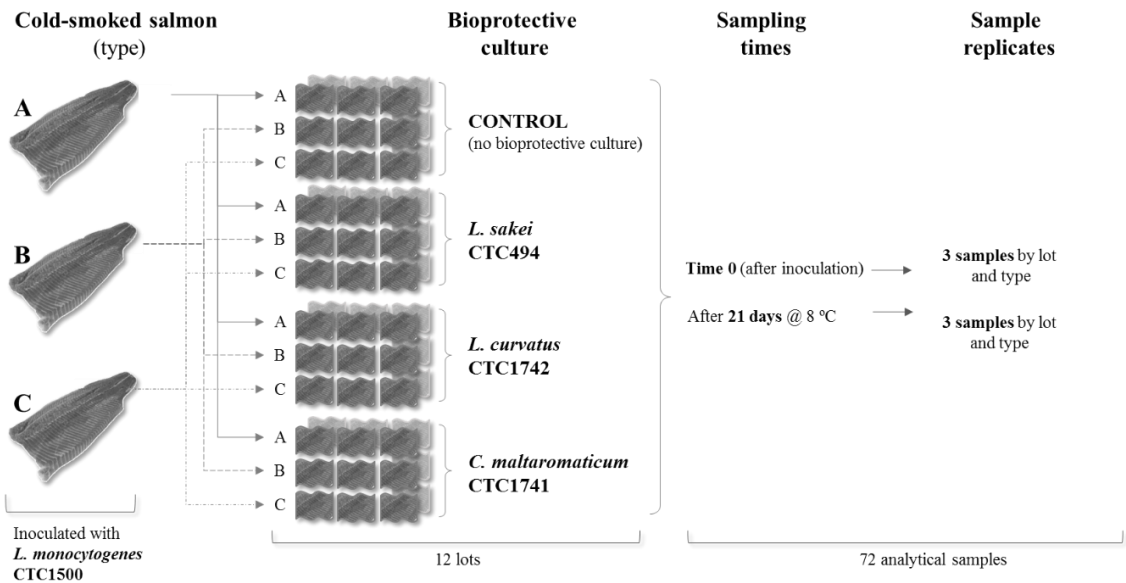
620 **Table 2:** Microbial counts (expressed in log CFU/g) of vacuum-packed cold-smoked salmon immediately after *L. monocytogenes* CTC1500  
 621 inoculum (Time 0) and after 21 days of storage at 8 °C.

Lot	Smoked salmon type	<i>L. monocytogenes</i>			Lactic acid bacteria			<i>Carnobacterium</i>		<i>Enterobacteriaceae</i>		
		Time (days)			Time (days)			Time (days)			Time (days)	
		0	21		0	21		0	21		0	21
		Mean ± SD	Mean	± SD	Mean ± SD	Mean	± SD	Mean ± SD	Mean	± SD	Mean ± SD	Mean ± SD
Control	A	2.68 <sup>A</sup> ± 0.05	6.43 <sup>Ba</sup>	± 0.36	1.45 <sup>b</sup> ± 0.58	2.63 <sup>b</sup>	± 1.93	2.17 ± 0.35	4.33 ± 2.73	0.95 ± 0.00	1.72 ± 1.20	
	B	2.65 <sup>A</sup> ± 0.13	5.85 <sup>Bab</sup>	± 2.44	1.45 <sup>b</sup> ± 0.58	2.19 <sup>b</sup>	± 2.47	2.87 ± 1.20	4.79 ± 3.23	0.96 ± 0.02	2.23 ± 1.44	
	C	2.69 <sup>A</sup> ± 0.11	4.93 <sup>Babc</sup>	± 0.70	2.35 <sup>b</sup> ± 1.68	2.74 <sup>b</sup>	± 2.06	2.16 ± 0.95	4.95 ± 1.78	0.95 <sup>A</sup> ± 0.00	2.97 <sup>B</sup> ± 0.86	
<i>L. curvatus</i> CTC1742	A	2.55 <sup>A</sup> ± 0.12	2.95 <sup>Bcde</sup>	± 0.17	4.65 <sup>Aa</sup> ± 0.23	8.68 <sup>Ba</sup>	± 0.18	2.64 ± 0.75	3.30 ± 1.51	0.95 ± 0.00	0.95 ± 0.00	
	B	2.56 <sup>A</sup> ± 0.11	3.49 <sup>Bbcde</sup>	± 0.60	4.73 <sup>Aa</sup> ± 0.08	8.80 <sup>Ba</sup>	± 0.07	2.71 ± 0.83	4.21 ± 2.60	0.96 ± 0.02	2.29 ± 1.55	
	C	2.63 <sup>A</sup> ± 0.04	4.00 <sup>Babcde</sup>	± 0.89	4.70 <sup>Aa</sup> ± 0.23	8.31 <sup>Ba</sup>	± 0.43	3.1 ± 0.84	4.69 ± 0.79	0.95 ± 0.00	2.07 ± 1.28	
<i>C. maltaromaticum</i> CTC1741	A	2.62 <sup>A</sup> ± 0.14	4.76 <sup>Babcd</sup>	± 0.71	1.45 <sup>b</sup> ± 0.58	0.95 <sup>b</sup>	± 0	3.91 <sup>A</sup> ± 0.33	6.73 <sup>B</sup> ± 1.28	1.08 ± 0.26	0.95 ± 0.00	
	B	2.67 <sup>A</sup> ± 0.09	5.22 <sup>Babc</sup>	± 0.26	1.45 <sup>b</sup> ± 0.58	3.48 <sup>b</sup>	± 1.92	3.99 <sup>A</sup> ± 0.42	7.69 <sup>B</sup> ± 0.56	0.95 ± 0.00	2.22 ± 1.46	
	C	2.63 ± 0.04	3.36 <sup>cde</sup>	± 1.03	2.28 <sup>b</sup> ± 1.53	4.22 <sup>b</sup>	± 3.77	3.7 ± 0.50	4.65 ± 1.13	1.52 ± 0.92	1.66 ± 0.82	
<i>L. sakei</i> CTC494	A	2.52 ± 0.03	2.27 <sup>e</sup>	± 0.20	4.86 <sup>Aa</sup> ± 0.03	8.51 <sup>Ba</sup>	± 0.06	2.68 ± 0.80	3.43 ± 1.81	0.95 ± 0.00	0.95 ± 0.00	
	B	2.67 ± 0.08	2.52 <sup>de</sup>	± 1.24	4.79 <sup>Aa</sup> ± 0.10	8.98 <sup>Ba</sup>	± 0.04	3.09 ± 1.28	4.48 ± 2.87	0.95 ± 0.00	2.21 ± 1.46	
	C	2.58 ± 0.10	2.10 <sup>e</sup>	± 0.90	4.89 <sup>Aa</sup> ± 0.11	8.88 <sup>Ba</sup>	± 0.08	2.66 ± 1.26	3.96 ± 0.84	1.31 ± 0.72	1.6 ± 0.63	

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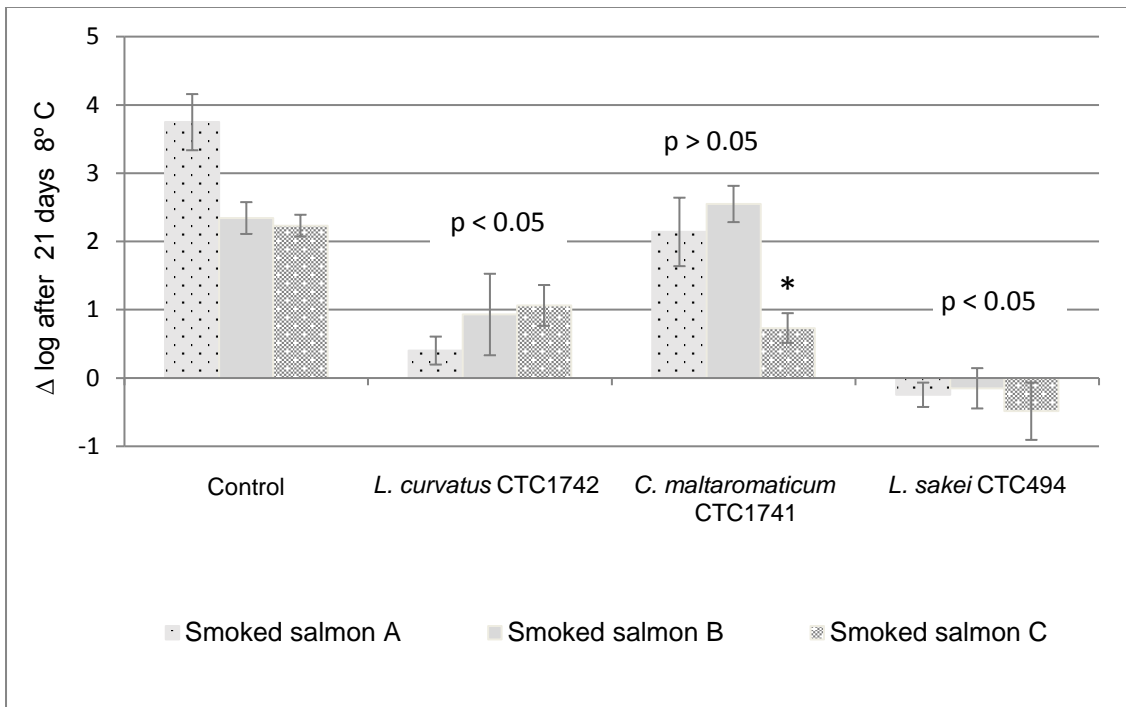
623 Significant differences in microbial counts among different types of cold-smoked salmon and lot are indicate by small letters (columns). Significant  
 624 differences in microbial counts between sampling times within each bacterial group are indicated by Capital letters (rows).

625 **Figure 1:** Challenge test experimental design for each independent trial.



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627 **Figure 2:** Growth potential of *L. monocytogenes* during the storage of vacuum-packed  
 628 cold-smoked salmon at 8 °C for 21 days, depending on the bioprotective culture and type  
 629 of salmon.  $p < 0.05$  (significant difference as compared with the control lot, according to  
 630 Dunnett's test). \* Significant differences among salmon types within each lot, according  
 631 to Tukey-Kramer test.



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