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

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Declarations of interest: none

ABSTRACT

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

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

Keywords: Food-borne pathogens; fish products; *Lactobacillus sakei* CTC494; listeriostatic.

1. Introduction

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

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

Biopreservation strategies are methods for preserving food using non-pathogenic safe microorganisms (protective cultures) that are selected to prevent the development of other undesirable microorganisms. Such strategies are considered natural and 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

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

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

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

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

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

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

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

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

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

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

2.2.1. Microbial analysis

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

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

2.2.2. Physicochemical analysis

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

2.3. Statistical analysis

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

3. Results

3.1. Identification and antimicrobial activity of isolates

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

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

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

3.2. Microbial and physicochemical characteristics of cold-smoked samples

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

219 C*arnobacterium* levels were under 2 log CFU/g in salmon A, and 2.15 ± 0.22 and 2.81
220 ± 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±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 ± 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 ºC (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 ± 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 ± 0.68 log CFU/g, while in the *C. maltaromaticum* CTC1741 lot, *L.*
256 *monocytogenes* achieved an average log increase of 1.81 ± 1.06 log CFU/g (almost
257 greater than a 2 log increase) (Figure 2), with average counts of 4.45 ± 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 ± 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 ± 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 ± 1.05 log

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

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

4. Discussion

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

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

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

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

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

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

In this context, while control samples and *C. maltaromaticum* CTC1741 lots should be classified at the higher risk Category 1 (products that could support the growth of *L. monocytogenes*), *L. curvatus* CTC1742 may be moved to Category 2A (products which enable limited growth of *L. monocytogenes* to levels not higher than 100 CFU/g throughout the stated shelf life). In addition, cold-smoked salmon with *L. sakei* CTC494 may be classified as Category 2B (RTE food products in which the growth of *L. monocytogenes* cannot occur throughout the expected shelf life of that food), which is a less risky category, not only benefiting consumer and public health but also the food enterprise, with low levels of monitoring priority and legislation constraints. Moreover, considering the USDA *Listeria* zero policy approach (FSIS, 2014), the bacteriostatic effect of *L. sakei* CTC494, and the capacity of *L. curvatus* CTC1742 to limit the growth of *L. monocytogenes*, these strains could potentially be classified as antimicrobial agents (AMAs). In addition, the total suppression of *L. monocytogenes* growth exerted by *L. sakei* CTC494 would make the product eligible for a labelling claim regarding enhanced protection on the RTE cold-smoked salmon.

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

Acknowledgements

This work was financially supported by the Spanish project, INIA-ENVASAC 2011 – 00030. We thank the CERCA Programme for its financial institutional support (Generalitat de Catalunya) and Raquel Rubio for the technical support.

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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 [a] ± 1.37	7.21 [a] ± 1.99	15.44 [b] ± 2.24
Protein (%)	20.48 [a] ± 0.85	22.50 [b] ±1.00	19.99 [a] ± 1.17
pH	6.03 ± 0.03	6.07 ± 0.06	6.10 ± 0.10
a_w	0.96 ± 0.00	0.96 ± 0.00	0.96 ± 0.00
Moisture (%)	67.42 [b] ± 0.67	64.47 [b] ± 0.15	58.57 [a] ± 0.31
NaCl (%)	3.90 ± 0.80	3.15 ± 0.86	3.32 ± 0.80
Total phenol content (mg/Kg)	37.80 [b] ± 15.77	42.59 [b] ± 11.52	12.35 [a] ± 2.85
Lactic acid (mg/Kg)	5267 ± 153	5551 ± 239	5277 ± 578
Acetic acid (mg/Kg)	667 [a] ± 104	652 [a] ± 242	1818 [b] ± 341

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612 [a,b:] 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.

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

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

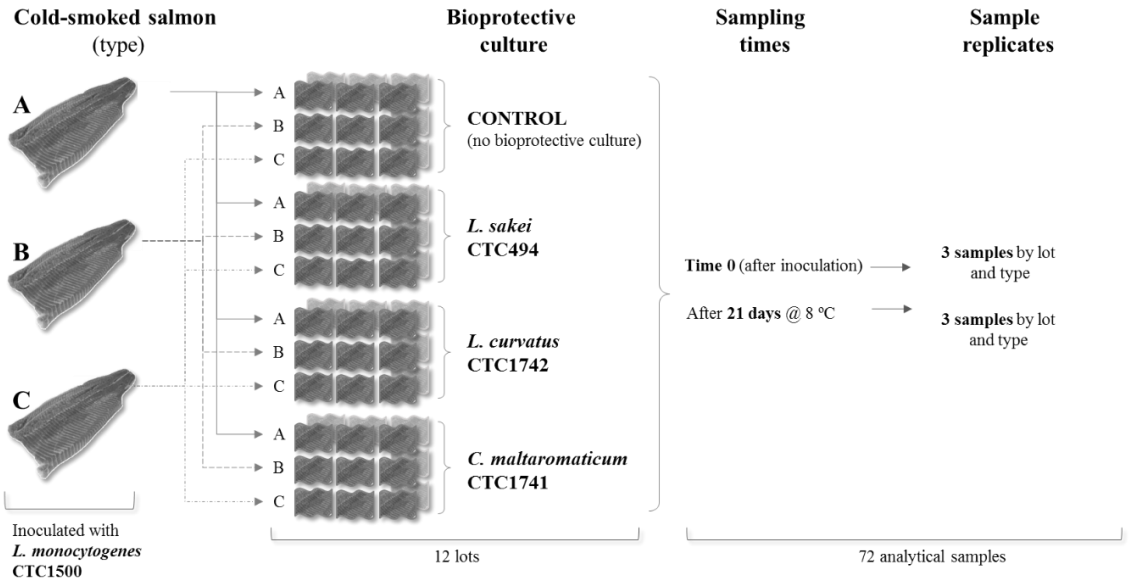

Figure 2: Growth potential of *L. monocytogenes* during the storage of vacuum-packed cold-smoked salmon at 8 °C for 21 days, depending on the bioprotective culture and type of salmon. $p < 0.05$ (significant difference as compared with the control lot, according to Dunnett's test). * Significant differences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