Anti-listerial inhibitory lactic acid bacteria isolated from commercial cold smoked salmon

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Abstract

The natural microflora of cold-smoked fish at the end of shelf-life are lactic acid bacteria (LAB). Some of these display a capacity to inhibit spoilage as well as several strains of pathogenic micro-organisms, e.g. *Listeria monocytogenes* which is isolated frequently from cold-smoked salmon (CSS).

Eight batches of sliced vacuum-packed CSS from Norway, Scotland and Spain were collected at retail. Packs were stored at 5 °C and examined for chemical and microbiological characteristics, at purchase date and at expiration date. pH, water activity and salt content were similar to available data on lightly preserved fish products. There was a consistent pattern in the development of the microflora on CSS; the initial level of LAB was low on freshly produced CSS (10^2 cfu g^{-1}); however, storage in vacuum packaging at refrigeration temperature was elective for LAB. At the end of the stated shelf-life these micro-organisms, represented mainly by *Lactobacillus* spp., attained ca.10^7 cfu g^{-1} while *Enterobacteriaceae* counts were consistently lower (10^5 cfu g^{-1}), which indicates the ability of LAB to grow and compete with few carbohydrates available and in the presence of moderate salt concentrations. *L. monocytogenes* was not found in any sample.

Forty-one percent of LAB strains isolated exhibited inhibitory capacity against *Listeria* innocua, in a plate assay. A majority of the inhibitory effects were non-bacteriocinogenic, but nevertheless were very competitive cultures which may provide an additional hurdle for improved preservation by natural means.

Keywords: Cold smoked fish; Lactic acid bacteria; Salmon; Bacteriocins; Listeria; Preservation; Competitive bacteria

Introduction

The consumer acceptance of minimally processed refrigerated foods has been increasing during the past several years (Montville and Kaiser, 1993). However, the microbiological safety of these foods is of concern due to the possible presence of non-proteolytic strains of *Clostridium botulinum* able to grow at 4 °C, and post-processing contamination with other psychrotrophic pathogens, such as *Listeria monocytogenes* (Wessels et al., 2004).

L. monocytogenes may be found in cold-smoked salmon (CSS), typically in low numbers (Cortesi et al., 1997; Jørgensen and Huss, 1998), and it is possible that the lactic acid bacterial microflora developing during storage of CSS, may play an important role in controlling the growth of this pathogen. Inhibition results from the production of natural preservatives such as organic acids, hydrogen peroxide and diacetyl, and antimicrobials such as bacteriocins, as well as competition for, or depletion of, specific nutrients (see for example, Devlieghere et al., 2004; Buchanan and Bagi, 1997; Nilsson et al., 1999).

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The purpose of this study was to characterize the LAB microflora of sliced vacuum-packed CSS from different countries and manufacturers, and specifically to test the antagonistic activity of LAB isolated from those products, against *L. innocua* and *L. monocytogenes*. The nature of the inhibition was also investigated, by neutralizing the acidity of LAB culture supernatants and treatment with catalase and trypsin.

Materials and methods

Eight batches (each one consisting of 6 × 100-g packs) of sliced vacuum-packed, CSS, from Norway (P1–P4), Scotland (P5–P6) and Spain (P7–P8) were collected at retail. Packs were stored at 5 °C and examined for chemical and microbiological characteristics, at the purchase date (T_0) and at the expiry date (T_1), just 35 days after T_0. The dates of manufacture of products were not stated on the packs. The country of origin of the raw salmon was stated as Norway except for Scottish CSS (P5–P6) in which case the salmon was from Scotland. Declared ingredients included smoked salmon (*Salmo salar*), salt and sugar. Two of the four samples of CSS from Norway, denoted as P1 and P3, were made without sugar.

Physico-chemical analyses

Salt concentration was determined according AOAC (1995), method 937.09. The salt content was expressed as % NaCl in water phase of muscle (Tomé et al., 1999).

Moisture contents of samples of CSS were evaluated according AOAC (1995), method 24.003.

Water Activity (a_W) was determined using a Rotronic Hygroskop DT Instrument (Rotronic AG, Zurich, Switzerland).

pH was measured in smoked fish homogenates (1:1 mix of CSS and distilled water, stomached for 2 min) with a micropH 2002 pH meter (Crison, Barcelona, Spain).

Microbiologic alanalyses

At T_0 and at T_1, 10 g of fillets were homogenized in 90 ml of 1/4 strength Ringer's solution (Lab M, Manchester, UK) for 2 min in a Stomacher 400 Lab Blender (Seward Medical, London, UK). Serial decimal dilutions in 1/4 strength Ringer's solution were prepared.

Total viable counts (TVC) were determined by spread-plating suitable dilutions on Long and Hammer's Medium, (L & H; as modified by van Spreekens, 1974) + 1% w/v NaCl (Merck, Darmstadt, Germany), and plates were incubated aerobically at 25 °C for 5 days.

Lactobacillus spp. were enumerated in pour plates of MRS (LAB M; De Man et al., 1960) agar medium, incubated aerobically (25 °C for 3 days).

Total lactic acid bacteria (LAB) were enumerated by pour-plating in nitrite actidione polymyxin (NAP) agar, pH 6.7 (Davidson and Cronin, 1973). All colonies were counted as presumptive LAB after 5 days of anaerobic incubation at 25 °C.

Enterobacteriaceae were determined using overlaid plates of violet red bile glucose agar (VRBGA; Biokar Diagnostics, Beauvais, France). Plates were incubated at 37 °C for 24 h.

Detection of L. monocytogenes was performed following the ISO11290-1 protocol (ISO, 1996). All tubes were streaked onto PALCAM agar (Merck); incubated at 35 °C, 48 h. Typical colonies were streaked onto Tryptone Soy Yeast Extract agar (TSA-YE; Tryptone Soy Broth (Lab M) + 6 g l^{-1} yeast extract (Lab M) + 12 g l^{-1} agar (Lab M); incubated at 37 °C for 24 h for further confirmation and identification by Gram staining, catalase activity, CAMP test with *Staphylococcus aureus* and *Rhodococcus equi* and also by mannitol, rhamnose and xylose fermentation.

Enumeration of *L. monocytogenes* was by the most probable number (MPN) technique. The values of the MPN for three tubes inoculated from each of three successive 10-fold dilutions were determined according to Harrigan and McCance (1986) MPN calculations.

Isolation and phenotypic characterizatio no fmicro-organisms

At T_0 and T_1, 10% of colonies overall were picked randomly from NAP plates containing 10–100 colonies. Presumptive LAB were subcultured on NAP agar without selective agents or on all-purpose Tween (APT, Difco) agar, examined for purity and characterized using Gram stain, cytochrome oxidase, and catalase tests. Organisms that were Gram positive, cytochrome oxidase negative and catalase negative were stored in APT broth with glycerol (30% v/v) at −80 °C.

Anti-listerial activity

Antimicrobial activity of the LAB isolates was investigated against *L. innocua* 2030c (Central Public Health Laboratory, Colindale, London), a tetracycline-resistant strain. Challenge studies with cold smoked fish have shown that *L. innocua* 2030c can be considered a suitable marker for replacing *L. monocytogenes* in experiments where anti-listerial properties of LAB strains are evaluated (Vaz-Velho et al., 2001). An overnight culture (37 °C) of *L. innocua* 2030c, was diluted in sterile APT broth to an OD (UNICAM 8620, Cambridge, England) between 0.2 and 0.3 (ca.10^6 cfu ml^{-1}) before use, and 200 µl were spread on

APT agar medium with a swab. Drops (10 µl) of each LAB culture, previously grown in APT broth at 25 °C for 48 h, were then inoculated. Plates were incubated at 25 °C for 48 h. Any clear zones of inhibition around the LAB culture spot were measured.

From the above assay, LAB cultures showing defined inhibition zones, >8 mm zone width, were selected. Zones of <8 mm tended to disappear after 48 h incubation. Cell-free culture supernatants were spotted against *L. innocua*. Anti-listerial LAB supernatants were also tested against the producing organisms already stated and four different strains of *L. monocytogenes* previously isolated from smoked salmon or trout.

In order to determine the nature of the inhibition, the pH of cell-free supernatants was adjusted to 6.5 with NaOH (1 N) and then treated with catalase (Sigma; 500 IU ml^{-1}) and trypsin (Sigma; 0.1 mg ml^{-1}). Neutralized cell-free supernatant, neutralized cell-free supernatant treated with catalase and neutralized cell-free supernatant treated with catalase and trypsin, were spotted against the producing organisms and against *L. innocua* and *L. monocytogenes*.

Lactobacillus sakei CTC 494 (CTC, IRTA Meat Technology Centre Collection Monells, Spain) was used as an anti-listerial reference strain in this study.

LAB identication

Those cultures of which the cell-free supernatant showed anti-listerial properties were identified using the API 50 CH kit (BioMerieux, Lyon, France) and analysed by APILAB PLUS software version 3.2.2.

Statistical analyses

One-way ANOVA and multiple range test were used to evaluate differences between means of pH, salt, aw and microbiological counts, using the least significance difference (LSD) test at a significance level of 5%. Calculations were carried out using Statgraphics Plus (Rockville, USA).

Results

Chemical and physico-chemical characteristics

Results of analyses of vacuum-packaged CSS are summarized in Table 1. The eight lots collected at retail from different producers were relatively homogeneous in their salt concentrations in the water phase, ranging between 4.12% and 4.99% with an average concentration of 4.50 ± 0.28%.

The pH, initially with an average value equal to 5.99 ± 0.10, remained relatively unchanged during the storage of all the samples, with an average value of 6.01 ± 0.14 at the end of the stated shelf-life.

Water activity (a_W) with an average value of 0.95 ± 0.01, had the lowest mean value in Norwegian smoked samples (0.94) and the highest (0.96) in one of the two Scottish samples.

Table 1
Physical and chemical characteristics of refrigerated vacuum-packed CSS (*S. salar*) from different producers, at purchase date (T_0) and at the expiry date (T_1)

Producer[A]	NaCl (%)[B]	pH		a_W
		T_0	T_1	
P1	4.31[a]	5.95[a]	6.07[a]	0.958[bc]
P2	4.75[a]	6.25[a]	6.22[a]	0.938[a]
P3	4.99[a]	5.94[a]	5.93[a]	0.938[a]
P4	4.28[a]	5.97[a]	5.91[a]	0.955[b]
P5	4.55[a]	5.97[a]	5.90[a]	0.951[b]
P6	4.12[a]	5.98[a]	5.92[a]	0.962[c]
P7	4.48[a]	5.93[a]	6.21[a]	0.951[b]
P8	4.52[a]	5.97[a]	5.93[a]	0.953[b]
Average	4.50[a]	5.995[a]	6.011[a]	0.951
SD	0.28[a]	0.104	0.136	0.009

Means in the same column with the same letter are not significantly different ($P > 0.05$).
[A]Six packages from each producer
[B]Salt content in water phase

Microbiological status

Results of microbiological analyses at the purchase date (T_0) and at the expiry date (T_1) are listed in Table 2.

The initial number of micro-organisms recovered on L & H medium ranged between 10^2 and 10^4 cfu g^{-1}, with statistically significant differences ($P < 0.05$) between the two Scottish and the two Spanish producers.

During the storage period, TVC increased significantly, independently of the manufacturer, reaching values between 10^5 and 10^7 cfu g^{-1} at the expiration date of the product.

The initial counts of *Lactobacillus* spp. recovered on MRS agar were, in general, low (10^2–10^3 cfu g^{-1}) and became dominant under refrigerated storage, increasing to 10^5–10^7 cfu g^{-1}. Higher counts of these micro-organisms were found in samples P4, P5, P6, P7 and P8. The lowest counts, samples P1 and P3, were both from products manufactured without addition of sugar.

Initial total LAB counts ranged between 10^2 and 10^4 cfu g^{-1}. Statistically significant differences between samples P6 and P8 were found between MRS and NAP counts at T_0. During vacuum-packed storage at 5 °C, total LAB increased. Counts on MRS agar became equal to counts on NAP agar except for P3, indicating that *Lactobacillus* was generally the dominant genus.

Table 2
Microbiological status of refrigerated vacuum-packed CSS at the purchase date (T_0) and after 5 weeks of refrigerated storage (T_1).

Microbial counts (Log cfu g^{-1})										
Producer[A]	TVC (L&H)		*Lactobacillus* spp. (MRS)		Total LAB (APT)		*Enterobacteriacea* (VRBG)		*L. monocytogenes* (MNP)	
	T_0	T_1	T_0	T_1	T_0	T_1	T_0	T_1	T_0	T_1
P1	2.69^{a}	5.67^{d}	3.51^{ab}	5.44^{d}	3.22^{a}	5.74^{de}	3.58^{b}	5.02^{c}	<0.3	<0.3
P2	2.69^{a}	7.14^{fg}	2.06^{a}	6.52^{ef}	2.23^{a}	6.42^{e}	2.32^{a}	5.02^{c}	<0.3	<0.3
P3	3.01^{a}	6.13^{e}	2.79^{a}	5.45^{d}	3.28^{a}	5.81^{e}	3.04^{a}	5.14^{cd}	<0.3	<0.3
P4	2.75^{a}	7.41^{g}	2.00^{a}	7.22^{g}	2.00^{a}	7.55^{g}	2.00^{a}	5.44^{d}	<0.3	<0.3
P5	2.98^{a}	7.43^{g}	2.00^{a}	7.58^{g}	2.22^{a}	7.76^{gh}	2.46^{a}	5.00^{c}	<0.3	<0.3
P6	3.56^{b}	7.78^{h}	2.90^{a}	7.35^{g}	3.68^{b}	7.69^{gh}	2.60^{a}	6.32^{e}	<0.3	<0.3
P7	3.10^{a}	6.61^{f}	3.27^{ab}	7.29^{g}	4.16^{bc}	7.05^{fg}	2.00^{a}	3.74^{b}	<0.3	<0.3
P8	3.96^{b}	7.78^{h}	3.74^{b}	7.49^{g}	4.59^{c}	7.09^{fg}	2.41^{a}	4.08^{b}	<0.3	<0.3

Means with the same letter are not significantly different ($P > 0.05$).
[A]Six packages from each producer

Counts of *Enterobacteriaceae* were initially low, ranging between 10^2 and 10^3 cfu g^{-1} at T_0, but increasing to ca. 10^5 cfu g^{-1} after 5 weeks of vacuum storage, except for sample P6 and P7, P8 in which slightly higher and lower counts were recorded, respectively.

L. monocytogenes was not found in any sample.

Anti-listerial activity and LAB identication

The anti-listerial activity of 299 presumptive LAB picked from NAP plates, at the purchase date (T_0) and 331 strains of LAB picked after 5 weeks of refrigerated storage in vacuo (T_1) was evaluated. Inhibition zones against *L. innocua* $\geqslant$ 8 mm were observed for 261 (41.4%) isolates. Fig. 1 shows the percentage of inhibitory organisms (as cultures) isolated at T_0 and T_1 from products from each manufacturer. Most of the inhibitory isolates, except notably in P_3 and P_4, were isolated at T_0.

From the cultures with antagonistic properties, only six cell-free supernatants showed inhibitory activity against *L. innocua* 2030c.

All six live culture isolates and their cell-free supernatants, were able to inhibit *L. monocytogenes* in an in vitro assay performed at 25 °C (Table 3), although the inhibitory activity was greater with live cells than with cell-free supernatants (data not shown). Anti-listerial activity was lost in two cell-free supernatants after adjusting their pH to 6.5, which suggests that their inhibitory activity was due to organic acids.

Another two cell-free supernatants were inactivated by catalase. Anti-listerial activity by proteolytic compounds was found in two strains which make them promising candidates for biopreservation of CSS stored at 5 °C.

According to the carbohydrate fermentation patterns, all of these inhibitory strains belong to the genus *Lactobacillus* or *Lactococcus*. The two strains considered as bacteriocin-producing LAB, were Gram-positive cocci, and successfully identified as *Lactococcus lactis* ($P = 99.9\%$). The others strains are short, Gram-positive rods, two of them (acid-producing LAB) identified as *Lactobacillus plantarum* and the last two strains (hydrogen peroxide-producing LAB) as *Lactobacillus brevis* ($P = 99.9\%$, respectively). These micro-organisms were previously isolated from CSS and other fish products (Antara et al., 2002; González-Rodríguez et al., 2002; Sharp and Pettipher, 1983).

No auto-inhibition was observed.

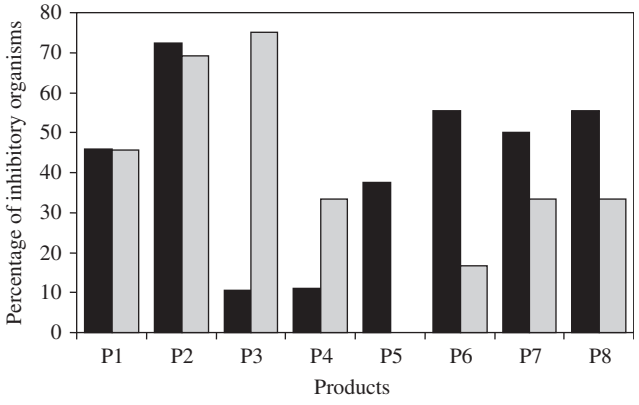

Fig. 1. Percentages of anti-listerial LAB cultures obtained from cold smoked salmon fillets produced by different manufacturers (P1–P8), during refrigerated storage (5 °C). T_0 ■; T_1 □.

Discussion

Chemical and physico-chemical characteristics

In all samples salt-on-water concentrations were higher than the minimum guidelines for vacuum-

Table 3
Inhibitory activity (25 ºC) of lactic acid bacteria against *L. innocua* 2030c and *L. monocytogenes* determined by a spot assay on APT agar

Producer strain	*L. monocytogenes* strains and *L. innocua* (2030c)				
	Live-cell supernatant	Cell-free supernatant	Cell-free supernatant[a]	Cell-free supernatant[a] treated with catalase[b]	Cell-free supernatant[a] treated with catalase[b] & trypsin[c]
E1	+	+	+	–	–
E2	+	+	–	–	–
E3	+	+	+	–	–
E4	+	+	–	–	–
E5	+	+	+	+	–
E6	+	+	+	+	–
L. sakei CTC 494	+	+	+	+	–

[a]Adjusted to pH 6.5.
[b]500 IU ml^{-1}.
[c]0.1 mg ml^{-1}; + inhibition zone; – no inhibition zone.

packaged cold-smoked fish processed without nitrites (3.5%) (ACMSF, 1992).

The pH remained relatively unchanged during the storage of all the samples, reflecting low sugar contents and a strong buffering effect of fish muscle. Although there were no statistically significant differences ($P > 0.05$) in mean pH values among the samples, pH values close to pH 5.9 were observed in most of the samples with higher lactobacilli counts (P4, P5, P6 and P8). Leroi et al. (1998) have shown that, among nine bacterial groups currently identified in CSS, *Lactobacillus* was the only genus which was able to acidify the products to these values.

Microbiological status

According to the samples analysed there was not a large variation in the microbial quality of smoked salmon offered on the Portuguese market.

Since the production date of these products was unknown (because they were acquired in the supermarket), the different storage temperatures to which the products were exposed before the purchase date, could explain at least in part, the variations in the quantitative microbiological composition found among them. Published data on the microbial contamination of CSS indicated that it is highly dependent on the storage temperature. According to Sirkorski (2002), the bacterial load on vacuum-packed CSS stored 4–7 days at 12 °C, may be about 2.5 log cycles higher than at 6 °C. Furthermore, to control the microbiological quality of the final product, the initial contamination of the fish must be assessed because it could represent a risk of bacterial food poisoning caused by fish smoked in mild conditions (< 30 °C).

The ability of *Lactobacillus* spp. to grow and compete, with only a low level of carbohydrates available and in the presence of moderate salt concentrations, is noteworthy. However, as shown in Table 2, the presence of sugar enhances the growth of lactobacilli, resulting from the fermentative nature of these micro-organisms. Previous studies (Leroi et al., 2001; Connil et al., 2002; Emborg et al., 2002) have demonstrated that LAB are potential spoilage organisms associated with higher total volatile basic nitrogen (TVBN) concentrations in CSS, reducing the shelf-life of this product.

According to Leroi et al. (1998, 2000), the carnobacteria count can be estimated by the difference between NAP and MRS counts; in T_0 samples P6 and P8, a proportion of the LAB probably belonged to the *Carnobacterium* genus. In T_1 samples, only P3 presented a difference in total LAB and MRS counts, suggesting that carnobacteria were a significant proportion of the LAB microflora developed in this sample. It is interesting to note that P3 was made without sugar.

Development of *Enterobacteriacea* seems to be correlated with a low sodium chloride concentration and higher a_w, e.g. in P6 with a low salt a 4 log increase, in P3 with highest sodium chloride concentration a 2 log increase in *Enterobacteriaceae* counts.

The *Enterobacteriaceae* counts always remained 1–3 logs lower than TVC, indicating that they were not the dominant flora in any of these products. According to Leroi et al. (1999), the shelf life of smoked salmon is closely linked to the initial *Enterobacteriaceae* count; the higher the initial total count on VRBG agar, the shorter the shelf life.

L. monocytogenes was not found in any sample. The absence of *L. monocytogenes* in these samples of vacuum-packed fish, with an average salt concentration in water phase of 4.50%, which would suppress the growth of *C. botulinum* in these products, could indicate good manufacturing practice and appropriate storage.

Anti-listerial activity

The percentage of inhibitory cultures isolated at T_0 and T_1 shows a large variation between them, including between producers in the same country (Fig. 1).

These results indicate large differences in the compositions of the LAB between CSS samples, probably as a result of the different processing and hygiene conditions between manufacturers. As can be seen in Fig. 1, isolates from T_0 showed the greatest percentages of inhibitory strains in most of the samples. The greater percentages of inhibitory LAB on freshly produced cold-smoked fish could protect the product to a considerable degree against some pathogenic micro-organisms like *L. monocytogenes*, in which case, counts of this pathogen could be maintained at a low level during the storage.

The antagonistic activity by live cells was only observed in the corresponding filter supernatants of six isolates. These results suggest that the antagonistic mechanism of most of the isolates could be due to competition for or depletion of specific nutrients as has been suggested by Nilsson et al. (2005).

Inhibitory activity against *L. innocua* and strains of *L. monocytogenes* was lost in two cell-free supernatants when the pH was neutralized. Similar antimicrobial results to those reported here, were obtained by Aslim et al. (2005) when testing 19 cell-free supernatants of LAB isolated from Turkish dairy products. Among the *Lactobacillus* spp. cultures assayed, it was observed that inhibitory activity against strains of *L. monocytogenes* and *S. aureus* was lost in 15 cell-free supernatants, including *L. plantarum* culture-free supernatants, when the pH was neutralized.

Production of lactic acid, the major metabolite of LAB, is responsible for the associated decrease in pH, which may be sufficient to antagonize many micro-organisms. The undissociated acid can cause the collapse of the electrochemical proton gradient of susceptible bacteria, leading to bacteriostasis and eventual death (Soomro et al., 2002).

Another two inhibitory cell-free supernatants were inactivated by catalase. Peroxide-producing LAB were identified as *Lactobacillus brevis*. Hydrogen peroxide is produced by a large number of LAB lacking the enzyme catalase—in particular by *Lactobacillus* spp.—and inhibits other micro-organisms such as *Staphylococcus aureus* and *Listeria* spp. The potential of H_2O_2 produced by LAB for food preservation, may be limited by the oxidizing nature of the molecule, and free radicals produced may have profound effects on the sensory quality, causing rancidity of fats and oils and discoloration reactions (Devlieghere et al., 2004). The production of acids and hydrogen peroxide by LAB and inhibitory properties have been registered by several authors (Juvnen et al., 1992; Tharrington and Sorrelis, 1992; Ocando et al., 1993; Guerra et al., 2001).

Anti-listerial activity by peptides or proteins was found in two strains identified as *Lact. lactis,* which make them promising candidates for biopreservation of cold-smoked salmon stored at 5 °C. Several bacteriocins produced by *Lactococcus lactis*, other than nisin, have been identified: Lacticin 481, Lactococcin A, Lactococcin B and Lactococcin M (O'Sullivan et al., 2002). The first one belongs to Class I encompassing the small, post-translationally modified, broad host range lantibiotics, of which nisin is the best-known example, and the last three belong to Class II which includes the small, heat-stable unmodified peptides (Cleveland et al., 2001).

In conclusion, although the nature of most of the inhibitions was unknown, the presence in vacuum-packed CSS of a high proportion of inhibitory LAB, at high cell densities, may provide an additional hurdle for improved preservation by natural means.

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