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Lactococcus lactis ssp. *lactis* as Protective Culture in Vacuum-Packed Raw Salmon (*Salmo salar*)

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This study evaluates the potential use of protective cultures to improve the microbial quality of vacuum-packed raw Atlantic salmon. The inhibitory properties of 16 selected lactic acid bacteria and bifidobacteria against 32 spoilage organisms were characterized. As the food matrix and natural microflora of the product can affect the inhibitory effect, the best inhibitory strain, *Lactococcus lactis* ssp. *lactis*, was also tested in vacuum-packed salmon. As a result, *L. lactis* treated products had 3-days prolonged shelf life when compared to nontreated fish. In addition, the usage of *L. lactis* did not change the organoleptical and textural properties of the fish. This study shows that *Lactococcus lactis* might be applied to increase shelf life of vacuum-packed raw fish stored at refrigeration temperatures.

Keywords: fish, salmon, spoilage, vacuum-packed, biopreservation, *Lactococcus lactis*

INTRODUCTION

Biopreservation is gaining commercial attention, as consumers prefer minimally processed food prepared without chemical preservatives. In biopreservation, the food storage life is extended and safety of foods is enhanced using the natural or controlled microflora and/or their antimicrobial products (Stiles, 1996). Lactic acid bacteria (LAB) and their metabolites as bacteriocins are often used in biopreservation (Devlieghere et al., 2004). Biopreservation of meat is much more common than that of fish products. Most fish studies are concentrated on the anti-*Listeria* activity of LAB in cold smoked salmon (Brillet et al., 2005; Duffes et al., 1999; Tome et al., 2006, 2008; Vescovo et al., 2006; Wessels and Huss, 1996) as well as shrimps and sardines (Fall et al., 2010; Paari et al., 2011, 2012) and are usually conducted with LAB isolated from the same product as the spoilage organisms. The aim of this study was to select commercial LAB or bifidobacteria strains with inhibition capacity against spoilage bacteria of vacuum-packed raw salmon. LAB and bifidobacteria are anaerobic organisms, so they can survive in vacuum-packed products where the atmospheric oxygen is removed. In addition, many of the selected bacteria have probiotic status. Probiotic bacteria as protective cultures offer additional marketing opportunities as they have health promoting effects, and more importantly, they are well characterized and have a long history of safe use (FAO/WHO, 2001).

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The first step in the selection of the protective cultures is the screening test, which is usually carried out in microbiological media. The common methods of inhibition assessment are the agar spot test, agar overlay method, and well and disk diffusion assays. Although widely used, they are time-consuming and do not take the full cycle of growth curve into account. As the effect of the protective culture depends on growth rates of both the spoiling and the protective strains (Rodgers, 2001), screening based on the analysis of the growth curve of the spoilage organism could be a better method for the selection of the best protective strains. Here, a fast method based on the growth rate and lag phase as determined by fitting the data to the reparameterized Gompertz model (Zwietering et al., 1990) was used to screen for biopreservative strains. The model has been used earlier in predicting the general growth of bacteria in foods (Baranyi and Roberts, 1994) and in the estimation of *Listeria monocytogenes* growth (Lu et al., 2005) as well as in the prediction of *Staphylococcus aureus* growth as a function of temperature, pH, and NaCl concentration (McCann et al., 2003). Finally, since the food matrix can affect the inhibitory effect of the protective culture, the best strain—*Lactococcus lactis* ssp. *lactis*—was incorporated into the vacuum-packed salmon to evaluate the inhibitory and sensory quality effects in the real product.

MATERIALS AND METHODS

Bacterial Strains

Sixteen bifidobacteria and lactic acid bacteria strains were tested for their inhibition activity against 32 indicator strains (Tables 1 and 2). The indicator strains were originally isolated from the spoilage microflora of vacuum-packed Atlantic salmon (Vesterlund, Submitted). Bifidobacteria and LAB were grown in de Man, Rogosa, and Sharpe (MRS; Oxoid, Basingstoke, UK) broth in anaerobic conditions at 37°C for 24 h (or for 48 h in the case of bifidobacteria). MRS was supplemented with 40 mM glycerol for *Lactobacillus reuteri* ING1 and with 0.05% L-cysteine for bifidobacteria. Cell-free supernatants (CFSs) of bacteria were collected by centrifugation (5,000 *g* at 4°C for 7 min)

TABLE 1
Strains used to screen the antimicrobial properties against spoilage organisms of vacuum-packed Atlantic salmon

No.	*Strain*	*Origin of the strain*
1	*Bifidobacterium lactis* Bb-12	Chr. Hansen A/S (Hørshom, Denmark)
2	*Bifidobacterium lactis* Bl-04	Danisco (Kantvik, Finland)
3	*Bifidobacterium longum* 46	DSM 14583
4	*Lactobacillus acidophilus* LA5	Chr. Hansen A/S (Hørshom, Denmark)
5	*Lactobacillus brevis* Lbr-35	Danisco (Kantvik, Finland)
6	*Lactobacillus casei* Shirota	Yakult® (Tokyo, Japan)
7	*Lactobacillus fermentum* ME3	University of Tartu (Tartu, Estonia)
8	*Lactobacillus plantarum*	ATCC 8014
9	*Lactobacillus plantarum* 299v	Valio (Helsinki, Finland)
10	*Lactobacillus plantarum* Lp-115	Danisco (Kantvik, Finland)
11	*Lactobacillus reuteri* ING1	Ingman Foods (Söderkulla, Finland)
12	*Lactobacillus rhamnosus* 1460	Danisco (Kantvik, Finland)
13	*Lactobacillus rhamnosus* GG	ATCC 53103; (Valio, Helsinki, Finland)
14	*Lactobacillus rhamnosus* LC-705	DSM 7061; (Valio, Helsinki, Finland)
15	*Lactobacillus rhamnosus* LR-32	Danisco (Kantvik, Finland)
16	*Lactococcus lactis* ssp. *lactis*	Valio (Helsinki, Finland)

TABLE 2
Spoilage bacteria isolated from vacuum-packed Atlantic salmon

Number of isolates	*Indicator strain, closest match (GenBank), homology (%)*
2	*Brochothrix* sp. 22L, FJ151397, 95%
2	*Brochothrix thermosphacta* isolate DSMZ 20599, AY543024, 97%
3	*Brochothrix thermosphacta* isolate MF 88, AY543029, 98%
1	*Buttiauxella* sp. 01WB04.1-50, FM161527, 91%
3	*Buttiauxella agrestis* strain Shia-ES148.4, FJ392809, 97%
3	*Carnobacterium* sp. sp. KH1, AB213026, 92-100%
1	*Carnobacterium divergens* strain LHICA_42_3, FJ656714, 100%
1	*Carnobacterium divergens* strain LHICA_42_5, FJ656715, 99%
1	*Carnobacterium divergens* strain LHICA_53_4, FJ656716, 91%
1	*Carnobacterium maltaromaticum* CLFP 196, DQ412705, 100%
1	*Carnobacterium maltaromaticum* LHICA_36_4, FJ656722, 98%
1	Enterobacter sp. ZJUPD5, EU430751, 91%
1	*Hafnia* sp. GC36, EU159563, 99%
1	*Hafnia* sp. SM1-48, AM268318, 99%
1	*Hafnia alvei*, AB435608, 99%
1	Uncultured *Kluyvera* sp. isolate, FJ719120, 96%
1	*Lactobacillus sakei* subsp. *sakei* 23K, NC_007576, 99%
1	*Obesumbacterium proteus* strain B8P3-1, EU888873, 99%
1	Marine bacterium CS-23, EF040544, 94%
1	Uncultured proteobacterium, AJ310685, 100%
1	*Serratia* sp. AKB-2008-HE59, AM989307, 98%
1	*Serratia* sp. CJB2, FJ545753, 99%
1	*Serratia proteamaculans* strain 2A-CDF, FJ811861, 99%
1	*Yersinia kristensenii* strain 991, FJ641894, 100%

and filter-sterilized through Pall Acrodisc PF Syringe Filter 0.8/0.2 μm membrane filters (Pall Corporation, Port Washington, NY, USA).

The indicator strains were grown in cooked meat broth (CMB; Merck, Darmstadt, Germany) in aerobic conditions at 37°C for 24 h. Bacteria were harvested by centrifugation (5,000 *g* for 7 min), washed twice with phosphate-buffered saline (PBS; pH 7.2), and the optical density (at 600 nm) of the cultures was adjusted spectrophotometrically to 0.5.

The Inhibitory Assay

In the inhibitory assay, the effect of CFSs on the growth curve parameters of the indicator strains was determined. Ten microliters of indicator strain, 100 μL of CFS, and 185 μL of CMB were mixed in each well of a 96-well microtiter plate. In the control samples, CMB broth was used instead of CFS. The plate was incubated in the Victor Multilabel counter (Perkin Elmer, Turku, Finland) at 37°C with shaking (200 rpm) for 24 h, and the optical density (at 595 nm) was measured every hour. Each experiment was repeated three times. The data were fitted to the Gompertz function as modified by Zwietering et al. (1990; see below) using the nonlinear regression procedure in the statistical analysis system (NLIN, SAS 9.1, SAS Inc., Chicago, IL, USA). The growth curve parameters for the 32 indicator strains were generated in the absence and in the presence of the CFS of the LAB and bifidobacterial strains. In the modified Compertz equation:

$$y = a\exp\{-\exp[(\mu_m e/a)(c - t) + 1]\},$$

where y = Ln (optical density at 595 nm), t = time, a = maximum bacterial growth at stationary phase, μ_m = maximal growth rate, and c = lag time. Reduction of the growth rate by ≥ 50% or prolongation of the lag phase by ≥ 200% was considered as inhibition.

Storage Experiment with Salmon

Fresh Atlantic salmon (*Salmo salar*) fillets (obtained 2 days after harvesting from Jokisen Eväät, Raisio, Finland) were cut to pieces of 400 g each. In the treatments, fillets were soaked in the *L. lactis* solution for 5 min. *L. lactis* was grown overnight in MRS broth at 37°C in anaerobic conditions, washed twice with 0.9% NaCl, and finally diluted with 0.9% NaCl to a concentration of 1×10^8 CFU/mL. Control fillets were soaked into 0.9% NaCl solution for 5 min. Treated and control fillets were vacuum-packed in the Supervac GK 113 machine (Vienna, Austria; vacuum 6–8 mbar) using polyamide/polyethylene bags (Opalen 75; UPM Pack, Valkeakoski, Finland). Packages were stored at 3°C and analyzed after 1, 4, 7, and 10 days. For microbiological analysis, a sample of 100 g was homogenized in 100 mL of peptone-water in a Stomacher Lab Blender (230 RPM, 7 min; Seward Ltd., Worthing, UK). For total microbial counts, appropriate 10-fold dilutions were pour plated on plate-count agar (Difco Laboratories, Sparks, MD, USA), and plates were incubated at room temperature for 5 days. For analysis of *Listeria*, samples were plated on chromogenic *Listeria* agar (Oxoid) supplemented with amphotericin (10 μg mL^{-1}), ceftazidime (6 μg mL^{-1}), nalidixic acid (26 μg mL^{-1}), polymyxin B (10 μg mL^{-1}) (supplement SR0227; Oxoid), and lecithin solution (SR0228; Oxoid); plates were counted after aerobic incubation at 37°C for 24 h. Five independent storage experiments were performed with two parallel samples. Student's *t*-test ($p < 0.05$) was used to find statistically significant differences between samples.

Sensory Analysis

Sensory analysis of treated and untreated vacuum-packed Atlantic salmon fillets was performed by a six-member trained sensory panel. The panelists did not know which samples were treated or which were control. The general guidelines for the selection, training, and monitoring of panelists (ISO 8586-1) were used. All samples were evaluated by their intensity of each attribute—i.e., color, texture, and odor, on a line scale of 0–10. Differences among samples were analyzed by Student's *t*-test. Sensory analysis was performed 11 days after treatment and packaging—i.e., 4 days after the shelf life of the product (shelf life 7 days). Samples were analyzed at room-temperature.

RESULTS AND DISCUSSION

Lactic acid bacteria are widespread in nature and commonly found in many food products (dairy, meat, fruits, vegetables, etc.). They have been used in the natural fermentation of milk, meat, vegetables, and fruits for centuries and thus offer a special promise for implementation as protective cultures. Their inhibitory effect against other microorganisms is based on competition of nutrients and production of antimicrobial metabolites as organic acids, hydrogen peroxide, antimicrobial enzymes, and bacteriocins (as nisin, pediocin, and sakacin; Ouwehand and Vesterlund, 2004). The protective culture properties of LAB have been extensively tested with meat but rarely with fish. Fish is a highly perishable food with many specific spoilage organisms (SSOs). The presence of SSOs is dependent on the fish species, origin of the fish (salt concentration and temperature of the water), as well as the atmosphere of the storage (on ice, vacuum-packed, or modified-atmosphere packed). Previously, we had determined the SSOs of vacuum-packed Atlantic salmon to be *Brochothrix thermosphacta, Buttiauxella agrestis, Enterobacteriaceae* (especially, *Hafnia*

alvei and *Serratia proteamaculans*), and LAB (especially, *Carnobacterium divergens* and *C. maltaromaticum*; Vesterlund, Submitted). The previous determination of SSOs allowed reasonable screening of the potential protective cultures.

When the lag phase and growth initiation of spoilage organisms was explored during exposure to cell-free supernatant of bifidobacteria or LAB strains, it was found that the lag phase correlated with the growth rate. This means that spoilage strains with longer lag phase also have a reduced growth rate. Among the tested strains, the best inhibitory effect was found in *Lactococcus lactis* ssp. *lactis* followed by *Lactobacillus plantarum* Lp-115 and *Bifidobacterium lactis* -04 (Table 3). *L. lactis* inhibited the growth of 27 indicator strains and delayed the lag phase of 25 indicator strains when 32 indicator strains were tested. The inhibitory effect was attributed to the secreted antimicrobial compounds of bifidobacteria and LAB as our selection procedure was based on the use of cell-free culture supernatants. Secreted compounds are typically organic acids (lactic and acetic acid), as all the strains acidify their culture medium. Moreover, other antimicrobial compounds such as hydrogen peroxide or bacteriocins (i.e., nisin) might be produced.

When salmon fillets were treated with *L. lactis*, the total microbial counts were always lower in the treated fillets throughout the entire storage period. When five independent experiments were performed, the treated fillets had 0.78 $\log_{10}$ units less bacteria than the control fillets at the end of shelf life (i.e., at the time-point 7 days; $p < 0.05$; Figure 1). Moreover, the treated fillets reached the microbial spoilage level of 1×10^7 CFU/g 3 days later than the control fillets (Figure 1). After 10 days of storage, the total microbial count in control fillets was 7.74 $\log_{10}$ units, while the treated fillet had 7.15 $\log_{10}$ units ($p < 0.001$). No *Listeria* was found in both samples, so the inhibitory effect of *L. lactis* against *Listeria* remains to be detected in future studies. The *L. lactis* strain used in this study produces nisin with a wide bacteriocidal spectrum against Gram-positive organisms, such as *Listeria monocytogenes* (Héchard and Sahl, 2002) and *Staphylococcus aureus* (Vesterlund et al.,

TABLE 3
The number of fish spoilage bacteria inhibited by cell-free supernatant of bifidobacteria or lactic acid bacteria strains. Results are based on the evaluation of the growth rate and the lag phase of the indicator strains (a reduction in the growth rate by 50% or more and a delay in lag phase by 200% or more compared to the control was considered as inhibition). Thirty-two indicator strains isolated from vacuum-packed salmon were studied

Strain	*The number of inhibited bacteria based on the reduction of the growth rate*	*The number of inhibited bacteria based on the delay in lag phase*
B. lactis Bb-12	2	15
B. lactis Bl-04	25	16
B. longum 46	14	13
L. acidophilus LA5	4	17
L. brevis Lbr-35	5	9
L. casei Shirota	14	9
L. fermentum ME3	6	5
L. plantarum	12	13
L. plantarum 299v	8	16
L. plantarum Lp-115	25	19
L. reuteri ING1	16	6
L. rhamnosus 1460	19	13
L. rhamnosus GG	14	11
L. rhamnosus LC-705	11	13
L. rhamnosus LR-32	12	8
L. lactis ssp. *lactis*	27	25

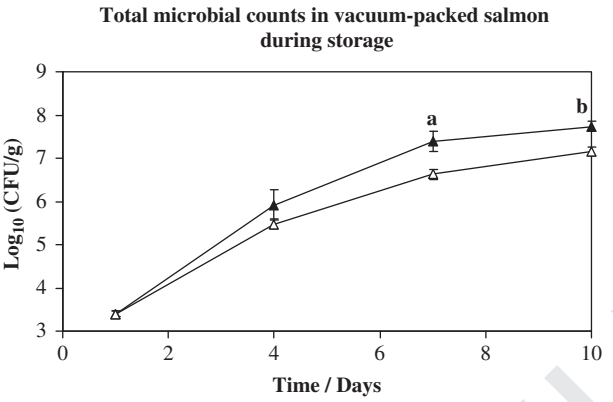

FIGURE 1 Total microbial plate counts of vacuum-packed salmon during storage. Average ± *SEM* of five independent experiments is shown. Open triangles represent *L. lactis*-treated fillets, and closed triangles represent control fillets. Time-point of 7 days represents normal shelf life of the product ($^{a}p < 0.05$, $^{b}p < 0.001$).

2004). However, production of nisin in the vacuum-packed salmon fillets appears to be unlikely due to a low storage temperature (3°C), which is not favorable to the growth of *L. lactis* as described by Wessels et al. (1996). In addition, there is limited diffusion of nisin in fatty fish, because the lipid phase of salmon reduces the bacteriocin's biological activity (Jung et al., 1992). The inhibitory properties of *L. lactis* were probably caused by the competition of nutrients in the product and the antimicrobial compounds other than nisin as organic acids.

The level of organic acids in the product was at a moderate level, as sensory analysis did not show acidification properties. Also color, texture, and odor were similar in the treated and un-treated fillets. This indicates that addition of *L. lactis* to salmon fillets did not cause apparent changes in sensory characteristics of the fillets.

CONCLUSION

Lactococcus lactis ssp. *lactis* exhibited inhibitory effects against growth of many spoilage organisms in vacuum-packed raw Atlantic salmon and extended shelf life of the fillets stored at 3°C with no apparent change of sensory characteristics. This lactic acid bacterium might be utilized for extending shelf life of vacuum-packed fish fillets during refrigerated storage.

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