Growth control of *Listeria innocua* 2030c on vacuum-packaged cold-smoked salmon by lactic acid bacteria

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Abstract

Five bacteriocin-producing lactic acid bacteria (LAB): *Enterococcus faecium* ET05, *Lactobacillus curvatus* ET06, *L. curvatus* ET30, *L. deldrueckii* ET32 and *Pediococcus acidilactici* ET34, selected by their capacity for growth and producing inhibition *in vitro* at high salt-on-water content, low temperature and anaerobic atmosphere, conditions simulating cold-smoked fish, were inoculated onto salmon fillets, in co-culture with *Listeria innocua* 2030c, and cold-smoked processed (dry salted for 6 h; drying for 6 h; smoke for 2 h).The finished product was then packed under vacuum and stored at 5 °C. Enumeration of LAB and *L. innocua* was performed during storage. Results showed that strain *E. faecium* ET05 was the best biopreservative candidate for controlling *L. innocua* growth in vacuum-packaged cold-smoked salmon (CSS) processed under the salting/drying/smoking parameters referred above. *L. curvatus* ET30 and *L. delbrueckii* ET32 also showed a good biopreservation potential for CSS although they were less effective than the former. *L. curvatus* ET06 and *P. acidilactici* ET34 showed a bacteriostatic mode of action against the target bacteria *in vitro* as well as when inoculated into the salmon fillets.

This study describes a potential application of five different LAB in the biopreservation of *Listeria* in CSS.

Introduction

Cold-smoked salmon (CSS) is a typical seafood product subjected to only a light preservation process before being purchased 'ready-to-eat' by the consumer. As a result, the human pathogen *Listeria monocytogenes*, is often isolated from this product, albeit in low numbers (Jinneman et al., 1999; Johansson et al., 1999). *L. monocytogenes* is the causative agent of listeriosis with 30% lethality rate in compromised individuals (Duffes et al., 1999). None of the preservation parameters, individually or combined, used in the manufacture of CSS, can control the presence or growth of this bacterium, which has been described as a ubiquitous microorganism capable of growth at pH values as low as 5, at refrigeration temperature, and at very high salt concentrations (Eklund et al., 1995).

Although many technologies have been applied successfully in CSS to prevent growth of *L. monocytogenes*, i.e. the addition of low concentration of lactic acid and/or acetic acid, potassium lactate and sodium diacetate (Yoon et al., 2004; Vogel et al., 2006), there is a growing demand by consumers for lightly preserved fish products (and other foods) that do not contain chemical preservatives but still have a long and safe shelf life. Interest in developing biopreservation techniques in lightly preserved foods has increased in recent decades and has focused on minimizing the presence of and/or suppressing the growth of *L. monocytogenes*. Biopreservation is the extension of the storage life and enhancing the safety of foods using the natural or controlled microflora and/or their antimicrobial products (Stiles, 1996). In CSS biopreservation techniques have involved the introduction of a competitive LAB microflora for their protective

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effects, including bacteriocin-producing LAB, and purified antilisterial bacteriocins (Hugas and Monfort, 1997). The advantages of protective cultures include additive-free preservation, natural image, and temperature-responsive inhibition — it can "switch on" only when temperature abuse takes place (Devlieghere et al., 2004). The addition of protective cultures directly into the product offers a controlled process in terms of desired safety outcomes and in the prevention of uncontrollable spoilage. Furthermore, the constant production of bacteriocin by viable cultures can overcome the problem of their decomposition and binding to food particles when used as an additive (Rodgers, 2001). Potential but very rare health risks associated with LAB include bacteremia, associated with vancomycin therapy (Stiles, 1996). Evidence of non-pathogenicity, such as being non-haemolytic and antibiotic sensitive (Embarek et al., 1994), should be demonstrated for cultures to be used as protective cultures in foods, especially in the case of antilisterial *Enterococcus* and *Bacillus* species in which the horizontal transfer of plasmids may dramatically alter their phenotypes in terms of their pathogenicity (Helgason et al., 2000).

Many studies have demonstrated that LAB comprises the dominant microflora in CSS (González-Rodríguez et al., 2002; Cardinal et al., 2004). Most of them are able to hamper the growth of *L. monocytogenes* due to the production of diverse compounds including antimicrobials produced at high cell density, or peptides called bacteriocins (Lindgren and Dobrogosz, 1990; Kleerebezem, 2004). There is strong evidence for their use as potential bioprotective cultures for CSS. However, the use of protective cultures in CSS has focused on the genus *Carnobacterium* (Nilsson et al., 1999; Nilsson et al., 2004; Duffes et al., 1999, 1999a; Connil et al., 2002; Yamazaki et al., 2003; Brillet et al., 2004; Vaz-Velho et al., 2005; Vescovo et al., 2006) and to a lesser extent on *L. sakei* (Katla et al., 2001), *L. curvatus* (Ghalfi et al., 2006), *L. casei* and *L. plantarum* (Vescovo et al., 2006). In this study, the ability of five strains isolated from CSS, identified as *E. faecium* ET05, *L. curvatus* ET06 and ET30, *L. delbrueckii* ET32 and *P. acidilactici* ET34 (capable of growth and producing listerial inhibition *in vitro*, under conditions prevailing in vacuum-packaged CSS fillets; sensitivity to vancomycin, unable to produce histamine and lacking haemolytic activity by *E. faecium* ET05 strain, (Tomé et al., 2007) to inhibit the growth of *L. monocytogenes* during CSS production was investigated. In parallel with the biopreservation study, the mode of action of the bacteriocins produced by the strains inoculated into the fillets, was assessed using *in vitro* assays.

Materials and methods

Efficacy of protective lactic acid bacteria in growth control of L. innocua 2030c on cold-smoked salmon

Raw fish

Fresh gutted farmed salmon (*Salmo salar*) from Norway were acquired at Matosinhos' Doca (Porto, Portugal). Salmon arrived by lorry (72 h travel) in a chilled container with the temperature controlled between 0 °C and 4 °C, inside polystyrene boxes (two layers of fish between two layers of ice). The fish was transported to the Escola Superior de Biotecnologia (Porto, Portugal) in chilled conditions, beheaded and filleted by hand on the same day.

Pure cultures

Five inhibitory LAB previously isolated from CSS, and capable of growth and producing inhibition *in vitro* at high osmolarity, low temperature and anaerobic atmosphere, conditions simulating cold-smoked fish, were selected for inoculation onto salmon fillets. Two overnight subcultures of *E. faecium* ET05, *L. delbrueckii* ET32 and *P. acidilactici* ET34 incubated for 30 h (yielding 10^{8-10} cfu ml^{-1}) and for *L. curvatus* ET06 and ET30 incubated for 48 h (yielding 10^{11-12} cfu ml^{-1} and 10^{9-10} cfu ml^{-1}, respectively) were made in APT broth at 30 °C before experimental use. *L. innocua* 2030c, a tetracycline resistant strain from Public Health Laboratory Services (PHLS, London) private collection, was used as target organism. Two overnight subcultures (30 °C, 18 h yielding 10^{8-9} cfu ml^{-1}) of *L. innocua* 2030c previously stored on Lab Lemco agar (Oxoid, Hampshire, UK) at ambient temperature, were made in TSB-YE (Lab M, Bury, UK) before experimental use. The selection of *L. innocua* 2030c as an alternative organism to *L. monocytogenes*, was due to the restriction on the use of this pathogen in the processing plant; this strain had previously been selected as a suitable marker organism for replacing *L. monocytogenes* in experiments where antilisterial properties of LAB strains were evaluated (Vaz-Velho et al., 2001).

Treatments

Overnight cultures (20 ml) of *L. innocua* 2030c and of LAB strains ET05, ET06, ET30, ET32, ET34 were centrifuged at 8670 ×*g*, for 10 min at 4 °C (Hettich, Rotina 35 R, Tuttlingen, Germany). Each pellet was re-suspended in 20 ml of sterile saline solution (1% w/v NaCl in water) and each cell suspension was diluted in 1 l (2% v/v) of water. Two fish fillets were immersed for 30 s in this volume as required by trials 1–4. Trials are described in Table 1. Five independent experiments were performed with each lactic acid bacterium (see Table 2) described previously, and two independent runs (batches) were performed in each experiment at the same time of the year. Salmon fillets were dry salted for 6 h (salt: fish::1:3) at 5 °C. Sugar was added to the salt in the proportion 1:3::sugar:salt. The total weight of salt and sugar corresponded to one third of the weight of the fillet. After salting, the fillets were washed/rubbed to remove the surplus salt and were drained/dried overnight at 5 °C. Then fish fillets were placed inside the smoking kiln for 6 h of drying and 2 h of exposure to smoke at <30 °C. The smoked samples were cooled overnight at 5 °C. The following

Table 1
Design of the trials used in the inoculation of the salmon fillets

Trial	*L. innocua* 2030c	LAB culture
1	No	No
2	No	Yes
3	Yes	Yes
4	Yes	No

Table 2
Lactic acid bacterium used as inoculum in trials 2 and 3 in each experiment

Experiment	LAB inoculated
1	*E. faecium* ET05
2	*L. curvatus* ET06
3	*L. curvatus* ET30
4	*L. delbrueckii* ET32
5	*P. acidilactici* ET34

day, lug and pin bones and belly flaps were removed, the fillets sliced and vacuum-packed. Packs were stored for 3 weeks at 5 °C and sampled weekly.

Sampling procedure

Two salmon fillets from each trial were sampled to determine LAB and *Listeria* populations. Ten grams of each fillet (fresh or smoked material), taken from the centre of each fillet, were homogenized in 90 ml of sterile BPW (Merck, Darmstadt, Germany) for 2 min in a Stomacher 400 Lab Blender (Seward Medical, London, UK). Serial dilutions in sterile 1/4 strength Ringer's solution (Lab M) were prepared. Enumeration of *L. innocua* 2030c and LAB was done on the raw material, after smoking, and weekly during the 3 weeks of storage at 5 °C. All samples in each trial were analysed in duplicate. Bacteriocin activity was measured in trial 2-samples from the homogenised (stomacher) filtered liquid after seven days of refrigerated storage. The samples were spotted onto indicator plates inoculated with *L. monocytogenes* 54 (ESB/UCP culture collection, Porto, Portugal). In a parallel study the bacteriocin activity adsorbed on plastic bags was tested by placing pieces of packaging material on indicator plates and incubated overnight at 25 °C.

Physical and chemical analyses

Salt concentration in the finished products was evaluated according to AOAC (1995), method 937.09 in samples taken from the negative control (trial 1) for two batches, in each experiment. The salt content was expressed as % NaCl in water phase of muscle (Tomé et al., 1999). Moisture content of the samples of CSS was determined according AOAC (1995), method 24.003. These analyses were performed at the beginning of storage.

Microbiological analyses

LAB were enumerated by pour-plating in APT (Difco, Detroit, USA) agar. All colonies were counted as presumptive LAB after 5 d of anaerobic incubation at 25 °C. Listeria counts were obtained by direct plating suitable dilutions of product on Agar Listeria Ottavani & Agosti (ALOA, bioMérieux, Marcy l'Etoile, France) medium. All blue-colored colonies without a halo around them were counted as presumptive *Listeria* spp. after 24–48 h of incubation at 37 °C.

Statistical analysis

ANOVA two-factors with replications and Multiple Range Test were used to evaluate differences of means between batches and treatments as well as the effect of the different treatments on *L. innocua* 2030c growth. The null hypothesis assumed that there was no difference between batches and between treated/untreated samples. Significant differences were considered when $P<0.05$. Analysis of variance and effects calculations were performed using Statistica 6.0 (StatSoft Inc., Oklahoma, USA).

Bacteriocin activity

Bacteriocin activity was measured in trial 2-samples from the homogenized stomacher filtered liquid after seven days of refrigerated storage. The samples were spotted onto plates inoculated with *L. monocytogenes* 54 (ESB/UCP). In parallel, activity adsorbed on plastic bags was tested by loading pieces of packaging material on indicator plates and incubated overnight at 25 °C.

Mode of action

The mode of action of the bacteriocins produced by strains ET05, ET06, ET30, ET32 and ET34, was studied *in vitro*. It was assessed by adding 20 ml aliquots of each bacteriocin-containing filter-sterilized and cell-free supernatant (CFSF; pH 6.0) to a 100 ml culture of *L. innocua* 2030c, at the onset of growth (at 37 °C) and again after 3 h and 5 h of growth. The

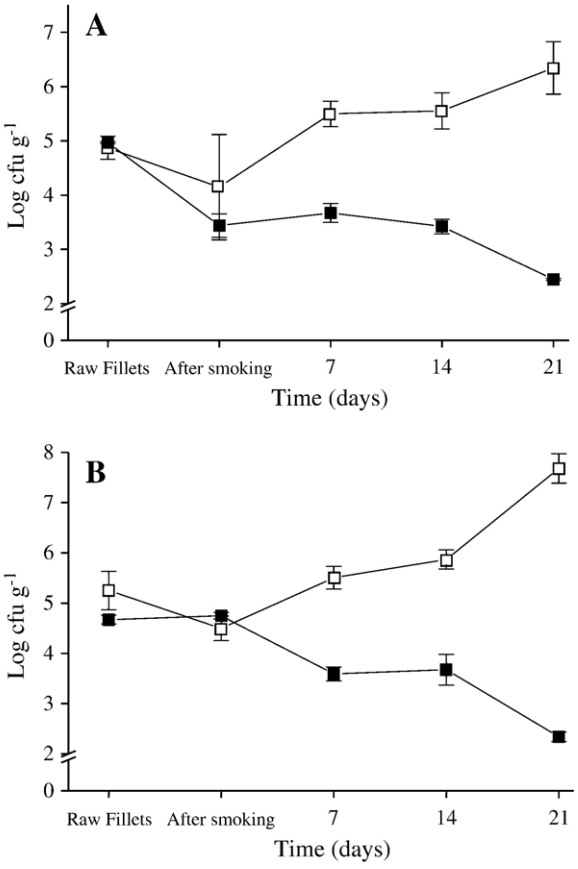

Fig. 1. Effect of *E. faecium* ET05 on *L. innocua* 2030c on salmon fillets before and after cold-smoking and during storage at 5 °C in vacuum packs for 21 days A) Batch 1, (B) Batch 2 □ *L. innocua* 2030c ■ *L. innocua* 2030c in a co-culture with *E. faecium* ET05.

optical density (600 nm) of the culture was determined at regular intervals. Three independent experiments were run in duplicate.

Results

Efficacy of protective lactic acid bacteria on growth control of L. innocua 2030c on cold-smoked salmon

The potential of five antimicrobial-producing LAB strains to inhibit *L. innocua* 2030c growth, was evaluated in experiments comprising artificial contamination of salmon fillets, which were then smoked and stored in vacuum packs at 5 °C for 21 days. The two smoking batches performed for each inoculated strain, were treated separately for statistical analysis, since significant differences ($P<0.05$) among batches were found in some of the assays, even though the smoking process for each batch was performed in the same month without great differences either in the temperature of the smoking process or in the relative humidity among batches in the same assay (results not shown).

A bactericidal-like effect on *L. innocua* 2030c was obtained in samples co-inoculated with *E. faecium* ET05 during the smoking stage (reduction of ≈ 1.5 log cycles, batch 1) and, after seven days of storage (reduction of ≈ 1.25 log cycles, batch 2) compared with the inoculum level. This effect was greater at the end of the storage period when counts of *L. innocua* were ≈ 2.5 log cycles lower with respect to the raw inoculated fillets and ≈ 4.5 log cycles lower than the control for both batches (Fig. 1). No significant differences ($P<0.05$) were found in *L. innocua* counts in co-cultured samples among the batches.

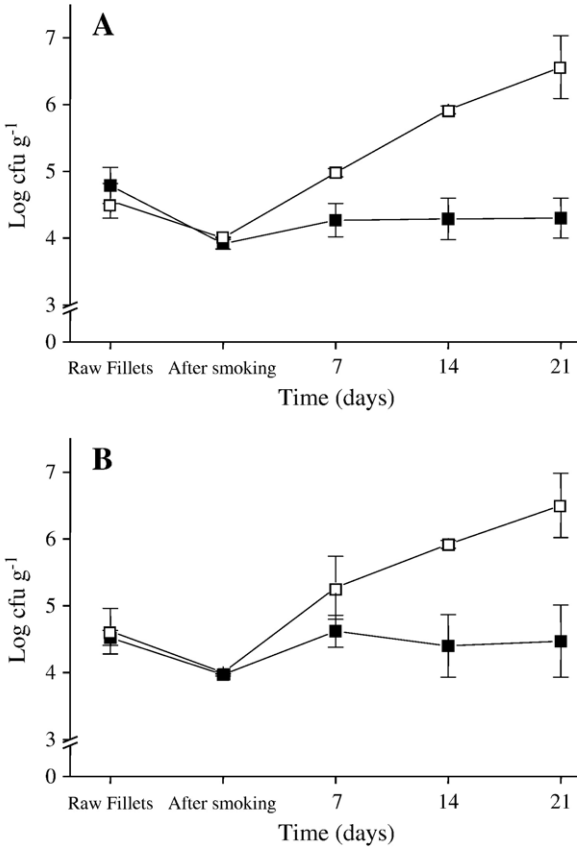

Fig. 2. Effect of *L. curvatus* ET06 on *L. innocua* 2030c on salmon fillets before and after cold-smoking processing and during storage at 5 °C in vacuum packs for 21 days (A) Batch 1, (B) Batch 2 □ *L. innocua* 2030c ■ *L. innocua* 2030c as a co-culture with *L. curvatus* ET06.

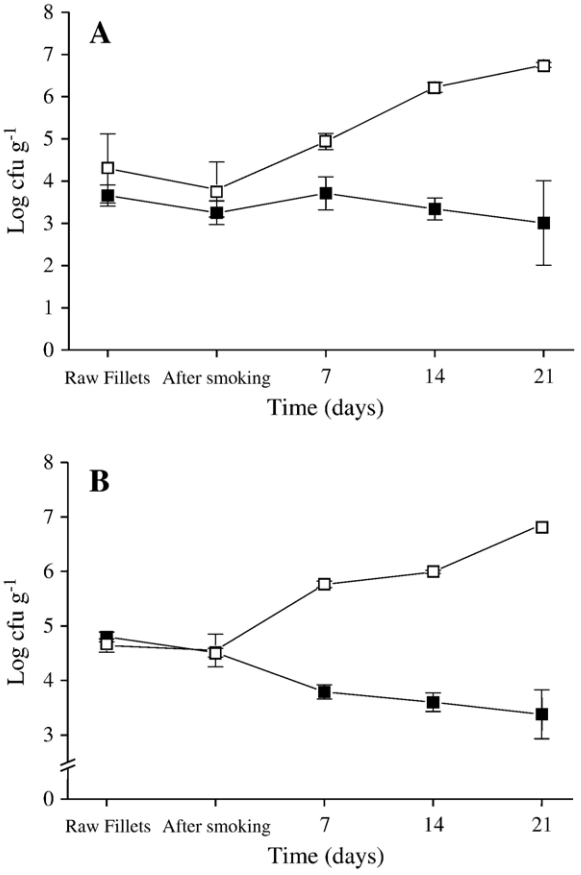

Fig. 3. Effect of *L. curvatus* ET30 on *L. innocua* 2030c on salmon fillets before and after cold-smoking processing and during storage at 5 °C in vacuum packs for 21 days (A) Batch 1, (B) Batch 2 □ *L. innocua* 2030c ■ *L. innocua* 2030c as a co-culture with *L. curvatus* ET06.

Counts of *L. innocua* 2030c in samples treated with *L. curvatus* ET06 (Fig. 2) showed a slight recovery of ≈ 0.5 log cycle from the beginning of storage (immediately after smoking) to the seventh day, compared to the control, and remained almost steady for the rest of the storage period. This treatment resulted in less than one log reduction of *L. innocua* numbers in batch 1 and no reduction in batch 2 at the end of the storage period, compared with the inoculum level. The *L. curvatus* ET06 culture showed a bacteriostatic-like effect on *L. innocua* growth. In batch 1 and batch 2, statistical analysis considered this treatment to be significantly different ($P<0.05$) from the control.

Samples treated with *L. curvatus* ET30 showed, in the first batch, a decrease in *L. innocua* 2030c numbers, compared with the control, from the 7th day of storage decreasing by ca. 2.3 log cycles at the end of the storage period. There was no reduction of *L. innocua* 2030c counts obtained with respect to the initial inoculum level after 7 days (Fig. 3A). In the second batch, after

smoking, a strong bactericidal effect on *L. innocua* 2030c compared to the inoculum level and to the control, was found in samples treated with *L. curvatus* ET30 culture. This reduction attained ca. 1.5 log cycles and ca. 3.4 log cycles in relation to the inoculum level and the control respectively, at the end of the storage period (Fig. 3B).

In the first batch, samples treated with *L. delbrueckii* ET32, showed during smoking, a reduction of ca. 1.3 log cycles of *L. innocua* 2030c numbers below the inoculum level. However, numbers recovered after seven days of storage and remained steady below the inoculum level during further storage (Fig. 4A). In contrast, in the second batch, although counts of *L. innocua* 2030c remained close to the inoculum level until the seventh day of storage, a strong bactericidal-like effect was observed during the remaining period of storage. A reduction upper than 4 log cfu g^{-1} was achieved in these samples after 21 days of incubation compared with the control (Fig. 4B).

Results for the CSS fillets inoculated with *P. acidilactici* ET34 are illustrated in Fig. 5. In the non-LAB-inoculated assay (control) *L. innocua* 2030c reached 2.2×10^{6} cfu g^{-1} and 3.4×10^{7} cfu g^{-1}, in batch 1 and batch 2 respectively, after 21 days of incubation in CSS vacuum-packaged at 5 °C. The presence of *P. acidilactici* ET34 caused a reduction in *L. innocua* 2030c numbers, during smoking, of ca. 0.5 log cycle compared

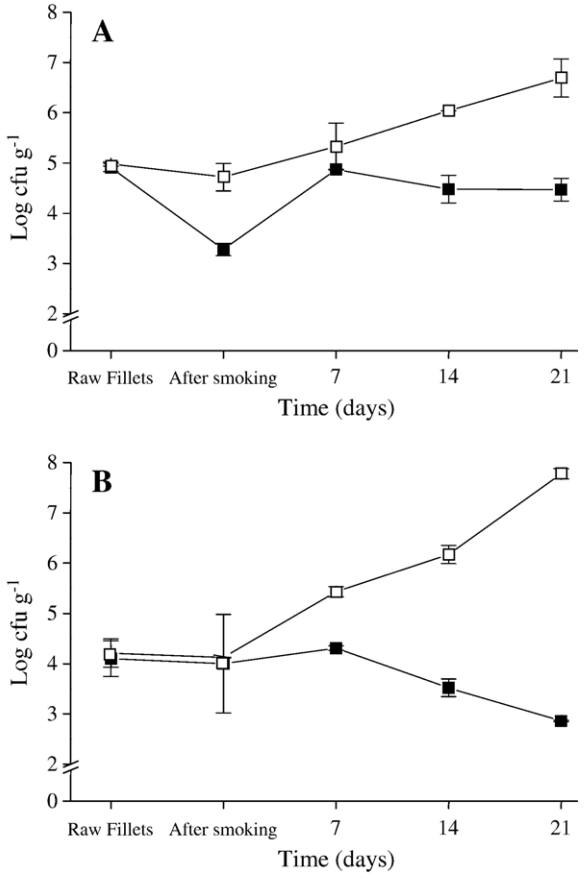

Fig. 4. Effect of *L. delbrueckii* ET32 on *L. innocua* 2030c on salmon fillets before and after cold-smoking and during storage at 5 °C in vacuum packs for 21 days A) Batch 1, (B) Batch 2 □ *L. innocua* 2030c ■ *L. innocua* 2030c in co-culture with *L. delbrueckii* ET32.

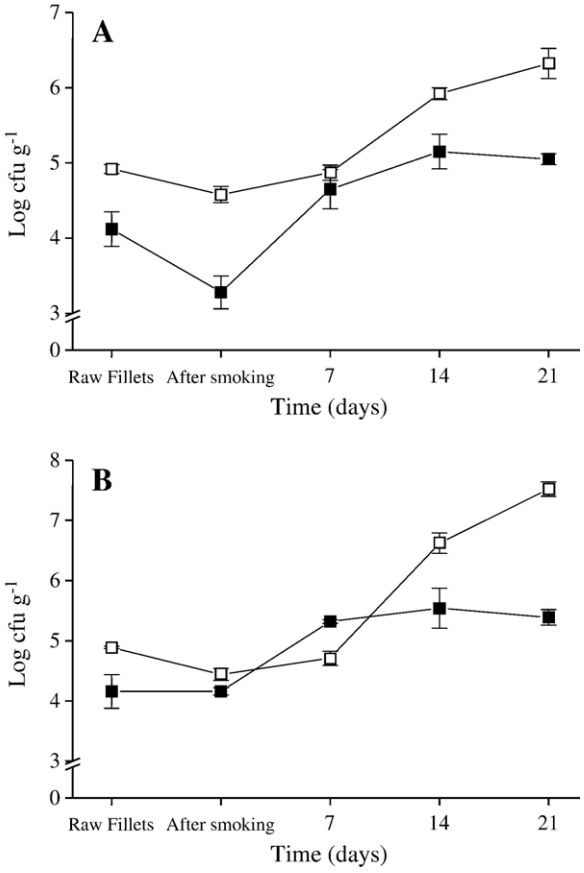

Fig. 5. Effect of *P. acidilactici* ET34 on *L. innocua* 2030c in salmon fillets before and after cold-smoking and during storage at 5 °C in vacuum packs for 21 days. (A) Batch 1, (B) Batch 2 □ *L. innocua* 2030c ■ *L. innocua* 2030c in co-culture with *L. acidilactici* ET34.

with the control in batch 1, and no reduction in batch 2. During storage, in the presence of the bacteriocinogenic culture, cell numbers of *L. innocua* 2030c increased, reaching a maximum of 10^{5} cfu g^{-1} after fourteen days of storage in both batches, and remained constant at that level during the rest of the incubation

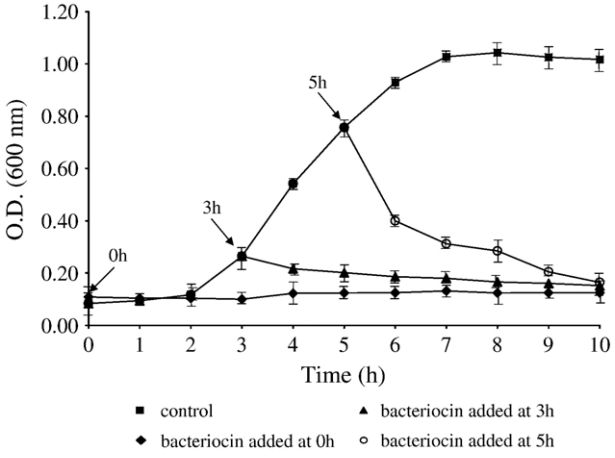

Fig. 6. Effect of bacteriocin ET05 addition on the growth of *L. innocua* 2030c. Data points represent the average of three experiments run in duplicate.

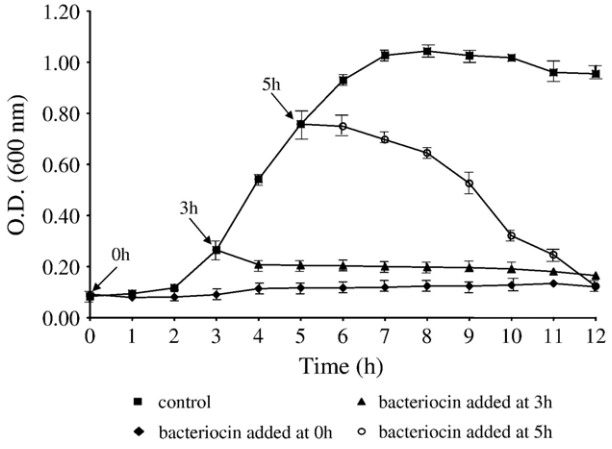

Fig. 7. Effect of bacteriocin ET06 addition on the growth of *L. innocua* 2030c. Data points represent the average of three experiments run in duplicate.

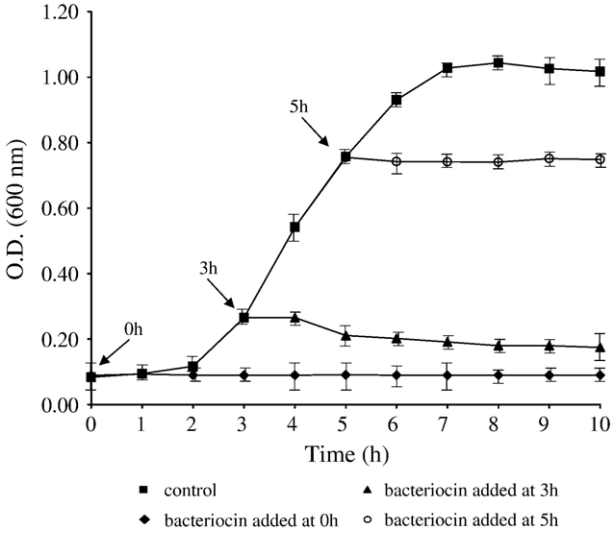

Fig. 9. Effect of bacteriocin ET32 addition on the growth of *L. innocua* 2030c. Data points represent the average of three experiments run in duplicate.

period. A weak bacteriostatic behavior seems to take place in these samples.

In general, an average initial cell concentration 10^{5-6} cfu g^{-1} of each of the LAB strains was obtained in each of the raw fillets of salmon inoculated independently, with *E. faecium* ET05, *L. curvatus* ET06, *L. curvatus* ET30, *L. delbrueckii* ET32 and *P. acidilactici* ET34, both in the first and the second batch (results not shown). The same pattern of increase in LAB numbers was observed both in inoculated and non-inoculated (*L. innocua*) samples, reaching 10^9 cfu g^{-1} during the storage. There was no statistical difference ($P<0.05$) in the count of LAB on CSS vacuum-packed fillets inoculated with each of the lactic acid strains ET05, ET06, ET30 and ET34 grown in co-culture with *L. innocua* 2030c (trial 3) and the LAB count in the control (trial 2) in both batches. A significant difference was observed between trials 2 and 3 in CSS vacuum-packed fillets inoculated with *L. delbrueckii* ET32.

The residual activity of bacteriocins produced by the five strains inoculated into salmon fillets was not detected in any of the samples analysed after seven days of storage. Transfer of activity of bacteriocins ET05 and ET30 to the plastic package material was observed.

A slight variation among batches was noticed in the chemical composition. However there was no significant difference ($P<$ 0,05) in the percentage of salt in water phase between batches inoculated with the same LAB (results not shown). The percentage of salt in the water phase among all batches ranged between 3.3% and 4.8% with an average concentration of 3.9% w/v.

Mode of action

Addition of CFSF from *E. faecium* ET05 at the onset of growth (0 h) and again after 3 h and 5 h of growth to the cell suspension of *L. innocua* 2030c resulted in a very marked decrease in optical density (Fig. 6). The same effect was observed after the addition of CFSFs from *L. curvatus* ET30 and *L. delbrueckii* ET32 (Figs. 7 and 8) The addition of bacteriocins

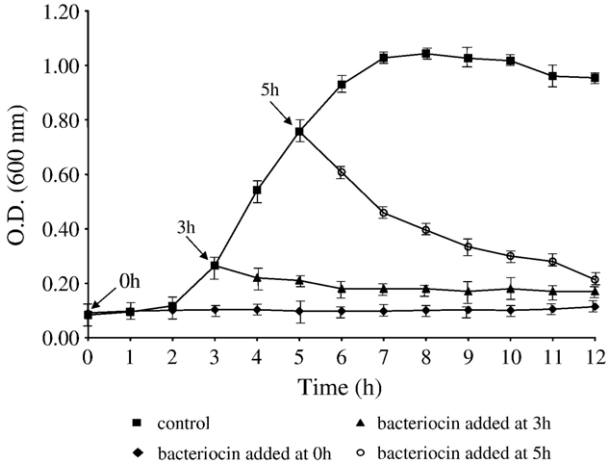

Fig. 8. Effect of bacteriocin ET30 addition on the growth of *L. innocua* 2030c. Data points represent the average of three experiments run in duplicate.

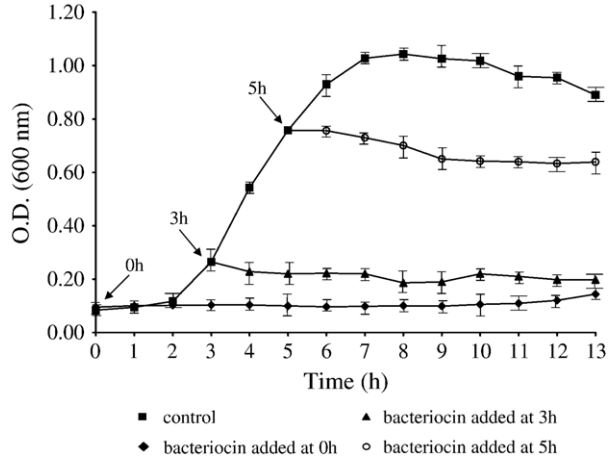

Fig. 10. Effect of bacteriocin ET34 addition on the growth of *L. innocua* 2030c. Data points represent the average of three experiments run in duplicate.

ET06 and ET34 after 3 h and 5 h to a cell suspension of *L. innocua* 2030c resulted in complete growth inhibition but no decrease in optical density (Figs. 9 and 10).

Discussion

Effect of protective lactic acid bacteria in growth control of L. innocua 2030c on cold-smoked salmon

The results of this study demonstrated a potential for application of five strains of LAB as biopreservative cultures against *L. innocua* 2030c on vacuum-packaged CSS stored at 5 °C. All the LAB strains suppressed the growth of the target microorganism. However, the highest reduction of *L. innocua* 2030c numbers at all sampling times was obtained with the *E. faecium* ET05 strain. It was demonstrated that 3.7% w/w of salt content (WPS) in fish fillets did not interfere with the growth of *E. faecium* ET05 in salmon fillets as well as bacteriocin production/activity, otherwise low levels of *L. innocua* 2030c would not be attained. On the other hand, neither the low temperature during the over-night storage before smoking, nor during the storage-time of CSS for 21 days at 5 °C, would repress enterocin production. In contrast with our findings, Aymerich, Artigas, Garriga, Monfort and Hugas, (2000) reported that bacteriocin production of *E. faecium* CTC492 in dry-fermented sausages, was affected by low temperatures with an optimal production at temperatures between 25 °C and 35 °C. The potential as antilisterial factors of enterococcal bacteriocins produced by *E. faecium* strains is well documented (Giraffa, 1995; Núñez et al., 1997; Foulquié Moreno et al., 2002). As result of their antimicrobial properties several *E. faecium* strains have already been proposed as starter culture or co-cultures in both dairy products and fermented foods (i.e. *E. faecium* T136, Casaus et al., 1997; *E. faecium* RZS C5, Leroy et al., 2003; *Enterococcus casseliflavus* IM 416K1, Sabia et al., 2002; *E. faecium* F58, Achemchem et al., 2005).

The results in this study have shown that *L. curvatus* ET06 had a bacteriostatic effect on *L. innocua* 2030c growth. In contrast, when *L. curvatus* ET30 culture was added to vacuum-packaged CSS, a bactericidal effect on *L. innocua* was observed. The inhibitory capacity of the bacteriocins produced by *L. curvatus* strains L442, LBPE and CWBI-B28 against *L. monocytogenes* were also observed by Mataragas, Drosinos and Metaxopoulos (2003), Benkerroum et al. (2005) and Ghalfi et al. (2006) in sliced, cooked, cured pork shoulder stored under vacuum at 4 °C, dry-fermented sausage and CSS, respectively.

The main interest regarding *L. delbrueckii* comes from the importance of the subspecies *bulgaricus* and *lactis* due to their fermentation patterns and their tolerance and adaptive acid stress response. In addition, both subspecies are able to ferment lactose and to degrade casein and grow in milk. *L. delbrueckii* has also been used as a starter culture in meat fermentation processes. As a result of its homofermentative metabolism, the acid formed reduces the pH, preventing the growth of food poisoning and spoilage bacteria. Kalalou, Faid and Ahami (2004) investigated the possibility of extending the shelf life of fresh camel meat using *L. delbrueckii* subsp. *delbrueckii* isolated from Moroccan foodstuffs, and selected for their inhibitory activity on Gram-positive and Gram-negative bacteria. Up to now, no data have been published on the preservation and extension of the shelf life of vacuum-packaged CSS by using *L. delbrueckii* and/or its metabolites. The addition of this LAB to salmon fillets in co-culture with *L. innocua* 2030c could be considered successful. Even though batch 1 and 2 of salmon fillets treated with *L. delbrueckii* ET32 showed significant differences ($P<0.05$) between them; when analysed separately, in each case statistical differences in *L. innocua* 2030c counts between treated and untreated samples were found during the storage period. As with *E. faecium* ET05, a considerable reduction in *L. innocua* 2030c numbers was observed with respect to the control samples, mainly in batch 2. As a negative aspect we should comment that *L. innocua* counts only began to decrease on the seventh day of storage which could allow the pathogen growth up to this time.

Production of pediocins by pediococci has been intensively investigated in view of the potential use of either the purified bacteriocins or the producer cultures in biopreservation of foods. Pediocins produced by *P. acidilactici* or *P. pentosaceus* strains include pediocin AcH (Bhunia et al., 1988; Biswas et al., 1991), pediocin PA-1 (Gonzalez and Kunka, 1987; Pucci et al., 1988), pediocin JD (Christensen and Hutkins, 1992), pediocin SJ-1 (Schved et al., 1993), pediocin A (Piva and Headon, 1994), pediocin PO_2 (Coventry et al., 1995), pediocin PD-1 (Green et al., 1997), pediocin Np5 (Manca de Nadra et al., 1998), and pediocin ACCEL (Wu et al., 2004).

The use of pediocin, mostly pediocin AcH (same as PA-1), mainly for biopreservation of meats, has been investigated in laboratory-scale, model studies. In studies with dry sausage fermentation, the use of bacteriocin-producing *P. acidilactici* JD1-23, *P. acidilactici* PAC 1.0 as fermenting agents, resulted in numbers of *L. monocytogenes* per gram of dry sausage that were 1–2 logs lower than those in control sausages (Berry et al., 1990; Foegeding et al., 1992; Luchansky et al., 1992; Baccus-Taylor et al., 1993; Työppönen et al., 2003). Pediocin PA-1 was also experimentally used as a dried powder preparation to successfully inhibit *L. monocytogenes* in food systems such as dressed cottage cheese, half-and-half cream, and cheese sauce (Pucci et al., 1988). Two commercial antimicrobial compounds, Microgard (Rhône-Poulenc, Courbevoie, France) and Alta 2341 (Quest BioTechnology Inc., Sarasota, Fla., USA), have found use in the food industry and are licensed for use. These are not purified bacteriocin compounds but rather starter cultures of pediocin-producing bacteria that impart antibacterial properties to the foods (Stiles, 1996). However pediococci, compared to *Lactobacillus* and *Pediococcus* spp., are used relatively rarely as probiotics. In the current study the use of *P. acidilactici* ET34 in salmon fillets artificially contaminated with *L. innocua* 2030c, prevented the growth of the indicator organism respect to the control. However a significant increase of the indicator pathogen was observed in LAB treated-samples during the first fourteen days of storage. *In vitro* assays showed that the bacteriocin ET34 produced by *P. acidilactici* ET34 has a bacteriostatic behavior towards *L. innocua* 2030c. It was also shown that this bacterium was tolerant of the stressful conditions

existing in vacuum-packaged CSS since it was able to produce an active bacteriocin under these unfavorable conditions in broth culture (results not shown). However its behavior was slightly different when it was incorporated into the fish muscle; perhaps the bacteriocin activity was destroyed by proteolytic activities of the fish.

In none of the co-cultured samples with LAB was revival of *L. innocua* 2030c observed during the 21 days of storage. This could be a positive aspect indicating non-acquisition of resistance by *L. innocua*, at least over this short period of storage. The development of resistant mutants of *Listeria* spp. has been reported to occur for pediocin (Pucci et al., 1988), piscicolin (Ming and Daeschel, 1993; Wan et al., 1997) and carnocin (Mathieu et al., 1994). However, previously reported studies by Vaz-Velho et al. (2005) and Duffes et al. (1999) with *L. innocua* 2030c, as well as other *L. monocytogenes* strains, indicated that the main types of Portuguese *L. monocytogenes* strains in CSS as well as *L. innocua* 2030c, were not associated with resistance to bacteriocins. On the other hand, the transference of bacteriocin activity to the plastic package material could be one of the reasons for non-detection of residual bacteriocin activity in the fish. According to Duffes et al. (1999) the attachment of the bacteriocins to packaging could be explained by the strongly hydrophobic properties of the molecules. Vaz-Velho et al. (2005) could not detect residual activity on samples of vacuum-packaged CSS previously treated with a 2% v/v suspension of a culture of *C. divergens* V41 analysed during 21 days of storage at 5 °C. In several studies it has been shown that the inhibition of the target organism by bacteriocins is less effective in foods compared to laboratory media. Possible reasons may include: binding of bacteriocins to other organic compounds present in the food, including lipids, or plastic packaging (Henning et al., 1986; Jung et al., 1992), and inactivation of bacteriocins by proteases of food and of microbial origin (Schillinger et al., 1991). Larsen, Vogensen and Josephsen (1993) mentioned that the presence of moderate NaCl concentrations could lead to the inactivation or reduced production of bacteriocin. This last reason was not the case in the current study since at least the five strains inoculated into the fish fillets, were able to produce active bacteriocin using *in vitro* assays at salt concentrations up 5% (Tomé et al., 2007). It seems most likely that the detection method used might not have been sufficiently sensitive to detect low levels of activity (1:10 dilution of fish) although any residual activity could have affected *L. innocua* growth. However, there was no evidence to indicate that the antilisterial effect of these five strains was due to anything other than bacteriocin activity, since *in vitro* experiments eliminated the possibility of other effective compounds — lactic acid, hydrogen peroxide (results not shown).

Concerning LAB counts, they were very homogeneous within the batches per treatment and intra-treatments (results not shown). No statistical differences between controls (Trials 1) and inoculated samples (Trials 2) were observed relative to LAB counts for strains ET05, ET06, ET30 and ET34. This means that in the presence of any of the inoculated LAB strains, the natural lactic acid flora grew as well as in the control, indicating that LAB are insensitive to the bacteriocin produced by each one of the bacteriocin-producing strains inoculated. Similar results were obtained by Brillet, Pilet, Prévost, Cardinal and Leroi (2005) when the inhibitory effect of *C. divergens* V41 against *L. monocytogenes* was assessed in CSS.

Mode of action

It is well known that the mode of inhibition of bacteriocins depends on the concentration, and on the nature and the physiological stage of the target strain. The bactericidal mode of action of bacteriocins on sensitive cells in most cases involves bacteriocin binding to cytoplasmic membranes, and formation of the poration complex (Abee 1995; Moll et al., 1999). This process finally leads to cell death that may occur with or without cell lysis, probably depending on concomitant activation of the cell autolysins. The enterocin of strain ET05 showed a bactericidal effect towards *L. innocua* 2030c (a rapid fall in OD). Similar results were achieved by Foulquié Moreno, Callewaert, Devreese, Van Beeumen and De Vuyst (2003) when the activity of six *E. faecium* strains isolated from different origins was compared.

A similar rapid bactericidal effect toward *L. innocua* 2030c was observed for bacteriocin of *L. curvatus* strain ET30, inducing cell lysis. An analogous pattern has been described for other bacteriocins produced by the genus *Lactobacillus* and *Pediococcus*, such as lactacin B produced by *L. acidophilus* (Barefoot and Klaenhammer, 1983), curvacin A from a strain of *L. curvatus* (Tichaczek et al., 1992), pediocin PA-1 from *P. acidilactici* and *L. plantarum* WHE 92 (Rodriguez et al., 2002). In contrast with the previous statement, the very similar viable cell counts of *L. innocua* 2030c at different time intervals (data not shown), suggest that the bacteriocin from *L. curvatus* ET06 strain, like pediocin produced by the isolate ET34 (Fig. 5), have a bacteriostatic action with respect to the indicator microorganism. It is not clear why a bacteriocin may act as a bacteriostat or as a bactericide, although Vescovo et al. (2006) investigating the potential growth of antimicrobial-producing LAB cultures in vacuum-packaged CSS, showed that *L. casei* was bacteriostatic against *L. innocua* when the former was inoculated at 6 log cfu g^{-1} but bactericidal at 8 log cfu g^{-1}. Similar findings were reported by Boris, Jiménez-Díaz, Caso and Barbés (2001) when partially-purified bacteriocin from *L. delbrueckii* was added to a sensitive *Listeria* strain; at high concentrations viable cell counts and optical density of the treated cultures decreased on continued incubation, indicating that it was bactericidal, whereas control cultures or samples treated with lower concentrations did not show a significant number of lysed cells.

In conclusion, this study has shown that particularly strains of *E. faecium* (ET05), *L. curvatus* (ET30) and *L. delbrueckii* (ET32) inoculated in CSS, exhibit some interesting properties that make them strong candidates for practical use as biopreservative adjunct cultures. However *in vivo* experiments must be designed to study what levels of these microorganisms are compatible with organoleptic qualities of CSS. Other positive features of these five LAB include their sensitivity to vancomycin and their lack of haemolytic activity (γ-haemolysis) by *E. faecium* strain ET05 (Tomé et al., 2007).

This application will not replace other measures for controlling *L. monocytogenes* in CSS, but will act as an extra hurdle specifically designed to minimize the risk of listeriosis. Additionally, it is important to mention that in spite of bacteriocins ET06 from *L. curvatus* and bacteriocin ET34 from *P. acidilactici* having a bacteriostatic effect against *L. innocua* 2030c, the challenge level of *Listeria* used in the present study was very high (ca. 10^5 cfu g^{-1}) considering the actual level of *Listeria* encountered in vacuum-packaged CSS (in most cases less than 10 cells g^{-1} (Huss et al., 1998).

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