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Growth control of *Listeria innocua* 2030c during processing and storage of cold-smoked salmon-trout by *Carnobacterium divergens* V41 culture and supernatant

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Abstract

The objectives of this study were to ascertain the efficacies of *Carnobacterium divergens* V41 and its supernatant V41 as treatments to control *Listeria innocua* 2030c during salmon-trout (*Oncorhynchus mykiss*) cold-smoking processes by treating the raw fish fillets. Fillets were dip-inoculated with 2% (v/v) culture of *L. innocua* 2030c and/or 2% (v/v) culture of *C. divergens* V41 culture and 2% or 5% (v/v) of culture supernatant V41. Fillets were dry-salted for 6 h and an average salt content of 5.3% (water phase salt) was obtained. Salted fillets were cold-smoked for 5 h (3 h of drying + 2 h of smoking) and stored at 5 °C for 3 weeks in vacuum packs. The % of salt in the water phase was determined for all fillets. Sensorial analyses were performed in order to compare treated/untreated cold-smoked salmon-trout fillets to ascertain potential spoilage odours due to the treatments.

All the treatments with *C. divergens* V41 or supernatant showed a significant inhibitory effect on *L. innocua* 2030c growth, but the 5% (v/v) *C. divergens* supernatant treatment, was by far the most effective against this species. No significant off-odours were found in the products with respect to the tested treatments.

Keywords: *Listeria* spp.; *Carnobacterium divergens*; Bacteriocins; Cold-smoked fish processing

1. Introduction

Listeria monocytogenes is an ubiquitous bacterium capable of growth at temperatures between 1 and 45 °C (Seeliger & Jones, 1986, pp. 593–596), at salt concentrations up to 12% (McClure, Roberts, & Oguru, 1989) and at pH values near 4.5 (Ahmad & Marth, 1990). Water activities between 0.93 and 0.96 did not significantly affect *L. monocytogenes* (Guyer & Jemmi, 1991). Unfortunately *L. monocytogenes* is the causative agent of listeriosis which leads to death in 20–30% of reported cases (Rocourt, 1996).

As reported by several authors, *L. monocytogenes* is commonly found in cold-smoked fish products although generally in low numbers. Guyer and Jemmi (1991) showed that, whatever the initial level of *L. monocytogenes*, salting and cold-smoking processes were not sufficient to inhibit growth of the pathogen.

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The enforcement of the zero-tolerance policy by the US Food and Drug Administration resulted in at least 39 Class I recalls issued by FDA from 1990 to April 2000 involving 53,000 kg of cold-smoked fish (Jørgensen, 2000).

The majority of the natural microflora of cold-smoked fish are lactic acid bacteria (LAB) that account for most of the total viable counts (TVC) found in these products. LAB are widely used as starter cultures in food processes and they display a capacity to inhibit spoilage as well as strains of the same or closely related species. They produce natural preservatives such as organic acids, hydrogen peroxide and diacetyl, and lower the pH in foods during fermentation (Larsen & Norrung, 1993). Furthermore, some LAB produce bacteriocins, peptides that have generated considerable interest for use as biopreservatives due to their bactericidal or bacteriostatic capacity against sensitive bacterial species (Giraffa, 1995). Nisin is the only natural anti-microbial bacteriocin permitted for use in some foods in EC but not for cold-smoked fish products (European Parliament & Council, 1995). Biopreservation methods have gained popularity in the area of food safety e.g. the use of protamines (Johansen, Gill, & Gram, 1996), monoglycerides (Wang & Johnson, 1997), bacteriocins (Duffes, 1999; Duffes, Corre, Leroi, Dousset, & Boyaval, 2000; Duffes, Leroi, Boyaval, & Dousset, 1999; Goff, Bhunia, & Johnson, 1996; Leisner, Greer, Dilts, & Stiles, 1995; McMullen & Stiles, 1996; Rodriguez, Gaya, Medina, & Nunez, 1997; Stecchini, Aquilli, & Sarai, 1995).

Only a few bacteriocin-producing strains have been isolated from fish products (Buchanan & Bagi, 1997; Jeppesen & Huss, 1993; Pilet et al., 1995; Stoffels, Nes, & Gudmundsdottir, 1992) and their potential for preservation of seafood products needs to be clarified. As far as we know, only Wessels and Huss (1996), Nilsson, Gram, and Huss (1999), Duffes (1999), Duffes et al. (2000) and Duffes et al. (1999) studied inhibition of *L. monocytogenes* by bacteriocin-producing strains on cold-smoked salmon.

In cold-smoked salmon, dramatic treatments to assure the absence of the pathogen may also change the organoleptic characteristics of the products and in most cases it will be impossible to reconcile the artisanal process with an adequate safety program. The application of selected LAB strains or their products, for inhibition/destruction of *L. monocytogenes*, may be an appropriate means for those products which are intended to be maintained as ''traditional'' but safe.

Carnobacterium divergens V41, isolated from trout intestinal tract (Pilet et al., 1995), and divercin V41 were applied to cold-smoked salmon fillets inoculated with several strains of *L. monocytogenes*, and both cultures and bacteriocins, were found to be effective against the pathogen (Duffes et al., 2000; Duffes et al., 1999). Carnobacteria are known to be both psychrotrophic (able to grow at temperatures from 0 to 10 °C) and halophilic, but are not able to grow in acid media (Collins, Farrow, Philips, & Jones, 1987).

Considering the inhibitory results on *L. monocytogenes* growth achieved in cold-smoked salmon with *C. divergens* V41 and divercin V41, it was decided to apply the same treatments but to the raw fish before salting, rather than to the finished smoked product. Although effective against *L. monocytogenes*, the treatment applied to the smoked finished product might change product appearance. It was considered that for industrial purposes, the best moment to apply these treatments to the fish was after washing step just before salting. It was less time-consuming and avoidance of changes on product appearance was assured. As far as we know, this was the first experiment of applying *C. divergens* and divercin V41 onto the raw fish rather than onto the finished smoked products.

A *Listeria innocua* strain was used for replacing *L. monocytogenes* in these cold-smoking studies due to processing laboratory constraints of using the pathogen. As all previous isolated naturally occurring *Listeria* were, to date, tetracycline sensitive (Vaz-Velho, Duarte, McLauchlin, & Gibbs, 2001), a *L. innocua* tetracycline resistant strain, was selected for this study. As, previous works (Vaz-Velho, Fonseca, Silva, & Gibbs, 2001) showed that the sensitivities of *L. innocua* 2030c, a tetracycline resistant strain, and the major biotypes of *L. monocytogenes* isolated from Portuguese cold-smoked fish products to *C. divergens* V41 and its divercin V41, were likely, this *L. innocua* strain was considered a suitable marker for replacing *L. monocytogenes* in this study.

2. Materials and methods

Fresh-water farm reared salmon-trout (*Oncorhynchus mykiss*) (one and half years old, 1–2 kg), slaughtered on the same day, obtained from a farm in the North of Portugal, were packed in ice and shipped in a refrigerated truck (3 h of travelling) to the laboratory. The following day, the fish were eviscerated, beheaded and filleted by hand.

2.1. Pure cultures

C. divergens V41 isolated from trout intestine by Pilet et al. (1995), stored at −20 °C in Elliker broth (Elliker, Anderson, & Hannesson, 1956) supplemented with 15% (v/v) sterile glycerol, was supplied by École Nationale d'Ingénieurs des Techniques des Industries Agricoles et Alimentaires (ENITIAA), Nantes, France. Two subcultures (30 °C, 30 h yielding 10^{8-9} CFU/ml) were made in Elliker before experimental use.

C. divergens V41 semi-purified supernatant of a *C. divergens* culture (30 °C, 30 h) grown in Elliker broth and stored at −20 °C was also provided by ENITIAA.

L. innocua 2030c from Public Health Laboratory Services (PHLS, London) private collection was stored on Lab Lemco agar (Oxoid, CM17) at ambient temperature. Two overnight subcultures (30 °C, 18 h yielding 10^{8-9} CFU/ml) were made in TSB-YE (Lab M) before experimental use.

2.2. Treatments

Overnight cultures (20 ml) of *L. innocua* 2030c and of *C. divergens* V41 (TSBYE, 30 °C) were centrifuged (Centromix P Selecta, Spain; 2400 g, 10 min) and the deposit resuspended in 20 ml of sterile saline solution (1% w/v NaCl in water). These cell suspensions and 20 and 50 ml of semi-purified supernatant V41, were each diluted in 1 l of water and fish fillets were immersed for 30 s in these 1 l volumes as required by trials 1–8:

Two independent runs (batches) were performed. In the first batch the trials were as follows:

Trial 1: Two fish fillets inoculated with a 2% (v/v) *L. innocua* 2030c culture.
Trial 2: Two fish fillets inoculated with 2% (v/v) *L. innocua* 2030c and treated with a 2% (v/v) *C. divergens* V41 culture.
Trial 3: Two fish fillets inoculated with 2% (v/v) *L. innocua* 2030c and treated with a 5% (v/v) *C. divergens* V41 supernatant.
Trial 4: Two fish fillets not inoculated nor treated (negative control).
Trial 5: Two fish fillets treated with 2% (v/v) of *C. divergens* V41 culture.
Trial 6: Two fish fillets treated with 5% (v/v) of *C. divergens* V41 supernatant.
In the second batch a treatment with 2% (v/v) *C. divergens* V41 supernatant was also assayed. Thus, two extra trials were performed.
Trial 7: Two fish fillets inoculated with 2% (v/v) *L. innocua* 2030c and treated with 2% (v/v) *C. divergens* V41 supernatant and,
Trial 8: Two fish fillets treated with 2% (v/v) *C. divergens* V41 supernatant.
Raw fillets were then salted and smoked as described below.

2.3. Sampling procedure

Twenty-five grams of each fillet (fresh or smoked material), taken from each centre of the fillet, were homogenised with 225 ml of sterile MRD (Lab M) broth in a Stomacher (Seward 400) for 2 min. Further serial decimal dilutions up to 10^{-5} were made in MRD. Enumeration of *L. innocua* 2030c was done in the raw material, after smoking, and weekly during the 3 weeks of storage at 5 °C. Two samples per treatment were analysed on each occasion. The serial dilutions were assayed for *L. innocua* 2030c counts (by spread plating, 0.1 ml) on PALCAM (Merck) with 8 μg/ml of tetracycline-HCl (Sigma). Plates were incubated at 30 °C for 48 h.

All samples in each trial were analysed in duplicate. An average cell concentration of $\approx 10^{3-4}$ CFU/g and 10^{2-3} CFU/g of *L. innocua* 2030c was obtained in the raw fillets in the first and the second batches respectively. As treatments with both, *L. innocua and C. divergens* cultures, were applied in the same conditions initial cell concentration was assumed to be the same.

2.4. Bacteriocin activity assay

Anti-listerial activity screening of the semi-purified supernatant V41 and of the supernatant obtained by homogenization of the inoculated fish samples in the Stomacher, was performed using the critical dilution assay (Pilet et al., 1995) and the agar spot test reported by Pilet et al. (1995). Elliker agar (containing 1.5% w/v agar) was used. For overlay, Elliker soft agar (1% w/v agar) was prepared. 0.1% (v/v) of *L. innocua* 2030c (30 °C, 18 h culture) was added to the soft agar. In order to eliminate the actions of lactic acid or of proteases on *L. innocua* 2030c, the pH of the tested supernatants was adjusted to 6.5 with 6 N NaOH and also heated at 90 °C for 10 min. Twofold dilutions of the supernatant in phosphate buffer (0.1 M, pH 6.5) were prepared. Ten microliters of each supernatant dilution were dropped onto the Elliker soft agar plates and further stored at 30 °C for 18 h. The activity of the supernatant was expressed in arbitrary units (AU/ml). One AU was defined as the reciprocal of the highest serial twofold dilution showing a clear zone of growth inhibition of the indicator strain (Barefoot & Kaenhammer, 1983).

In fish fillets, sampling for bacteriocin activity assay was done after inoculation of the fresh fillets, after smoking, and weekly during the 21 days of storage.

2.5. Salting and cold-smoking processes

Two independent experiments were performed. For both batches dry salting was done for 6 h at 5 °C. Sugar was added to the salt in the proportion 1:6, 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 of salt and were drained/dried overnight at 5 °C. Fish filets were placed inside the smoking kiln for 5–3 h of drying and 2 h of exposure to smoke. A mechanical vertical smoker (AGK, Type 135/12, Wallersdorf, Germany) was used. A more sensitive thermostat and timer (lae® electronic, MTR12, Oderzo, Italy) replaced the one

supplied with the smoker. Smoke was produced by pyrolysis of beech wood chips in contact with an electrical résistance (Räucher Gold®, type Kl 2/16, Rettenmaier & Söhne, Rosenberg, Germany). Time/temperature profiles of the two processes were recorded on a portable microprocessor (Hanna instruments, HI 92804c, Bedfordshire, UK). Smoke temperature reached the 33 and 25 °C respectively in the first and the second batch. Humidity, recorded by an hygrometer (Rotronic AM3, Knoxville, USA) varied from 80% to 70% during the first three hours decreasing to 50% to 52% at the end of the smoking process.

The smoked samples were cooled overnight at 5 °C. The following day, lug and pin bones and belly flaps were removed and the fillets were then sliced by hand (clean plastic gloves and sterile knives for each trial) and vacuum packed (Multivac Sepp Haggenmüller KG, A300/41/42, Germany; 1 mbar/10 s). The permeability of the plastic packs to O_2, CO_2 and N_2 were respectively 4, 13 and 4 mol/m^2 d bar. Packs were stored for 3 weeks at 5 °C for weekly analysis.

2.6. Chemical analysis

Salt content and moisture of the finished products were evaluated following respectively, the NP 2929-1988 and NP 2282-1991 protocols for fish products (Institute Portuguese for Quality). These analyses were performed on smoked fillets after 3 weeks of storage.

2.7. Sensory evaluation

Sensory evaluation, regarding the presence of off-odours, was carried out with 10 semi-trained judges presented with a pooled sample from each trial (trials 4, 5, 6 and 8). A scale for the following features: no off-odours, slight off-odours and strong off odours was presented to each judge and scored, respectively, with 3, 2 and 1 points. Samples scored with totals of $\geqslant 20$ points were considered acceptable. These analyses were performed after 3 weeks of storage.

2.8. Statistical analysis

The effect of the different treatments on *L. innocua* 2030c growth and on sensory properties of the smoked fillets was evaluated by analysis of variance (ANOVA). It was accepted there was a significant difference between treated/untreated samples if $P < 0.05$.

3. Results

Two smoking batches were performed: The first batch took place in summer whereas the second was performed in winter. In summer, with a room temperature of ≈21 °C, it was difficult to keep smoke temperatures below 30 °C during the whole process. For about 30 min the fish fillets temperature in the first batch reached the 33 °C. In winter, as room temperature was ≈13 °C, the temperature along the smoking process never reached the 30 °C. Due to these differences in temperatures, and not knowing if it influenced results, the batches were treated separately for statistical analysis.

Counts of *L. innocua* 2030c of the two batches are present in Figs. 1 and 2.

In the first batch, a strong bactericidal-like effect (reduction of ≈2 log cycles) was obtained on samples

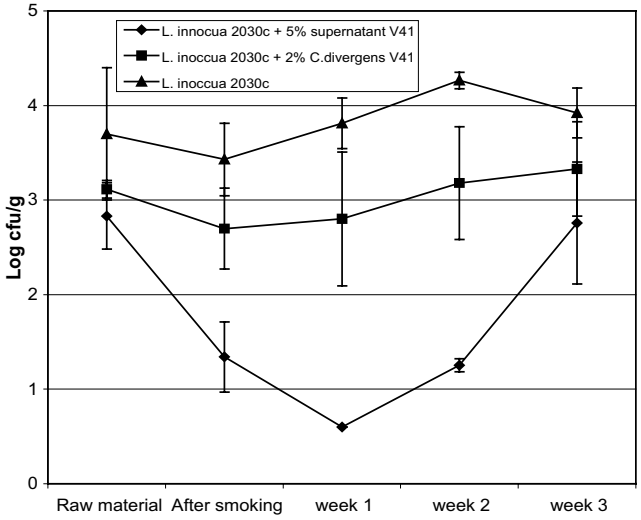

Fig. 1. First batch: Effect of *C. divergens* V41 and its supernatant V41 on *L. innocua* 2030c numbers during cold-smoking processing and storage of salmon-trout.

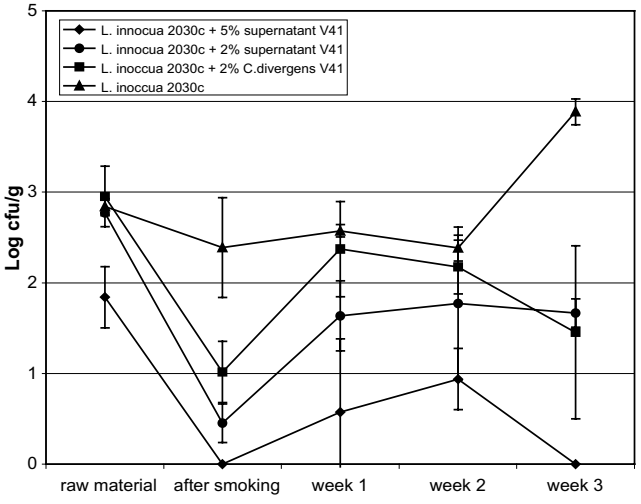

Fig. 2. Second batch: Effect of *C. divergens* V41 and its supernatant V41 on *L. innocua* 2030c numbers during cold-smoking processing and storage of salmon-trout.

treated with 5% supernatant V41 after smoking and, at week 1, this difference was even greater (reduction of 3 log cycles). However, this treatment resulted in only one log reduction of *L. innocua* numbers at the end of the storage period. In the same batch, the 2% *C. divergens* culture showed a bacteriostatic-like effect on *L. innocua* growth, numbers being ≈1 log lower than the control during the 3 weeks of storage (Fig. 1).

In the second batch, after smoking, a strong bactericidal effect on *L. innocua* 2030c, was found in samples treated with 5% (v/v) of *C. divergens* V41 supernatant compared to the control. No cells of *L. innocua* 2030c were found after smoking nor at the end of the storage period (Fig. 2). With the 2% (v/v) supernatant V41, a reduction of ≈2 log CFU/g was found after smoking, but numbers recovered during 1 week's storage and remained steady below inoculum level during further storage. With the 2% (v/v) *C. divergens* V41 culture a reduction of ≈2 log was found after smoking compared to the inoculum level but numbers recovered to ≈0.5 log CFU/g below inoculum level and declined to ≈1.5 log CFU/g below inoculum level after 3 week's storage. In contrast, numbers of *L. innocua* in the control trial, declined slightly after smoking (≈0.3 log CFU/g), remained steady for 2 weeks storage and increased by ≈1.5 log CFU/g in the final week (Fig. 2).

In batch 1 and batch 2, statistical analysis considered treatments 5% supernatant V41 and 2% *C. divergens* V41 culture to be significantly different from each other and from the control ($P < 0.05$). However, in the second batch, statistical analysis did not differentiate the 2% supernatant V41 and 2% *C. divergens* V41 treatments ($p > 0.05$).

The supernatant V41 showed an activity of 13,107,200 AU/ml against *L. innocua* 2030c pure culture. Bacteriocin activity in the raw fillets just after treatment was 204,800 AU/ml for trials with 5% (v/v) supernatant and 102,400 for trials with 2% (v/v) supernatant in both batches. Despite the dramatic fall of *L. innocua* numbers after smoking in supernatant-treated samples compared to the control and to the other treatments, no bacteriocin activity could be detected in those samples during chilled storage. After smoking, bacteriocin activity was just detectable on samples treated with 2% (v/v) of *C. divergens* culture at a level of 400 AU/ml. In all the other trials for the same sampling time and in the remaining samples during storage no bacteriocin activity was detectable.

As shown in Table 1, all the samples presented sensory scores $\geqslant 20$, and therefore were considered acceptable with respect to spoilage. However, it is noticeable that samples from the first batch were always better scored compared to batch 2. In both batches, the best score was obtained by the control but statistical analysis did not differentiate the products ($P > 0.05$). The highest difference between scores was 2 and 3 points respectively in batches 1 and 2. In the first batch the treatment with 5% (v/v) supernatant got 2 points less than the control; in the second batch, the 2% (v/v) *C. divergens* treated samples were scored with 3 points less than the control samples. In the second batch, the 2% *C. divergens* treated samples presented the minimum level of acceptability considered for this study ($\geqslant 20$ points). In the second batch the scores obtained by the control and by the 2% (v/v) supernatant treatment were the same.

The % of salt in the water phase varied from 4.6% to 6% and 3.7% to 7.9%, respectively for the first and second batches. The samples, tested for sensory analysis, presented levels of salt in the water phase between

Table 1
% of salt in the water phase (WPS) and sensory scores of batches 1 and 2

Samples	% WPS		Sensory scores	
	Batch 1	Batch 2	Batch 1	Batch 2
2% *L. innocua* + 5% supernatant V41	5.0	4.1	nd	nd
2% *L. innocua* + 5% supernatant V41	4.6	3.7	nd	nd
2% *L. innocua* + 2% supernatant V41	nt	6.8	nd	nd
2% *L. innocua* + 2% supernatant V41	nt	5.3	nd	nd
2% *L. innocua* + 2% *C. divergens* V41	5.9	6.7	nd	nd
2% *L. innocua* + 2% *C. divergens* V41	5.7	6.7	nd	nd
2% *L. innocua*	5.1	6.7	nd	nd
2% *L. innocua*	4.7	3.8	nd	nd
2% *C. divergens V41*	**5.2**	**5.0**	**26**	**19**
2% *C. divergens* V41	4.8	7.9	nd	nd
2% supernatant V41	nt	**4.7**	nd	**23**
2% supernatant V41	nt	6.0	nd	nd
5% supernatant V41	**5.0**	**4.2**	**25**	**22**
5% supernatant V41	4.7	6.0	nd	nd
Not inoculated nor treated	**6.0**	**4.3**	**29**	**23**
Not inoculated nor treated	5.5	6.4	nd	nd

nd: not determined; nt: not tested.

5.2% and 6% and 4.2% and 5% in the first and in the second batches, respectively (Table 1).

4. Discussion

Despite the different temperatures of smoking of the two batches and consequently in *L. innocua* growth in treated samples, the best treatment for reducing *L. innocua* numbers, at all sampling times, was the 5% (v/v) supernatant V41. Treatments were more effective for reducing *L. innocua* levels in the second batch compared to the first. Somehow, activities of *C. divergens* culture and supernatant were reduced in the first process. The higher temperature of the first process might have been the reason for this result. Duffes et al. (1999) reported *C. divergens* V41 bacteriocin production in co-culture with *L. monocytogenes* in Elliker medium, was higher at 20 °C than at 30 °C the stationary phase was reached earlier at 20 °C, but the inhibitory activity decreased after 24 h. No interference of *L. monocytogenes* presence on the growth rate of *C. divergens* was found by Duffes et al. (1999), but after 21 days a slight growth of *L. monocytogenes* occurred probably because bacteriocins were destroyed by proteolytic activities released into the broth. In the present study, lower smoke temperatures of batch 2 (15–24 °C) seemed to favour the anti-listerial activity of *C. divergens* V41 and supernatant V41, compared to temperatures of batch 1 (15–32 °C). In batch 2, *L. innocua* 2030c numbers of control samples increased dramatically at week 3 whereas in batch 1, for the same week, a slight fall was detectable.

L. innocua 2030c recovery of treated samples after smoking (week 1) was higher in batch 2 samples (lower temperature exposure) compared to batch 1 but, in the former batch, numbers declined during storage whereas in the latter, growth occurred. It is possible that *L. innocua* revival in treated samples from batch 1 could be linked to acquired resistance of *L. innocua* 2030c. However, this strain was previously studied (Duffes et al., 2000) and no acquired resistance was noted. Furthermore, as no revival occurred in batch 2 treated samples, the process temperature of process 1 seems to be the only factor responsible for that occurrence by decreasing growth and/or bacteriocin production or activity. Thus, in this study, temperatures of fish fillets maintained below 25 °C during smoking clearly favoured the production and activity of divercin V41.

The generally high salt content of samples could also have interfered with *C. divergens* V41 growth; these strains are more salt-sensitive than *Listeria* strains. As reported by Pilet (1994) *C. divergens* V41 pure cultures grew slowly when media had a level of 6.5% (w/v) NaCl and it was not able to grow at 8% (w/v) NaCl. In batch 2, the inoculated samples treated with 2% (v/v) of *C. divergens* V41 culture contained a 6.7% of salt in the water phase, whereas in batch 1, the percentage of salt for the same type of samples was lower (5.7–5.9%). Nevertheless, after smoking and at the end of storage, treated samples from batch 2 despite their higher salt content, showed much lower numbers of *L. innocua* than treated samples from batch 1. Thus, the overall differences in growth of *L. innocua* between the two treatments seems to be due to the temperature of smoking, that affected the anti-listerial activity of *C. divergens* culture and supernatant, and not to the salt content of the samples. However, looking at the greater efficacy of the 5% (v/v) supernatant treatment on batch 2, compared to the other treated samples from both batches, in this particular case the lower levels of salt of these samples (3.7% and 4.1%) might have had an additive effect.

In this study no marked spoilage odours were detected in the tested samples. This was in agreement with the results of Leroi, Arbey, Joffraud, and Chevalier (1996) and Duffes et al. (1999) studies with *C. divergens* V41. Paludan-Müller, Dalgaard, Huss, and Gram (1998), supported by Nilsson et al. (1999), showed that at 5 °C, *Carnobacterium piscicola* inoculated at 10^5 CFU/g did not accelerate the spoilage process of cold-smoked salmon.

At 5 °C the natural lactic acid bacteria (LAB) flora seemed able to control growth of *L. monocytogenes* in naturally contaminated cold-smoked fish samples (Jørgensen, 2000). At higher temperatures *L. monocytogenes* out-competed the LAB and became the dominant microflora, resulting in suppression of the LAB. For cold-smoked fish, a maximum period of 3 weeks storage at temperatures <5 °C is now recommended by Danish and French authorities as regarded stabilisation of numbers of *L. monocytogenes* in such products.

Truelstrup-Hansen, Gill, Røntved, and Huss (1996) reported standard deviations on salt content being higher in dry-salted salmon samples compared to injection-brined samples. In another study of comparison of salting methods (Vaz-Velho et al., 2001) the standard deviations on salt content of dry-salted samples (0.93) were higher than those of brined samples (0.78). In the study reported here, all samples were dry-salted but standard deviations were low in the first batch (0.48) and very high in the second batch (1.29).

There are no legal requirements but, according to Huss, Ben Embarek, and Jeppesen (1995), approximately 5% NaCl (WPS) is the upper limit for consumer acceptability. Cold-smoked salmon normally contains 3–6% water phase salt but 7–8% was often detected by Jørgensen (2000) but values of water phase salt in commercial Danish cold-smoked salmon ranging from 3% to 12.5% (Jørgensen et al., 2000). Portuguese commercial cold-smoked swordfish presented always levels of salt (water phase salt) ≈8% (w/w) and *L. monocytogenes* has been consistently isolated from this kind of products (Vaz-Velho, Duarte, & Gibbs, 2001). Thus, in the

present study, the variation on levels of salt content of smoked fish samples is unlikely to affect *L. monocytogenes* growth.

In this study, samples were not homogenised before inoculation and treatment as is usually done in laboratory experiments. For samples to undergo further smoking it would not have been sensible to smoke minced fish. Thus, variation in counts and other analytical parameters were to be expected. The choice of working with the whole fillets was to reproduce as closely as possible the normal smoking process. Also, if treatments are to be applied in an industrial process it would be difficult to reconcile costs with application of treatments to the fillets one by one, the easier way being the immersion of the whole batch of fillets in a bath containing the bacteriocin producers or the bacteriocins. The treatment of fillets after smoking is not considered here because it will change the characteristics of the products.

There are intrinsic and extrinsic factors associated with a food product which can interfere with effectiveness of bacteriocins in foods: they can be inactivated by food components (proteases, lipids, other microorganisms) or their activity could be affected during processing (temperature, drying) (Duffes et al., 1999).

The loss of bacteriocin activity on samples treated with 2% (v/v) culture during storage and on samples treated with supernatant after smoking might have been due to the interference of raw fish proteases, adsorption to packaging or to target or endogenous microflora competition. On the other hand, production and persistence of bacteriocin activity on foods is very difficult to measure probably due to interactions with the product and packaging as transfer of activity could be observed from the flesh of the salmon to the packaging throughout storage (Duffes et al., 2000). Ming, Weber, Ayres, and Sandine (1997) reported that more than 90% of the activity was retained on casings and that pediocin-coated bags were effective against *L. monocytogenes* growth on meat at 4 °C. Furthermore, in the current work, the detection method used might not have been sufficiently sensitive to detect low levels of bacteriocins but residual bacteriocin activity still affect *L. innocua* weakened by process conditions. As no published scientific information regarding the behaviour of artificially contaminated *Listeria* spp. samples related to their sensitivity to LAB and bacteriocins during the smoking process is available, further experiments should be performed to ascertain whether or not the salting and smoking steps are responsible for the non-detection of bacteriocin activity. However, due to the reduced bacteriocin activity after the smoking process found in this experiment, comparison of the treatments applied either to the raw fish or to the fish after salting or after smoking should be performed to ascertain the possible interference of process factors on bacteriocin production or activity. From a commercial point of view it seems easier to apply the treatment before processing but there is no point if effectiveness of the treatment is not achieved.

According to Huss et al. (1995), the complete exclusion of *L. monocytogenes* from cold-smoked salmon is unrealistic and the so-called "zero tolerance" would lead to this product being excluded from the market. The presence of *L. monocytogenes* in cold-smoked fish cannot be completely controlled but should be reduced to a minimum by good manufacturing practice (GMP). Furthermore, as growth of the pathogen cannot at present be controlled by a standard preservation method, the shelf-life of the cold-smoked salmon stored at temperatures <5 °C, should also be reduced to 3 weeks (Huss et al., 1995). The final report of the European Fair Project CT/95-1207 "Spoilage and safety of cold-smoked fish" concluded that numbers of *L. monocytogenes* are usually low in cold-smoked fish samples probably because of the LABs competition. They also recommended for safety assurance of these food products, a maximum period storage of 3 weeks at temperatures below 5 °C to keep *L. monocytogenes* numbers below 10^2 CFU/g taking into account that, until so far, food samples related with listeriosis outbreaks always presented numbers above 10^3 CFU/g.

Any contra-indication about safety for use of *C. divergens* V41 in foodstuffs was commented upon by Duffes et al. (2000). These authors reported no spoilage odours occurred on product, no detection of putrescine, cadaverine, phenylethylamine, spermidine, agmatine or histamine and levels of tyramine, although high, they were not very different from the control samples.

Thus, taking into account the preliminary results obtained in this study with *C. divergens* supernatant V41 and the results of Duffes et al. (2000) with 97% purified divercin V41, the use of these bacteriocins, particularly if pure molecules are employed, could be considered as an alternative means of controlling *L. monocytogenes* growth without any other additives and of being readily accepted by consumers.

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