Biodiversity of Listeria monocytogenes sensitivity to bacteriocin-producing Carnobacterium strains and application in sterile cold-smoked salmon

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Abstract:

The aim of this study was to demonstrate the inhibitory capacity of *Carnobacterium* strains against a collection of *Listeria monocytogenes* strains in cold-smoked salmon (CSS).

Methods and Results: Three bacteriocin-producing strains, *Carnobacterium divergens* V41, *C. piscicola* V1 and *C. piscicola* SF668, were screened for their antilisterial activity against a collection of 57 *L. monocytogenes* strains selected from the French smoked salmon industry, using an agar spot test. All the *Listeria* strains were inhibited but three different groups could be distinguished differing in sensitivity to the three *Carnobacterium* strains. However, *C. divergens* V41 always had the highest inhibitory effect. The antilisterial capacity was then tested in sterile CSS blocks co-inoculated with *Carnobacterium* spp. and mixtures of *L. monocytogenes* strains. *C. divergens* V41 was the most efficient strain, maintaining the level of *L. monocytogenes* at <50 CFU g^{-1} during the 4 weeks of vacuum storage at 4 and 8°C, whatever the sensitivity of the set of *L. monocytogenes* strains.

Conclusions: *C. divergens* V41 may be a good candidate for biopreservation in CSS.

Significance and Impact of the Study: A biopreservation strategy for CSS against the risk of *L. monocytogenes* was investigated using bacteriocin-producing lactic acid bacteria.

Keywords: bacteriocin, biopreservation, Carnobacterium, cold-smoked salmon, Listeria monocytogenes.

INTRODUCTION

Listeria monocytogenes is the pathogenic bacterium responsible for listeriosis, which is a food-borne disease. Listeriosis is generally associated with a high mortality rate (20-40%) and is regarded as the most fatal foodborne infection (Feldhusen, 2000; Rocourt *et al.*, 2000). Populations at greatest risk are pregnant women, newborn infants, the elderly and people with a weak immune system. However, it has recently been established that *L. monocytogenes* may cause febrile gastro-enteritis in healthy adults not associated with the above mentioned risk groups (Miettinen *et al.*, 1999).

A whole range of food categories have been associated with listeriosis outbreaks and fish or ready-to-eat fish products have also been incriminated (Rocourt *et al.*, 2000). Lightly preserved fish products such as cold smoked salmon (CSS) are classified as high risk merchandises. Indeed, the raw material is frequently contaminated by *L. monocytogenes*, the processing parameters i.e. salting, drying, smoking and vacuum-packaging are insufficient to inactivate the bacterium or to prevent growth during chilled storage, and contamination during processing can occur. Since this product has an extended shelf-life and is consumed without further cooking, it represents a health risk for consumers. Although no listeriosis outbreak due to consumption of CSS has been reported in France, the detection of *L. monocytogenes* in CSS has led to recalls, destruction, cleaning, adverse publicity and sometimes closure of factories. In France (DGAL/SDHA/N98/N°8088, 1998) and in many European countries, the guidelines for presence of *L. monocytogenes* in CSS is less than 100 CFU g^{-1} till the sell-by date. Last years, many efforts have been done in clean-up and sanitation procedures, reducing significantly the prevalence of *L. monocytogenes* in this product (Rorvik *et al.*, 1997; Autio *et al.*, 1999). However, production of CSS consistently free of the bacterium is impossible because no elimination step for bacteria exists in the

smoking process (Huss *et al.*, 2000). In parallel, many publications about *Listeria* inhibitory treatments on food products, such as gamma irradiation (Savvaidis *et al.*, 2002), ultra high pressure (Ritz *et al.*, 2000; Lakshmanan *et al.*, 2003), antimicrobial agents (Antunes *et al.*, 2002), sodium lactate (Nykanen *et al.*, 2000; Glass *et al.*, 2002), sodium chloride (Peterson *et al.*, 1993), sodium nitrite (Lyhs *et al.*, 1998), lactoperoxydase (Boussouel *et al.*, 1999) have been reported. Nevertheless, many of these treatments have a negative incidence on the quality of the CSS and some of them are not allowed in the European regulation. Biopreservation, which consists in inoculating food product by selected bacteria to inhibit the growth of undesired micro-organisms, seems to be an interesting strategy to master *L. monocytogenes* risk in CSS. Many studies report the use of lactic acid bacteria (LAB) as protective cultures in a range of ready-to-eat food products (Kelly *et al.*, 1996) and a variety of refrigerated meat (Schillinger *et al.*, 1991; McMullen and Stiles, 1996; Hugas, 1998; Bredholt *et al.*, 1999; Budde *et al.*, 2003), vegetable (Vescovo *et al.*, 1996; Schillinger *et al.*, 2001), dairy (Eppert *et al.*, 1997; Benkerroum *et al.*, 2002; Foulquié Moreno *et al.*, 2003) and fish products (Nilsson *et al.*, 1999; Katla *et al.*, 2001). In CSS, the natural microflora is frequently dominated by LAB, in which *Carnobacterium* genus is often represented (Leroi *et al.*, 1998; Truelstrup Hansen and Huss, 1998; Jorgensen *et al.*, 2000; Leroi *et al.*, 2000). *Carnobacterium* species are good candidates for a biopreservation strategy since many strains secrete antimicrobial compounds called bacteriocins, capable of inhibiting related bacteria such as *L. monocytogenes*. Moreover, they are not believed to have any adverse effect on sensory properties of CSS (Stohr *et al.*, 2001). In previous studies, antilisterial activity of three bacteriocin-producing strains isolated from seafood, *C. divergens* V41, *C. piscicola* V1 and *C. piscicola* SF668, has been demonstrated in model culture media (Pilet *et al.*, 1995; Duffes *et al.*, 1999a). Results have been confirmed by Duffes *et al.* (1999b) in CSS against

one strain of *L. monocytogenes* chosen for its high sensitivity to each bacteriocin-producing strain.

The aim of this study was to evaluate the robustness of this bioprotective technology. This should include an assessment of the inhibitory spectrum against a range of *L. monocytogenes* strains. Target strains have been chosen out of a wide collection representative for *L. monocytogenes* strains encountered in the French CSS industry. Sensitivity of each *L. monocytogenes* strain to *C. divergens* V41, *C. piscicola* V1 and *C. piscicola* SF668 was tested in plate medium and in CSS so as to select the bacteriocin-producing strain with the strongest inhibitory effect for a potential application as safety agent in fish products.

MATERIALS AND METHODS

Bacterial strains and subcultures conditions

C. divergens V41 and *C. piscicola* V1 were isolated from trout intestine and characterized by Pilet *et al.* (1995). *C. piscicola* SF668 was isolated from commercial Norwich CSS by Leroi *et al.* (1998), and studied by Duffes *et al.* (1999a).

152 strains of *L. monocytogenes* were isolated from the environment and salmon products of 5 French CSS manufacturers (ASEPT, Laval, France). After phenotypic (serotype, resistance to Cadmium, Arsenic and Tetracycline) and genotypic typing (Pulsed Field Gel Electrophoresis), 57 different strains were selected as representative for the collection and kindly provided by ASEPT for this study.

Subculture media used were Elliker broth (BK 054, Biokar, Beauvais, France) for *Carnobacterium* spp. and Brain Heart Infusion (BK015, Biokar) with 3 % (w/v) NaCl for *L. monocytogenes*. *Carnobacterium* and *Listeria* were subcultured for 24 h at 30°C. All strains were stored at -80°C in their growth medium with 20 % (v/v) sterile glycerol.

Preparation of sterile CSS model

Five whole-gutted salmon (*Salmo salar*) of approximately 4-5 kg, stored in ice, were bought from a Norwich salmon farm. According to Joffraud *et al.* (1998), the fishes were washed with different washing and sanitizing solutions and fillets were collected and skinned respecting the highest hygienic conditions. Fillets were dry-salted for 140 min at 12°C with heat-treated salt (1 h, 160°C), rinsed in sterile water for salt removal, dried for 90 min (65 % RH) and smoked for 120 min at 20°C in a smoking kiln previously cleaned and heated to 70°C. Cold-smoked fillets were then vacuum-packed and stored for 40 h at 2°C before being aseptically cut into small blocks (about 1 cm^3). Salmon blocks were dispatched in 34 parts of 300 g and stored in polyamide polyethylene bags purchased from Bourdeau (St-Etienne-de-Montluc, France), vacuum-packed and frozen at -80°C. Then, frozen bags were ionised (1.6 kGy) in a plant equipped with electron beam facilities (Gradient Ouest, Berric, France). Sodium chloride was measured with a Chloride Analyser 926 (Corning, Halstead, England) and total phenols were quantified by the method described in the French standard for smoked salmon (NF V 45-065).

Biodiversity of *L. monocytogenes* sensitivity to the three *Carnobacterium* spp.

The antibacterial activity of the three *Carnobacterium* bacteriocin-producers was tested on 57 *L. monocytogenes* strains by a standardised agar spot test with critical dilution assay (Pilet *et al.*, 1995). The three *Carnobacterium* spp. were separately cultivated in fermentors (SGI 2L, Setric, Toulouse, France). One litter of Elliker broth was inoculated with 20 ml of a *Carnobacterium* spp. subculture. Cultures were run at 20°C under agitation with pH adjusted to 6.5 by automatic addition of NaOH (6N). Growth was controlled by regular DO$_{600\,nm}$ measurements, and when stationary phase was reached (approx. 30 h), 100 ml of cell free

supernatant of each *Carnobacterium* was obtained by centrifugation (8000 g, 5 min at 4°C). Supernatants, containing the thermoresistant bacteriocins, were also heated for 15 min at 80°C to inactivate protease activity, and stored at -80°C until used. Two successive individual subcultures of *L. monocytogenes* target strains were run as previously described and 1 ml of the 100-fold dilution were poured in a plate with Elliker containing 1% agar. For each *Carnobacterium* strains, 10 µl of the treated supernatant and their 2-fold successive dilutions in phosphate buffer (0.1 M ; pH 6,5) were spotted onto the 57 indicator plates. After overnight incubation at 30°C, a translucent zone corresponding to absence of *L. monocytogenes* growth (inhibition zone) revealed sensitivity. The first spot corresponding to the $1/2^n$ dilution showing no inhibition zone was retained for data treatment.

Antibacterial activity of *Carnobacterium spp*. in CSS

Three groups of *L. monocytogenes* strains were designed according to their sensitivity to *Carnobacterium* strains observed in the agar spot test. According to ASEPT information, some strains of their collection were persistent in the plant (they have been isolated from environment at least at two different times, and some of them were also found in the product) and others were sporadic. In each group, four to five strains were chosen to represent this diversity. Set 1, corresponding to group 1, was constituted of strains RF107, RF114, RF119, RF129, RF148, set 2 (group 2) of RF100, RF120, RF122, RF123, RF140, and set 3 (group 3) of RF131, RF132, RF133, RF151. Table 1 summarizes the sensitivity of the different sets to the three *Carnobacterium* strains. *C. piscicola* SF668 and *C. piscicola* V1 were co-inoculated individually with *L. monocytogenes* set 1 and set 3 separately (strains respectively fairly and highly sensitive to the two *C. piscicola*). Set 2 composed of strains fairly sensitive to the three *Carnobacterium* was not tested because it was similar with set 1. In the same way, *C. divergens* V41 was co-inoculated with *L. monocytogenes* strains of set 1 and set 2 separately

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(strains respectively fairly and highly sensitive to this strain). Each set of *L. monocytogenes* was tested in at least three independent experiments in CSS, inoculated alone (control) and in co-culture with *Carnobacterium* spp.

For one set, each *L. monocytogenes* strain was individually twice subcultured in 10 ml tubes (24 h, 30°C). All final cultures were adjusted to the same optical density (600 nm) using fresh medium and mixed (v/v) in a sterilized test tube. Cultures of *C. divergens* V41, *C. piscicola* V1 and SF668 were grown in 100 ml of Elliker broth for 24 h at 30°C before cells were centrifuged and washed in physiologic salt solution (0.1 % (w/v) tryptone (Biokar) and 0.85 % (w/v) NaCl). Immediately, appropriate dilutions of mixed *L. monocytogenes* and *Carnobacterium* strains were mixed and inoculated (2 % v/w) in parts of 30 g of thawed sterile CSS pieces distributed in polyamide polyethylene bags (Bourdeau). Pieces were gently mixed with the inoculating solution and samples were then vacuum-packed and incubated for 28 days using the following conditions : 9 days at 4°C followed by 19 days at 8°C as specified in the French standard for shelf-life validation of perishable and refrigerated food (NF V 01-003), with a break during 2 h at 20°C after 19 days of storage (to mimic a break in the cold chain during distribution and sale). The initial desired levels in the flesh for *L. monocytogenes* and *Carnobacterium* were 20 and 10^5 CFU g^{-1} respectively. For each *L. monocytogenes* set, a control was prepared by inoculating CSS pieces with *L. monocytogenes* alone (*Carnobacterium* subculture being replaced by sterile physiologic salt solution). Microbial analysis was done weekly in triplicates (three different bags analysed).

Listeria monocytogenes strains	*C. divergens* V41	*C. piscicola* V1	*C. piscicola* SF668
Group 1 **RF107, RF114, RF119, RF129, RF148**	++	+	+
Group 2 **RF100, RF120, RF122, RF123, RF140**	+	+	+
Group 3 **RF131, RF132, RF133, RF151**	++	++	++

Table 1 Sensitivity of *Listeria monocytogenes* strains to the three *Carnobacterium* strains
++ : *Listeria monocytogenes* strains highly sensitive to *Carnobacterium* spp. (inhibited by a supernatant diluted more than 256 times)
+ : *Listeria monocytogenes* strains fairly sensitive to *Carnobacterium* spp. (inhibited by a supernatant diluted less than 256 times)

Bacterial enumeration

Salmon samples (30g) were transferred aseptically into a stomacher bag containing 120 ml of chilled physiologic salt solution and homogenised for 2 min in a stomacher (Lab Blender, London, UK). Homogenate was left at room temperature for 30 min for resuscitation. *Carnobacterium* spp. was enumerated on Elliker plates incubated aerobically for 5 days at 20°C. *L. monocytogenes* was enumerated on Palcam agar (BK145, Biokar) with selective supplement (BS00408, Biokar) incubated for 48 h at 30°C. The detection threshold of *L. monocytogenes* was lowered to 1 CFU g^{-1} by pour plating 5 ml of the mother solution in five Palcam plates.

Statistical Analysis

The 57 strains of *L. monocytogenes* were clustered on their sensitivity to the three *Carnobacterium* spp. using the Ward's hierarchical clustering method with the squared Euclidean distance (Uniwin software, Uniwin Plus, version 3.01, Sigma Plus, Paris, France). Comparison of the inhibition potential of the three *Carnobacterium* spp. against 57 *L. monocytogenes* strains (test in Petri dish) was done with a paired-sample Student test. The effect of the three *Carnobacterium* on the different sets of *L. monocytogenes* (test in sterile

CSS) was treated with one-way variance analysis. Means were compared by the least significance difference (LSD) test at the 0.05 level of probability (Statgraphics Plus, version 4, Sigma Plus).

RESULTS

Biodiversity of *L. monocytogenes* sensitivity to the three *Carnobacterium* spp.

A total of 57 *L. monocytogenes* strains representative for five French smoked-salmon factories (from product and environment) were screened for their sensitivity to the three *Carnobacterium* supernatants. All *L. monocytogenes* strains were inhibited by each supernatant, but sensitivity was different between *Listeria* strains. A clustering method permitted to draw three groups of *Listeria* with different behaviours against the three *Carnobacteria* supernatants. Sensitivity was defined as follows : a target strain inhibited by a supernatant diluted more than 256 times was considered as "highly sensitive", inhibition by a lower dilution led the strain to be considered as "fairly sensitive". Results are summarized in Table 1. The first group was constituted of 38 *Listeria* strains (67 %) which were highly sensitive to *C. divergens* V41 and fairly sensitive to *C. piscicola* V1 and SF668. 11 strains (19 %) were fairly sensitive to the three *Carnobacteria* supernatant (group 2), and 8 strains (14 %) were highly sensitive to the three *Carnobacteria* supernatant (group 3).

Inhibiting capacity of *Carnobacterium* spp.

Paired Student test revealed that there was no significant difference between the effect of *C. piscicola* SF668 and *C. piscicola* V1, showing that their inhibitory effect was equivalent on all the target strains considered. On the other hand, a significant difference was observed between the inhibitory effect of *C. divergens* V41 and the two *C. piscicola* strains. *C.*

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divergens V41 had always the highest inhibitory effect. In presence of *C. divergens* V41 supernatant, an inhibition zone was always observed with 3 to 8 two-fold dilutions more than with the two *C. piscicola*.

Inhibition test in sterile CSS

The aim of this part was to compare the inhibitory effect of the three *Carnobacterium* strains against *L. monocytogenes* in CSS matrix. The number of experiments has been reduced by testing a set of four to five strains of *L. monocytogenes* per each of the three groups previously described.

The salt and phenol concentration of the smoked salmon used in these experiments were 5.0 % (water phase) and 0.81 mg 100 g^{-1} respectively. Fat content was 14.5 % (w/w), dry matter 40.0 % (w/w) and pH 6.0. Results showed that *L. monocytogenes* alone grew very easily in CSS, from 20 CFU g^{-1} at the beginning to 10^4-10^5 CFU g^{-1} at the end of the experiment (Fig. 1). No difference was observed between the three sets, except at the end of the experiment where strains of set 3 reached a level significantly but slightly higher than sets 1 and 2 (respectively 8 x 10^5, 7 x 10^4 and 2 x 10^5 CFU g^{-1}). Concerning *Carnobacteria*, the three strains colonised very well the product, with a slight advantage for *C. divergens* V41. For this strain, the growth began at 4°C, from 5x10^4 to 10^5 after 1 week (difference statistically significant). The growth increased considerably at 8°C and *C. divergens* V41 reached 10^8 CFU g^{-1} after 1-2 weeks at 8°C. The break at 20°C did not seem to modify the growth curve but this break occurred when *C. divergens* V41 had already reached its maximum level. Growth of the two *C. piscicola* strains were a little weaker, *C. piscicola* V1 reaching 10^8 CFU g^{-1} after three weeks of storage and *C. piscicola* SF668 only 10^7 CFU g^{-1}. The presence or the absence of *L. monocytogenes* did not influence the growth of *Carnobacterium* spp. (data not shown).
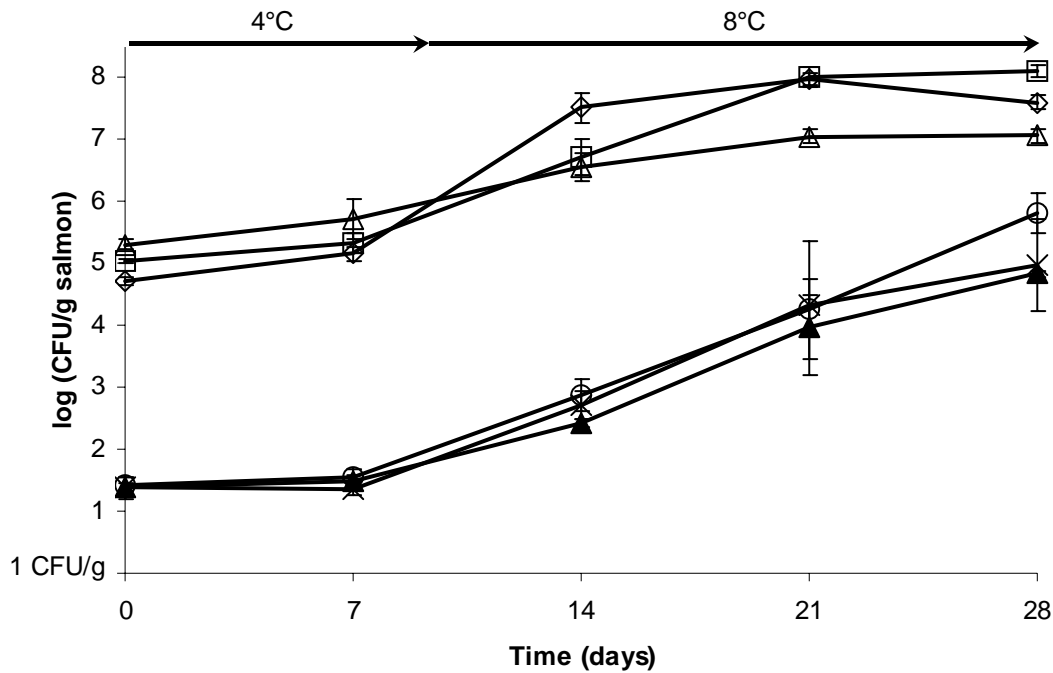
Fig. 1 : Growth of *Listeria monocytogenes* alone : set 1 (▲ : highly sensitive to *Carnobacterium divergens* V41 and fairly sensitive to *C. piscicola* V1 and SF668), set 2 (✗ : fairly sensitive to the three *Carnobacteria*), set 3 (○: highly sensitive to the three *Carnobacteria*) and growth of *C. divergens* V41 (◇), *C. piscicola* V1 (□) and *C. piscicola* SF668 (△). Bars indicate 95% confidence intervals.

Experiments in CSS confirmed that *C. divergens* V41, *C. piscicola* SF668 and *C. piscicola* V1 were able to inhibit partly or totally the growth of *Listeria* strains, whatever the set considered. Inhibitory effect of *C. piscicola* SF668 was significant but weak. The strongest decrease observed at the end of the experiment was 1 log (CFU g^{-1}) for *L. monocytogenes* set 1 (fairly sensitive strains) and 2 log for set 3 (highly sensitive strains) (Fig. 2). *C. piscicola* V1 had a higher effect (Fig. 3). For "highly sensitive" strains of *L. monocytogenes* (set 3), *C. piscicola* V1 had a bactericidal effect, the number of *Listeria* decreasing from 20 to 1 CFU g^{-1} after 3 weeks, this level being maintained till the end of the experiment. For *L. monocytogenes* "fairly sensitive" (set 1), the level never exceeded 10^2 CFU g^{-1}, whereas *L. monocytogenes* reached 10^4 CFU g^{-1} in control. Finally, *C. divergens* V41 had the strongest inhibitory effect. For the two sets tested, *C. divergens* V41 had a bactericidal or bacteriostatic effect and this strain was able to maintain the number of *L. monocytogenes* inferior to 50

CFU g^{-1} during the 4 weeks of vacuum storage whatever the sensitivity of the target strains (Fig. 4).
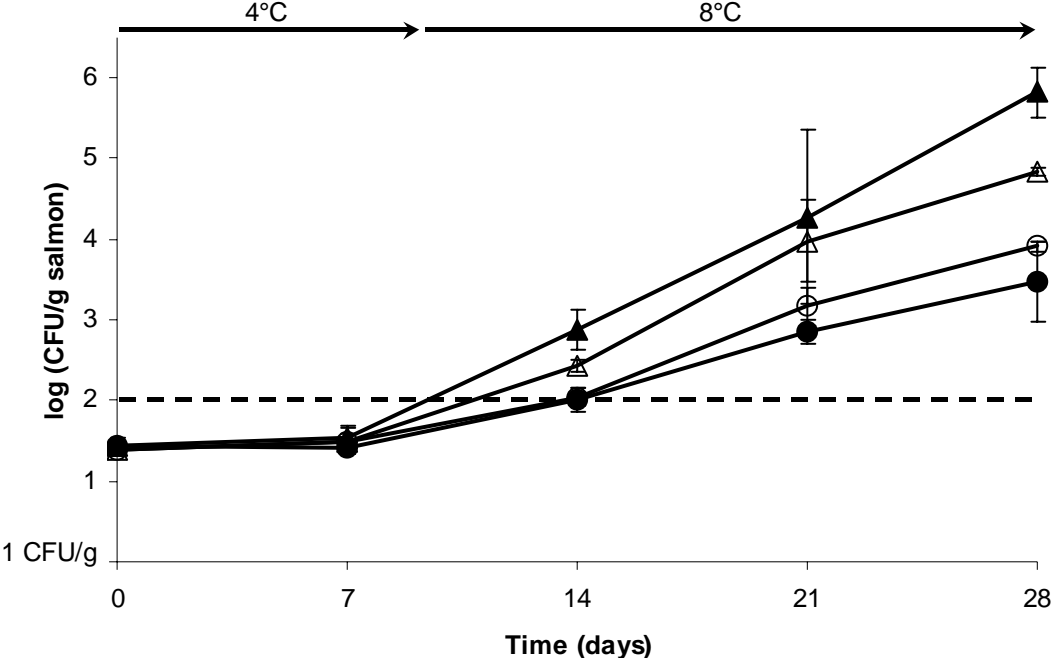

Fig. 2 : Growth of *L. monocytogenes* alone (△,▲) and growth of *L. monocytogenes* in co-culture with *C. piscicola* SF668 (○,●). Open symbol : *L. monocytogenes* set 1, fairly sensitive to *C. piscicola* SF668 ; closed symbol : *L. monocytogenes* set 3, highly sensitive to *C. piscicola* SF668. Bars indicate 95% confidence intervals.
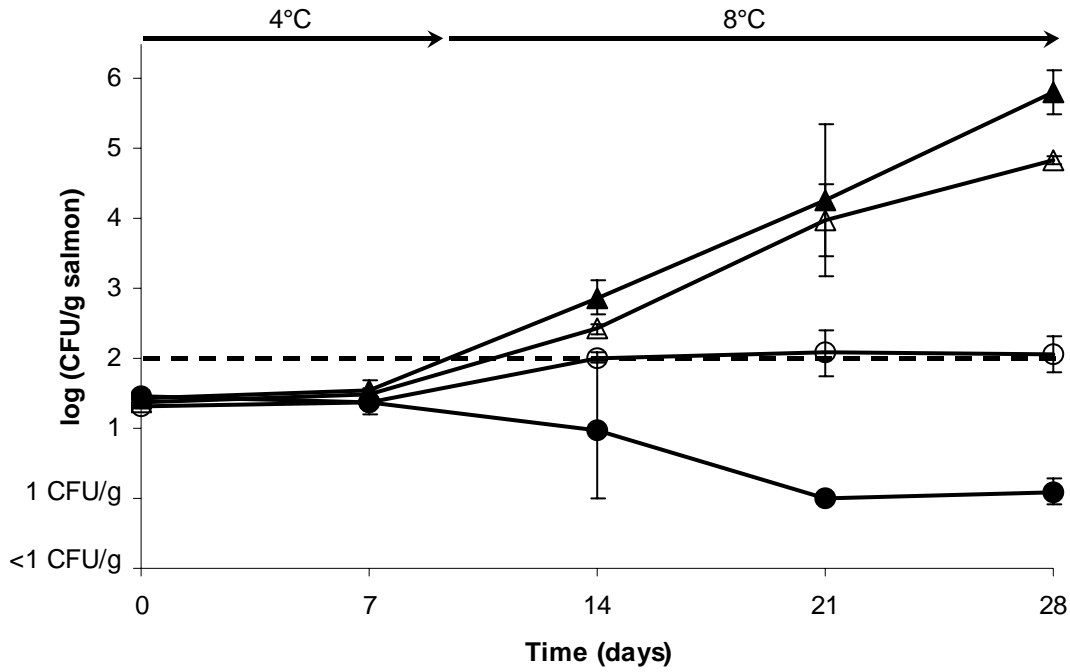
Fig. 3 : Growth of *L. monocytogenes* alone (△,▲) and growth of *L. monocytogenes* in co-culture with *C. piscicola* V1 (○,●). Open symbol : *L. monocytogenes* set 1, fairly sensitive to *C. piscicola* V1 ; closed symbol : *L. monocytogenes* set 3, highly sensitive to *C. piscicola* V1. Bars indicate 95% confidence intervals.
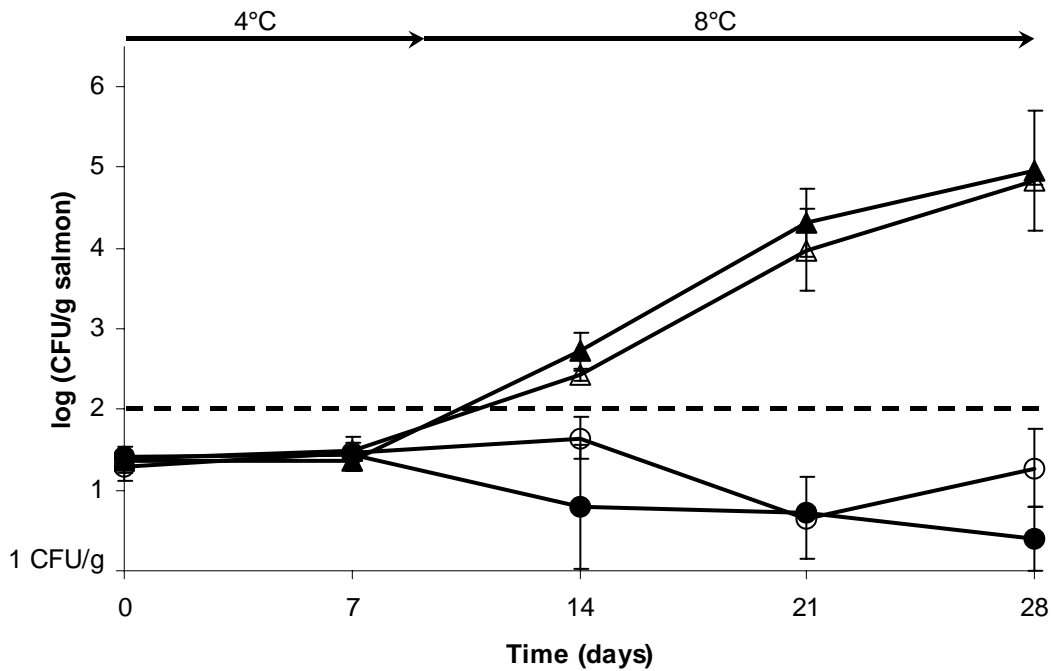

Fig. 4 : Growth of *L. monocytogenes* alone (△,▲) and growth of *L. monocytogenes* in co-culture with *C. divergens* V41 (○,●). Open symbol : *L. monocytogenes* set 1, highly sensitive to *C. divergens* V41 ; closed symbol : *L. monocytogenes* set 2, fairly sensitive to *C. divergens* V41. Bars indicate 95% confidence intervals.

DISCUSSION

The inhibitory activity of three bacteriocin-producing *Carnobacteria* was demonstrated against a collection of 57 *L. monocytogenes* strains being representative for the french smoked salmon industry by the agar spot test. All the *Listeria* strains tested were sensitive to the three *Carnobacterium* spp. supernatants. It has been shown in previous studies that one or two class IIa-bacteriocins are produced by the *Carnobacterium* strains used in this experiment : divercin V41 secreted by *C. divergens* V41 (Metivier *et al.*, 1998), piscicocin V1a (identical to piscicolin 126, characterized by Jack *et al.*, 1996) and piscicocin V1b (identical to carnobacteriocin BM1) produced by *C. piscicola* V1 (Bhugaloo-Vial *et al.*, 1996), and probably one bacteriocin identical to piscicolin 126 for *C. piscicola* SF668 (not published data). The results of inhibition are in agreement with those of Katla *et al.* (2003) who have shown the activity of several bacteriocins of class IIa against large numbers of *L. monocytogenes* isolated from food and food industry environment. Nevertheless, differences in the susceptibilities of *Listeria* strains to the three *Carnobacteria* could be observed : 67 % of *Listeria* strains (group 1) were highly sensitive to the supernatant of *C. divergens* V41 whereas fairly sensitive to the supernatant of *C. piscicola* V1 and *C. piscicola* SF668. Katla *et al.* (2003) have reported differences in susceptibility of *L. monocytogenes* strains to class IIa bacteriocins, such as sakacin P, sakacin A and pediocin PA-1, partly due to large strain-to-strain differences such as surface properties of target strains. Further experiments with *L. monocytogenes* collection from ASEPT are actually done to search for any correlation between sensitivity to bacteriocin and other properties such as serotyping, pulsotyping, adhesion properties, sensitivity to disinfectants or production of monocine.

Among our collection, 81% of *Listeria* strains were highly sensitive to supernatant of *C. divergens* V41, and 86% were fairly sensitive to supernatant of *C. piscicola* V1 and *C. piscicola* SF668. Divercin V41 produced by *C. divergens* V41 has been characterized and

contains two disulfide bridges (Metivier *et al.*, 1998), whereas piscicocins V1a and V1b produced by *C. piscicola* V1 and SF668 have a single disulfide bridge (Bhugaloo-Vial *et al.*, 1996). According to several studies, the number of disulfide bridges could explain the higher activity of certain class IIa bacteriocins (Fimland *et al.*, 2000; Guyonnet *et al.*, 2000). However comparison of the exact sensitivity of *L. monocytogenes* strains to these bacteriocins should require the complete purification of each peptide.

The application of purified antimicrobial agent in fish products or any type of food products is submitted to food preservatives legislation. In Europe, nisin is the only bacteriocin admitted in a limited number of food products, e.g. semolina puddings, cheese refined and melted, and coated cream (directive 95/2/CE). The use of food preservatives in french CSS is not allowed and previous studies have shown that nisin activity in CSS is limited during the time of storage (Nilsson *et al.*, 1997). In addition, the use of bacteriocin-producing strains directly on CSS was shown to be more effective than the application of bacteriocin alone (Duffes *et al.*, 1999b). Therefore, our strategy was to add bacteriocin-producing strains in CSS just before vacuum-packaging. This approach has already been used in several studies on CSS (Wessels and Huss, 1996; Nilsson *et al.*, 1999; Katla *et al.*, 2001; Yamazaki *et al.*, 2003). In these studies, inhibition was tested against one or few strains of *L. monocytogenes* that are usually very sensitive to the bacteriocin tested. According to strain-to-strain differences of this pathogenic bacterium in their bacteriocin susceptibility, validation of antilisterial activity on a wide collection of representative strains encountered in the industry seems essential for the development of a biopreservation strategy. In order to lower the number of experiments in CSS, three sets of four to five *L. monocytogenes* strains were selected, representing the different susceptibility classes of the three bacteriocin-producing *Carnobacterium* strains. Our experiments demonstrate the ability of the different sets of *L. monocytogenes* to grow in CSS even when it was inoculated at very low levels (around 20 CFU g^{-1}). The chemical

characteristics of CSS, particularly phenol and salt concentrations, are known to be determinant to limit the growth of *L. monocytogenes* in this product (Dalgaard and Jorgensen, 1998). However, Thurette *et al.* (1998) demonstrated that phenol concentration required for effective inhibition of the pathogenic bacterium was 2 mg 100 g^{-1} at 8°C whereas the concentration of phenol in CSS used in our experiments was representative of the classic levels encountered in french commercial CSS (< 1 mg 100 g^{-1}; Leroi, 2002). Temperature of storage is another important factor for growth limitation of *L. monocytogenes* in CSS. Our results showed that the number of *L. monocytogenes* in the product increased from the 9th day to the end of the experiment, which corresponds to the period of storage at 8°C. These results are in agreement with previous studies showing that when inoculated at low levels (< 200 CFU g^{-1}), *L. monocytogenes* exhibited a slight growth at 4°C in CSS, but reached 10^6 to 10^8 CFU g^{-1} in 2-4 weeks at 8 or 10°C (Duffes *et al.*, 1999b; Thurette *et al.*, 1998; Peterson *et al.*, 1993). *Carnobacterium* strains grew very easily on CSS from 10^4-10^5 CFU g^{-1} at the beginning to 10^7-10^8 CFU g^{-1} at the end of storage, showing their capacity of colonisation of smoked-products at refrigerated temperatures, with high NaCl concentration and without sugar, as it was previously demonstrated by Duffes *et al.* (1999b).

With each of the three *Carnobacteria* tested, inhibition of the growth of *L. monocytogenes* was effective all over the 4 weeks storage. For *C. piscicola* V1, as observed in agar spot test, inhibition was higher for highly sensitive strains than for fairly sensitive strains. A quite 2 logarithm difference at the end of the experiment was observed between counts of *L. monocytogenes* set 1 (fairly sensitive) and set 3 (highly sensitive) in presence of *C. piscicola* V1 (Fig. 3). Katla *et al.* (2002) have also shown that susceptibility differences to bacteriocins measured in microtiter plate assays were comparable to differences seen for the same strains of *L. monocytogenes* in food model systems. However, for *C. piscicola* SF668 and *C. divergens* V41, difference of sensitivity between sets of *L. monocytogenes* were not so

pronounced in CSS and no statistical difference could be noticed between counts of highly and fairly sensitive strains (Fig. 2 and 4). Gänzle *et al.* (1999) have shown that intrinsic factors could affect the activity of a bacteriocin in a food product.

In CSS, *C. piscicola* SF668 was less active than *C. piscicola* V1 to maintain the level of the different sets of *L. monocytogenes* below 100 CFU g^{-1}. This result had already been observed in sterile CSS blocks by Duffes *et al.* (1999b) when working with a highly sensitive strain of *L. monocytogenes*. Contrary, in agar spot test no difference was observed between the inhibitory effect of supernatant from *C. piscicola* V1 and *C. piscicola* SF668. This could be attributed to difference of bacteriocin production in CSS by *Carnobacterium* spp. *C. divergens* V41 showed a greater inhibition capacity in CSS during the course of the storage whatever the sensitivity of the *Listeria* set recorded on the agar spot test. In presence of *C. divergens* V41, the number of *L. monocytogenes* was maintained around 50 CFU g^{-1}, which is lower than the 100 CFU g^{-1} tolerated level for lightly preserved products such as CSS (DGAL/SDHA/N98/N°8088, 1998). Bacteriocin production by this strain was detected in CSS extract (Connil *et al.*, 2002). Moreover, evidence of inhibition of *L. monocytogenes* by divercin V41 on CSS has recently been demonstrated using a divercin V41 deficient mutant of *C. divergens* V41 (Richard *et al.*, 2003). In the study of Duffes *et al.* (1999b) in sterile CSS model, the inhibitory activity of *C. piscicola* V1 against *L. monocytogenes* was higher than the activity of *C. divergens* V41. The fact that this activity was demonstrated against only one highly sensitive strain of *L. monocytogenes* is one of the possible explanations of these differences, showing the interest to use different strains with various sensitivities for inhibition assessment.

Results of this study show a promising application of at least two strains, *C. divergens* V41 and *C. piscicola* V1 as natural food preservatives against the pathogenic bacteria *L. monocytogenes* on vacuum-packed CSS stored at 4°C and abused temperatures such as 8°C.

Carnobacteria are frequently isolated from CSS during storage, and several studies showed that these bacteria are not believed to affect the sensory quality of the product (Nilsson *et al.*, 1999; Paludan-Muller *et al.*, 1998; Stohr *et al.*, 2001). Further work should therefore be carried out to determine the real effect of our strains on the sensory quality of CSS and their potential inhibitory effects on the endogenous flora of these products.

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