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

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## ABSTRACT

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**Aims:** 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 food-borne 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 gastroenteritis in healthy adults not associated with the above-mentioned risk groups (Miettinen *et al.* 1999).

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A whole range of food categories have been linked to listeriosis outbreaks, including fish and ready-to-eat fish products (Rocourt *et al.* 2000). Lightly preserved fish products, such as cold-smoked salmon (CSS), are classified as high risk merchandise. Indeed, the raw material is frequently contaminated by *L. monocytogenes* and the processing conditions, i.e. salting, drying, smoking and vacuum packaging, are insufficient to inactivate the bacterium or to prevent growth during chilled storage. Thus, contamination during or after processing can occur. As 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 the closure of factories. In France

(DGAL/SDHA/N98/N°8088, 1998) and in many European countries, the guidelines for the presence of *L. monocytogenes* in CSS is <100 CFU $g^{-1}$ until the sell-by date. In recent years, many efforts have been made 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, the production of CSS consistently free of the bacterium is impossible because there is no elimination step for bacteria in the smoking process (Huss *et al.* 2000). In parallel, many publications about *Listeria* inhibitory treatments of 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), and lactoperoxidase (Boussouel *et al.* 1999), have been reported. Nevertheless, many of these treatments have a negative incidence on the quality of the CSS and most of them are not allowed by the European regulation.

Biopreservation, which consists of inoculating food products with selected bacteria to inhibit the growth of undesirable micro-organisms, seems to be an interesting strategy to control the risk of *L. monocytogenes* 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 are frequently dominated by LAB, in which the *Carnobacterium* genus is often represented (Leroi *et al.* 1998, 2000; Truelstrup Hansen and Huss 1998; Jorgensen *et al.* 2000). *Carnobacterium* species are good candidates for a biopreservation strategy as 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 the sensory properties of CSS (Stohr *et al.* 2001). In previous studies, the 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). The 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 included an assessment of the inhibitory spectrum against a range of *L. monocytogenes* strains. Target strains were chosen out of a wide collection representative for *L. monocytogenes* strains encountered in the French CSS industry. The 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 a safety agent in fish products.

## MATERIALS AND METHODS

### Bacterial strains and subcultures conditions

*Carnobacterium divergens* V41 and *C. piscicola* V1 were isolated from salmon and 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).

A total of 152 strains of *L. monocytogenes* were isolated from the environment and from salmon products of five 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 were kindly provided by ASEPT for this study.

The 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* strains were subcultured for 24 h at 20°C and 15°C respectively. 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 approx. 4–5 kg, stored in ice, were bought from a Norwich salmon farm. According to Joffraud *et al.* (1998), the fish 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 distributed into 34 parts of 300 g and stored in polyamide polythene bags purchased from Bourdeau (St-Etienne-de-Montluc, France), vacuum packed and frozen at −80°C. Then, frozen bags were ionized (1·9 kGy) in a plant equipped with electron beam facilities (Gradient Ouest, Berric, France). Sodium chloride was measured with a Chloride Analyser 926 (Corning,

Halstead, UK) and total phenols were quantified by the method described in the French standard for smoked salmon (NF V 45-065 1995).

### 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 standardized agar spot test with a critical dilution assay (Pilet *et al.* 1995). The three *Carnobacterium* spp. were separately grown in fermentors (SGI 2L, Setric, Toulouse, France). One litre of Elliker broth was inoculated with 20 ml of a *Carnobacterium* spp. subculture. Cultures were run at 20°C under agitation with the pH adjusted to 6·5 by automatic addition of NaOH (6 N). Growth was controlled by regular $O.D._{600\ nm}$ measurements and, when the 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 then 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 was poured onto a plate with Elliker containing 1% agar. For each *Carnobacterium* strain, 10 μl of the treated supernatant and their twofold 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 the absence of *L. monocytogenes* growth (inhibition zone), revealed sensitivity. The first spot of 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, some strains of their collection were persistent in the plant (they have been isolated from the environment at two different times at least, and some of them have also been 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 fairly and highly sensitive respectively to the two *C. piscicola*). Set 2, composed of strains fairly sensitive to the three *Carnobacterium*, was not tested because it was similar to set 1 (Table 1). In the same way, *C. divergens* V41 was co-inoculated with *L. monocytogenes* strains of set 1 and set 2 separately (strains fairly and highly sensitive respectively 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 subcultured twice 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

**Table 1** Sensitivity of *Listeria monocytogenes* strains to the three supernatants of bacteriocin-producing *Carnobacterium* strains, determined by agar spot test

| *Listeria monocytogenes* strains | *C. divergens* V41 | *C. piscicola* V1 | *C. piscicola* SF668 |
|---|---|---|---|
| Group 1 | | | |
| **RF107**, **RF114**, **RF119**, **RF129**, **RF148**, RF96, RF97, RF98, RF99, RF101, RF102, RF103, RF104, RF105, RF106, RF108, RF109, RF110, RF111, RF112, RF117, RF121, RF126, RF127, RF128, RF130, RF134, RF136, RF137, RF139, RF141, RF143, RF144, RF145, RF146, RF147, RF149, RF150 | ++ | + | + |
| Group 2 | | | |
| **RF100**, **RF120**, **RF122**, **RF123**, **RF140**, RF113, RF115, RF116, RF118, RF135, RF138 | + | + | + |
| Group 3 | | | |
| **RF131**, **RF132**, **RF133**, **RF151**, RF124, RF 125, RF152, RF142 | ++ | ++ | ++ |

++: *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 <256 times).
The sets of strains representative for each group are in bold.

grown in 100 ml of Elliker broth for 24 h at 30°C before cells were centrifuged and washed in physiological 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 polythene bags (Bourdeau). The pieces were gently mixed with the inoculating solution and samples were then vacuum packed and incubated for 28 days under 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 2004), with a break of 2 h at 20°C after 19 days of storage (to imitate a break in the cold chain during distribution and sale). The initial desired levels in the salmon 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 (the *Carnobacterium* subculture being replaced by sterile physiological salt solution). Microbial analysis was carried out weekly in triplicate (three different bags analysed).

### Bacterial enumeration

Salmon samples (30 g) were transferred aseptically into a stomacher bag containing 120 ml of chilled physiological salt solution and homogenized for 2 min in a stomacher (Lab Blender, London, UK). The homogenate was left at room temperature for 30 min for resuscitation. *Carnobacterium* spp. was counted on Elliker plates incubated aerobically for 5 days at 20°C. *L. monocytogenes* was counted on Palcam agar (BK145, Biokar) with a 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 on five Palcam plates.

### Statistical analysis

The 57 strains of *L. monocytogenes* were clustered in their sensitivity to the three *Carnobacterium* spp. using Ward's hierarchical clustering method with the squared Euclidean distance (Uniwin software, Uniwin Plus, version 3.01, Sigma Plus, Paris, France).

A comparison of the inhibition potential of the three *Carnobacterium* spp. against 57 *L. monocytogenes* strains (agar spot test) was made with a paired-sample Student's 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. Mean values were compared by the least significant difference 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 the environment) were screened for their sensitivity to the three *Carnobacterium* supernatants. All *L. monocytogenes* strains were inhibited by each supernatant, but the sensitivity was different between *Listeria* strains. A clustering method distinguished three groups of *Listeria* showing different behaviour towards 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'. The 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. Eleven strains (19%) were fairly sensitive to the three *Carnobacteria* supernatants (group 2), and eight strains (14%) were highly sensitive to the three *Carnobacteria* supernatants (group 3).

### Inhibitory capacity of *Carnobacterium* spp.

Paired Student's tests 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. Whichever *L. monocytogenes* strain was considered, *C. divergens* V41 always had the highest inhibitory effect. In the presence of *C. divergens* V41 supernatant, an inhibition zone was always observed with three to eight twofold dilutions more than with the two *C. piscicola* strains.

### 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 a CSS matrix. The number of experiments was reduced by testing a set of four to five strains of *L. monocytogenes* for each of the three groups previously described.

The salt and phenol concentrations of the smoked salmon used in these experiments were 5·0% (w/w, water phase) and 0·81 mg/100 g respectively. Fat content was 14·5% (w/w), dry matter 40·0% (w/w) and pH 6·0. The 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

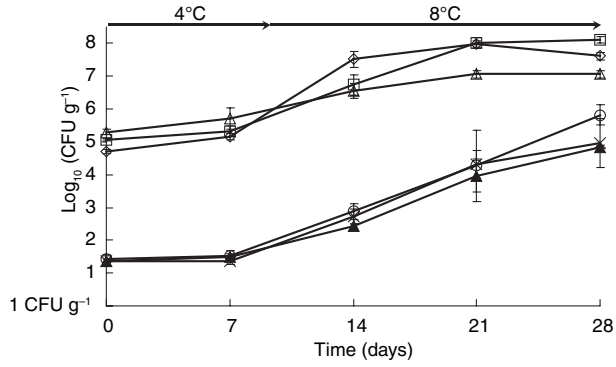


**Fig. 1** Growth of *L. monocytogenes* strains alone (▲) set 1, highly sensitive to *C. divergens* V41 and fairly sensitive to *C. piscicola* V1 and SF668; ×: set 2, fairly sensitive to the three *Carnobacteria*; ○: set 3, highly sensitive to the three *Carnobacteria*) and growth of *Carnobacterium* spp. (◇: *C. divergens* V41; □: *C. piscicola* V1; △: *C. piscicola* SF668) in sterile cold-smoked salmon during storage (9 days at 4°C and 19 days at 8°C, with a break of 2 h at 20°C after 19 days of storage). Bars indicate 95% confidence intervals

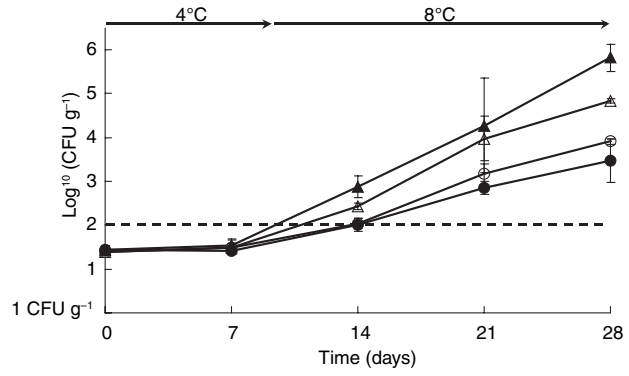


**Fig. 2** Growth of *L. monocytogenes* strains inoculated alone (△, ▲) and in co-culture with *C. piscicola* SF668 (○, ●) in sterile cold-smoked salmon during storage (9 days at 4°C and 19 days at 8°C, with a break of 2 h at 20°C after 19 days of storage). Open symbol: *L. monocytogenes* set 1, fairly sensitive to *C. piscicola* SF668; closed symbol: *L. monocytogenes* set 3, highly sensitive to *C. piscicola* SF668. Bars indicate 95% confidence intervals

experiment when strains of set 3 reached a level slightly but significantly higher than sets 1 and 2 (respectively $8 \times 10^5$, $7 \times 10^4$ and $2 \times 10^5$ CFU $g^{-1}$). Concerning *Carnobacteria*, the three strains colonized the product very well, with a slight advantage for *C. divergens* V41. For this strain, the growth began at 4°C, increasing from $5 \times 10^4$ to $10^5$ CFU $g^{-1}$ after 1 week (statistically significant difference). 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 was a little weaker, *C. piscicola* V1 reaching $10^8$ CFU $g^{-1}$ after 3 weeks of storage and *C. piscicola* SF668 reaching 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).

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, whichever set was considered. The inhibitory effect of *C. piscicola* SF668 was significant but weak. The biggest 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 greater 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 was maintained till the end of the experiment. For fairly sensitive *L. monocytogenes* (set 1), the level never exceeded $10^2$ CFU $g^{-1}$, whereas *L. monocytogenes* reached $10^4$ CFU $g^{-1}$ in the

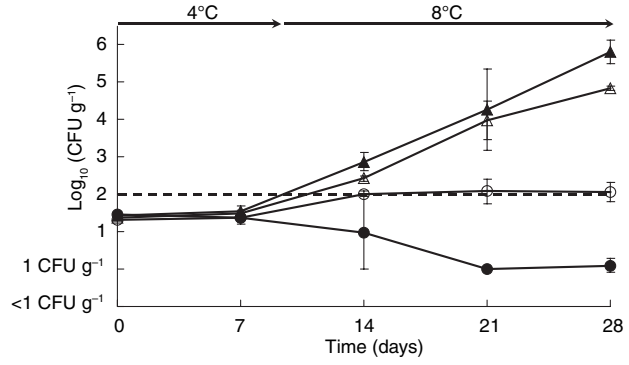


**Fig. 3** Growth of *L. monocytogenes* strains inoculated alone (△, ▲) and in co-culture with *C. piscicola* V1 (○, ●) in sterile cold-smoked salmon during storage (9 days at 4°C and 19 days at 8°C, with a break of 2 h at 20°C after 19 days of storage). Open symbol: *L. monocytogenes* set 1, fairly sensitive to *C. piscicola* V1; closed symbol: *L. monocytogenes* set 3, highly sensitive to *C. piscicola* V1. Bars indicate 95% confidence intervals

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* at lower than 50 CFU $g^{-1}$ during the 4 weeks of vacuum storage, whatever the sensitivity of the target strains (Fig. 4).

## DISCUSSION

The inhibitory activity of three bacteriocin-producing *Carnobacteria* was demonstrated against a collection of 57 *L. monocytogenes* strains representative for the French

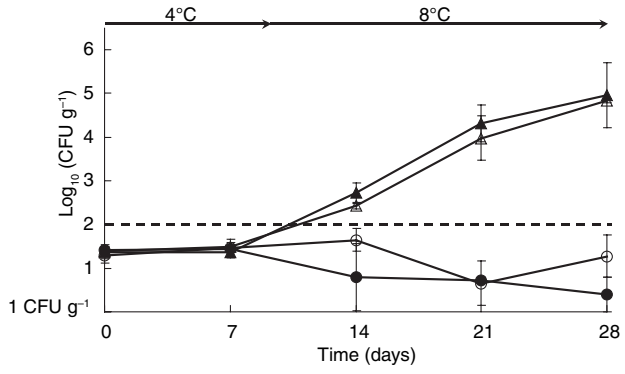


**Fig. 4** Growth of *L. monocytogenes* strains inoculated alone (△, ▲) and in co-culture with *C. divergens* V41 (○, ●) in sterile cold-smoked salmon during storage (9 days at 4°C and 19 days at 8°C, with a break of 2 h at 20°C after 19 days of storage). Open symbol: *L. monocytogenes* set 1, highly sensitive to *C. divergens* V41; closed symbol: *L. monocytogenes* set 2, fairly sensitive to *C. divergens* V41. Bars indicate 95% confidence intervals

smoked salmon industry using 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 (Pellé *et al.*, unpublished 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 the food industry environment. Nevertheless, differences in the sensitivity 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 but fairly sensitive to the supernatant of *C. piscicola* V1 and *C. piscicola* SF668. Katla *et al.* (2003) have reported differences in sensitivity of *L. monocytogenes* strains to class IIa bacteriocins, such as sakacin P, sakacin A and pediocin PA-1. This is partly due to large differences, such as surface properties, between target strains. Further experiments with the *L. monocytogenes* collection from ASEPT are currently being carried out to search for any correlation between sensitivity to bacteriocin and other properties such as serotyping, pulsotyping, adhesion properties, sensitivity to disinfectants or the production of monocine.

Among our collection, 81% of *Listeria* strains were highly sensitive to the supernatant of *C. divergens* V41 and 86% were fairly sensitive to the supernatant of *C. piscicola* V1 and *C. piscicola* SF668. Divercin V41, produced by *C. divergens* V41, has been characterized and contains two disulphide bridges (Metivier *et al.* 1998), whereas piscicocins V1a and V1b, produced by *C. piscicola* V1 and SF668, have a single disulphide bridge (Bhugaloo-Vial *et al.* 1996). According to several studies, the number of disulphide bridges could explain the higher activity of certain class IIa bacteriocins (Fimland *et al.* 2000; Guyonnet *et al.* 2000). However, a comparison of the exact sensitivity of *L. monocytogenes* strains to these bacteriocins would require the complete purification of each peptide.

The addition of a purified antimicrobial agent to fish products or any type of food product is subject to food preservative legislation. In Europe, nisin is the only bacteriocin permitted in a limited number of food products, e.g. semolina puddings, refined and melted cheese, and coated cream (Directive 95/2/CE 1995). 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 to 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 a few strains of *L. monocytogenes* that are usually very sensitive to the bacteriocin tested. Due to the strain-to-strain differences in bacteriocin sensitivity of this pathogenic bacterium, the validation of antilisterial activity on a wide range of representative strains encountered in the industry seems essential for the development of a biopreservation strategy. In order to decrease the number of experiments in CSS, three sets of four to five *L. monocytogenes* strains were selected, representing the different sensitivity 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 in limiting the growth of *L. monocytogenes* in this product (Dalgaard and Jorgensen 1998). However, Thurette *et al.* (1998) demonstrated that the phenol concentration required for effective inhibition of the pathogenic bacterium was 2 mg/100 g at 8°C whereas the concentration of phenol in CSS used in our experiments was representative of the typical levels encountered in French commercial CSS (<1 mg/100 g; Leroi 2002). The 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 ninth 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 $10^6$–$10^8$ CFU $g^{-1}$ in 2–4 weeks at 8 or 10°C (Peterson *et al.* 1993; Thurette *et al.* 1998; Duffes *et al.* 1999b). *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 for colonization of smoked products at refrigerated temperatures, with high NaCl concentration and without sugar, as was previously demonstrated by Duffes *et al.* (1999b).

With each of the three *Carnobacteria* tested, inhibition of the growth of *L. monocytogenes* was effective throughout the 4-week storage. For *C. piscicola* V1, as observed in agar spot tests, inhibition was higher for highly sensitive strains than for fairly sensitive strains. An almost two 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 the presence of *C. piscicola* V1 (Fig. 3). Katla *et al.* (2002) have also shown that differences in sensitivity to bacteriocins measured in microtitre plate assays were comparable with differences seen for the same strains of *L. monocytogenes* in food model systems. However, for *C. piscicola* SF668 and *C. divergens* V41, the differences in sensitivity between sets of *L. monocytogenes* were not so pronounced in CSS and no statistical difference was observed between counts of highly and fairly sensitive strains (Figs 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*. In contrast, in agar spot tests no difference was observed between the inhibitory effect of supernatant from *C. piscicola* V1 and from *C. piscicola* SF668. This could be attributed to differences in bacteriocin production in CSS by *Carnobacterium* spp. *C. divergens* V41 showed a greater inhibitory capacity in CSS throughout the storage time, whatever the sensitivity of the *Listeria* set recorded on the agar spot test. In the presence of *C. divergens* V41, the number of *L. monocytogenes* was maintained at 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 has been 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 a 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 the activity was demonstrated against only one highly sensitive strain of *L. monocytogenes* is one of the possible explanations of these differences, showing the importance of using different strains with various sensitivities for inhibition assessment.

The 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 excessive temperatures such as 8°C. *Carnobacteria* are frequently isolated from CSS during storage, and several studies have shown that these bacteria are not believed to affect the sensory quality of the product (Paludan-Muller *et al.* 1998; Nilsson *et al.* 1999; 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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