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Effect of inoculation of Carnobacterium divergens V41, a biopreservative strain against Listeria monocytogenes risk, on the microbiological, chemical and sensory quality of cold-smoked salmon

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Abstract: The aim of this study was to develop a biopreservation strategy for cold-smoked salmon (CSS) by the use of lactic acid bacteria previously selected for their capability to inhibit the growth of Listeria monocytogenes in the product. The spoiling potential of three Carnobacterium strains (Carnobacterium divergens V41, Carnobacterium piscicola V1 and SF668) was tested in sterile CSS blocks inoculated by 104–5 CFU g− 1 and stored under vacuum for 9 days at 4 °C followed by 19 days at 8 °C. C. divergens V41 grew a little faster than the other strains and none of the three carnobacteria showed any adverse effect on quality of the product, i.e. no off-odour detected by a trained panel, no total volatile basic nitrogen (TVBN) production, no acidification and no biogenic amine except a slight production of tyramine. An application on commercial CSS was tested by spraying C. divergens V41 (104–5 CFU g− 1) on slices of four batches freshly processed in different smokehouses. Microbial, chemical and sensory characteristics were weekly compared to a control during 4 weeks of vacuum storage. When the natural microflora was initially weak (two batches < 20 CFU g− 1), C. divergens V41 quickly reached 107–8 CFU g− 1 and a slight inhibition of endogenous Enterobacteriaceae, lactobacilli and yeasts was observed. The presence of C. divergens V41 was slightly detected (odour and flavour) but none of the sample was considered as spoiled by the sensory panel. When the natural microflora was initially high (2 batches > 104–5 CFU g− 1), no effect on the microflora, TVBN and biogenic amine production, nor on the sensory characteristics was observed in presence of C. divergens V41. In conclusion, biopreservation of CSS using lactic acid bacteria such as C. divergens V41 is a promising way to inhibit the growth of pathogenic bacteria such as L. monocytogenes with low effect on the quality of the product.

Keywords: Cold-smoked salmon; Biopreservation; *Carnobacterium*; Spoilage microflora; Biogenic amine; Sensory analysis

1. Introduction

The production of CSS within the European community supplies nearly 80% of the world market with an annual production rising 70000 tonnes (FAO, 2003). France is the first worldwide producer with approx. 19000 tones/year but other countries such as Denmark, Germany and United Kingdom have recently increased there production. Salting and smoking treatments which were traditionally used as preservation technology are nowadays lighter leading to highly perishable product, with an average salt content of around 5 % (water phase) in Europe and phenol content less than 1 mg 100 g^{-1} (Cardinal et al., 2004). The major risk associated with this product is *Listeria monocytogenes* (Bledsoe et al., 2001). *L. monocytogenes* is of special concern to the CSS industry because it is able to grow at low temperature and in presence of high NaCl concentration. Indeed, a high prevalence of *L. monocytogenes* in CSS has been reported (Ben Embarek, 1994; Rorvik et al., 1995; Heinitz and Johnson, 1998; Jorgensen and Huss, 1998) and the processing steps (i.e. salting and smoking) are insufficient to inactivate this pathogenic bacterium (Guyer and Jemmi, 1991; Ribeiro Neunlist, in press). Moreover growth in the final product stored at chilled temperature is possible (Jorgensen and Huss, 1998; Duffes et al., 1999a; Nykanen et al., 2000) and *L. monocytogenes* can reach 100 CFU g^{-1} which is the maximum tolerable limit till the sell-by date in many European countries. Recently, a biopreservation strategy for this product has been developed. Biopreservation consists in inoculating food product by selected bacteria to inhibit the growth of undesired micro-organisms, without changing quality of the product. The inhibition capacity of three bacteriocin-producing strains isolated from seafood, *Carnobacterium divergens* V41, *C. piscicola* V1 and *C. piscicola* SF668, has been demonstrated against a wide collection of *L. monocytogenes* both in agar plate and in sterile CSS blocks artificially contaminated (Brillet et al., 2004). The three carnobacteria strains gave promising results, *C. divergens* V41 being the most efficient strain, maintaining the level of *L. monocytogenes* lower than 50 CFU g^{-1} (initial contamination : 20 CFU g^{-1}) during the four weeks of vacuum storage at 4° and 8°C.

In order to bring the biopreservative technology to a stage where it can become available for the CSS industry, it is necessary to evaluate the effect of the *Carnobacterium* strains on the quality of CSS. It has been clearly stated that the early quality deterioration of vacuum-packed CSS during vacuum storage at chilled temperature, resulting in the occurrence of off-odours and pasty textures (Stohr et al., 2001), is due to microbial activity (Joffraud et al., 1998). However, spoilage mechanisms are still incompletely understood. Several authors have identified the CSS microflora (Truelstrup Hansen et al., 1998; Stohr et al., 2001) which is highly variable both qualitatively and quantitatively due to difference in the final product composition and in hygienic conditions in the smokehouses (Truelstrup Hansen and Huss, 1998; Leroi et al., 2001). Just after the process, the microflora is often composed of a mixture of Gram-negative marine bacteria such as *Shewanella putrefaciens, Photobacterium phosphoreum, Vibrio* spp. and psychrotrophic *Enterobacteriaceae* (Truelstrup Hansen et al., 1995; Leroi et al., 1998; Jorgensen et al., 2000a). During the vacuum storage at refrigerated temperatures, Gram-positive bacteria especially lactic acid bacteria (LAB), represented by *Lactobacillus* spp. and *Carnobacterium* spp. become by far the most common variety (Truelstrup Hansen and Huss, 1998; Leroi et al., 1998). *Brochothrix thermosphacta* and yeasts can also be found in CSS but generally at lower level (Leroi et al., 2001). When inoculated in pure culture in sterile cold-smoked blocks, those micro-organisms do not all participate in spoilage. *Lb. sakei*, *B. thermosphacta*, *Serratia liquefaciens* and *P. phosphoreum* have been shown to be responsible of off-odours whereas the spoilage potential of *C. piscicola* is promisingly weak depending on the strain tested (Stohr et al., 2001).

The aim of the present study was to evaluate the effect of three biopreservative *Carnobacterium* strains showing a high inhibition potential of *L. monocytogenes* (Brillet et al., 2004) on microbiological, chemical and sensory quality of CSS. First, the spoilage potential of *C. divergens* V41, *C. piscicola* V1 and *C. piscicola* SF668, was studied on a sterile CSS system by sensory evaluation of off-odour production and analysis of some physico-chemical indices. Following experiments one of the strains, *C. divergens* V41, was sprayed on slices of commercial CSS batches freshly processed, provided by four different French producers. The effect of inoculation on the sensory characteristics (odour, flavour, texture and colour), physico-chemical parameters (pH, TVBN, biogenic amines) and natural microflora was evaluated during the four weeks of storage at refrigerated temperature.

2. Materials and methods

2.1. Bacterial strains and subcultures conditions

C. divergens V41 and *C. piscicola* V1 were isolated from salmon and trout intestine respectively and characterized by Pilet et al. (1995). *C. piscicola* SF668 was isolated from commercial Norwegian CSS by Leroi et al. (1998), and studied by Duffes et al. (1999b). All strains were stored at -80°C in their growth medium with 20 % (v/v) sterile glycerol.

Elliker broth (BK 054, Biokar Diagnostics, Beauvais, France) was used as subculture medium to cultivate the *Carnobacterium* strains for 24 h at 20°C before inoculation in sterile CSS. As commercial CSS inoculated with *C. divergens* V41 was eaten by the panellists for sensorial analysis, the broth used to cultivate *C. divergens* V41 before inoculation was prepared without protein of animal origin, and contained wheat trypton (20 g L^{-1}), glucose (5 g L^{-1}), saccharose (5 g L^{-1}), yeast extract (5 g L^{-1}), NaCl (4 g L^{-1}), ascorbic acid (0.5 g L^{-1}), $MgSO_4$ (0.4 g L^{-1}), $MnSO_4$ (0.1 g L^{-1}). This medium was named WAP (Without Animal Protein) broth.

2.2. Cold-smoked salmon

Sterile CSS blocks were prepared as described by Joffraud et al. (1998) and modified by Brillet et al. (2004), from five Norwegian farmed salmon (*Salmo salar*) of approximately 4-5 kg, processed at IFREMER. Vacuum-packed bags of 300 g of CSS blocks were sterilised under frozen conditions (–80°C) by ionising treatment at 1.2 kGy (Gradient Ouest, Berric, France). Bags were stored frozen until inoculation.

The commercial salmons (Norwegian farmed *Salmo salar*), traditionally processed (dry-salting, traditional smoking with smoke from beech and oak shaving combustion), were provided by four different French CSS producers. Each batch, at least constituted of 10 fillets, was freshly processed, sliced in constant 40 g weight slices and vacuum-packed just before conveying in refrigerated conditions at our laboratory. Sliced fillets were stored at 4°C just after reception and slices were inoculated in the following hours.

2.3. Sample preparation and storage

2.3.1. Sterile CSS blocks

Cultures of *C. divergens* V41, *C. piscicola* V1 and SF668 were carried out twice successively in 10 and then 100 ml of Elliker broth for 24 h at 20° before cells were centrifuged and washed in sterile saline water (NaCl : 0.85 % w/v). Immediately, appropriate dilutions of *Carnobacterium* strains were inoculated separately (2 % v/w) in 15 replicate

samples of 50 g (for microbial and chemical analysis) and 28 samples of 20 g (for sensory analysis) of thawed sterile CSS blocks distributed in polyamide polyethylene bags (Bourdeau, St Etienne de Montluc, France). Pieces were gently mixed with the inoculating solution and samples were then vacuum-packed and incubated for 28 days using the following conditions : nine 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 during 2 h at 20°C after 19 days of storage (industrial recommendations). The initial desired level in the flesh for *Carnobacterium* spp. was 10^5 CFU g^{-1}. A control made of CSS blocks inoculated with 2 % of sterile saline was also prepared.

2.3.2. Commercial CSS slices

Cultures of *C. divergens* V41 were carried out twice successively in 10 and then 100 ml of WAP broth for 24 h at 20°C and cultures were washed as previously described for sterile CSS experiments. Immediately, appropriate dilution of *C. divergens* V41 was aseptically sprayed (1 % v/w) on each side of 70 slices per batch of commercial CSS with an aerographer (Paasches V, Paasche Airbrush Company, Illinois, USA). Slices were sprayed in a laminar flow hood. Slices were then aseptically installed on carbon tray (two slices per tray) and vacuum-packed in bags provided by each producer, to be in the same packing conditions as in supermarkets. The initial desired level in the flesh for *C. divergens* V41 was 10^5 CFU g^{-1}. For each batch, a control was made of 70 slices packed in the same fashion as inoculated samples but without spraying saline, to be exactly as in commercial processing conditions. Samples and controls were stored for 28 days in the conditions previously described for sterile CSS experiments.

2.4. Microbiological analysis

For both inoculated CSS blocks and commercial slices, microbial analysis was done weekly in triplicate samples. Salmon samples (30 g) were homogenised and diluted in 120 ml chilled physiological saline containing 0.85 % (w/v) NaCl and 0.1 % (w/v) tryptone (Biokar Diagnostics) for 2 min in a stomacher (Lab. Blender, London, UK). After 30 min at room temperature, the homogenate was 10-fold serially diluted in physiological saline, and 0.1 ml or 1 ml of each appropriate dilution was spread-plated or pour-plated respectively in duplicate. For commercial CSS slices, total viable counts (TVC), total LAB, lactobacilli, Enterobacteriaceae, *B. thermosphacta* and yeasts were enumerated with appropriate agar medium and incubation conditions, as described by Cardinal et al. (2004). *Listeria* spp. was enumerated in pour plates of Palcam agar (BK145, Biokar Diagnostics) with selective supplement (BS00408, Biokar Diagnostics). Plates were incubated at 30°C for 48 hours. In order to lower the threshold to one *Listeria* spp. per gram, five Palcam plates per sample were inoculated. For CSS inoculated blocks, only total LAB were enumerated, corresponding to the inoculated carnobacteria.

2.5. Chemical analysis

For both inoculated blocks and commercial CSS, 200 g of flesh were sampled just after processing, and homogenised in a Warring Blender. Dry matter content was analysed by oven drying 10 g of homogenate smoked salmon at 103°C until reaching a constant weight. Total fat content was determined by hexane extraction, and sodium chloride content was measured with a Chloride Analyser 926 (Corning, Halstead, England). Total phenols were quantified by the colorimetric method described by Cardinal et al. (2004).

Each week during storage, the remaining flesh in each package opened for microbiological analysis was homogenised in a Warning Blender. TVBN was determined in duplicate by the Conway micro-diffusion method (Conway and Byrne, 1933). The pH was measured in the homogenate of CSS used for microbiological analysis with a pH-meter (Mettler Delta 320, AES, Combourg, France.

2.6. Bacteriocin activity

For all samples (both inoculated CSS blocks and commercial slices) 1 ml of the five-fold dilution flesh was heated for 15 min at 80°C to inactivate protease and was stored at –80°C until used. Bacteriocin concentration was semi quantified against *L. monocytogenes* RF76 target strain (IFREMER collection) by a standardised agar diffusion test with critical dilution assay (Connil et al., 2002).

2.7. Biogenic amines

Another fraction of 1 ml of the five-fold dilution flesh was kept at –80°C for biogenic amines analysis (tyramine, histamine, cadaverine and putrescine). According to Eerola et al. (1993), quantification of biogenic amines requires a step of derivatization of sample extract. The protocol has been modified as follow : 100 µl of NaOH (2N) were added to 300 µl of thawed-homogenate of CSS. Next, 150 µl of saturated sodium bicarbonate were added, followed by 1 ml of freshly prepared dansyl chloride solution (10 mg dansyl chloride (Sigma Aldrich, L'Isle d'Abeau Chesnes, France) in 1 ml of acetone. Reaction mixture was incubated at 40°C during 45 min safe from the light. Then, residual dansyl chloride was removed by adding 50 µl ammonia. After 30 min in darkness, 700 µl of acetonitrile were added, and reaction mixture was centrifuged 10 min at 4000 g. Finally, supernatant was collected in glass tube and stored at –20°C before analysis. Dansylated amines in homogenate of CSS were then separated by reversed-phase liquid chromatography (Eerola et al., 1993) on a C18 ODS2 Equisorb column (4.6x250 mm, particle size 5 µm, CIL Cluzeau, France) mounted with a C18 Bondapack guard column (Waters, France) with a HPLC apparatus (Waters 600E Multisolvent Delivery System and Waters 2487 Dual λ Absorbance Detector, Waters, France). The gradient elution was carried out with eluent A (ammonium acetate, 0.1 mol l^{-1}) and eluent B (acetonitrile) as describe by Connil et al. (2002). Dansylated amines were detected by u.v. absorption at 254 nm and their concentrations in each homogenate of CSS were calculated by a calibration curve for each amine (Borwin software).

2.8. Sensory analysis

The method used was a quantitative descriptive analysis with conventional profiling performed by an internal regularly trained panel of 14 people (IFREMER). Panellists were required to make a comparative evaluation of the products by scoring each descriptor on an unstructured line scale from zero (low intensity) to ten (high intensity). For CSS blocks, the four samples (three inoculated batches with different *Carnobacterium* spp. and control) were evaluated in one session. Nineteen odour descriptors were quoted after one and three weeks of storage, using 20 g of blocks per panellist. For commercial CSS slices, 34 sensory attributes (odour, appearance, flavour and texture) were weekly scored by the panel. Two sessions per week were necessary to evaluate all the samples (two commercial batches inoculated with *C. divergens* V41 and their control per session). The day of analysis, each package was opened and slices were cut in two portions individually repacked in aluminium foils. The main relevant and discriminative descriptors of smoked salmon slices were : *odour* : global

intensity, smoke, raw salmon, herring, amine, acid, green aroma, rancid, ham, plastic, fruity, sour, butter, rubber, cheese, musty, hydrogen sulphide, cabbage, feacal ; *appearance* : pink colour, orange colour, homogeneity of colour ; *flavour* : global intensity, smoke, raw salmon, herring, rancid, salty taste, acid, amine, rubber ; *texture* : firmness, melting texture, pasty texture.

Another objective of the sensory evaluation was to assess the sensory rejection time. Samples were scored by the same 14 trained panellists who classified them into the following three classes defined by spoilage level on the basis of odour, flavour, texture and colour evaluation : class 1, no spoilage noted, class 2 : weak spoilage, class 3 : strong spoilage. A quality coefficient (QC) was calculated as follow (Leroi et al., 2001): QC = [(1 x % class 1) + (2 x % class 2) + (3 x % class 3)]/100. A sample was rejected when QC was higher than 2, corresponding to sample scored in class 3 by at least 50 % of the panellists.

2.9. Statistical analysis

Results are expressed as mean of three measures ± 95% Confidence Interval (CI = 1.96 x $\sqrt{\text{standard deviation}^2/3}$). For microbial and chemical data, means were compared by one-way analysis of variance with the least significance difference (LSD) test at the 0.05 level of probability (Statgraphics Plus, version 4, Sigma Plus). For CSS blocks, results of the sensory profiling test were treated by two-way analysis of variance (ANOVA) to test the effect of the carnobacteria strains and panellists with Fizz 2.00 software (Fizz system, Biosystèmes, Dijon, France). In case where significant differences occurred, the means were compared by the Duncan test at the 0.05 level of probability. For each batch of commercial CSS, slices inoculated with *C. divergens* V41 were compared to their respective control. Score for each descriptor was compared using a paired-sample Student test (Fizz system).

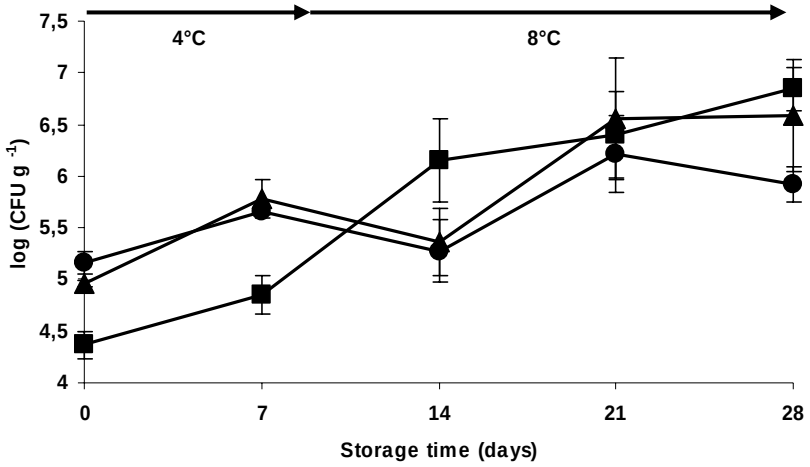
3. Results

3.1. Effect of Carnobacterium spp. in sterile CSS blocks

The salt and total phenol concentrations of CSS blocks used in these experiments were respectively 3.2% (w/w, total phase) corresponding to 5.0% (w/w, water phase) and 1.33 mg 100 g^{-1}. Dry matter and total fat content were 40.1% (w/w) and 15.1 % (w/w) respectively.

Fig. 1 represents growth of the three carnobacteria in inoculated CSS during the four weeks of vacuum storage at chilled temperature. All strains grew in CSS blocks. For the two *C. piscicola* strains, growth curves were similar during the first three weeks but final count was slightly higher for *C. piscicola* V1 than for *C. piscicola* SF668 (5.9 ± 7.2 x 10^6 and 8.7 ± 3.6 x 10^5 CFU g^{-1} respectively). For *C. divergens* V41, although the initial inoculation level was lower by approximately 1 $\log_{10}$ (CFU g^{-1}), the growth increased considerably at 8°C and this strain reached 7.4 ± 3.1 x 10^6 CFU g^{-1} at the end of storage.

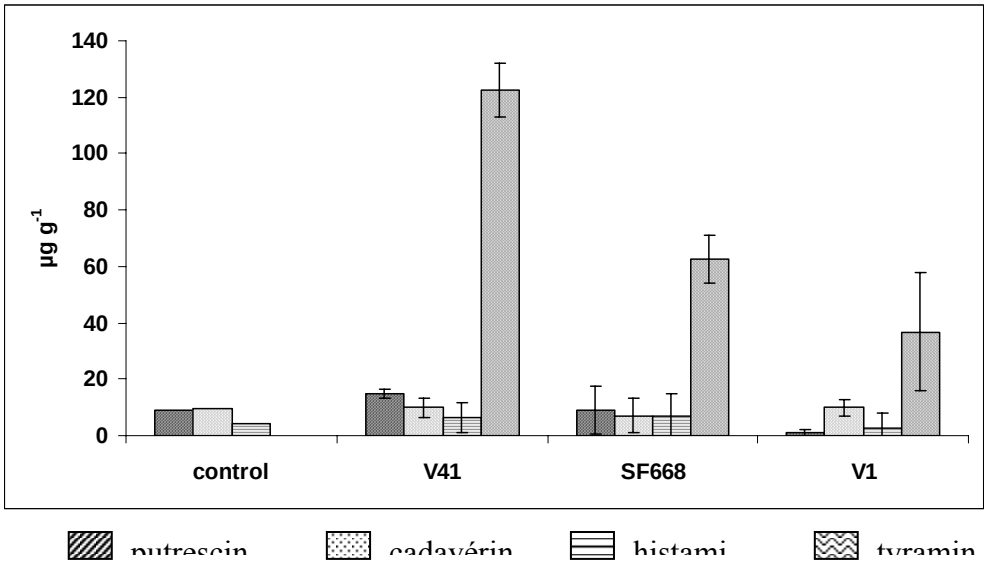
Fig. 1 Growth of *Carnobacterium divergens* V41 (■), *Carnobacterium piscicola* SF668 (●) and *Carnobacterium piscicola* V1 (▲) in sterile cold-smoked salmon blocks during vacuum storage at 4 and 8°C. Each growth curve represents the mean of three enumerations (bars : 95% confidence interval).



No TVBN was produced in CSS blocks, both in control and in inoculated samples. The average initial concentration was 13.3 ± 0.9 mg-N 100 g^{-1} and remained stable (data not shown) during the four weeks of storage whatever the *Carnobacterium* strain considered (the average concentration was 15.3 ± 1.7 mg-N 100 g^{-1} at the end of experiment). The pH was constant ranging between 5.95 and 6.03 ± 0.02 during the four weeks of storage and was not significantly different in control and inoculated samples (data not shown). Bacteriocin was never detected by the agar diffusion test.

Fig. 2 shows biogenic amines production in CSS blocks in presence of each *Carnobacterium* strain after four weeks of storage. The three carnobacteria strains produced tyramine, *C. divergens* V41 being the most important producer with 122 ± 9 µg g^{-1} of tyramine after four weeks. *C. piscicola* SF668 and V1 produced two to four times less tyramine concentrations, with 63 ± 8 and 37 ± 21 µg g^{-1} respectively. None of the carnobacteria produced histamine, putrescine nor cadaverine.

Fig. 2 Biogenic amines concentration after four weeks of storage (nine days at 4°C and 19 at 8°C) in sterile cold-smoked salmon blocks (control) and in inoculated blocks with *Carnobacterium divergens* V41, *Carnobacterium piscicola* SF668 and V1 (bars : 95% confidence intervals).



After one week of storage, no significant difference between control and inoculated samples was observed for any of the nineteen sensory descriptors quoted and samples were always considered as not spoiled (data not shown). Same results were observed after three weeks of storage, except a slight but significant note of cheese/feet detected in samples inoculated with *C. divergens* V41. However, the average score of the 14 panellists for this descriptor was very low (0.6 on a scale ranging from 0 to 10).

3.2. Effect of C. divergens V41 in commercial CSS slices

In the first part of this study, no significant difference was observed between the three strains of carnobacteria concerning their spoilage potential in CSS. As *C. divergens* V41 had showed the highest inhibition capacity against a wide collection of *L. monocytogenes* (Brillet et al., 2004), this strain was selected for an application in naturally contaminated CSS slices. Chemical composition of each CSS batch is shown in Table 1. A wide variation among batches was noticed but all results satisfied with the French CSS standard (NF V 45-065, 1997).

Table 1 Chemicals analyses of the four commercial cold-smoked salmon (CSS) batches

CSS batches	sodium chloride (% w/w WP)*	total phenols (mg 100 g^{-1})	dry matter (% w/w)	total fat (% w/w)
A	5.0	0.47	35.7	8.4
B	4.6	0.68	38.8	11.8
C	3.8	0.85	40.6	15.4
D	5.6	0.93	41.7	14.6

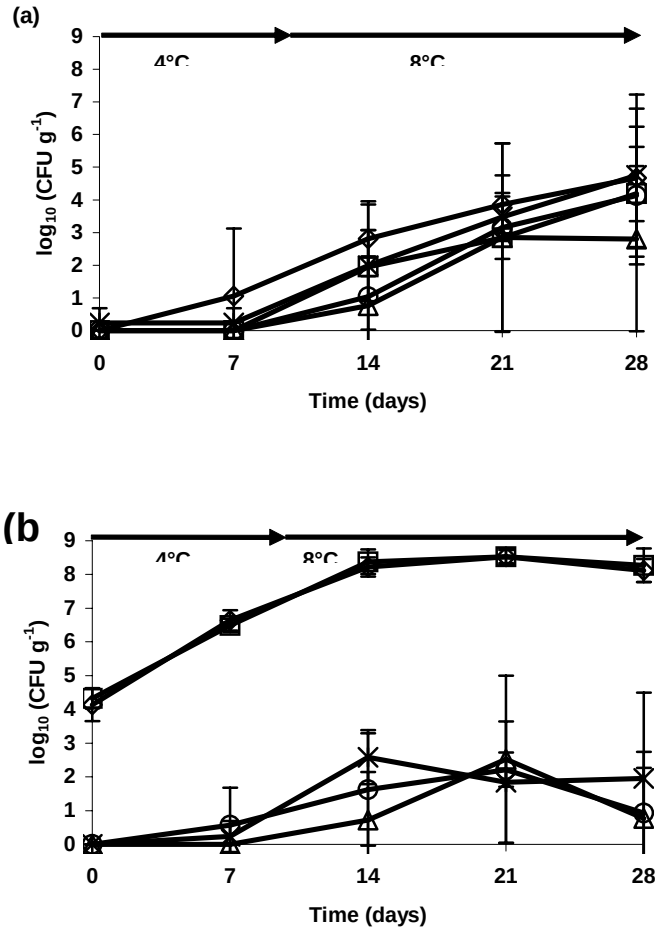
* WP : in water phase = % NaCl (w/w in CSS) x 100 / % NaCl (w/w in CSS) + % water in CSS

3.2.1. Effect of C. divergens V41 on microbiology

Just after reception, natural microflora of CSS was very different from one batch to another and two cases were observed : two batches (smokehouses A and D) were of high hygienic quality whereas two other batches (B and C) were strongly contaminated. In the first case (A and D), just after processing and reception of the batches at the laboratory, TVC was below the detection threshold (25 CFU g^{-1}). In the control A, the microflora increased gradually to reach around 10^5 CFU g^{-1} at the end of storage and was composed of a mixture of *Enterobacteriaceae*, yeasts and LAB partially represented by *Lactobacillus* (Fig. 3a). No *B. thermosphacta* nor *Listeria* spp. was detected on the plates. In the control D after four weeks of storage, a surprisingly very weak growth of the initial microflora was observed (data not shown) ; TVC never exceeded 10^2 CFU g^{-1} and was represented by LAB probably belonging to the *Carnobacterium* specie (no count on Rogosa agar). When *C. divergens* V41 was sprayed on batches A and D, TVC was only represented by the inoculated bacterium which grew from 10^{4-5} to 10^{7-8} CFU g^{-1} in three weeks. The other groups of flora were slightly inhibited by presence of *C. divergens* V41 (Fig. 3b). Average counts of Enterobacteriaceae, *Lactobacilli* and yeasts never exceeded 4 x 10^2, 3 x 10^2 and 2 x 10^2 CFU g^{-1} whereas they reached 6 x 10^4, 7 x 10^2 and 2 x 10^4 CFU g^{-1} in control. However, due to high heterogeneity in naturally contaminating flora within packages, differences between control and inoculated samples were not statistically significant.

In the second case (strongly spoiled CSS), TVC at the beginning of the storage was around 10^5 CFU g^{-1} and was probably dominated by marine gram-negative bacteria such as *Photobacterium* spp., *Vibrio* spp., *Shewanella* spp. or *Pseudomonas* spp.. TVC increased rapidly in both controls B and C and reached their maximum level (10^{7-8} CFU g^{-1}) after one and three weeks respectively. LAB, mainly represented by lactobacilli, became rapidly the dominating flora but *Enterobacteriaceae*, *Brochothrix thermosphacta* and yeasts were also present at levels varying from 10^2 to 10^4 CFU g^{-1}. As an example, Fig. 4a represents the evolution of naturally contaminating microflora of CSS for batch C. *Listeria* spp were also detected in this batch, at a level never exceeding 8 CFU g^{-1}, whereas in batch B, no colony was detected on Palcam plates. When *C. divergens* V41 was sprayed on batches B and C, the inoculated LAB represented the dominating flora during the first two weeks. However, it rapidly became difficult to distinguish the inoculated bacteria from the natural LAB contaminating slices because the level of LAB in control was the same as in inoculated samples (Fig. 4b). No statistical reduction of the average count of the spoiling microflora was observed, for both batch B and C during the four weeks storage period. Count of *Listeria* spp. in batch C was lowered (< 1 CFU g^{-1} after three and four weeks of storage) but inhibition was not significant due to very low level of contamination in control.

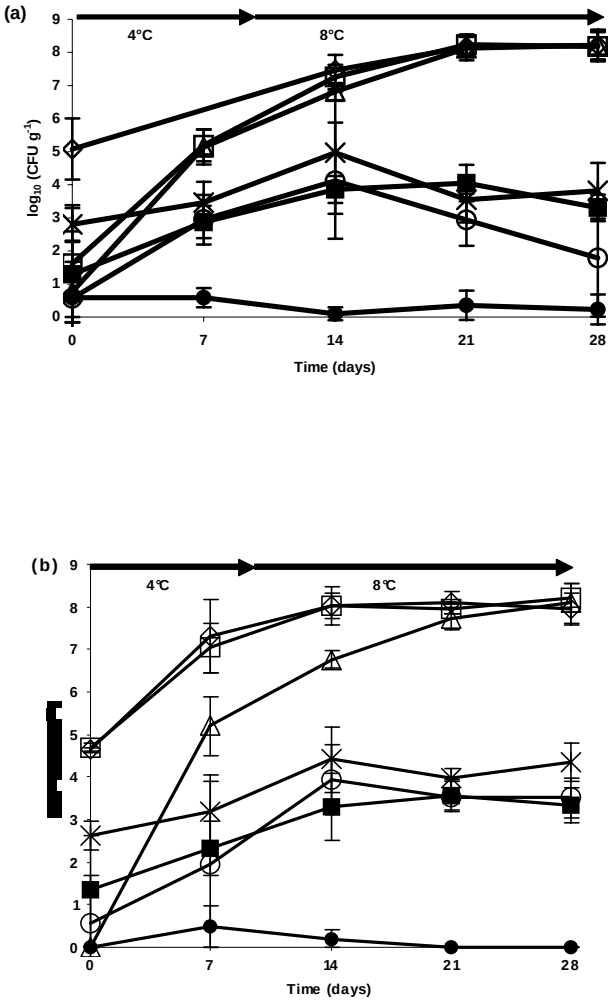
Fig. 3 Growth of natural microflora (batch A) in control (a) and in the presence of *C. divergens* V41 (b) during vacuum storage at 4°C for nine days and 8°C for 19 days : (◊) total viable count, (□) lactic acid bacteria, (Δ) *Lactobacillus* spp., (*) Enterobacteriaceae, (○) yeast. Neither *Listeria* spp. nor *Brochothrix thermosphacta* were detected. Each growth curve represents the mean of three enumerations.



3.2.2. Effect of C. divergens V41 on physico-chemical parameters

All over the storage, the pH was rather stable in controls and in batches inoculated with *C. divergens* V41, ranging between 5.9 to 6.2, and no statistical difference was observed between samples. Results for TVBN production are shown in Table 2. In the first case (smokehouses A and D, high hygienic quality) no TVBN was produced, TVBN concentration ranging from 16 ± 0 mg-N $100g^{-1}$ and 16 ± 1 mg-N $100g^{-1}$ just after processing to 18 ± 2 and 21 ± 2 mg-N 100 g^{-1} in samples A and D respectively, after four weeks of storage. In the presence of *C. divergens* V41, TVBN production was slightly but significantly increased with 29 ± 0 and 25 ± 2 mg-N 100 g^{-1} after 4 weeks for samples A and D respectively. In the second case (smokehouses B and C, poor hygienic quality), important TVBN production was noticed in control (42 ± 2 and 51 ± 9 mg-N 100 g^{-1} respectively, at the end of experiment) and there was no significant difference with inoculated samples (45 ± 1 and 50 ± 9 mg-N 100 g^{-1} respectively).

Fig. 4 Growth of natural microflora (batch C) in control (a) and in the presence of *C. divergens* V41 (b) during vacuum storage at 4°C for nine days and 8°C for 19 days : (◊) total viable count, (□) lactic acid bacteria, (Δ) *Lactobacillus*, (*) Enterobacteriaceae, (○) yeast, (×) *Listeria* spp., (+) *Brochothrix thermosphacta*. Each growth curve represents the mean of three enumerations.



No bacteriocin activity was detected during storage by agar diffusion test, both for control and inoculated samples.

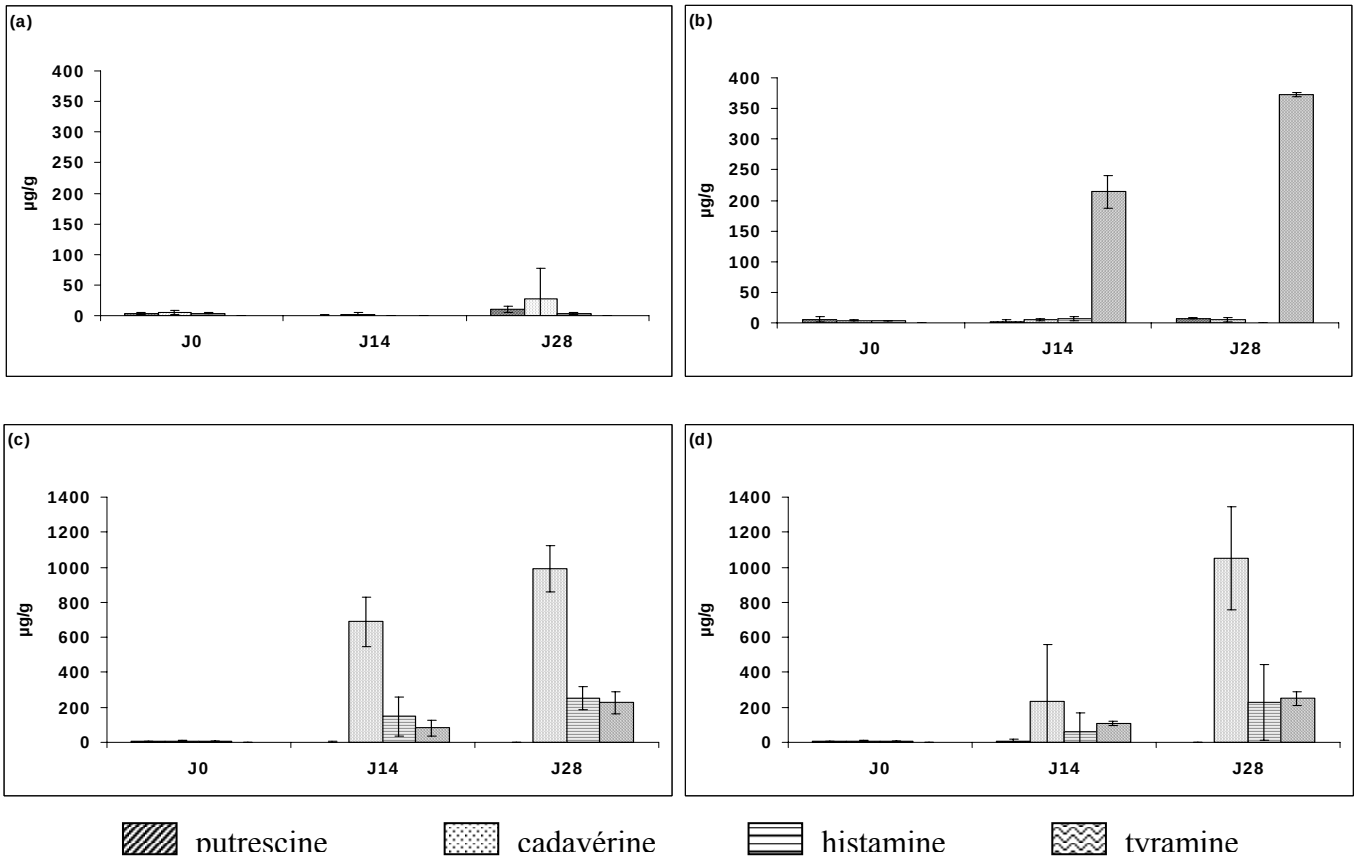
The concentration of putrecine, cadaverine, histamine and tyramine produced during the storage is shown in Fig. 5 for batch A and C. In weakly spoiled CSS (batches A), no biogenic amine was detected except a weak quantity of putrecine and cadaverine in control (Fig. 5a) at the end of storage (10 ± 5 and 28 ± 9 µg g^{-1} respectively). In the presence of *C. divergens* V41, tyramine was produced (372 ± 3 µg g^{-1} after four weeks), as it had previously been observed in inoculated CSS blocks(Fig. 5b). In strongly spoiled CSS (batches B and C), biogenic amine were produced early during storage. As an example for batch C after two weeks, 689 ± 140, 147 ± 110 and 81 ± 3 µg g^{-1} of cadaverine, histamine and tyramine were respectively produced, reaching 992 ± 131 , 254 ± 66 and 226 ± 61 µg g^{-1} after four weeks (Fig. 5c). In the presence of *C. divergens* V41, a slight inhibition was observed in cadaverine and histamine production after two weeks (respectively 235 ± 322 and 59 ± 110 µg g^{-1}) but similar concentrations were observed at the end of storage (1050 ± 295 and 231 ± 216 µg g^{-1} respectively) (Fig. 5d). No significant difference between tyramine produced in naturally

contaminated product (control) and products inoculated with *C. divergens* V41 was noticed (respectively 226 ± 61 and 251 ± 38 µg g^{-1} at the end of experiment). Similar results were observed in batch B (data not shown), excepted a slight putrescine production detected in control and assay at the end of storage (respectively 70 ± 130 and 58 ± 53 µg g^{-1}).

Table 2 Total volatile basic nitrogen production (mg-N 100 g^{-1}) in commercial batches of cold-smoked salmon alone and in presence of *Carnobacterium divergens* V41, stored under vacuum during nine days at 4 and 19 days 8°C. Each result represents the mean of three measures (95% confidence interval).

		Time (week)				
	CSS batch	0	1	2	3	4
A	Control	16.4 (± 0.0)	16.6 (± 0.8)	18.8 (± 0.4)	16.7 (± 0.4)	18.2 (± 1.8)
	C. divergens V41	17.6 (± 0.2)	17.2 (± 0.9)	20.6 (± 1.0)	24.7 (± 1.9)	28.8 (± 0.0)
B	Control	18.0 (± 3.2)	26.7 (± 9.4)	34.3 (± 3.1)	33.6 (± 2.7)	41.9 (± 2.0)
	C. divergens V41	19.5 (± 4.3)	32.3 (± 1.7)	38.1 (± 3.7)	39.5 (± 1.1)	44.8(± 0.6)
C	Control	16.2 (± 0.9)	26.6 (± 0.8)	29.5 (± 7.4)	44.0 (± 2.6)	51.3 (± 9.5)
	C. divergens V41	15.0 (± 0.8)	20.6 (± 6.8)	28.2 (± 6.8)	40.4 (± 1.9)	49.6 (± 8.6)
D	Control	16.2 (± 0.9)	17.1 (± 1.9)	19.1 (± 1.2)	18.9 (± 1.2)	21.0 (± 2.0)
	C. divergens V41	15.9 (± 1.1)	17.0 (± 1.3)	19.4 (± 2.6)	27.4 (± 1.5)	25.0 (± 2.2)

Fig. 5 Biogenic amines concentration in commercial CSS during vacuum storage at 4°C for nine days and 8°C for 19 days : (a) batch A, control ; (b) batch A inoculated with *Carnobacterium divergens* V41, (c) batch C, control (d) batch C inoculated with *Carnobacterium divergens* V41 (bars : 95% confidence intervals).

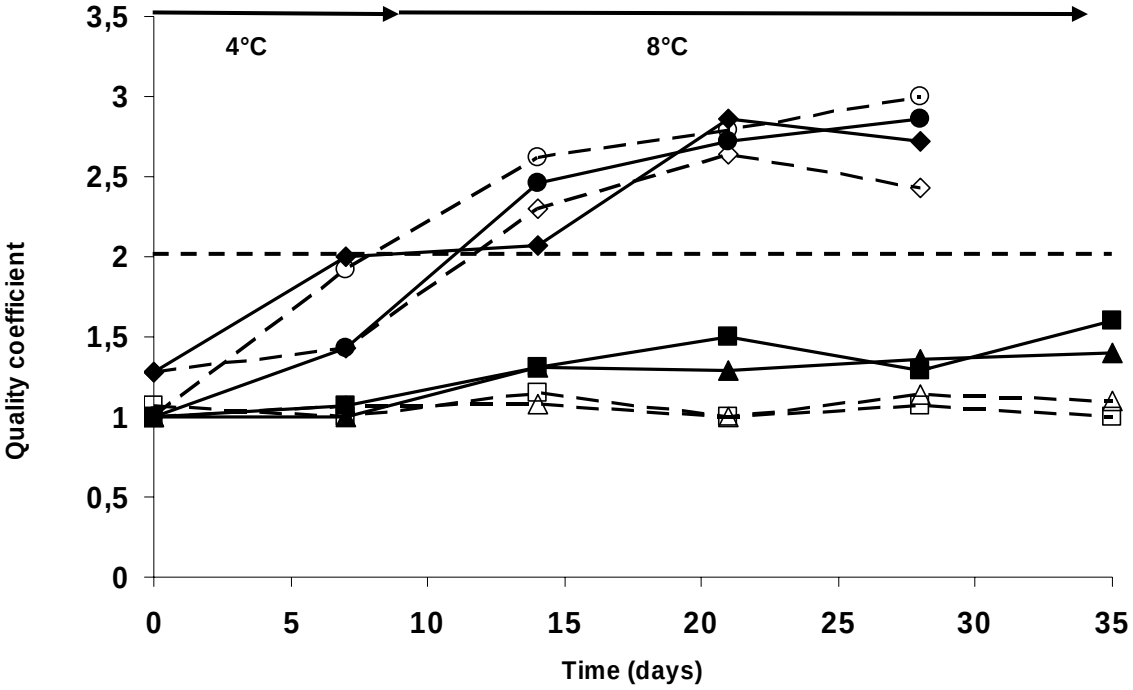


3.2.3. Effect of C. divergens V41 on sensory quality

Profiling sensory analysis were performed weekly on each batch of CSS (control and inoculated samples). In the first case (high hygienic quality, batches A and D), control were of very high sensory quality all over the storage, with no degradation of the initial score for the 34 descriptors. Statistical analysis at 95% level of confidence revealed very few difference between control and inoculated products for the descriptors of appearence, odour, flavour, and texture (data not shown). Inoculated products presented a slightly more intensive global and plastic odour, pink colour and firm texture but those differences were not observed regularly during storage. At 90% level of confidence, some odours such as sour, acid and feet and amine flavour were significantly higher in inoculated product than in control, but always with a very low score (less than one on a scale ranging between zero and ten). The quality coefficient (QC) resulting of classification in three spoiling classes is shown on Fig. 6. Results confirm that control A and D were of very high quality during the four weeks of storage (QC = 1). Presence of *C. divergens* V41 was slightly detected by the trained panel after two to three weeks but QC remained always less than 1.5, which corresponds to products not or weakly spoiled. In the second case (batches B and C strongly spoiled) controls were rejected by the panel (QC > 2) after 12 and 8 days respectively (Fig. 6). After three and two weeks for

batches B and C respectively, panellists refused to eat sample and only scored odour and appearance descriptors. The main sensory descriptors highly scored for these batches were amine and, to a lesser extend, sour, acid, cabbage, rancid and feacal odours. Presence of *C. divergens* V41 was not detected by the panel, for both batches B and C. There was no statistical difference between control and assays in any of the 34 sensory descriptors even at 90% level of confidence (data not shown) all over the storage period. Accordingly, low differences in QC between control and inoculated CSS slices were observed (Fig. 6).

Fig. 6 Quality coefficient for commercial cold-smoked salmon batches. Open symbol : control ; closed symbol : inoculated with *Carobacterium divergens* V41. Batch A (□, ■), batch B (◇, ◆), batch C (○, ●), batch D (Δ, ▲).



4. Discussion

A strategy of biopreservation of CSS against *L. monocytogenes* risk by the use LAB has been developed by Brillet et al. (2004). In order to allow implementation of this technology in the industry, the effect of the selected LAB on CSS quality had to be considered. The three *Carnobacterium* strains tested grew in inoculated CSS blocks, with a slight advantage for *C. divergens* V41. This result confirmed previous observations done by Brillet et al. (2004). However in the present study, final count of carnobacteria was always one to two $\log_{10}$ (CFU g^{-1}) lower than in the former study. This could be explained by a higher level of total phenols in this experiment (1.33 *vs* 0.81 mg 100 g^{-1}) (Thurette et al., 1998 ; Leroi et al., 2000), whereas salt concentrations were similar. No pH acidification was noticed in CSS blocks when inoculated with *C. divergens* V41, *C. piscicola* V1 and SF668, confirming the none aciduric status of carnobacteria. The three strains did not produce TVBN, which has been identified as one of the major quality index for CSS (Leroi et al., 2001). None of the three *Carnobacterium* showed any spoiling capacity according to the profiling test performed on odours by 14 trained panellists. These results are in agreement with previous works in which *Carnobacterium* spp. were not considered as specific spoilage organisms (Leroi et al., 1998; Joffraud et al., 2001; Stohr et al., 2001) and were probably not involved in

CSS spoilage (Paludan-Muller et al., 1998). Vaz-Velho et al. (in press) also have shown that *C. divergens* V41 did not produced off-odour in cold-smoked trout.

Concerning safety aspects, none of the three strains tested were able to produce histamine which is regarded as the main agent for scombrotoxic fish poisoning (Taylor, 1986), nor putrecine and cadaverine, often correlated to spoilage (Jorgensen et al., 2000a). However, the three carnobacteria produced small amount of tyramine. Tyramine production by *Carnobacterium* spp. has already been observed by Edwards et al. (1987), Leisner et al. (1994) and Jorgensen et al. (2000b). In our set of experiments, *C. divergens* V41 was shown to be the greatest tyramine producer (122 µg g^{-1} *vs* 63 and 37 µg g^{-1} for *C. piscicola* SF668 and V1 respectively) but this concentration is to be related to the highest *C. divergens* V41 count at the end of the experiment. To correlate tyramine production with cell numbers during bacterial growth, the apparent yield factors for production of tyramine, $pY_{TYR/CFU}$, was calculated according to Jorgensen et al. (2000b). The $pY_{TYR/CFU}$ values were 5.1 ± 0.3, 4.8 ± 0.2 and 4.1 ± 0.1 respectively for *C. piscicola* V1, *C. divergens* V41 and *C. piscicola* SF668 showing that *C. divergens* V41 and *C. piscicola* V1 had a lower activity of tyramine production than *C. piscicola* SF668 (i.e., lower $pY_{TYR/CFU}$ value). Tyramine may cause migraine headaches and hypertensive effects, and in some cases can act as a potentiator of histamine effects (Ten Brink et al., 1990). However, tyramine concentration found in CSS blocks inoculated with *C. divergens* V41 is lower than natural tyramine concentrations currently reported in commercial CSS (Jorgensen et al., 2000b; Connil et al., 2002) and no legal upper limit exists for tyramine in fish products in the European legislation. Finally, *C. divergens* V41 have shown the highest *L. monocytogenes* inhibitory capability (Brillet et al., 2004) which lead us to select it for an application on naturally contaminated CSS slices.

As the microbial ecology of CSS is very complex and may vary among smokehouses (Leroi et al., 2001), potential application of biopreservation on commercial CSS was tested by spraying *C. divergens* V41 at a level of 10^{4-5} CFU g^{-1} on the slices of four batches provided by different smokehouses. According to several studies (Truelstrup Hansen and Huss, 1998; Leroi et al., 2001; Gram and Dalgaard, 2002; Cardinal et al., 2004), initial TVC just after processing were very heterogeneous, with two batches presenting a very good hygienic quality (TVC < 25 CFU g^{-1}) and two batches being highly contaminated (TVC > 10^{4-5} CFU g^{-1}).

On weakly spoiled CSS slices (batches A and D), *C. divergens* V41 grew very well, from 10^4 to 10^8 CFU g^{-1} in two weeks. Growth was more obvious than in inoculated CSS blocks, probably due to a lower total phenol concentrations in batches A and D (respectively 0.47 and 0.93 mg 100 g^{-1}) than in CSS blocks (1.33 mg 100 g^{-1}) (salt contents were similar). It is also possible that bacterial growth on thin slices was enhanced compared with growth on blocks due to higher surface and nutrient availability. In the presence of *C. divergens* V41, growth of the different microflora specifically enumerated were slightly inhibited, but due to high heterogeneity of contamination of the slices inside a batch, those differences were not significant at the 95% probability level. Inhibition of lactobacilli could be explained by sensitivity of some *Lactobacillus* strains to divercin V41 produced by *C. divergens* V41 (Pilet et al., 1995). Inhibition of Enterobacteriaceae and yeasts could be due to nutrient competition but has not been further investigated. Concerning the TVBN production, an increase of around 10 mg-N $100g^{-1}$ in samples containing the inoculated strain was observed in comparison with controls. This production had not been observed when *C. divergens* V41 was inoculated in CSS blocks, suggesting particular interaction between the LAB starter and the natural contaminating bacteria. For example, high level production of TVBN has been observed in sterile CSS blocks inoculated with co-culture of *C. piscicola* and *B. thermosphacta* while no production was noticed by any of the strains when inoculated in pure culture (unpublished data). Nevertheless, the level of TVBN at the end of storage was always

lower than the 30 mg-N 100 g^{-1} spoilage limit defined by Leroi et al. (2001) for CSS. No production of biogenic amines was detected in the control whereas tyramine was produced in the samples inoculated with *C. divergens* V41 as it was expected. The amount of tyramine detected after four weeks of storage was three times higher than the amount measured in inoculated CSS, probably due to the high count of carnobacteria at the end of the storage. It is unlikely that production of tyramine by *Carnobacterium* was enhanced in the presence of indigenous flora as no particular metabolic interaction was detected when *Carnobacterium* spp. were co-inoculated with different species isolated from CSS (Jorgensen et al., 2000b). The final level of tyramine could be reduced by respecting the chilled storage temperature (4°C). Indeed, Connil et al. (2002) have showed that tyramine production by *C. divergens* V41 was delayed at low temperatures. During the first two weeks of storage, no sensory difference between control and inoculated products was noticed. After that, trained panellists were able to discriminate the products, which is not really surprising because they compared CSS inoculated with high level *C. divergens* V41 to CSS which natural contamination do not contain many bacteria. Some slight off-odours and taste were detected but always at very low level (less than one on a scale from zero to ten whereas in highly spoiled batches B and C, those descriptors reach five or six) and products were never rejected by the panel until the end of storage.

On extensively spoiled CSS batches, all flora grew very quickly and reached their maximum level after two to three weeks. Batches B and C had a low level of salt (respectively 4.6 and 3.8 % in WP) compared to batches A and D (5.0 and 5.6 % in WP), and were fairly smoked. As demonstrated by Leroi et al. (2000) these characteristics may explain the quicker growth of the natural microflora. LAB which were in the minority at the beginning quickly became dominant, and were represented mainly by lactobacilli (count on Rogosa agar at pH 5.5 identical to count on Nitrite Actidione Polymixine agar used for total LAB enumeration). This predominance has already been observed in several studies and specially at the end of the storage period (Leroi et al., 1998; Lyhs et al., 1998, Jorgensen et al., 2000a) probably because a lot of lactobacilli are well adapted to cold-smoked salmon condition, being psychrotrophic facultative anaerobic bacteria and able to grow in the presence of up to 8-10 % of NaCl. Both controls from batch B and C were rejected by the panellists, after three and two weeks respectively, because of very strong amine off-odours. Those odours are clearly linked with the presence of lactobacilli at high levels. Although unspecifically identified in this study, *Lb. curvatus* and *Lb. sakei* species frequently dominate the LAB colonizing lightly preserved fish product (Leroi et al., 1998; Truelstrup Hansen and Huss, 1998; Lyhs et al., 1999, Jorgensen et al., 2000a). *Lb. sakei* was identified as a great spoilage organism in CSS and often releases strong sulphurous and acidic odours (Truelstrup Hansen et al., 1995; Stohr et al., 2001, Joffraud et al., 2001). *Lb. curvatus* was also identified as a specific spoilage organism of CSS because of its production of biogenic amines (Jorgensen et al., 2000b). It is also possible that psychrotrophic Enterobacteriaceae and *B. thermosphacta*, although present at a lower level, participate in spoilage because those micro-organisms can produce strong off-odours when inoculated in CSS blocks (Joffraud et al., 2001; Stohr et al., 2001). In the presence of *C. divergens* V41, none of the natural flora enumerated were inhibited and grew as well as in the control. No difference in TVBN production was observed. However, after two weeks of storage, cadaverine and histamine concentrations were significantly reduced in inoculated products. This could be explained by an inhibition of strains from the Vibrionaceae family which are not specifically enumerated in this study. Indeed, Jorgensen et al. (2000b) have shown that *P. phosphoreum* was the only species isolated from CSS that produced histamine below 10°C. This species also produced cadaverine, as well as some Enterobacteriaceae (*S. liquefaciens*). During the first two weeks of storage, *C. divergens* V41 was dominant in the inoculated product (count of LAB one to two $\log_{10}$ (CFU g^{-1}) higher than in control), which

may explain the inhibition. After three weeks, no difference in biogenic amine concentration was observed between control and inoculated CSS. At this time, LAB count in inoculated CSS is the same as in control and the ratio *C. divergens* V41 / spoilage micro-organism may be too small to observe an inhibition. It is also possible that strains (particularly *Lactobacillus* spp.) present in batches B and C differ from batch A and are unsensitive to divercin V41. Concentration of histamine in naturally contaminated batches B and C after 4 weeks of storage (respectively 397 ± 45 and 254 ± 58 µg g^{-1}) exceeded the European tolerated limit in Scombroids and Clupeids fish (mean of 9 samples < 100 µg g^{-1} and no sample higher than 200 µg g^{-1}, ECC 91/493). Free histidine, which is the substrate for histamine production, can be found in sufficient concentration in *Salmo salar* flesh to allow such production (5860 µg g^{-1} USDA nutrient database) and high levels of histamine have been found in routine control of smoked salmon in Denmark (Huss et al., 1995). The most effective methods for preventing biogenic amine formation are handling and processing under sanitary condition and temperature control (< 5°C) throughout the process. In our experiment, samples were stored at abuse temperatures (8°C for 19 days with a 2 h break at 20°C). Concerning the sensory aspect, low differences between control and inoculated CSS was detected. Presence of *C. divergens* V41 in highly contaminated CSS slices was not detected by the trained panellists. On the other hand, this strain did not improved the quality of the product, but it clearly had been selected for its targeted antilisterial activity and not for controlling spoilage micro-organisms. The inhibitory effect of *C. divergens* V41 against *L. monocytogenes* has been shown to be specifically related to the divercin V41 production (Richard et al., 2003). Although no bacteriocin was detected in the inoculated flesh in this set of experiment, the inhibitory effect of *L. monocytogenes* is effective (Brillet et al., 2004). Indeed, Duffes et al. (1999a) have emphasis those difficulties to measure divercin V41 in CSS, probably due to interaction with product and packaging, although inhibition was observed.

In conclusion, this study has shown that the strain *C. divergens* V41 inoculated in CSS in a biopreservation goal exhibits some interesting properties : it is able to grow at high level without giving major sensory changes in the product. This solution will not replace other measures for controlling *L. monocytogenes* (and spoiling microflora) in cold-smoked salmon but will act as an extra hurdle specifically designed to eliminate the listeriosis risk. To our knowledge, application of protective LAB is not common in fish products mainly because of their limited growth in chilled foods (Wessels and Huss, 1996) and production of off-odour (Nilsson et al., 1999). In CSS batches of high hygienic quality, some slight off-odours and flavours were detected in inoculated samples by a trained panel. Differences are so slight that it is believed that *C. divergens* V41 probably would not be detected by a non trained consumer panel. Some negative effects of *C. divergens* V41 , such as slight productions of TVBN and tyramine, could be reduced by optimising *C. divergens* V41 level of inoculation while keeping an efficient inhibition of *L. monocytogenes* and by avoiding abused temperature storage. Lastly, work is currently under way to select tyramine negative mutants of *C. divergens* V41 for an application in CSS.

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