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International Journal of Food Microbiology 112 (2006) 51 – 61
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Effect of bacterial interactions on the spoilage of cold-smoked salmon

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Received 28 September 2005; received in revised form 24 February 2006; accepted 14 May 2006

Abstract

Cold-smoked salmon is a lightly preserved fish product in which a mixed microbial flora develops during storage and where the interactive behaviour of micro-organisms may contribute to their growth and spoilage activity. The aim of this study was to assess the effect of the bacterial interactions between the main species contaminating the cold-smoked salmon on bacterial growth, chemical and sensory changes, and spoilage. First, *Carnobacterium piscicola*, *Photobacterium phosphoreum*, *Lactobacillus sakei*, *Vibrio* sp., *Brochothrix thermosphacta* and *Serratia liquefaciens*-like were inoculated as pure cultures on sterile cold-smoked salmon. All bacterial species grew well; *Vibrio* sp. was the fastest and *L. sakei* strains developed very rapidly as well with a high maximum cell density on cold-smoked salmon blocks (up to 10^9 cfu g^{-1} after 10 days at 8 °C). Based on sensory analysis, *Vibrio* sp. was identified as non-spoilage bacteria, *C. piscicola* as very lightly and *B. thermosphacta* as lightly spoiling. *L. sakei* and *S. liquefaciens*-like were found to be the most spoiling bacteria. Secondly, *C. piscicola* and *L. sakei*, two species frequently occurring in the lactic flora of the product, were inoculated together and each of them in mixed cultures with respectively *P. phosphoreum*, *Vibrio* sp., *B. thermosphacta,* and *S. liquefaciens*-like. The growth of *L. sakei* was shown to strongly inhibit most of the co-inoculated strains i.e. *P. phosphoreum*, *B. thermosphacta, S. liquefaciens*-like and, to a lesser extent, *Vibrio* sp. The growth of *C. piscicola* seemed to be enhanced with *B. thermosphacta* and to develop earlier with *P. phosphoreum* and *Vibrio* sp. Conversely, *S. liquefaciens*-like and *P. phosphoreum* were weakly inhibited by *C. piscicola*. The main observation resulting from the sensory evaluation was the delay in the appearance of the spoilage characteristics in the mixed cultures with *L. sakei*, in particular *L. sakei/ S. liquefaciens*-like. On the other hand, the spoilage activity of the non-spoiler strains *Vibrio* sp. or the moderate spoilage strains *B. thermosphacta* and *C. piscicola* was increased when they were associated together.

It is concluded that the spoilage behaviour of micro-organisms in mixed culture is significantly different from pure culture and explain the difficulty to find robust quality indices for this product.

Keywords: Cold-smoked salmon; Microbial flora; Interaction; Spoilage

1. Introduction

Vacuum-packed cold-smoked salmon (CSS) is of considerable economic importance worldwide, and particularly in Europe. This foodstuff is produced by a light salting and smoking process and is typically consumed as ready-to-eat with no heat treatment. CSS, usually stored at chilled temperature, is very sensitive to deterioration and, based on sensory evaluation, has a limited shelf life ranging from 3 to 6 weeks at 5 °C (Ward, 2001). Microbial activity has been found to be responsible for off-flavours (Truelstrup Hansen et al., 1995, 1996; Joffraud et al., 1998; Leroi et al., 2001).

In recent years, the specific bacterial flora of CSS or cold-smoked trout have been extensively studied and the main taxonomic groups that occur frequently and dominate the spoilage microflora have been determined (Truelstrup Hansen et al., 1995; Gram and Huss, 1996; Leroi et al., 1998; Lyhs et al., 1998; Paludan-Müller et al., 1998; Truelstrup Hansen et al., 1998; Truelstrup Hansen and Huss, 1998; Jorgensen et al., 2000a; Leroi et al., 2000b, 2001; Gonzalez-Rodriguez et al., 2002). Moreover, with the aim of identifying specific spoilage organisms (SSOs), the spoilage potential of bacteria belonging to these different taxonomic groups isolated from CSS by Leroi et al. (1998) was assessed by investigating off-odour production from inoculated CSS using sensory analysis at the end of storage (Stohr et al., 2001) and by analyzing the composition of the volatiles released (Joffraud et al.,

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doi:10.1016/j.ijfoodmicro.2006.05.014

2001; Jorgensen et al., 2001). These inoculation studies allowed some bacterial species to be characterized as active spoilers when inoculated in single culture into sterile CSS blocks. Thus, *Lactobacillus sakei*, *Brochothrix thermosphacta*, *Photobacterium phosphoreum*, *Aeromonas* spp. and *Serratia liquefaciens*-like were found to produce specific off-odours/flavours associated with CSS spoilage (Stohr et al., 2001). However, large variations in the quantitative and qualitative microbiological composition of commercial products have previously been observed (Truelstrup Hansen et al., 1998; Truelstrup Hansen and Huss, 1998; Jorgensen et al., 2000a,b; Leroi et al., 2001; Cardinal et al., 2004). As a result, several frequently occurring situations have been described: domination by lactic acid bacteria (LAB), *Carnobacterium* sp. and/or *Lactobacillus* sp., at levels of 10^7–10^9 cfu g^{-1}; domination by a mixture of LAB and Enterobacteriaceae at levels of 10^7–10^8 cfu g^{-1}; domination by *Photobacterium*/marine *Vibrio*-type at levels of 10^6–10^7 cfu g^{-1} occasionally with high levels of LAB; domination by a mixture of total LAB and *B. thermosphacta*. Under these conditions, interactions between dominating bacterial species may play an important role in sensory spoilage.

In the present study, in order to investigate the interactions, sterile CSS blocks were inoculated with mixed cultures in pairs combining the two main LAB occurring on commercial CSS, *L. sakei* and *C. piscicola*, together and each one of them with *B. thermosphacta*, *S. liquefaciens*-like, *P. phosphoreum* and *Vibrio* sp. respectively.

Contrary to previous studies where samples inoculated with single bacterial cultures were analysed for sensory changes and volatile production only at the end of the storage period (Joffraud et al., 2001; Stohr et al., 2001), the aim of the present work was to investigate chemical, bacterial and sensory changes over the whole storage period to provide a kinetic insight and to examine the interactions between predominating species.

2. Materials and methods

2.1. Sterile CSS model

58 kg of sterile CSS model system produced in two batches by an aseptic process and ionization as described by Joffraud et al. (1998) was used as substrate. In this process, CSS was diced into approximately 5-mm-sided cubes, vacuum-packed and frozen at −80 °C before the ionization treatment. Based on three determinations, the composition of this model system was 60.4%±0.8 water, 4.4%±0.3 NaCl in water phase and 0.82 mg 100 g^{-1} ±0.14 phenols for the trial 1 and 61.6%±0.9 water, 4.8%±0.4 NaCl in water phase, 0.88 mg 100 g^{-1} ±0.16 for the trial 2.

2.2. Bacterial strains

Strains, selected from the collection of the laboratory, were previously isolated from vacuum-packed CSS and identified (Leroi et al., 1998). Two inoculation trials were performed at different periods and each trial was divided into two successive sets. The different strains inoculated in these trials and the corresponding labels used in graphs are shown in Table 1. The first two sets (A and C) of each trial used pure cultures from *C. piscicola*, *P. phosphoreum*, *L. sakei*, *Vibrio* sp., *B. thermosphacta* and *S. liquefaciens*-like. Except for the two isolates for *C. piscicola*, each group was represented by a mixture of five strains. All isolates, apart from *Vibrio* sp, had already been individually tested for spoilage potential in a previous study (Stohr et al., 2001). The second sets (B and D)

Table 1
Bacterial strains (reference numbers in IFREMER collection) inoculated as pure or mixed cultures in cold-smoked salmon for the different sets of trials

Inoculation trials	Set	Inoculated strain	Label
Trial 1	Set A pure cultures	Control	T
		Brochothrix thermosphacta: (SF 674, SF 677, SF 678, SF 713, SF 712)	B
		Serratia liquefaciens-like: (SF 1460, SF 1461, SF 1467, SF 1463, SF 1464)	S
		Carnobacterium piscicola: (SF 644, SF 692)	C
		Photobacterium phosphoreum: (SF 680, SF 686, SF 684, SF 714, SF 723)	P
		Lactobacillus sakei: (SF 811, SF 696, SF 699, SF 841, SF 771)	L
		Vibrio sp.: (SF 1766, SF 1803, SF 1805, SF 1813, SF 1816)	V
	Set B mixed cultures	Control	T
		Lactobacillus sakei/*Carnobacterium piscicola*	L/Ca
		Lactobacillus sakei/*Brochothrix thermosphacta*	L/B
		Lactobacillus sakei/*Serratia liquefaciens*-like	L/S
		Carnobacterium piscicola/*Brochothrix thermosphacta*	C/B
		Carnobacterium piscicola/*Serratia liquefaciens*-like	C/S
Trial 2	Set C pure cultures	Control	T
		Carnobacterium piscicola	C
		Photobacterium phosphoreum	P
		Lactobacillus sakei	L
		Vibrio sp.	V
	Set D mixed cultures	Control	T
		Lactobacillus sakei/*Carnobacterium piscicola*	L/Cb
		Lactobacillus sakei/*Photobacterium phosphoreum*	L/P
		Lactobacillus sakei /*Vibrio* sp.	L/V
		Carnobacterium piscicola/*Photobacterium phosphoreum*	C/P
		Carnobacterium piscicola/*Vibrio* sp.	C/V

consisted of mixed cultures in pairs, combining *L. sakei* and *C. piscicola* together and each one of them with *B. thermosphacta*, *S. liquefaciens*-like, *P. phosphoreum* and *Vibrio* sp. respectively (Table 1).

2.3. Culture, sample inoculation and packaging

Bacterial strains were revived from frozen storage by inoculation of a loop full into 10 ml of appropriate culture medium. *C. piscicola* strains were grown in Elliker broth (Elliker et al., 1956) (Biokar Diagnostics, Beauvais, France), *L. sakei* in Man, Rogosa and Sharpe (MRS) broth (de Man et al., 1960) (Merck, Darmstadt, Germany), and the other strains in brain–heart infusion (BHI) (Difco Laboratories, Detroit, MI, USA). Strains were pre-cultured individually at 20 °C, except for *P. phosphoreum* and *Vibrio* sp. (15 °C), for 24–48 h and then cultured in the same conditions until an absorbance-estimated concentration level of approx. 10^8 cells ml^{-1} was achieved. Isolates belonging to the same genus or species were mixed; for combinations, the mixtures of two taxonomic groups were pooled; the final mixture was thousand-fold diluted in cooled sterile peptone water (0.85% NaCl and 0.1% peptone). To achieve an inoculation concentration of approx. 10^3–10^4 cells g^{-1}, 6 ml of culture dilution (around 10^5 cells ml^{-1}) was added to 300 g of sterile CSS blocks in a sterile plastic bag, followed by a careful manual mixing of the bag contents. This action was repeated eight times until the amount needed was achieved (300 g × 8) for each bacterial group tested, alone or in combination. For each set, a control was created by replacing the inoculum with sterile peptone water. Blocks were then allotted and vacuum-packed in polyamide/polyethylene bags (PA/PE 20/70, Euralpac, Alfo, Germany). Permeability of the packaging film was 40–50 cm^3/m^2 for O_2 and 146 cm^3/m^2 for CO_2 at 24 h (1 atm at 23 °C, 75% r.F.). Bag weight differed depending on the type of analysis (20 g for sensory analysis and 60 g for microbial and chemical analysis). Despite French regulations prescribing a storage temperature range of 0–4 °C for chilled CSS (AFNOR, 1997), 8 °C was chosen to mimic temperature abuse at different stages from production to consumption (transport, commercial and domestic refrigeration, etc.). Immediately after inoculation and then twice a week for 5–6 weeks, bags from each batch (i.e. each taxonomic group or combination) were opened for sensory, microbiological and chemical analysis.

2.4. Quantitative microbiological analysis

Twice a week, inoculated strains were enumerated by a direct plating technique. Flesh samples (15 g) were homogenized with 60 ml of chilled peptone water in a stomacher bag filter for 2 min using a stomacher 400 (Lab Blender, London, UK) and left at room temperature for 30 min for resuscitation. The homogenate was serially diluted 10-fold in chilled peptone water and 0.1 ml of each appropriate dilution was spread on duplicate petri plates. When inoculated in pure culture, enumeration of strains was performed by using the following media: plate count agar (PCA; Biokar) incubated aerobically for 3–5 days at 20 °C for *B. thermosphacta* and *S. liquefaciens*-like; Long and Hammer's medium containing 1% NaCl (van Spreekens, 1974) after 5 days of aerobic incubation at 15 °C for *P. phosphoreum* and *Vibrio* sp.; MRS agar (Merck) for *L. sakei* and Elliker broth (Biokar) with 1.5% of agar for *C. piscicola*, MRS and Elliker plates were both placed in anaerobic jars (20% CO_2, 80% N_2) with Anaerocult A (Merck) at 20 °C for 5 days. For strains inoculated in combination, *L. sakei*, *B. thermosphacta* and *S. liquefaciens*-like were selectively enumerated by means of suitable media. Spread plates of Rogosa agar (Biokar) incubated anaerobically at 20 °C for *L. sakei*, Streptomycin Thallium Acetate Actidion (STAA) (Gardner, 1980) (2% peptone, 0.2% yeast extract, 0.1% KH_2PO_4, 0.1% $MgSO_4$ $7H_2O$, 1.5% glycerol, 1.3% agar, 0.05% streptomycin, 0.005% cycloheximide and 0.005% thallium acetate) incubated at 20 °C for *B. thermosphacta* and pour plates of CASO agar (Merck) overlaid by Violet Red Bile Glucose agar (VRBG; Oxoid, Basingstoke, England) incubated at 30 °C for 2 days for *S. liquefaciens*-like. *P. phosphoreum* and *Vibrio* sp. were differentially counted on Long and Hammer's medium on the basis of colony size, colonies of *L. sakei* or *C. piscicola* being smaller. In combination with *P. phosphoreum* or *Vibrio* sp., *C. piscicola* was selectively enumerated on Elliker plates.

2.5. Chemical analysis

The remaining flesh in the bags opened for microbiological analysis was homogenized in a Waring Blender (New Hartford, CO, USA). Total volatile basic nitrogen (TVBN) and trimethylamine (TMA) were measured in duplicate by the Conway microdiffusion method (Conway and Byrne, 1933). The pH value was measured in the five-fold-diluted flesh with a pH meter (Mettler Delta 320, AES, Combourg, France).

2.6. Sensory analysis

A trained panel of 6 people experienced in smoked salmon evaluation (IFREMER), was required to smell each sample and to identify, among an established list of seven descriptors (Table 2), the main detected odour. Before starting the experiment, the panel was trained during six sessions with aromatic molecules, either in

Table 2
Characteristic odours of spoiled cold-smoked salmon, description and chemical reference

Categories of odour	Label	Description and associated molecules
No specific odour	Nso	No note other than smoke or raw fish odour
Grass/green	Grass	Odours from freshly cut grass (hexanal)
Fruity/plastic	Fru	Odour of citrus fruit or plastic (nonylaldehyde or decylaldehyde)
Butter	Butt	Odour developed by fresh butter (diacetyl)
Feet/cheese	Feet	Odour of feet (isovaleraldehyde)
Amine/sour/vinegar	Amin	Odour related to urine or curdled milk or vinegar (trimethylamine for amine)
Sulphur	Sulph	Odour characterized by sulphur notes like garlic, cabbage, mud, (dimethylsulphide, thiobarbituric acid)

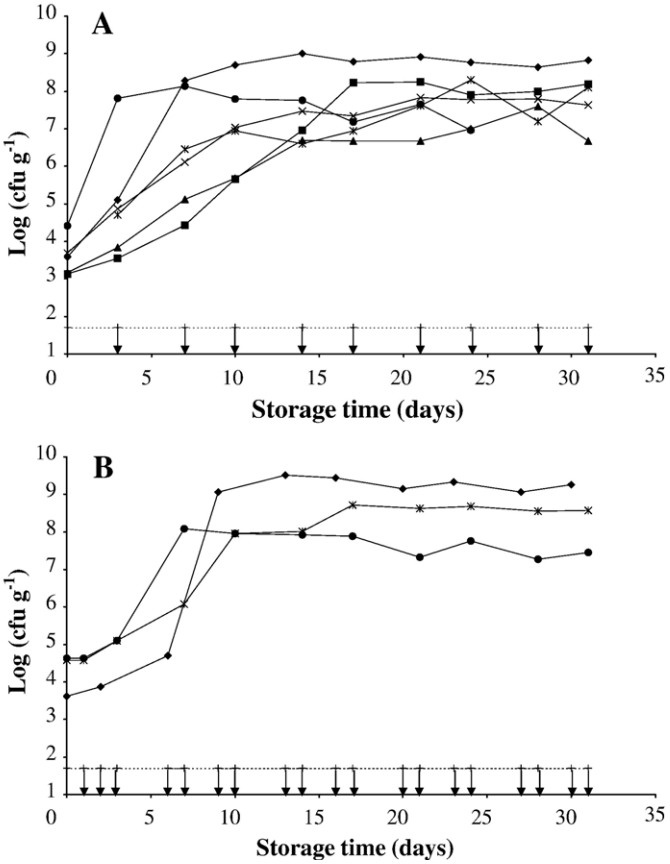

Fig. 1. Bacterial growth of pure cultures inoculated in vacuum-packed cold-smoked salmon stored at 8 °C. a: Trial 1, set A; b: trial 2, set C (♦): *Lactobacillus sakei*; (■): *Serratia liquefaciens*-like; (▲): *Brochothrix thermosphacta*; (×): *Photobacterium phosphoreum*; (*): *Carnobacterium piscicola*; (●): *Vibrio* sp; (+): control, no colony being detected, the symbol "↓" indicates that counts are below the detection threshold.

a water solution or added to smoked salmon blocks, to recognize the odours of spoiled CSS. These molecules have previously been identified in CSS as related to odours of spoiled products (Joffraud et al., 2001; Stohr et al., 2001). Samples of a same set were evaluated twice a week, for 31 days in the first trial and for 42 days in the second trial (see Table 1). Sessions were performed in individual partitioned booths, as described in the procedure NF V-09-105 (AFNOR, 1987) and equipped with a computerised system (Fizz, Biosystèmes, Couternon, France). Products were assigned 3-digit numbers, randomised and served simultaneously after 15 min at room temperature. 20 g of blocks of CSS was used for each sample and for each panelist. Before testing, each assessor opened the packaging himself. Assessors identified the main odour criteria by ticking the appropriate descriptor and, at the end of the evaluation, panelists classified each sample according to the spoilage level (NS: non-spoiled; LS: lightly spoiled; SS: strongly spoiled).

2.7. Statistical analysis

Quotation frequencies obtained from the panel for each sensory criteria (spoilage level and main odour) allowed correspondence factorial analysis (CFA) on Uniwin software (Uniwin Plus, version 3, Sigma Plus), respectively for single cultures and mixed cultures.

3. Results

3.1. Pure cultures

3.1.1. Bacterial kinetics

Fig. 1A and B shows the growth patterns of the different groups of bacteria as pure cultures during vacuum storage at 8 °C performed in the two trials. Samples inoculated with *P. phosphoreum* in the second period were found to be contaminated so these results will not be shown further or commented on. Most of the cultures were inoculated in a range of 10^3–10^4 cfu g^{-1} except for *Vibrio* sp. in the two trials and *C. piscicola* in the second one, inoculated at 10^4–10^5 cfu g^{-1}. All bacterial species grew well on the sterile CSS model. They reached their maximum level after 1 to 2 weeks of storage with final counts ranging between 10^7 cfu g^{-1} and 10^9 cfu g^{-1}. The

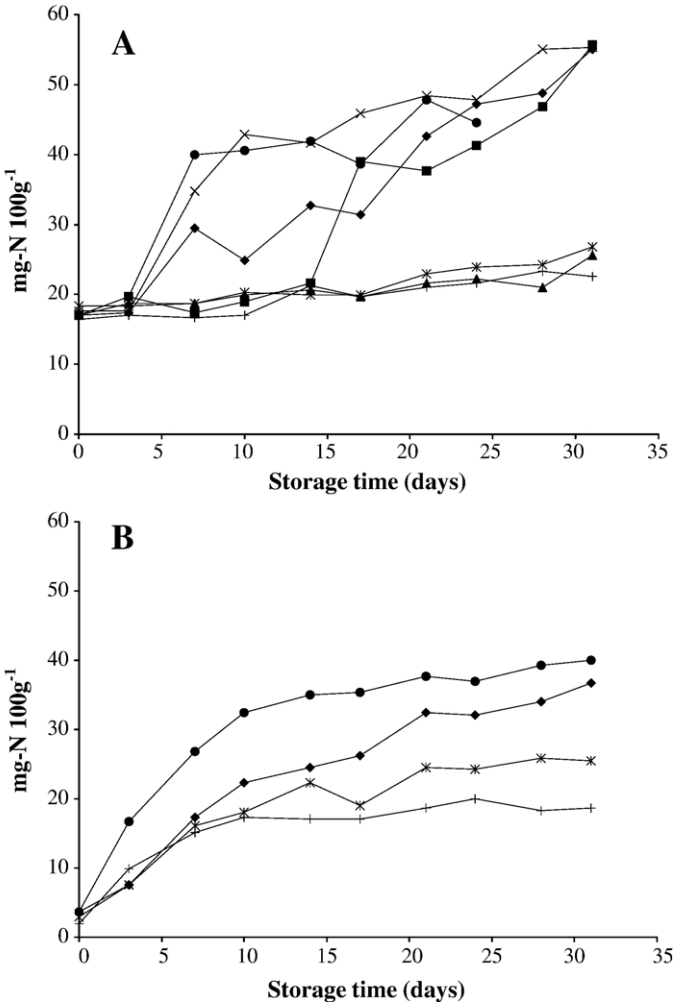

Fig. 2. Total volatile base nitrogen production of pure cultures inoculated in vacuum-packed cold-smoked salmon stored at 8 °C. a: Trial 1, set A; b: trial 2, set C (+): control; (♦): *Lactobacillus sakei*; (■): *Serratia liquefaciens*-like; (▲): *Brochothrix thermosphacta*; (×): *Photobacterium phosphoreum*; (*): *Carnobacterium piscicola*; (●): *Vibrio* sp.

number of *L. sakei* increased rapidly and reached 10^9 cfu g^{-1} after 10 days. *Vibrio* sp. had quite the same growth rate, attaining its maximum level between 3 and 7 days depending on the trial. However, although inoculated at higher level than *L. sakei*, the maximum level (10^8 cfu g^{-1}) was always 1 log lower than for *L. sakei*. The growth of the other species was slower (Fig. 1A), reaching 10^{7-8} cfu g^{-1} after 2 or 3 weeks of storage. No colony was detected on petri plates regarding control samples throughout the storage so they were considered sterile.

3.1.2. Chemical changes

TVBN concentration in the controls of both trials did not exceed 20 mg-N 100 g^{-1}. Most of the bacterial groups produced a significant amount of TVBN except *B. thermosphacta* and *C. piscicola* (Fig. 2A, B). In the first trial, concentration increased from 17–18 to 55–56 mg-N 100 g^{-1} for *L. sakei* and *Vibrio* sp. and from 4 to 37 and 40 mg-N 100 g^{-1} respectively in the second trial. In the first trial, two patterns could be observed in the production of TVBN. The maximum production rate was exhibited during the first two weeks for *P. phosphoreum* and *Vibrio* sp. and during the last two weeks for *L. sakei* and *S. liquefaciens*-like. This observation was confirmed by the data obtained in the second trial with *L. sakei* and *Vibrio* sp.

TVBN production is correlated with cell numbers during bacterial growth. Thus, the apparent yield factor proposed by Dalgaard (1995) for production of TVBN by the different bacterial groups in pure culture has been calculated. $pY_{TVBN/CFU} = -\log(TVBN_{final} - TVBN_{initial}/CFU_{final} - CFU_{initial})$ where $CFU_{initial}$ is the initial cell concentrations and CFU_{final} (cfu g^{-1}) is the cell concentrations at the end of the exponential growth phase, $TVBN_{initial}$ and $TVBN_{final}$ are the initial amount of TVBN and the amount of TVBN at the end of the exponential growth phase. Based on $pY_{TVBN/CFU}$, *P. phosphoreum* was found to be the strongest producer of TVBN with 6.1 log (mg-N TVBN cfu^{-1}) followed by *Vibrio* sp. (6.7), *S. liquefaciens*-like (6.9) and *L. sakei* (7.8).

Only *Vibrio* sp., *P. phosphoreum* and *S. liquefaciens*-like produced TMA; concentrations increased from 2 to 20–23 mg-N 100 g^{-1} in the first trial (data not shown). The pH was fairly stable during storage, ranging from 6.10 to 6.30 except for *L. sakei*, which showed a decrease from 6.13 to 5.73. This drop in pH in samples inoculated with *L. sakei* was confirmed in the second period, when the pH decreased from 6.13 to 5.63.

3.1.3. Sensory changes

Correspondence factorial analysis (CFA) performed on the seven sensory descriptors and the three levels of spoilage allowed the separation, on the first axis, of strongly spoiled samples (right part of the Fig. 3) from non-spoiled and lightly spoiled samples (left part of the Fig. 3). The first axis restored 40.4% of the total information and was a "spoilage axis" separating the strongly spoiled samples from the others. Non-spoiled (upper part) and

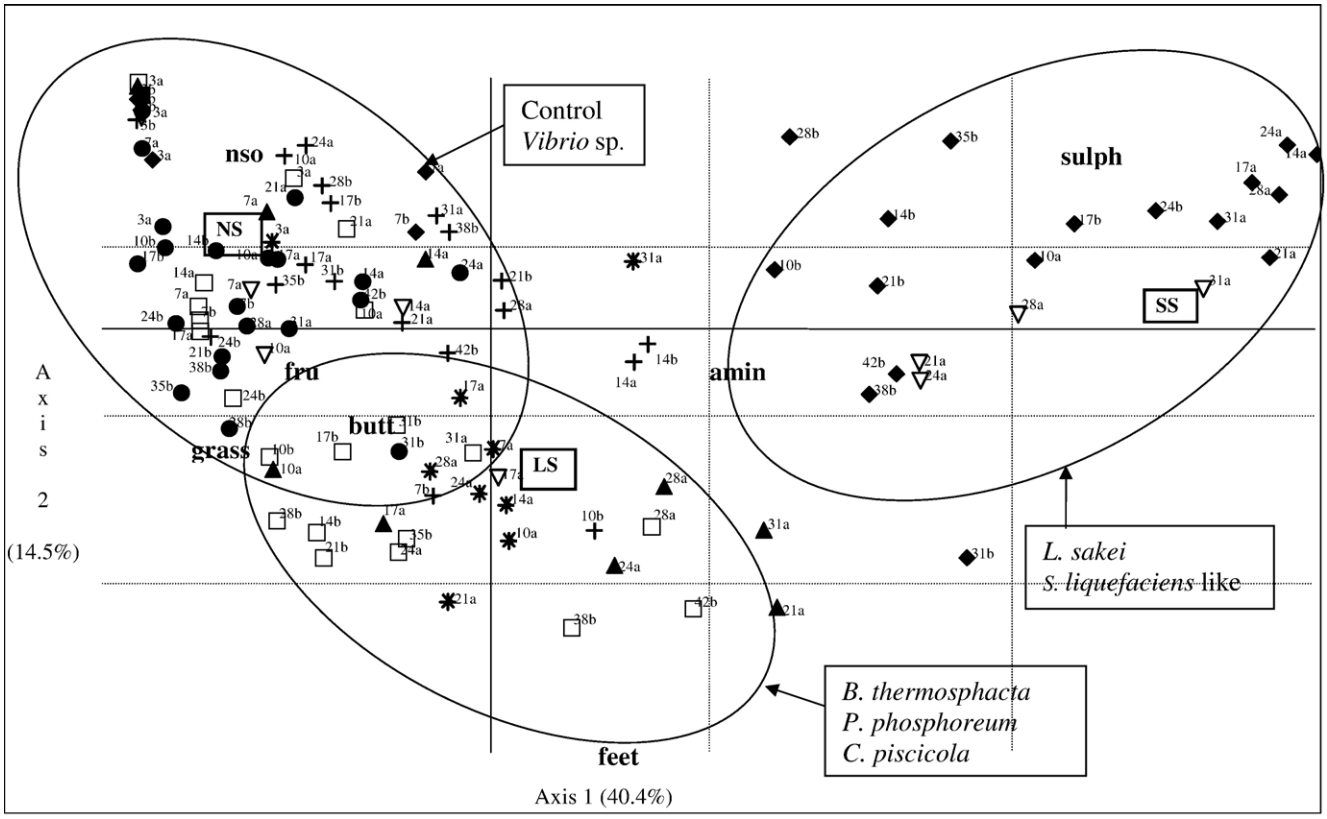

Fig. 3. Simultaneous loading of pure cultures, sensory descriptors and spoilage levels on the plane 1–2 of correspondence factorial analysis (CFA). Numbers express duration of storage (in days); a: first trial, b: second trial. (NS): Non-spoiled; (LS): lightly spoiled; (SS): strongly spoiled. (●): Control; (▲): *Brochothrix thermosphacta*; (□): *Carnobacterium piscicola*; (+): *Vibrio* sp; (*): *Photobacterium phosphoreum*; (▽): *Serratia liquefaciens*-like; (♦): *Lactobacillus sakei*.

lightly spoiled samples (lower part) were discriminated on the second axis (14.5% of the information). Three different evolution patterns were observed according to the strains. Control samples were located in the area of non-spoiled samples with no specific odour apart from smoke and salmon notes, except for a slight grassy note at the end of the storage period. *Vibrio* sp. and *C. piscicola* had characteristics similar to the control samples and presented a limited spoilage level during storage. *Vibrio* sp. was mainly classified in the non-spoiled group, even though a light amine note was detected, whereas *C. piscicola* presented grassy and fruity notes and samples were identified as lightly spoiled after three weeks of storage. *P. phosphoreum* and *B. thermosphacta* constituted the second group of bacteria. These strains developed some notes of spoilage during storage and reached a level of light spoilage more rapidly than the previous group. Their main odour characteristics were amine and feet notes. The third group identified according to the spoilage intensity included *L. sakei* and *S. liquefaciens*-like. These two bacteria were the most spoiling micro-organisms and were classified, very early, in the strong spoilage category. After three weeks of storage for *S. liquefaciens*-like and only ten days for *L. sakei*, samples were rejected by the panel. At this moment, more than 50% of the panelists classified samples in the category "strong spoilage". Odours were described as amine and sulphur notes.

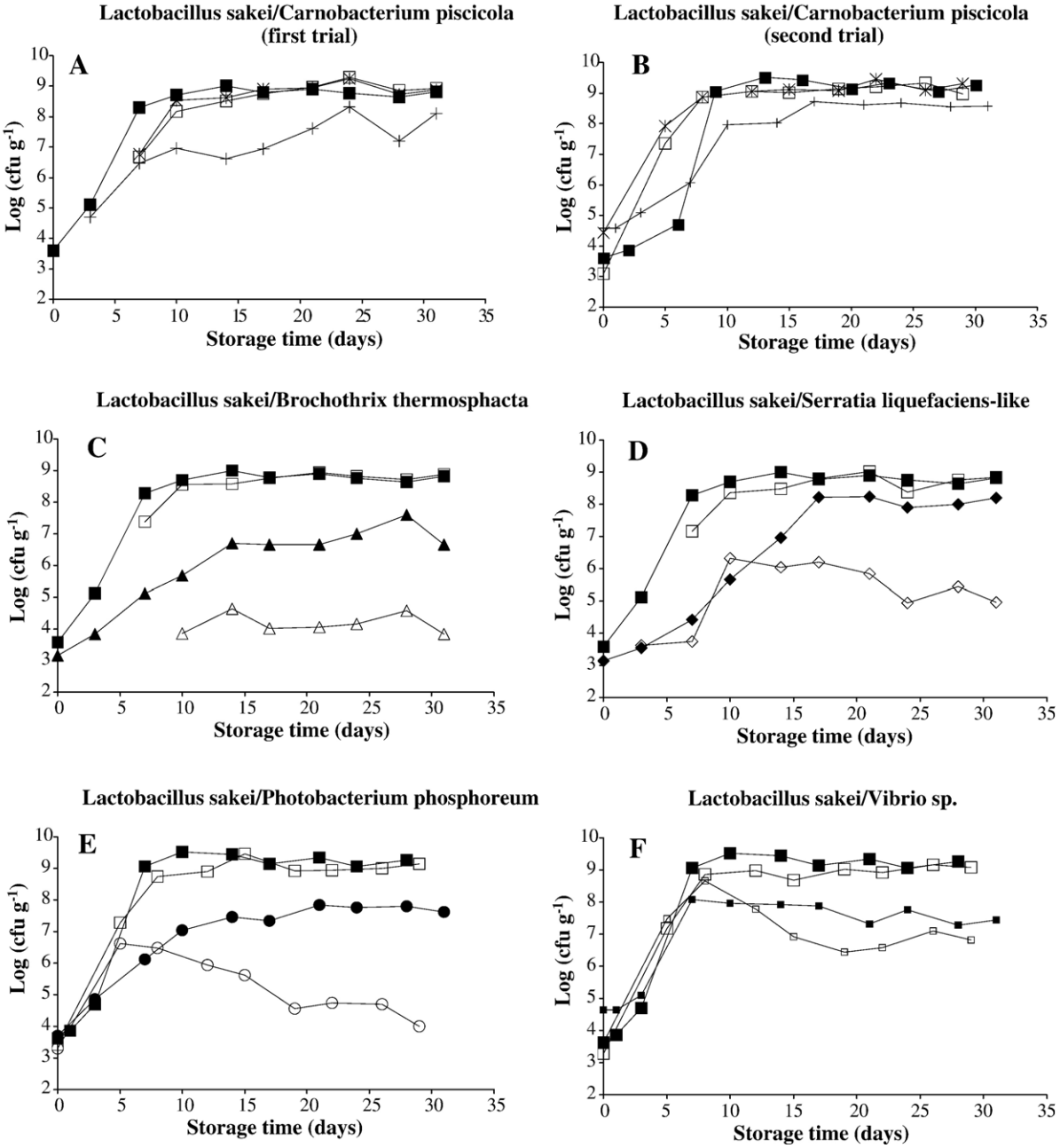

Fig. 4. Growth of the different bacterial groups in pure and mixed cultures with *Lactobacillus sakei* inoculated in vacuum-packed cold-smoked salmon stored at 8 °C. (■, □): *Lactobacillus sakei*; (◆, ◇): *Serratia liquefaciens*-like; (▲, △): *Brochothrix thermosphacta*; (●, ○) : *Photobacterium phosphoreum*; (■, □): *Vibrio* sp.; (full symbol: pure culture; empty symbol: mixed culture); (+): *Carnobacterium piscicola* (pure culture); (*): *Carnobacterium piscicola*+*Lactobacillus sakei*.

3.2. Mixed cultures

3.2.1. Bacterial kinetics

3.2.1.1. Combination L. sakei/other species. Fig. 4 presents the growth kinetics of *L. sakei* when inoculated in mixed cultures with respectively *C. piscicola*, *B. thermosphacta*, *S. liquefaciens*-like, *P. phosphoreum* and *Vibrio* sp. Growth of *L. sakei* strains did not seem to be affected by the other co-inoculated strains in comparison with growth in pure culture. Conversely, growth of *B. thermosphacta*, *S. liquefaciens*-like, *P. phosphoreum* and *Vibrio* sp. was clearly inhibited by *L. sakei.* It was not possible to know the specific growth of *C. piscicola* because an efficient selective medium was not available. A Cresol Red Thallium Acetate Sucrose Inulin medium proposed by Wasney et al. (2001) was unsuccessfully tested (this medium underestimated the number of Carnobacteria in pure culture when compared to a less selective medium such as Elliker, data not shown). The difference in growth level between pure and mixed culture mostly occurred after 15 days of storage when *L. sakei* had reached its maximum level. The differential was about 3 log except for *Vibrio* sp., which showed a weaker inhibition of growth (around 1 log).

3.2.1.2. Combination C. piscicola/other species. Fig. 5 represents the growth kinetics of the different bacterial groups in combination with *C. piscicola*. *B. thermosphacta* growth was not affected by the presence of *C. piscicola* (Fig. 5A) whereas a weak inhibition (1 log) was observed for *S. liquefaciens*-like and *P. phosphoreum* and less clearly for *Vibrio* sp. On the other hand, the growth of *C. piscicola* seemed to be lightly enhanced by the presence of *B. thermosphacta*, *P. phosphoreum* and *Vibrio* sp. but unchanged with *S. liquefaciens*-like.

3.2.2. Chemical changes

3.2.2.1. TVBN production — combination L. sakei/other species. When inoculated with non-TVBN-producing strains *C. piscicola* or *B. thermosphacta*, *L. sakei* produced TVBN at approximately the same level as in pure culture (55 mg-N 100 g^{-1}). In pure culture, the amount of TVBN generated by *S. liquefaciens*-like, *P. phosphoreum* and *Vibrio* sp. was in the same range as *L. sakei* (40–55 mg-N 100 g^{-1}). This level was hardly reached in the mixed culture *L. sakei*/*S. liquefaciens*-like (40 mg-N 100 g^{-1}) but was much higher in *L. sakei*/*Vibrio* sp. (60 mg-N 100 g^{-1}). TVBN production generated by the mixed culture *L. sakei*/*P. phosphoreum* followed the same pattern as *P. phosphoreum* in pure culture.

3.2.2.2. TVBN production — combination C. piscicola/other species. *C. piscicola* strains used in our study did not produce TVBN in pure culture. In combination with another non-TVBN-

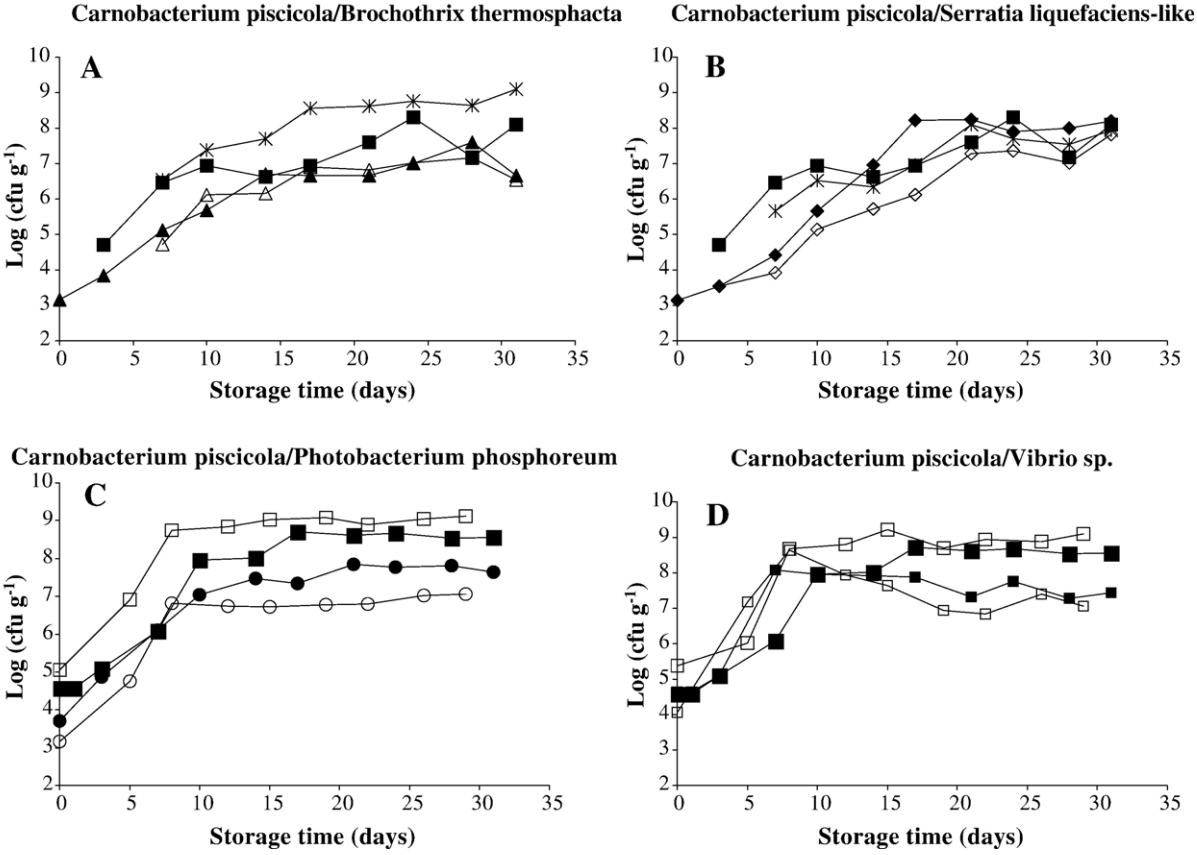

Fig. 5. Growth of the different bacterial groups in pure and mixed cultures with *Carnobacterium piscicola* inoculated in vacuum-packed cold-smoked salmon stored at 8 °C. (♦, ◇): *Serratia liquefaciens*-like; (▲, △): *Brochothrix thermosphacta*; (●, ○): *Photobacterium phosphoreum*; (■, □): *Vibrio* sp.; (■, □): *Carnobacterium piscicola*; (full symbol: pure culture; empty symbol: mixed culture); (*): *Carnobacterium piscicola*+*Lactobacillus sakei*.

producing species (*B. thermosphacta*), a significant amount was found (40 mg-N 100 g^{-1}) only at the end of storage. When inoculated with TVBN-producing species in pure culture (*S. liquefaciens*-like, *P. phosphoreum* and *Vibrio* sp.), 3 situations could be observed: (i) the final level in mixed culture remained unchanged as in pure culture (*P. phosphoreum*, *L. sakei* whose results have been described previously), (ii) the final level was higher (56 mg-N 100 g^{-1}) (*Vibrio* sp.), (iii) the final level was lower (27 mg-N 100 g^{-1}) (*S. liquefaciens*-like).

3.2.2.3. TMA production and pH changes. Production of TMA by *S. liquefaciens*-like was inhibited in mixed cultures with *L. sakei* or *C. piscicola* which are non-TMA-producing in pure cultures. The amount of TMA generated by *Vibrio* sp. was identical in pure culture and in mixed cultures with the two LAB species. *P. phosphoreum* produced the same level of TMA in pure culture and in mixed culture with *C. piscicola* (16 mg-N 100 g^{-1}) but this level was reduced in mixed culture with *L. sakei* (18 mg-N 100 g^{-1} versus 23 mg-N 100 g^{-1}) pH evolution in mixed cultures containing *L. sakei* showed the same pattern as *L. sakei* in pure culture i.e. a slight acidification (data not shown). For mixed cultures with *C. piscicola*, no change of pH was noted compared to the pure cultures except for the combination with *B. thermosphacta* where the pH dropped (from 6.10 to 5.70) at the end of the storage period.

3.2.3. Sensory changes

CFA performed on the frequencies of perception of the sensory characteristics and the three levels of spoilage, obtained for the mixed cultures, gave the same kind of representation as for pure cultures. The first axis of Fig. 6 allowed the spoilage evolution of each association of bacteria to be followed, from non-spoiled samples to strongly spoiled samples. However, new developments regarding the spoilage during storage at +8 °C appeared in some cases, when strains belonging to different species were associated together. When the most spoiling strains were simultaneously in culture (*L. sakei*/*S. liquefaciens*-like), an interaction was observed regarding the spoilage level since the classification in the strong spoilage group was delayed. Samples were only classified in the stronger spoilage group after four weeks whereas *L. sakei* in pure culture reached the same level in ten days and *S. liquefaciens*-like in three weeks. The association of the spoiler strains of *L. sakei* with the moderate spoilage activity strains, such as *P. phosphoreum* and *B. thermosphacta*, the very lightly spoiling strains of *C. piscicola* or the non-spoiler strains of *Vibrio* sp. led to two different evolution patterns: either a decrease in the spoilage power or an effect similar to the strongest spoiler strain. Cultures *L. sakei*/*C. piscicola*, *L. sakei*/*P. phosphoreum* or *L. sakei*/*B. thermosphacta* reduced the effect of *L. sakei* on spoilage, delaying both the appearance of spoilage descriptors and the moment when samples were detected as strongly spoiled. In contrast, samples inoculated with the

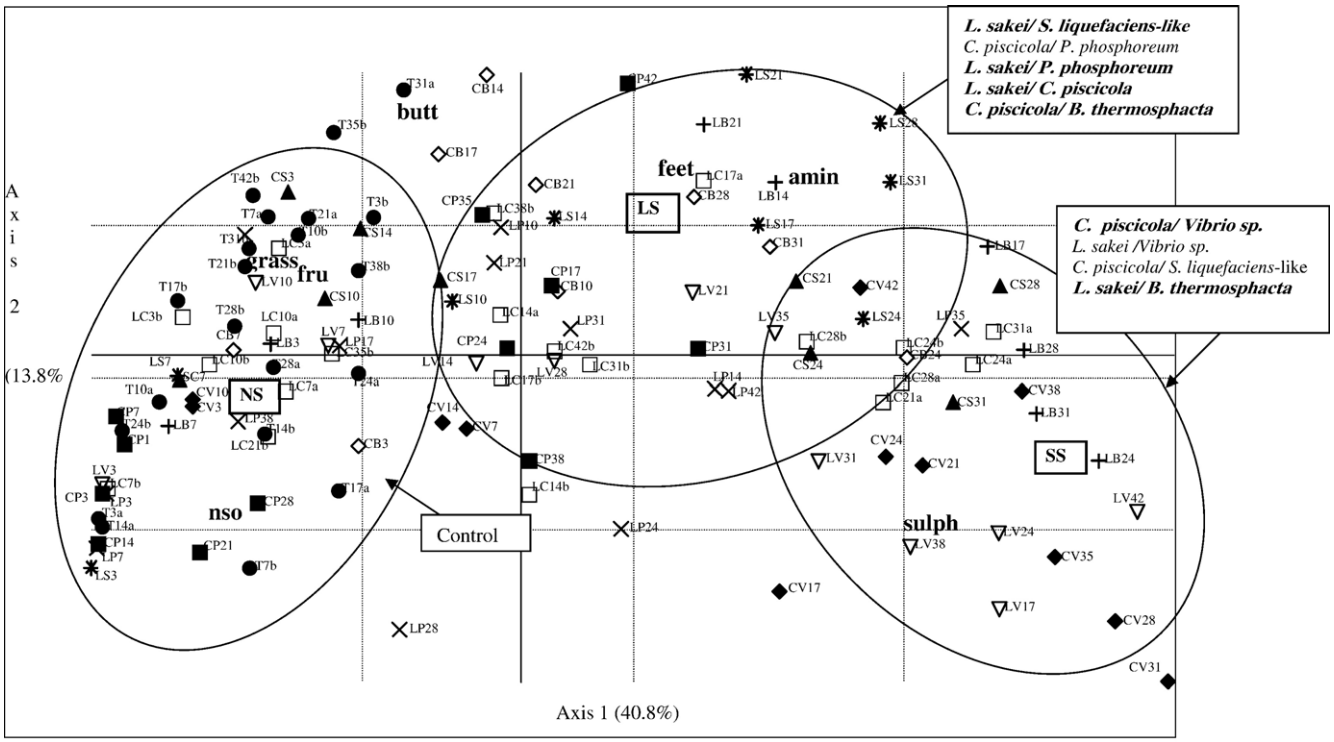

Fig. 6. Simultaneous loading of mixed cultures, sensory descriptors and spoilage levels on the plane 1–2 of correspondence factorial analysis (CFA). Numbers express duration of storage (in days); a: first trial, b: second trial. (NS): non-spoiled; (LS): lightly spoiled; (SS): strongly spoiled. Mixed cultures showing an interaction are expressed in bold characters. (●) : Control; (□): *Lactobacillus sakei*/*Carnobacterium piscicola*; (+): *Lactobacillus sakei*/*Brochothrix thermosphacta*; (*): *Lactobacillus sakei*/*Serratia liquefaciens*-like; (×): *Lactobacillus sakei*/*Photobacterium phosphoreum*; (▽): *Lactobacillus sakei*/*Vibrio* sp.; (◇): *Carnobacterium piscicola*/*Brochothrix thermosphacta*; (▲): *Carnobacterium piscicola*/*Serratia liquefaciens*-like; (■): *Carnobacterium piscicola*/*Photobacterium phosphoreum*; (◆): *Carnobacterium piscicola*/*Vibrio* sp.

associations *L. sakei*/*Vibrio* sp. were as spoiled as when inoculated with *L. sakei* alone. Samples kept the same strong spoilage characteristics, described as sulphur and amine notes. When two non-spoiler, very light or moderate spoiler strains were associated, such as *C. piscicola*/*Vibrio* sp., *C. piscicola*/*B. thermosphacta* or *C. piscicola*/*P. phosphoreum,* the spoilage either followed the same evolution during the storage as in pure culture (*C. piscicola*/*P. phosphoreum*) or, conversely, increased as observed with the mixed culture, *C. piscicola*/*Vibrio* sp. and the culture *C. piscicola*/*B. thermosphacta*. In this latter case, samples became highly spoiled after 24 days whereas they never reached this level of spoilage in pure culture. When *C. piscicola* was co-inoculated with the strong spoiler strains of *S. liquefaciens*-like, samples exhibit the same sensory characteristics as when inoculated with *S. liquefaciens*-like alone.

4. Discussion

Some results obtained in this study confirm the findings of previous works performed in the field of CSS quality, shelf life and spoilage. The rapid growth of *L. sakei* strains on CSS has already been reported in previous studies (Jorgensen et al., 2000a; Joffraud et al., 2001; Stohr et al., 2001). The five strains of each group had shown an heterogeneous spoilage potential when individually tested by Stohr et al. (2001). In this study, sensory changes assigned to each group and resulting from the interaction of the five mixed strains corresponded to the spoilage potential of the strongest spoiling strains except for *B. thermospacta* and *P. phosphoreum*, classified as weak spoiling group in our study whereas some strains were found to be strongly spoiling in the previous study. For *S. liquefaciens*-like and *L. sakei*, the inoculated products were rejected just after the exponential stage and at the beginning of the stationary phase of the growth curves. This behaviour was described by Gram and Huss (1996) as typical of SSOs. These findings are not completely in agreement with those of Jorgensen et al. (2000a). Based on biogenic amines production, shown to correlate closely with sensory scores of sliced CSS (Jorgensen et al., 2000b), they concluded that *P. phosphoreum* was the main specific spoilage organism in CSS whereas *S. liquefaciens*-like, although part of the spoilage microflora, was not able individually to deteriorate the product. The production of metabolites leading to spoilage has been shown to be often strain-dependent (Jorgensen et al., 2000a, Stohr et al., 2001) and can explain such a discrepancy. The spoilage potential of *Vibrio* sp. in CSS had never been tested previously. Although this species is suspected to be frequently present in this product (Bourrain et al., 2000; Cardinal et al., 2004), it was not responsible for any off-odours' production. Almost similar results were obtained for *C. piscicola*, which confirm the potential interest of this LAB species for a biopreservation application, currently tested by different research teams (Yamazaki et al., 2003; Brillet et al., 2004; Nilson et al., 2005; Vaz-Velho et al., 2005). Results from pure cultures also allowed us to confirm that TVBN alone is not a good indicator for estimation of the sensory spoilage. Indeed, *Vibrio* sp. produced a significant amount of TVBN (40–45 mg-N 100 g^{-1}) although no off-odour was detected. TVBN had been shown to be a useful quality index for naturally contaminated CSS only when associated to the count of *Lactobacillus* on Rogosa agar (Leroi et al., 2001).

This work has displayed some evidence of interaction between inoculated strains in terms of growth patterns, chemical changes and sensory evolution.

Inhibition of *P. phosphoreum* and Enterobacteriaceae co-inoculated with *L. sakei* has already been mentioned by Jorgensen et al. (2000a). TVBN concentrations in co-cultures involving *L. sakei* strains and TVBN-producing bacteria (*Vibrio* sp., *P. phosphoreum* and *S. liquefaciens*-like) were lower than the addition of TVBN produced by both the single cultures. This observation cannot be explained only by the growth inhibition of the co-inoculated strains by *L. sakei*. Indeed, except for *L. sakei*/*S. liquefaciens*-like, TVBN production observed in the mixed culture did not correspond to the addition of the predicted TVBN production by the two groups when using the yield factors (data not shown). Production was higher than that predicted for *P. phosphoreum*/*L.sakei*, suggesting a stimulation of the TVBN production. On the other hand, TVBN production in the mixed culture *L. sakei*/*Vibrio* sp. was lower than that predicted, suggesting an inhibition of the TVBN production. The delay observed in the appearance of the spoilage characteristics in most mixed cultures with *L. sakei*, in particular samples inoculated with *L. sakei*/*S. liquefaciens*-like which were the greatest spoilers when cultivated alone, was not in agreement with the findings of Jorgensen et al. (2000a). Based on biogenic amine production, they showed, on the contrary, that *L. sakei* enhanced the spoilage activity of *S. liquefaciens*-like.

S. liquefaciens-like and *P. phosphoreum* were weakly inhibited by the presence of *C. piscicola* and a limited suppression of the cell count within 1 log was observed. Jorgensen et al. (2000a) have also observed an inhibition of *P. phosphoreum* and Enterobacteriaceae by *C. divergens*. A slight

Table 3
Spoilage potential of bacterial groups inoculated in pure (diagonal) or mixed culture in cold-smoked salmon and stored at 8 °C under vacuum

Bacterial group	*L. sakei*	*C. piscicola*	*B. thermosphacta*	*Vibrio* sp.	*P. phosphoreum*	*S. liquefaciens*-like
L. sakei	++	+ (↓)	++ (↓)	++ (→)	+ (↓)	+ (↓)
C. piscicola		±	+ (↑)	++ (↑)	+ (→)	++ (→)
B. thermosphacta			+			
Vibrio sp.				–		
P. phosphoreum					+	
S. liquefaciens-like						++

(−): No spoiling; (±): very light spoiling; (+): light spoiling; (++): strong spoiling potential. (↑), (↓), and (→) indicate that spoilage potential is enhanced, decreased or similar, respectively, when compared to the highest spoiler bacterial group of the association.

production of TVBN was observed when *C. piscicola* and *B. thermosphacta* were inoculated together. The TVBN production in the association with *Vibrio* sp. was also higher than that in the pure culture. This is in concordance with the observation that the spoilage potential of *C. piscicola* was increased when associated together with *Vibrio* sp. or *B. thermosphacta*. In a previous study, Brillet et al. (2005) had shown that *C. piscicola* (different strains than in the present work) did not produce TVBN when inoculated in pure culture in sterile CSS blocks; however, when inoculated in naturally contaminated products, TVBN production was significantly enhanced. This phenomenon could be explained by the presence of *Brochothrix* and/or *Vibrio* in the product. The pH drop in samples with *C. piscicola*/*B. thermosphacta* was surprising as none of the strains acidified the product in pure culture and as a stimulation of the TVBN production was observed which should have had the opposite effect.

An understanding of the microbial ecology is essential for the development of preservation techniques aimed at reducing excessive food losses. Identifying the specific spoilage organisms of lightly preserved fish products such as CSS has proved difficult (Gram et al., 2002) probably because different groups of bacteria are involved under different conditions. As reviewed by Gram et al. (2002), substrate base, nutrient composition, chemical and physical parameters are very important for the selection, growth and activity of spoilage micro-organisms. Moreover, microbial food spoilage occurs when micro-organisms reach a high level (10^7–10^9 cfu g^{-1}) and at this level the micro-organisms are thought to interact by influencing the growth of one another (Gram et al., 2002; Malakar et al., 2003). Thus, interactive behaviour between the micro-organisms determines selection and metabolism and subsequently renders the understanding of spoilage mechanisms even more difficult. Malakar et al. (1999) stated that, in non-fermented products, microbial interactions could be neglected because concentrations of micro-organisms are generally low and a high level indicates that spoilage has already occurred. The case of CSS seems to be different; indeed, it has been shown many times that a high level of micro-organisms does not always lead to spoilage (Truelstrup Hansen et al., 1996; Truelstrup Hansen and Huss, 1998; Truelstrup Hansen et al., 1998; Leroi and Joffraud, 2000a; Leroi et al., 2001). Thus, interactions between the main groups of micro-organisms developing in CSS should be taken into account to better explain the spoilage process and to predict the shelf life and safety of the products.

The aim of our study was to point out the main interactions between the major components of the bacterial flora and not to elucidate the mechanisms of these interactions. However, some hypotheses can be suggested. Several types of interaction have been described in food ecosystems, including antagonistic or cooperative behaviour (Gram et al., 2002). Competition for nutrients is considered to be one of the major causes of interaction amongst bacteria and may select for the organisms best capable of using the limiting compounds. Such mechanisms could be involved in the mixed cultures *C. piscicola/S. liquefaciens*-like and *C. piscicola/P. phosphoreum*. Changes in environmental conditions, such as lowering the pH, can be a powerful way to create a selective advantage and thus to antagonize other bacteria. This is probably the case of co-cultures involving *L. sakei* that decreased the pH from 6.2 to 5.6–5.7 leading to the inhibition of the co-inoculated species. On the other hand, cooperative behaviour, where the growth or a particular metabolism of one organism is favoured by the growth of another organism, seemed to be the cause of the growth enhancement of *C. piscicola* co-inoculated with *B. thermosphacta*, *P. phosphoreum* or *Vibrio* sp. This behaviour could be compared to metabiosis (interspecies microbial metabolism of arginine) described by Jorgensen et al. (2000a) where production of putrescine was strongly enhanced when cultures of *S. liquefaciens*-like or *Hafnia alvei* were grown with *C. divergens* or *L. sakei*.

The present study has contributed to characterise the spoilage potential of different bacterial associations and the three possible scenarios (enhancement, decrease or no effect) have been observed and are summarised in Table 3. Those results emphasize that the spoilage of CSS is very difficult to predict. Moreover, results have been obtained with bacterial groups co-inoculated in pair, in equicellular concentrations, but the spoilage process could be more complex in naturally contaminated products. Further investigations are needed to make progress in the understanding of antagonistic or cooperative interactions between the main bacterial species occurring in the spoilage flora of CSS. Knowledge of these interactions is of great importance for the potential development of novel preservation techniques and for the predictive microbiology of foods like CSS in which a mixed flora develops during storage and several microbial species contribute to spoilage.

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