

Project Report  
20 - 02



# Rannsóknastofnun fiskiðnaðarins

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**SPOILAGE AND SAFETY OF  
COLD-SMOKED FISH –**  
Topic 3: Development of biological  
control measures for *Listeria* spp. in  
the manufacture of cold-smoked fish

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<i>Titill / Title</i>	<b>Spoilage and Safety of Cold-Smoked Fish -</b> Topic 3: Development of biological control measures for <i>Listeria</i> spp. in the manufacture of cold-smoked fish (FAIR CT96-1207)		
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<i>Ágrip á íslensku:</i>	<p>Markmið verkefnisins var þrjúþætt og skiptist það í 3 meginverkhætti:</p> <ol style="list-style-type: none"> <li>1. að ákvarða og þróa hlutlægt mat á skemmdum í kaldreyktum fiski þannig að auðveldara verði að koma upp innra eftirliti og gæðatryggingu við framleiðsluna;</li> <li>2. að ákvarða tíðni og smitleiðir <i>Listeria monocytogenes</i> í kaldreyktum fiski til að hægt verði að lágmarka áhættuna á smiti;</li> <li>3. að finna leiðir (náttúrulega rotvörn) til að halda <i>Listeria</i> í skefjum og tryggja þannig geymsluþol og öryggi vörunnar.</li> </ol> <p>Rf tók einungis þátt í seinni verkþáttum, en í þessari skýrslu er fjallað um möguleika á notkun náttúrulegrar rotvarnar til að stjórna/ hindra vöxt <i>Listeria</i> í kaldreyktum laxi.</p>		
<i>Lykilorð á íslensku:</i>	<i>náttúruleg rotvörn - kaldreyktur lax - Listeria - Carnobacterium - bakteríósín</i>		
<i>Summary in English:</i>	<p>This FAIR project on the spoilage and safety of cold-smoked fish was divided in 3 main topics which aimed at:</p> <ol style="list-style-type: none"> <li>1. establishing objective indices for determining the quality/ acceptability of cold-smoked fish products for effective quality assurance programmes, by understanding the mechanisms of spoilage;</li> <li>2. determining the degree of contamination of cold-smoked fish products in Europe with <i>Listeria monocytogenes</i> and to develop means to minimise this contamination; and</li> <li>3. establishing the efficacy and appropriate means of application, of selected strains of lactic acid bacteria (LAB) or their products, for inhibition/destruction of <i>L. monocytogenes</i> in cold-smoked fish products.</li> </ol> <p>IFL participated in the 2 latter topics. This report discusses the use of biopreservative techniques to control/ hinder the development of <i>Listeria</i> in cold-smoked salmon.</p>		
<i>English keywords:</i>	<i>biopreservation - cold-smoked salmon - Listeria - Carnobacterium - bacteriocin</i>		

## FAIR CT96-1207

### Spoilage and Safety of Cold-Smoked Fish

#### TOPIC 3: Development of biological measures for *Listeria* spp. in the manufacture of cold-smoked fish

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## Table of contents

Summary .....	3
Chapter 1 Introduction.....	6
Chapter 2 Materials and Methods.....	7
TASK 3A: Preliminary screening of inhibitor-producing LAB strains .....	8
TASK 3B: Secondary screening of inhibitor-producing LAB strains.....	9
TASK 3C: Inhibition studies on inoculated cold-smoked fish.....	10
TASK 3D: Control using antimicrobial agents .....	12
TASK 3E: Control using combinations of selected LAB and antimicrobial agents	13
Chapter 3 Results.....	15
TASK 3A: Preliminary screening of inhibitor-producing LAB strains .....	15
TASK 3B: Secondary screening of inhibitor-producing LAB strains.....	17
TASK 3C: Inhibition studies on inoculated cold-smoked fish.....	20
TASK 3D: Control using antimicrobial agents .....	24
TASK 3E: Control using combinations of selected LAB and antimicrobial agents	29
Chapter 4 Discussion .....	36
Chapter 5 References.....	44

## Summary

Cold-smoked fish is a ready-to-eat commodity which has been found to be contaminated by *L. monocytogenes*. This organism is able to grow at refrigerated temperatures and its control is therefore essential to ensure the safety of consumers. The prevalence of *L. monocytogenes* in such products has been reported to range from low levels (Guyer & Jemmi, 1990; Dillon *et al.*, 1994) to much higher percentages, 11-34% (Rørvik *et al.*, 1995; Heinitz & Johnson, 1998; Jørgensen & Huss, 1998). This suggests that the technological parameters involved in the production of cold-smoked fish are not sufficient to hinder *Listeria* proliferation, especially in the case of post-processing contamination occurring later after smoking. This third part of the FAIR Project CT96-1207, Spoilage and Safety of Cold-Smoked Fish, aimed at establishing the efficacy and appropriate means of application, of selected strains of lactic acid bacteria (LAB) or their products, for inhibition of *L. monocytogenes* in cold-smoked fish products.

Initially, a preliminary screening of inhibitor-producing LAB strains was conducted by testing eight LAB strains, both commercial and previously owned isolates, for their inhibitory action towards *L. innocua* P1 and/or *L. monocytogenes* P1 in a simulated cold-smoked fish system (SCSFS). Only two *Carnobacterium piscicola* strains were found to be promising *Listeria* inhibitors at low temperatures (4 and 8°C). These two strains, CpV1 and SF644, were tested for their potential use as protective cultures against *Listeria* spp. in cold-smoked salmon (CSS). CpV1 strain had been previously found to be a bacteriocin-producer and inhibitory towards *L. monocytogenes* (Pilet *et al.*, 1995), while the inhibitory action produced by SF644 strain was confirmed in this study to be of proteinaceous nature as well (bacteriocin). However, bacteriocin production of CpV1 was found to be greater than that of SF644 when tested in SCSFS at 4 and 8°C. Both *Carnobacterium* strains were bacteriostatic to *L. innocua* P1, the effect lasting for about 2 weeks at 4°C but 6-9 days at 8°C, after which slow growth occurred. Interestingly, *L. monocytogenes* P1 was found to be more sensitive than *L. innocua* P1 to CpV1 which had a bactericidal effect at 4°C, but was bacteriostatic at 8°C. However, the effect was found to be bactericidal when tested against a lower inoculation level of *L. monocytogenes* P1 (log 2.5/ml) at 8°C. Otherwise, monocultures of *Listeria* strains were shown to proliferate steadily in SCSFS at 4°C, but much faster at 8°C.

The ability of these *Carnobacterium* strains to inhibit or delay *Listeria* growth at low temperatures in a smoked salmon extract containing 4% (w/w) salt (SCSFS) indicated their possible use as protective cultures. However, it was felt necessary to verify whether their addition at high levels (log 4-5/g) to newly processed smoked salmon would accelerate the spoilage process. Sensory evaluation (smell, appearance and texture) of the inoculated products compared to a control (uninoculated smoked salmon) confirmed that these strains did not promote spoilage.

A challenge study of cold-smoked salmon, inoculated with *C. piscicola* strains and *L. monocytogenes* P1, demonstrated the ability of the *Carnobacterium* strains, especially CpV1, to grow in such a product at low temperatures (4 and 8°C) and delay the proliferation of *L. monocytogenes* P1 in spite of its rather high inoculation level (log 2.5/g). However, bacteriocin production was apparently lesser than that seen in SCSFS or it was undetected by the method used due to binding or inactivation in CSS. This probably explains the reduced antilisterial effect of the strains in CSS as compared to SCSFS. However, CpV1 was found to be more efficient than SF644 in delaying the growth of *L. monocytogenes* P1 in CSS. *Listeria* was otherwise found to proliferate well and at a similar rate when inoculated alone into SCSFS and on the surface of CSS. CpV1 could be a promising protective culture, but did not

provide a total control of high levels (log 2.5/g) of *L. monocytogenes* P1 at chilled temperatures in cold-smoked salmon.

In the search of an antilisterial system, various antimicrobial compounds were assessed in SCSFS at low temperatures. Nisin A (nisaplin) was listericidal at 4 and 8°C while nisin Z, its variant, only provided a short bacteriostatic effect towards *L. monocytogenes* P1. Growth of *L. monocytogenes* P1 was reduced in SCSFS acidified by organic acids (pH 5.4), citric acid being more efficient than lactic acid at 8°C. In fact, the bacteriostatic effect observed for the organic acids lasted longer than that seen with nisin Z. However, acidification of the nisin Z treatment to pH 5.4, using either lactic or citric acid, led to a better control of the pathogen at both temperatures than when using the antimicrobial compounds separately. The effect of acidification on the sensory quality of CSS was evaluated and it was found possible to lower the pH down to 5.6, using either citric or lactic acid, without interfering with its quality. The antilisterial effect of ALTA™ 2341 (ALTA) and FARGO™ 23 (FARGO) at 3 concentration levels (0.25, 0.5 and 1% w/v) on growth of *L. monocytogenes* P1 was assessed in SCSFS at 4°C. At the lower concentrations (0.25 and 0.5%) of FARGO, *Listeria* was suppressed for 1-2 weeks after which steady growth occurred. The other levels of FARGO and ALTA were listericidal throughout storage (28 days), with the exception of 1% ALTA for which the effect lasted only for 3 weeks. Interestingly, the antilisterial effect observed and the bacteriocin activity measured in the treatments during this trial did not agree, i.e. increased antilisterial effect did not imply a higher bacteriocin activity as it would be expected. But generally, a drop in activity was observed with increasing incubation (storage) time.

Based on these findings, further testing was done in SCSFS where the use of combined biopreservatives to suppress *Listeria* growth was evaluated. It was aimed at finding a combined system which would stimulate the bacteriocin production of *C. piscicola* strains (CpV1 and SF644) and/or act synergistically. Acidification by organic acids (lactic and citric acid) had a negative effect on growth and bacteriocin production of both *C. piscicola* strains, leading to a reduced antilisterial effect, even at pH 5.8. Growth and bacteriocin production of the *C. piscicola* strains in presence of ALTA or FARGO (0.25, 0.5 and 1% w/v) was evaluated as well as their effect on *L. monocytogenes* P1 proliferation in SCSFS at low temperatures. The combination with either compound was profitable to the *C. piscicola* strains, rendering their effect listericidal at both temperatures, the lowest concentration (0.25%) being just as effective as 1%. The addition of the compounds did not affect the growth of CpV1 as it did for SF644. However, the compounds stimulated bacteriocin production for both strains, with earlier detection of bacteriocin and generally at lower cell counts. Also, enhanced production was observed as storage progressed, except for CpV1 at 4°C. Overall, nisin A, ALTA and FARGO produced an antilisterial effect in the simulated CSS system and had to be tested in the product itself for confirmation.

A second challenge study of CSS was conducted to evaluate the use of nisin A (800 IU/g), ALTA (0.5 and 1% w/w) as well as the combination of ALTA and CpV1 to control the growth of a *L. monocytogenes* cocktail in CSS, as compared to the use of CpV1 alone at 4 and 8°C. *Listeria* proliferation in the control was found to be suppressed (8°C) or slowed down (4°C) from day 14, coinciding with a high level of an indigenous LAB microflora and the detection of an inhibition factor of less than 400 AU/g. This was in fact a good example of a naturally occurring inhibitory microflora in food products. Similarly to the previous CSS trial, the addition of CpV1 (log 5.6/g) to *Listeria* contaminated CSS only delayed the development of the *Listeria* cocktail. But this time, bacteriocin production was detected earlier, at both temperatures and in greater concentrations. This could be due to the presence of an indigenous and competitive LAB microflora that stimulated bacteriocin production of CpV1, especially at 8°C as evidenced by the higher bacteriocin titer. This led to a much slower growth of *Listeria* at 8°C than expected.

The use of the antimicrobial compounds, either ALTA or nisin A, in spiked CSS stored at 4°C retarded the growth of the *Listeria* cocktail, similarly to CpV1. However, their antilisterial effect was lesser at 8°C compared to that caused by CpV1. In fact, the effect of nisin was apparently lost during late storage at 8°C. Nisin delayed the development of the indigenous CSS microflora. On the other hand, ALTA did not apparently interfere with the natural microflora but seemed to have benefited of its inhibitory action on *Listeria*. The antilisterial effect of 0.5% ALTA was not lesser than that with the 1% concentration. Based on smelling of the samples, nisin and ALTA did not promote spoilage of CSS. The use of a combined system, including CpV1 and 0.5% or 1% ALTA, in spiked CSS provided a total control of *Listeria* as it suppressed the proliferation of the cocktail at 4°C, *Listeria* counts being at or below the detection level (8 CFU/g). At 8°C, the combined system required 1% ALTA to obtain an antilisterial effect greater than that seen with CpV1 alone. Enhancement of bacteriocin production of CpV1 due to the addition of ALTA, as reported in SCSFS trials, was only evidenced at 4°C with 0.5% ALTA. Hence, the increased inhibitory effect observed for the combined system (1% ALTA) which was not due to enhanced bacteriocin production must have been caused by a synergistic effect of both counterparts.

The selection of *L. monocytogenes* strains for the preparation of the cocktail was based on a study assessing the sensitivity of 30 strains, isolated from CSS products from 5 European countries, towards 6 antimicrobial compounds (nisin A and Z, ALTA, FARGO, CpV1 and SF644 supernatants). The results were compared to the sensitivity of the Portuguese strain, *L. monocytogenes* P1, used during the project. The strains had been biotyped by partner 01 and belonged to 8 different biotypes. It was found that sensitivity to the compounds was more related to the origin of the strains than to their biotype. Portuguese strains were the most sensitive, *L. monocytogenes* P1 being very sensitive to all compounds, while Danish strains were the most tolerant. Thought, variations in strain sensitivity/tolerance to the compounds were found. For instance, there were least variations for the nisin compounds, but most for the CpV1 and SF644 supernatants. These variations must be related to the mechanism of action of the antilisterial compounds as well as to the inherent characteristics of the different *L. monocytogenes* strains tested. The *L. monocytogenes* cocktail included 5 strains, one from each country. The main criteria for selection was an average sensitivity to all compounds when compared to all the other strains.

Finally, it can be concluded that CpV1 can be used as a protective culture to delay *Listeria* proliferation in CSS. However, the antilisterial effect obtained will depend on factors such as presence of an indigenous LAB microflora and the importance of its load, the *Listeria* contamination level and its sensitivity to the CpV1 bacteriocin as well as the temperature profile of the product during storage. The use of the commercial antimicrobial compounds ALTA and nisin A did not provide a better inhibition than that seen with CpV1, especially at 8°C. In fact, the use of nisin A should be avoided if the product is to be temperature-abused (> 4-5°C) which is likely to occur in the course of its shelf life. Total control of *Listeria* in CSS was only achieved at 4°C by combining ALTA and CpV1. Therefore, our findings demonstrated that due to the intrinsic and extrinsic parameters of CSS, the use of a combined antimicrobial system appears to be necessary to ensure a proper control of *Listeria* in such products.

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## Chapter 1 Introduction

This FAIR project on the spoilage and safety of cold-smoked fish aimed at:

1. establishing objective indices for determining the quality/acceptability of cold-smoked fish products for effective quality assurance programmes, by understanding the mechanisms of spoilage;

2. determining the degree of contamination of cold-smoked fish products in Europe with *Listeria monocytogenes* and to develop means to minimise this contamination; and

3. establishing the efficacy and appropriate means of application, of selected strains of lactic acid bacteria (LAB) or their products, for inhibition/destruction of *L. monocytogenes* in cold-smoked fish products.

Three main topics had been defined:

**Topic 1:** Shelf-life and spoilage of cold-smoked fish products

**Topic 2:** Contamination with *L. monocytogenes*

**Topic 3:** Development of biological control measures for *Listeria* spp. in the manufacture of cold-smoked fish

The participation of Icelandic Fisheries Laboratories (IFL), i.e. participant 05, implied work in Topics 2 and 3 over a 38-month period, and included tasks 2A, 2C, 3A, 3B, 3C, 3D, 3E and 3F.

**TASK 2A:** Isolation of *Listeria* spp. (mo 0 to 18)

**TASK 2C:** Sanitation procedures & *Listeria* ( mo 12 to 32)

**TASK 3A:** Preliminary screening of inhibitor-producing LAB strains (mo 0 to 9)

**TASK 3B:** Secondary screening of inhibitor-producing LAB strains (mo 6 to 18)

**TASK 3C:** Inhibition studies on inoculated cold-smoked fish (mo 12 to 24)

**TASK 3D:** Control using antimicrobial agents (mo 18 to 36)

**TASK 3E:** Control using combinations of selected LAB and antimicrobial agents (mo 24 to 32)

**TASK 3F:** Resistance of *L. monocytogenes* to LAB and natural inhibitory agents (mo 24 to 36)

However, only the work relating to Topic 3 will be reported here.



## Chapter 2            Materials and Methods

**Media and ingredients.** The following media and ingredients were used: Bacto Agar, Brain Heart Infusion (BHI) broth, Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA), Yeast Extract (YE), Peptone, Gelatin, Proteose Peptone and Beef Extract from Difco; Peptonized Milk, Listeria Selective Agar Base-Oxford formulation with supplement SR140E (LOX) and Maximum Recovery Diluent (MRD) from Oxoid; sodium hydroxide (NaOH), sodium chloride (NaCl), dipotassium phosphate ( $K_2HPO_4$ ), D(+)-glucose, magnesium sulfate ( $MgSO_4 \cdot 7 H_2O$ ), manganese sulfate ( $MnSO_4 \cdot H_2O$ ) and lactic acid (90% solution) from Merck; ferric ammonium citrate, Tween 80, polymyxin B sulphate, actidione (cycloheximide), sodium nitrite ( $NaNO_2$ ) and nisaplin (2.5% nisin with denaturated milk solids) from Sigma; ALTA™ 2341 (ALTA) and FARGO™ 23 (FARGO) kindly provided by Quest International; citric acid (food grade).

TSB-YE and TSA-YE were prepared by adding 0.6% YE to TSB and TSA, respectively. Nitrite Actidione Polymyxin Agar (NAP) was slightly modified from Davidson & Cronin (1973): peptone (10g per liter), peptonized milk (10g), yeast extract (10g), glucose (7.5g), beef extract (2.5g),  $MgSO_4 \cdot 7 H_2O$  (0.58g),  $MnSO_4 \cdot H_2O$  (0.04g), Tween 80 (1g), agar (15g); pH adjusted to 6.1, medium autoclaved and supplemented with 6% sodium nitrite (10 ml), 0.1% (w/v) actidione (10 ml) and 0.03% polymyxin B sulphate (10 ml) prior to pouring. Modified Long & Hammer's medium (LH) was prepared according to van Spreekens (1972) with 1% NaCl, and Butterfields' buffer as described by Vanderzant & Splittstoesser (1992).

**Bacterial cultures.** *Lactococcus lactis* SIK-83 (SIK, Sweden; R. Anderson), *Carnobacterium piscicola* UI49 (Science Institute of University of Iceland, Á. Gudmundsdóttir), and *Lactobacillus bavaricus* MI401 (Royal Veterinary & Agriculture University, Denmark; J. Josephsen & A.G. Larsen) had been used by IFL in a previous nordic biopreservation project.

*Carnobacterium piscicola* V1 (CpV1) was kindly provided by Xavier Dousset (ENITIAA, Nantes, France) and *C. piscicola* SF644 (SF644) by Françoise Leroi and Jean-Jacques Joffraud (IFREMER, Nantes, France). These Carnobacteria strains of French origin were isolated from trout intestine and cold-smoked salmon, respectively. *Listeria innocua* P1 and *L. monocytogenes* P1 were obtained from Manuela vaz Velho (Escola Superior de Biotecnologia, Porto, Portugal) and had been isolated from cold-smoked salmon trout and salmon, respectively. Between experiments, these strains were maintained frozen (-18°C) with 20% (v/v) sterile glycerol in 1-ml aliquots in sterile vials. Working cultures of LAB strains were made by transferring the content of thawed vials to 3 ml BHI and incubating at 30°C overnight. Prior to storage trials, a second-step culture was done by transferring a loopful of the BHI culture to SCSFS and incubating at 22°C for 24h before it could be used for experiments. Stock cultures of *Listeria* strains were thawed, transferred to 3 ml of TSB-YE and grown overnight at 35°C. Similarly, a second-step culture was prepared in SCSFS prior to inhibition studies. *Listeria* strains were maintained on TSA-YE during the course of an experiment.

Three commercial strains, ALC 01 (*Lb. plantarum* usually used in cheese production), FishFlor® and N-8 (*Lc. lactis* for meat and fish products), were kindly provided by Wiesby BioFermentation (Germany).

The following *L. monocytogenes* strains, that had been isolated by project's partners from cold-smoked salmon and biotyped by partner 1, were used: IS 2-1 and 2-2; FR 1-1, 1-2, 2-1, 2-2, 3-1, 3-2, 4-1 and 4-2; PO 7-1, 7-2 and 9-1; NL 1-1, 2-1, 2-2, 4-1, 5-1, 5-2, 7-1, 8-1 and 9-1; DK 1-1, 1-2, 2-1, 2-2, 3-1, 3-2, 4-1 and 4-2. The strain code used is as follows: IS is the origin of the strains and stands for Iceland, FR = France, PO = Portugal, NL = The Netherlands, DK = Denmark; the first number indicates the biotype number while the second is the strain number belonging to the same biotype.

**Analysis of data.** All data were analysed and plotted using Microsoft Excel 97.

### **TASK 3A: Preliminary screening of inhibitor-producing LAB strains**

A preliminary screening of inhibitor-producing LAB strains was conducted by testing both commercial and previously owned isolates for their production of an antilisterial factor in a sterile cold-smoked fish system (SCSFS).

**Preparation of a sterile cold-smoked fish system (SCSFS).** Newly produced cold-smoked salmon fillets were obtained from Edalfiskur Ltd. (Borganes, Iceland) and minced. One part of minced salmon was mixed with 2 parts of deionized water in an erlenmeyer flask, heated and boiled for 2 min and filtered. It was then supplemented with 3 g of NaCl/ 100 g filtrate to reach a salt concentration of about 4% (w/w) and portioned into glass bottles prior to autoclaving (121°C, 15 min).

**Evaluation of antilisterial factor produced by LAB strains in SCSFS (low salt) and microbiological media.** LAB strains were cultured overnight in BHI at 30°C and 100 µl of each culture were transferred to 9 ml SCSFS containing only 1% NaCl as well as to 9 ml BHI and MRS, and incubated at 30°C for 24 h. The production of an antilisterial factor was evaluated using the agar spot method. Each culture (1 ml) was centrifuged in an Eppendorf tube at 8000 rpm (Eppendorf, Centrifuge 5415C) at 4°C for 15 min and 10 µl of the supernatant spotted onto BHI plates pre-lawn with *L. innocua* P1. The plates were then incubated at 30°C for 24 h. Detection of inhibition zones around the spots indicated the presence of an antilisterial factor.

### **Evaluation of antilisterial activity of LAB strains in SCSFS at low temperatures.**

The following strains were tested: 3 commercial strains (ALC 01, Fishflor® and N-8), *C. piscicola* V1 and SF644 as well as *Lc. lactis* SIK-83. Dilutions of 24-h SCSFS cultures were prepared in Butterfields' buffer to reach an inoculation level of 10<sup>5</sup> CFU/ml for LAB strains and 10<sup>3</sup> CFU/ml for *L. innocua* P1 in SCSFS. *C. piscicola* strains were inoculated alone and co-inoculated with *L. innocua* P1 and incubated at 4 and 8°C. The commercial strains were co-inoculated with *L. innocua* P1 and incubated at 4 and 8°C. Growth and bacteriocin production of *Lc. lactis* SIK-83 was followed at 8°C. *C. piscicola* V1 was also tested in a second trial against *L. monocytogenes* P1. Sampling was done at frequent intervals.

LAB counts were followed by plating serial dilutions using NAP medium and a Spiral Plater (model D; Spiral Systems Inc., Cincinnati, Ohio), incubating at 30°C for 3 days. Enumeration was done using a Laser Bacteria Colony Counter (model 500A; Spiral System Instruments). The presence of bacteriocin and its activity against *L. innocua* P1 was evaluated by using the agar spot method. *Listeria* counts were assessed by

spread-plating and Spiral-plating serial dilutions on LOX medium and incubating at 35°C for 2 days. *Listeria* detection limit was 10 CFU/ml.

**Assay of bacteriocin activity.** The agar spot method was used to estimate bacteriocin activity. An overnight culture (100 µl) of *L. innocua* P1 or *L. monocytogenes* P1 in TSB-YE was transferred to 6 ml of molten BHI soft agar to be poured onto the surface of a BHI agar plate. Inoculated SCSFS (1 ml) was transferred to sterile Eppendorf tubes, centrifuged at 8000 rpm for 15 min at 4°C and the supernatant heated at 80°C for 10 min. After drying of the pre-lawn BHI plates, 10 µl of the supernatant to be tested was spotted onto the plate. Successive twofold dilutions, done in Butterfields' buffer, were spotted as required. Bacteriocin activity (titre) was defined as the reciprocal of the lowest dilution which showed inhibition of the indicator strain and was expressed in arbitrary units per ml (AU/ml). The detection limit was 100 AU/ml.

**Nature of antilisterial factor produced by *C. piscicola* SF644.** *C. piscicola* SF644 was grown in SCSFS for a week at 8°C. It was centrifuged at 8000 rpm for 15 min in a Sorvall RC-5B centrifuge with a GSA rotor (Du Pont Company, Willington, Delaware, USA) at 2-4°C and the inhibitory effect of the supernatant tested using the agar spot method on a BHI plate pre-lawn with *L. monocytogenes* P1. The rest of the supernatant was neutralized with sterile NaOH (1N) to reach a pH of 6.4. The inhibitory effect of the supernatant was tested again on a pre-lawn BHI plate to verify whether previously observed inhibition was due to the presence of organic acids. The supernatant was then heated at 80°C for 10 min and the inhibitory effect of the supernatant tested to ensure that obtained inhibition was not due to enzymatic action. Finally, the proteinaceous nature of the inhibition factor was verified as described in Einarsson & Lauzon (1995).

### **TASK 3B: Secondary screening of inhibitor-producing LAB strains**

#### **Evaluation of the spoilage potential of *C. piscicola* strains in cold-smoked salmon.**

(i) **Experimental preparation:** Newly produced cold-smoked salmon fillets (unskinned) were obtained from Edalfiskur Ltd. (Borganes, Iceland), diced into skinned cubes of about 1 cm<sup>3</sup>, everything pooled together and mixed in a sterile plastic bag. Ninety grams of diced salmon were weighed into 48 plastic bags (WIPAK high gloss, 140 x 270 mm, Valdimar Gíslason Ltd, Iceland). These bags were divided into 3 treatments: one control, one spiked with *C. piscicola* V1 and the third one with *C. piscicola* SF644. One milliliter of a proper dilution was pipetted into the bags to reach an initial contamination level of 10<sup>4</sup> CFU/g. One milliliter of sterile deionized water was added to the control treatment. The bags were vacuum-packed (Super-Vac®, Austria) and stored at 4 and 8°C for 29 days.

(ii) **Microbiological and chemical evaluation:** The microbiological quality of the raw material was evaluated. Total viable counts (TVC) were assessed using LH medium (Spiral-plated; 15°C for 4-5 days). Lactic acid bacteria counts were estimated by pour-plating serial dilutions using NAP medium (22°C, 4 days) and adding an overlay. Further microbiological evaluation of the samples was done on days 7, 15, 22 and 29. Each treatment was evaluated in duplicate. Forty grams of each sample were weighed, diluted with 120 g of cooled MRD, stomached (Stomacher Lab-Blender 400, A.J. Seward Lab., London, UK) for 2 min and proper serial dilutions plated.

Fifteen colonies were isolated from NAP plates of the controls and checked for catalase production with 3% H<sub>2</sub>O<sub>2</sub> to confirm the presence of lactic acid bacteria. The proportion of catalase negative colonies was used to get an estimate of the lactic acid bacteria counts. The pH of the salmon samples was measured by a PHM 80 pH meter (Radiometer, Copenhagen, Denmark) at each sampling time by mashing 5 g of sample with 5 ml of deionized water and allowing the mixtures to stand (30-60 min) prior to measurement by a glass electrode.

**(iii) Sensory evaluation:** Sensory evaluation of the different cold-smoked salmon treatments was conducted on days 15, 22 and 29 by a trained panel consisting of 7 to 9 judges. The parameters evaluated included the presence of a spoilage smell (its intensity ranging from 0 = no off-odours to 4 = very smelly, and its description) and whether the samples (inoculated) differed in their appearance (colour) and texture when compared to the control (uninoculated). Treatments were examined based on their storage temperature. Upon opening of the packages, forty grams of each sample were weighed in glass petri dishes and evaluated.

**(iv) Headspace analysis by electronic nose:** An electronic nose was used to assess whether any distinct variations became apparent during storage due to the addition of *C. piscicola* strains to the cold-smoked salmon, when compared to a control. The electronic nose used is a gas sensor instrument (FreshSense; Ólafsdóttir *et al.*, 1997) developed at the Icelandic Fisheries Laboratories in collaboration with Element Sensor Systems (Sauðárkrókur, Iceland). This instrument consists of a glass container (5.2 l) closed with a plastic lid, an aluminium sensor box fastened to the lid and a PC computer running a measurement program. The sensor box contains a temperature sensor and 9 different electrochemical gas sensors (CO, H<sub>2</sub>S, NO, NO<sub>2</sub>, SO<sub>2</sub> sensors from Dräger (Germany); NH<sub>3</sub> A7AM, H<sub>2</sub>S, SO<sub>2</sub>, NH<sub>3</sub> sensors from City Technology (Portsmouth, Britain). A converter and a microprocessor (Electronics, A/D) situated in the box are involved in the measurement reading which is sent to the PC. A fan is positioned in the container to ensure gas circulation. The sensors are not selective towards different compounds, but some of them have certain selectivity for specific classes of compounds. For example, the CO sensor responds well to alcohols as well as to other classes of compounds. On the other hand, the NH<sub>3</sub> sensors are selective for amines (Ólafsdóttir *et al.*, 1996).

Upon completion of the sensory evaluation, two glass petri dishes (80 g of diced salmon) of the same sample were put (lid off) into the glass container of the electronic nose and a static headspace analysis performed at room temperature. Measurements were taken every 10 seconds for 20 min. In the data analysis, the reported value (current) is calculated as follows: the final measurement (average of the last 3 measurements of the 20-min. measurement cycle) minus the initial measurement (average of 18 measurements prior to introduction of the sample into the container).

### **TASK 3C: Inhibition studies on inoculated cold-smoked fish**

#### **Challenge studies – Part A**

**(i) Experimental preparation:** Diced cold-smoked salmon was prepared as described above, everything pooled together in 2 sterile plastic bags and well mixed. Fifty grams of diced salmon were weighed into 108 plastic bags. These bags were divided into 3 treatments: spiked with (1) *L. monocytogenes* P1, (2) *L. monocytogenes* P1 and *C. piscicola* V1, and (3) *L. monocytogenes* P1 and *C. piscicola* SF644. A proper dilution of each 24-h culture was pipetted (0.5 ml) into the bags to reach an initial

contamination level of  $10^{2-3}$  CFU/g for *L. monocytogenes* P1 and  $10^5$  CFU/g for the *C. piscicola* strains. The bags were vacuum-packed and stored at 4°C (6 weeks) and 8°C (4 weeks).

**(ii) Microbiological evaluation:** The microbiological quality of the raw material was evaluated as 2 samples from the 2 pooling bags, as one bag was used to prepare the 4°C treatments and the other one for the 8°C treatments. Total viable and lactic acid bacteria counts were estimated as previously mentioned. Further microbiological evaluation of the samples was done twice a week for 4 weeks, as well as on day 43 for the 4°C treatments. Each treatment was evaluated in duplicate. *Listeria* growth was evaluated on LOX medium (35°C, 48 h), with a detection limit of 8 CFU/g.

**(iii) Bacteriocin activity:** Bacteriocin production was checked as previously described (but supernatant not centrifuged) by pipetting 0.5 ml of the first dilution from the stomaching bag, and testing against *L. monocytogenes* P1 as the indicator strain on BHI plates. Due to the dilution factor, the detection limit of the bacteriocin activity measurement was 400 AU/ml. Salmon dices from each treatment were directly put onto BHI plates pre-lawn with *L. monocytogenes* P1 to verify whether growth inhibition of *Listeria* would be noticed. Similarly, a piece of the packaging material was aseptically cut (1-2 cm<sup>2</sup>) and put onto a pre-lawn BHI plate. The plates were incubated overnight at room temperature. Growth inhibition of *L. monocytogenes* P1 underneath/around the bag piece indicated the presence of an inhibitory factor on the packaging material.

### **Study on the sensitivity of *L. monocytogenes* strains of different origins and biotypes to various antimicrobial compounds. Selection of a *Lm* cocktail.**

Thirty *L. monocytogenes* strains isolated from CSS by 5 partners were assessed for their sensitivity towards 6 antimicrobial compounds (nisin A and Z, ALTA, FARGO, CpV1 and SF644 supernatants) and compared to the *L. monocytogenes* P1 strain. These strains had been biotyped by partner 1 and were found to belong to 8 different biotypes. The strains were listed earlier in this report (see Bacterial cultures). CpV1 and SF644 supernatants were prepared by growing either strain in 15 ml BHI at 8°C for 4 days, centrifuging (8000 rpm, 15 min at 4°C) the cultures in Eppendorf tubes and heating (80°C for 10 min). The bacteriocin activity of the supernatants was 102400 AU/ml for CpV1 and 256 000 AU/ml for SF644 when tested against *L. monocytogenes* P1. The agar spot method on BHI plates pre-lawn with each *Lm* strain to be tested was used to evaluate their sensitivity to the diluted compounds.

Selection of strains for the preparation of the *Lm* cocktail was based on the following criteria: there should be one strain from each country and the chosen strains should have an average sensitivity to all compounds when compared to all the other strains.

### **Challenge studies - Part B**

**(i) Experimental preparation:** Diced cold-smoked salmon was prepared as described above, everything pooled together in 2 sterile plastic bags and well mixed. Forty-nine grams of diced salmon were weighed into 154 plastic bags. These bags were divided into 7 treatments: spiked with (1) *L. monocytogenes* cocktail, (2) *L. monocytogenes* and *C. piscicola* V1, (3) *L. monocytogenes* and nisin A (800 IU/g), (4) *L. monocytogenes* and 0.5% (w/w) ALTA, (5) *L. monocytogenes* and 1% ALTA, (6) *L. monocytogenes*, *C. piscicola* V1 and 0.5% ALTA, and (7) *L. monocytogenes*, *C. piscicola* V1 and 1% ALTA. (Notice that treatments 3, 4 and 5 were part of Task 3D, while treatments 6 and 7 were part of Task 3E.) A proper dilution of each 24-h culture was pipetted (0.5 ml) into the bags to reach an initial contamination level of  $10^{1-2}$

CFU/g for *L. monocytogenes* cocktail and  $10^{5-6}$  CFU/g for *C. piscicola* V1, while 1 ml of the ALTA™ 2341 and nisin A solutions prepared for treatments 3 to 7 was added to diced CSS. The bags were vacuum-packed and stored at 4°C (5 weeks) and 8°C (4 weeks). The *L. monocytogenes* cocktail included 5 strains originating from 5 different countries and belonging to 4 different biotypes: IS 2-1, FR 3-1, PO 9-1, NL 9-1 and DK 4-2.

**(ii) Microbiological evaluation:** The microbiological quality of the raw material was evaluated as 2 samples from the 2 pooling bags. Total viable and lactic acid bacteria counts were estimated using the Spiral-plating technique and the media previously mentioned. Colonies on NAP medium were checked for catalase using 3% H<sub>2</sub>O<sub>2</sub> to confirm the presence of lactic acid bacteria. The proportion of catalase negative colonies was used to get an estimate of the lactic acid bacteria counts. Further microbiological evaluation of the samples was done on days 1, 7, 14, 21 and 28, as well as on day 35 for the 4°C treatments. Each treatment was evaluated in duplicate. *Listeria* growth was evaluated on LOX medium, with a detection limit of 8 CFU/g.

**(iii) Bacteriocin activity:** Bacteriocin production was evaluated as described in part A.

**(iv) Sensory evaluation:** The samples were smelled following opening and sampling of the packages for microbiological analysis by 2 analysts.

### **TASK 3D: Control using antimicrobial agents**

**Effect of acidification of CSS by organic acids on its sensory quality.** Six packs of sliced cold-smoked salmon were minced into a paste, deionised water added (15% w/w) and well mixed. Seven portions were prepared: one as the control (pH 6.1), 3 to be acidified with 1 M citric acid to pH 5.8, 5.6 and 5.4, and 3 to be acidified with 2.5 M lactic acid to the same pH values. Five panellists were asked to assess the appearance/colour, taste and smell of the acidified CSS paste compared to the control. The pH of each treatment was reassessed following sensory evaluation to ensure the stability of the adjustment.

**Use of nisin A, nisin Z and organic acids to inhibit *Listeria* in SCSFS at 4 & 8°C.**

**(i) Preparation of acidified SCSFS:** 11 ml of sterile SCSFS were portioned to 18 sterile vials and pH adjustments were made to 12 of them, using 40 µl of 2.5 M lactic acid to half of them and 45 µl of 1 M citric acid to the other half to reach a pH of 5.4. The acid solutions had been previously sterile-filtered. The pH of unadjusted SCSFS was 6.0.

**(ii) Preparation of a purified nisin A solution:** 1 g of nisaplin was dissolved in 10 ml 0.02N HCl and stirred for 2 hours at room temperature. The mixture was centrifuged at 8500 RPM for 10 min at 4°C, the pH of the supernatant adjusted to 5.0 at room temperature and the purified nisin filter-sterilised (0.22 µm) and stored at 4°C till used (El-Khateib *et al.*, 1993). This bacteriocin solution was equivalent to 40 000 IU/ml, and 3200 AU/ml when tested against *L. monocytogenes* P1.

**(iii) Preparation of nisin Z solution:** it was prepared according to Einarsson & Lauzon (1995) and was equivalent to 200 AU/ml when tested against *L. monocytogenes* P1.

**(iv) SCSFS inoculation and follow-up:** 1 ml of either nisin solution was added to 12 of the vials, while 1 ml of sterile deionised water was added to the 6 vials left. The pH of unacidified SCSFS was checked upon addition of the nisin solutions. This created 2 sets of 9 treatments (control, nisin A and Z x 3 pH groups) to be stored at 4 and 8°C.

Dilution of 24-h SCSFS culture of *L. monocytogenes* P1 was prepared in Butterfields' buffer to reach an inoculation level of  $10^{2-3}$  CFU/ml into each vial. Sampling was done on days 5, 13, 20 and 25 by taking aliquots of 0.1 and/or 0.5 ml with further dilution as required. *Listeria* growth was followed using TSA-YE medium (35°C, 2 days). *Listeria* detection limit was 10 CFU/ml. The activity of nisin A once incorporated to SCSFS was expected to be about 265 AU/ml, while that of nisin Z was around 16 AU/ml.

**Use of ALTA™ 2341 and FARGO™ 23 to inhibit *Listeria* in SCSFS at 4°C.** A 10% solution of each commercial product was prepared as recommended by the supplier. ALTA was dissolved in deionised water and heated to 100°C for 10 min and transferred aseptically to a sterile bottle. As FARGO contains a live culture as well, it was weighed aseptically, dissolved in sterile deionised water at room temperature and used on the same day. The solutions were added to 9 ml SCSFS to reach a final concentration of 0.25, 0.5 and 1% (w/v). Sterile deionised water was added when necessary to adjust the SCSFS volume to 10 ml. The pH of SCSFS was checked upon addition of ALTA (1%). All tubes were inoculated with a 24-h SCSFS culture of *L. monocytogenes* P1 diluted in Butterfields' buffer to reach an inoculation level of  $10^{2-3}$  CFU/ml. One control tube (0% commercial product) was prepared to follow the growth of *L. monocytogenes* P1 at 4°C. Sampling was done at frequent intervals by taking aliquots of 0.1 and/or 0.5 ml with further dilution as required. Both the spread-plating and Spiral techniques were used as required. *Listeria* growth was followed using LOX medium (35°C, 2 days). *Listeria* detection limit was 10 CFU/ml. Growth of the FARGO culture was followed using NAP medium (30°C, 2 days). Bacteriocin activity was evaluated against *L. monocytogenes* P1 as previously described.

**Effect of ALTA™ 2341 and nisin A on growth of *L. monocytogenes* cocktail in CSS at 4 and 8°C.** As previously described in Task 3C – Part B, a trial was run assessing, among others, the effect of antimicrobial compounds on the growth of a *L. monocytogenes* cocktail in CSS. Two commercial products were tested: ALTA and nisaplin (nisin A, which was purified according to El-Khateib *et al.*, 1993). ALTA was applied at 2 levels: 0.5 and 1% (w/w), while the nisin A solution added was equivalent to 800 IU/g of CSS. The pH of CSS upon addition of the compounds was checked.

### **TASK 3E: Control using combinations of selected LAB and antimicrobial agents**

**Effect of pH on growth and bacteriocin production of *C. piscicola* strains in SCSFS at 4 and 8°C.**

(i) **Preparation of acidified SCSFS:** 18 ml of sterile SCSFS were portioned to 28 sterile tubes and pH adjustments were made to 24 of them, using 35, 50 and 65 µl of 2.5 M lactic acid or 1 M citric acid to reach pH of 5.6, 5.4 and 5.2, respectively. The acid solutions had been previously sterile-filtered. The pH of unadjusted SCSFS was 6.1.

(ii) **SCSFS inoculation and follow-up:** Dilutions of 24-h SCSFS culture of *C. piscicola* V1 and SF644 were prepared in Butterfields' buffer to reach an inoculation level of  $10^{4-5}$  CFU/ml into each tube. This created 2 sets of 14 treatments (control, 3 pH groups x 2 acids for each of the 2 LAB strains tested) to be stored at 4 and 8°C.

Sampling was done on days 5, 9, 13, 16, 21 and 29 by taking aliquots of 0.5 ml with further dilution as required. *C. piscicola* growth was followed using NAP medium and the Spiral technique. Bacteriocin production was evaluated by the agar spot method against *L. monocytogenes* P1.

**Effect of pH (citric acid) on growth/bacteriocin production of *C. piscicola* V1 and its inhibition against *L. monocytogenes* P1 in SCSFS at 4 and 8°C.**

(i) **Preparation of acidified SCSFS:** 15 ml of sterile SCSFS were portioned to 6 sterile tubes and pH adjustments were made to 4 of them, using 25 and 40 µl of 1 M citric acid to reach pH of 5.8 and 5.6, respectively. The acid solution had been previously sterile-filtered. The pH of unadjusted SCSFS was 6.1.

(ii) **SCSFS inoculation and follow-up:** Dilutions of 24-h SCSFS culture of *C. piscicola* V1 as well as *L. monocytogenes* P1 were prepared in Butterfields' buffer to reach an inoculation level of  $10^{3-4}$  CFU/ml for LAB culture and  $10^{2-3}$  CFU/ml for *Listeria* into each tube. This created 2 sets of 3 treatments (one control and 2 pH groups) to be stored at 4 and 8°C. Sampling was done on days 4, 7, 10, 14, 17, 21 and 28 by taking aliquots of 0.1 and/or 0.5 ml with further dilution as required. *C. piscicola* V1 growth was followed using NAP medium and the Spiral technique. *Listeria* growth was followed using LOX medium. *Listeria* detection limit was 10 CFU/ml. Bacteriocin production was evaluated by the agar spot method against *L. monocytogenes* P1.

**Effect of ALTA™ 2341 and FARGO™ 23 on growth/bacteriocin production of *C. piscicola* strains and their inhibition against *L. monocytogenes* P1 in SCSFS at 4 and 8°C.**

Ten percent ALTA and FARGO stock solutions were prepared as previously described and added to 9 ml SCSFS to reach a final concentration of 0, 0.25, 0.5 and 1% (w/v). Sterile deionised water was added when necessary to adjust the SCSFS volume to 10 ml. All tubes were inoculated with a 24-h SCSFS culture of *L. monocytogenes* P1 diluted in Butterfields' buffer to reach an inoculation level of  $10^{2-3}$  CFU/ml and with a dilution of a 24-h SCSFS culture of either *C. piscicola* V1 or SF644 ( $10^{5-6}$  CFU/ml). Two control tubes containing solely the *Listeria* strain were prepared to follow its growth at 4 and 8°C. This created 2 sets of 15 treatments (one *Listeria* control, each LAB strain co-cultured with *Listeria* and then supplemented with 3 concentrations of ALTA and FARGO) to be stored at both temperatures. Sampling was done at frequent intervals by taking aliquots of 0.1 and/or 0.5 ml with further dilution as required. Both the spread-plating and Spiral techniques were used as required. *C. piscicola* growth was followed using NAP medium, but LOX medium for *Listeria*. *Listeria* detection limit was 10 CFU/ml. Bacteriocin activity was evaluated against *L. monocytogenes* P1 as previously described.

**Effect of ALTA™ 2341 on growth/bacteriocin production/antilisterial action of *C. piscicola* V1 against *L. monocytogenes* cocktail in CSS at 4 and 8°C.** As previously described in Task 3C – Part B, a trial was run assessing, among others, the effect of combining *C. piscicola* V1 and ALTA on the growth of a *L. monocytogenes* cocktail in CSS. ALTA was applied at 2 levels: 0.5 and 1% (w/w).



## Chapter 3 Results

### TASK 3A: Preliminary screening of inhibitor-producing LAB strains

A preliminary screening of inhibitor-producing LAB strains was conducted by testing both commercial and previously owned isolates for their production of an antilisterial factor in SCSFS.

#### Evaluation of antilisterial factor produced by LAB strains in SCSFS (low salt) and microbiological media

Five strains were tested for the production of an antilisterial factor in SCSFS at 30°C (24 h) in comparison to BHI and MRS broth, two microbiological media. Growth was slightly better in microbiological media than SCSFS, but similar results were obtained for the production of an antilisterial factor (Table 1). Only 2 strains, *C. piscicola* SF644 and *Lc. lactis* SIK-83, were found to produce an antilisterial factor. *Lc. lactis* SIK-83 is a nisin Z producer (Andersson *et al.*, 1988; Einarsson & Lauzon, 1995). Interestingly, the inhibitory factor produced by *C. piscicola* SF644 was not detected when grown in MRS broth. The nature of the inhibitory factor produced by this strain must be confirmed. *C. piscicola* V1 had been previously studied by partner 4 and was known to be a bacteriocin producer. However, no antilisterial factor was detected when grown under the conditions studied. This is believed to be due to the high incubation temperature. Lower temperature would be preferable for its bacteriocin production, as demonstrated by Pilet *et al.* (1995). *C. piscicola* V1 and SF644 as well as *Lc. lactis* SIK-83 were therefore chosen for further trials in SCSFS at lower temperatures.

**Table 1.** Growth / production of an antilisterial factor at 30°C (24 h)

Strain	MRS	BHI	SCSFS
<i>Carnobacterium piscicola</i> UI49	++ /-	+++ / -	+ / -
<i>Lactococcus lactis</i> SIK-83	+++ / +	+++ / +	++ / (+)
<i>Lactobacillus bavaricus</i> MI401	+++ /-	+ / -	+ / -
<i>Carnobacterium piscicola</i> SF644	+++ /-	+++ / +	++ / +
<i>Carnobacterium piscicola</i> V1	+++ /-	+++ / -	++ / -

+ to +++ : little to much growth / antilisterial factor

-: no growth / antilisterial factor; (+) very little antilisterial factor

#### Evaluation of antilisterial activity of LAB strains in SCSFS at low temperatures

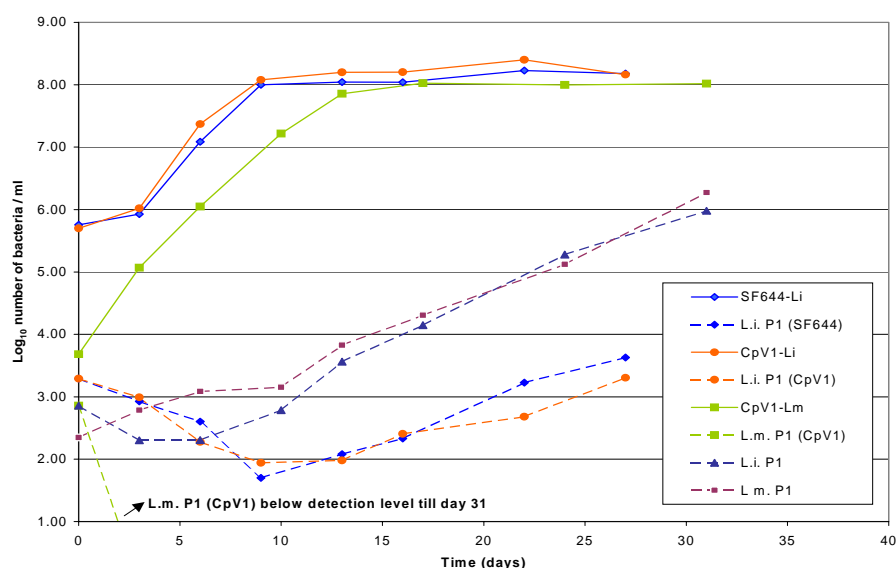
*C. piscicola* strains were tested for their antilisterial effect in SCSFS, simulating cold-smoked salmon. Table 2 shows the bacteriocin production occurring in inoculated SCSFS over the incubation period at 4 and 8°C. The highest bacteriocin activity detected for *C. piscicola* V1 was 1600 AU/ml, but 400 AU/ml for strain SF644. The higher temperature contributed to an earlier production of bacteriocin, while the amount produced (resulting activity) was similar. It was not evident whether the presence of *Listeria* influenced the bacteriocin production as similar results were obtained most of the time.

**Table 2.** Bacteriocin production of *C. piscicola* strains and co-inoculated with *L. innocua* P1 in SCSFS at 4 and 8°C

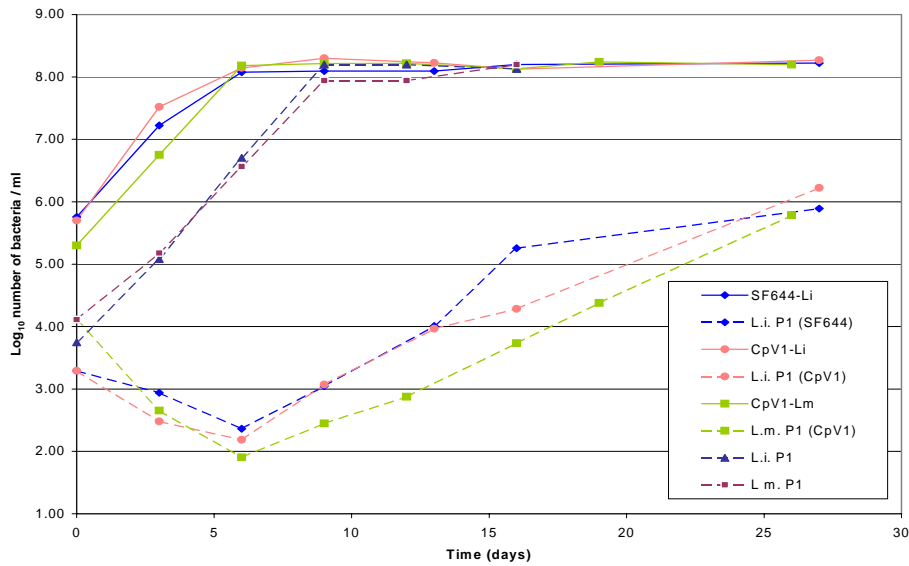
Strains	Bacteriocin activity <sup>a</sup> (AU/ml) detected after days of incubation								
	4°C	<i>Listeria</i>	3	6	9	13	16	22	27
<i>CpV1</i>	-	-	nd	nd	nd	400	800	1600	800
	+	+	nd	nd	nd	200	800	800	800
<i>SF644</i>	-	-	nd	nd	nd	nd	nd	400	200
	+	+	nd	nd	nd	nd	200	400	200
<b>8°C</b>									
<i>CpV1</i>	-	-	200	400	400	400	400		1600
	+	+	200	200	400	400	400		800
<i>SF644</i>	-	-	200	400	200	200	200		200
	+	+	nd	400	200	400	400		400

a: activity estimated by spotting agar method against *L. innocua* P1  
 nd: not detected (limit of detection = 100 AU/ml)

Figures 1 and 2 show the growth of *C. piscicola* strains in SCSFS at 4 and 8°C, respectively, inoculated with or without *Listeria*, as well as the growth behaviour of monocultures of *L. innocua* P1 and *L. monocytogenes* P1. *Listeria* growth increased by about 3 log after 31 days at 4°C, but after a week at 8°C. At 4°C, both *C. piscicola* strains had a bacteriostatic effect on *L. innocua* P1 growth for about 2 weeks. Initial inoculation level of *L. innocua* P1 (10<sup>3</sup> CFU/ml) was reached back after about 4 weeks, in comparison to a 3-log increase for monocultures of *Listeria* strains. When co-inoculated with *C. piscicola* V1, *L. monocytogenes* P1 was more sensitive than *L. innocua* P1 as a bactericidal effect was observed for the whole incubation period (31 days). In that case, the maximum bacteriocin titer measured was 6400 AU/ml against *L. monocytogenes* P1.



**Figure 1.** Growth curves for *C. piscicola* and *Listeria* strains in monocultures or co-inoculated during incubation in SCSFS at 4°C



**Figure 2.** Growth curves for *C. piscicola* and *Listeria* strains in monocultures or co-inoculated during incubation in SCSFS at 8°C

At 8°C, the bacteriostatic effect of *C. piscicola* V1 and SF644 on *L. innocua* P1 only lasted for 6-9 days, after which proliferation of *L. innocua* P1 occurred steadily with a 3-log increase between days 9 and 27, but this was observed to occur at a slower growth rate than that seen with *Listeria* monocultures. *L. monocytogenes* P1 was similarly inhibited when co-inoculated with *C. piscicola* V1 at 8°C, the bacteriostatic effect lasting for 6-13 days after which a 3-log increase took place during the succeeding 13 days of incubation. The bacteriocin titer was observed when tested against *L. monocytogenes* P1 (12 800 AU/ml) was similar to that at 4°C.

The commercial strains tested did not grow at 4°C, but did grow very poorly at 8°C. No antilisterial factor could be detected and the co-inoculated *Listeria* strain proliferated as seen with the control (data not shown). Similarly, *Lc. lactis* SIK-83 grew slowly at 8°C and did not produce its bacteriocin under these conditions.

#### **Nature of antilisterial effect produced by *C. piscicola* SF644**

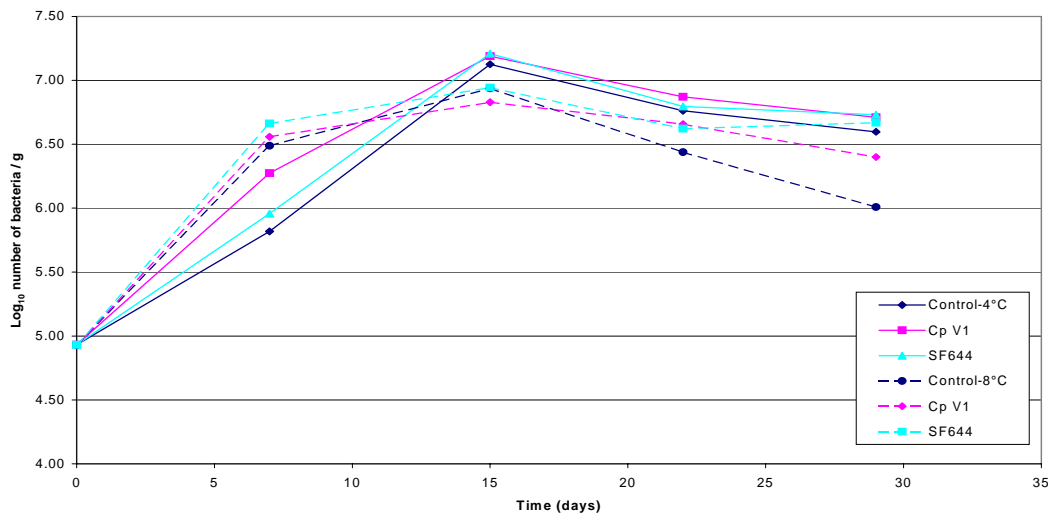
The inhibition factor produced by *C. piscicola* SF644 towards *L. monocytogenes* P1 was still observed following neutralization of the supernatant (pH 6.4) and heating to 80°C for 10 min. However, it disappeared after a protease treatment, indicating its proteinaceous nature (bacteriocin).

### **TASK 3B: Secondary screening of inhibitor-producing LAB strains**

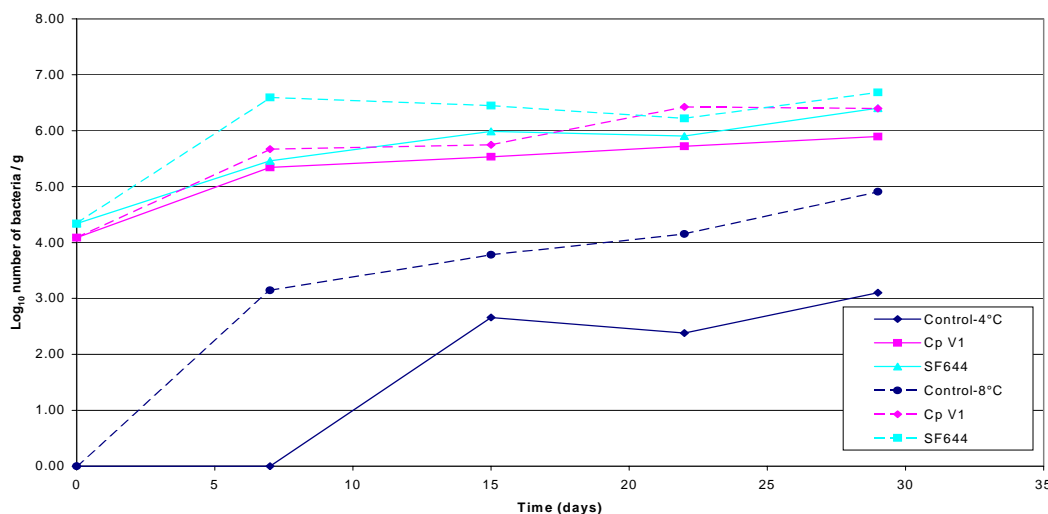
#### **Evaluation of the spoilage potential of *C. piscicola* strains in cold-smoked salmon**

Because of the observed antilisterial activity at low temperatures, the *C. piscicola* strains could possibly be used as protective cultures in cold-smoked fish. Before challenge studies could be conducted, it was necessary to assess whether these strains would promote spoilage in such products when inoculated at high levels. Newly smoked salmon was inoculated with the *C. piscicola* strains, repacked (vacuum), stored at 4 and 8°C, and the microbiological and sensory quality evaluated weekly

over a 29-day period. Figure 3 shows the psychrotrophic counts (TVC) over the storage period. As expected, bacterial proliferation occurred faster at 8°C than 4°C, with a difference of about 1 log after 7 days, after which a slowdown was observed at 8°C, followed by a decrease after the 15th day of storage at both incubation temperatures.



**Figure 3.** TVC (15°C) of diced, vacuum-packed cold-smoked salmon stored at 4°C (full line) and 8°C (dashed line)



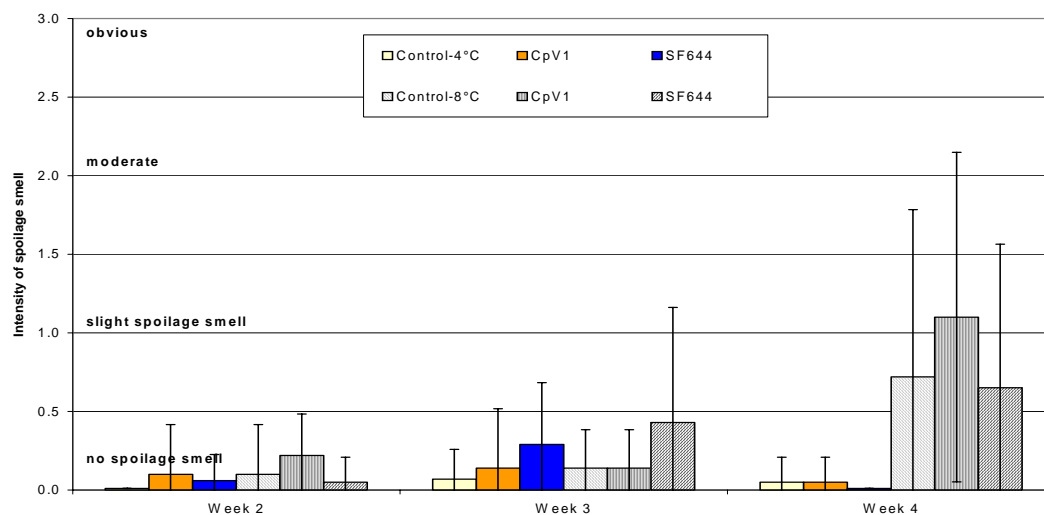
**Figure 4.** Lactic acid bacteria counts of diced, vacuum-packed cold-smoked salmon stored at 4°C (full line) and 8°C (dashed line)

Figure 4 shows how lactic acid bacteria (LAB) proliferated in the product. It is interesting to notice that in the control treatment where no initial LAB inoculation took place, LAB counts had increased by almost 5 logs at 8°C and 3 logs at 4°C after 29 days of storage. The inoculated LAB strains showed a 3-log increase during this period to reach 6-6.5 log, growing slightly faster at 8°C. The pH was evaluated and found to vary for all treatments, starting at 6.0. An initial decrease in pH value was

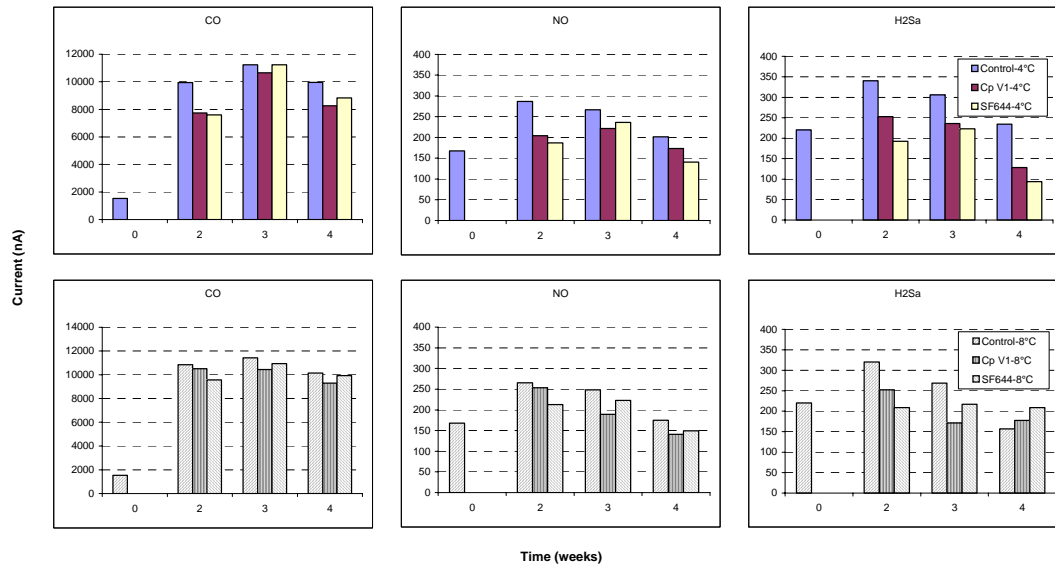
seen during the first 2 weeks, reaching an average value of 5.92 at 8°C and 5.95 at 4°C, after which it increased to the initial values or slightly higher. These observed variations were lesser at 4°C than at 8°C.

Based on sensory evaluation results (Figure 5), the *C. piscicola* strains were not found to accelerate spoilage in cold-smoked salmon (CSS). At 4°C, neither the control CSS samples nor the *C. piscicola* inoculated ones were found to be spoiled (score < 0.5) after 4 weeks of storage, except that the controls were softer. At 8°C, no spoilage smell was detected after 3 weeks of storage whereas at week 4, slight off-odours were detected from all the treatments. *C. piscicola* V1 inoculated samples had a significantly higher score ( $p=0.03$ ) than the controls and were found to be softer at that temperature. Notice that after 3 weeks of storage at 8°C, the average spoilage score of samples inoculated with strain SF644 was higher than that of the control and CpV1 inoculated samples as one judge gave it a score of 2 as opposed to 0 - 0.5 given by most others.

The headspace analysis of CSS samples performed by the electronic nose after weeks 2, 3 and 4 of storage at 4 and 8°C (Figure 6) demonstrated the similarity seen in the spoilage process taking place in the controls and *C. piscicola* inoculated samples. Three sensors reflected most of the changes occurring: CO, NO and H2Sa. The low response (< 100 nA) seen with the other sensors was only a measure of the noise occurring (data not shown). A large increase in the response of sensor CO was noticed between weeks 0 and 2, which stabilized for the rest of the storage period. On the other hand, a slight increase in response was observed with sensors NO and H2Sa between weeks 0 and 2, generally followed by a decrease during the succeeding weeks.



**Figure 5.** Sensory evaluation of the spoilage smell of CSS samples stored at 4 and 8°C for a 4-week period



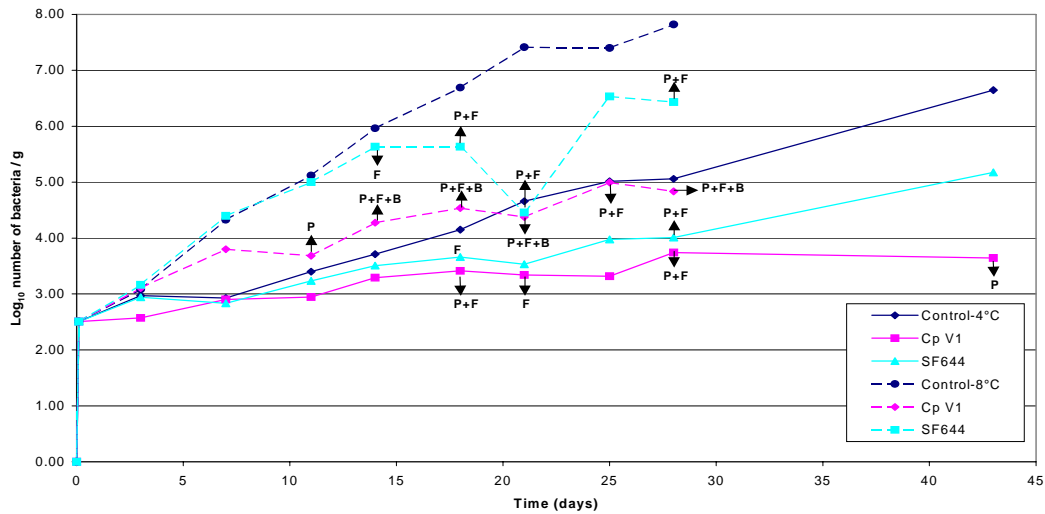
**Figure 6.** Characteristic response of 3 sensors to the different treatments at 4 (upper) and 8°C (lower)

### **TASK 3C: Inhibition studies on inoculated cold-smoked fish**

#### **Challenge studies – Part A**

A challenge study was conducted to assess the inhibitory effect of the *C. piscicola* strains V1 and SF644 on *L. monocytogenes* P1 when added to cold-smoked salmon, vacuum-packed and stored at 4 and 8°C. TVC and LAB counts (data not shown) of *C. piscicola* inoculated samples were similar throughout storage at both temperatures tested due to the high LAB inoculation level. The natural LAB flora of the control treatments became dominant (> log 6) after 3 weeks at 4°C and 2 weeks at 8°C, where a similar level of *Listeria* was reached.

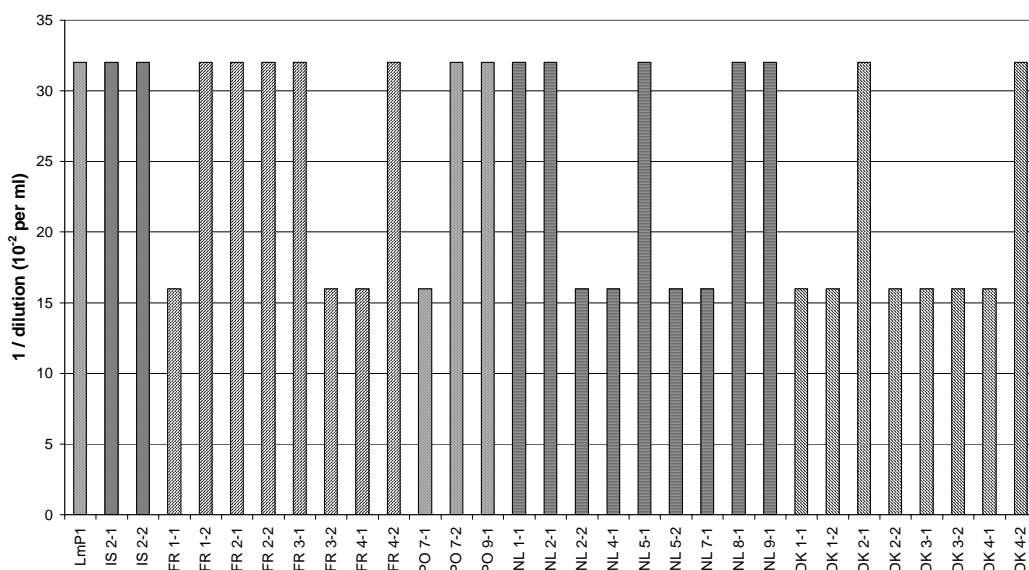
*L. monocytogenes* P1 proliferated slowly in the control treatment at 4°C, with a 4-log increase after 6 weeks of storage. Slight bacteriostatic effect against *Listeria* was seen with *C. piscicola* inoculated treatments during the first 4 weeks, where *Listeria* increased by only 1 log. This *Listeria* level was maintained during the 2 succeeding weeks for the CpV1 treatment while no further bacteriostatic effect was observed with the SF644 treatment during this period, as shown by the additional log increase in *Listeria*. At 8°C, *L. monocytogenes* P1 grew steadily in the control treatment, with a 5-log increase after ca. 3 weeks. *Listeria* growth was affected differently by the *C. piscicola* strains, SF644 showing a lesser bacteriostatic effect (4-log increase after 4 weeks) compared to CpV1 (2-log increase). Bacteriocin production (B) could only be detected from the CpV1 treatment (diluted samples) stored at 8°C, from day 14 to day 28, with an activity of 800 AU/ml on day 14, 1600 AU/ml on day 18 and 400 AU/ml from day 21 (when tested against *L. monocytogenes* P1 as an indicator strain). *L. monocytogenes* P1 inhibition factor was otherwise detected on packaging material (P=plastic) and/or fish (F) from both *C. piscicola* inoculated treatments stored at both temperatures during the storage trial (Figure 7). However, total *Listeria* inhibition was not achieved during this trial.



**Figure 7.** *Listeria* counts of diced, vacuum-packed cold-smoked salmon stored at 4°C (full line) and 8°C (dashed line). *L. monocytogenes* P1 inhibition factor seen on P = plastic, F = fish; and B = bacteriocin activity of 400-1600 AU/ml (detected from diluted samples).

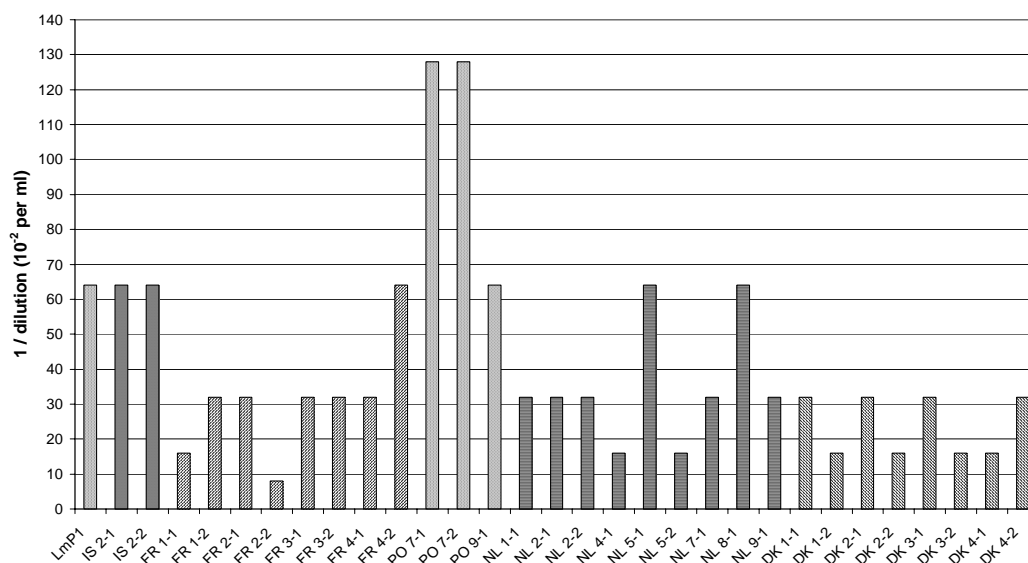
**Study on the sensitivity of *L. monocytogenes* strains of different origins and biotypes to various antimicrobial compounds. Selection of a *Lm* cocktail**

Thirty *L. monocytogenes* strains isolated from CSS by 5 partners were assessed for their sensitivity towards 6 antimicrobial compounds (nisin A and Z, ALTA, FARGO, CpV1 and SF644 supernatants) and compared to the *L. monocytogenes* P1 strain used during most of the project. Sensitivity of the strains was not found to be related to their biotype, but rather to their origin.

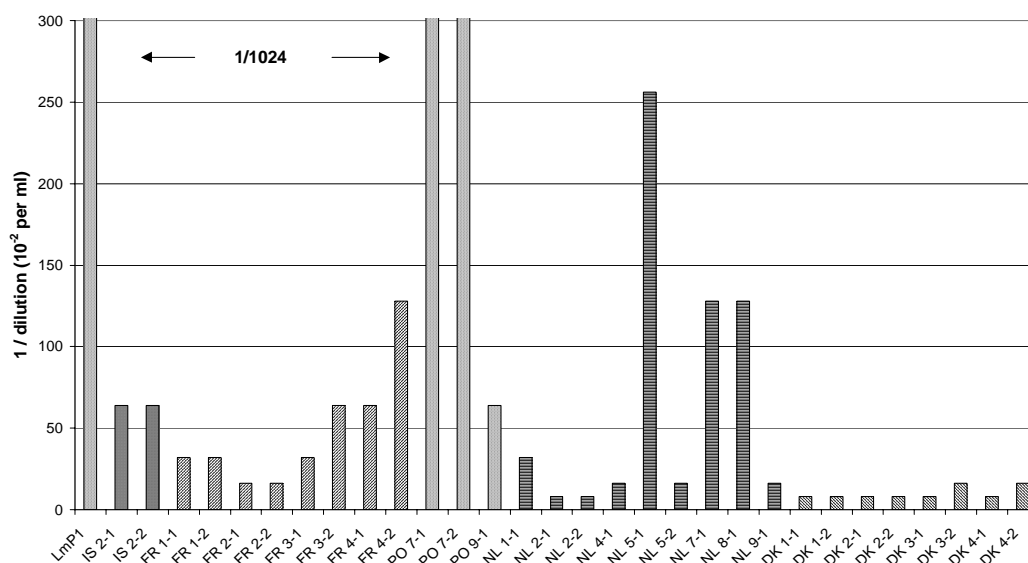


**Figure 8.** Sensitivity of various *L. monocytogenes* strains to nisin A (highly diluted means highly sensitive)

Generally, the most sensitive strains to the least ones ranked as follows: Portugal, Iceland, France, Netherlands and Denmark. Still, the differences observed in sensitivity varied among the compounds tested. For instance, there was little difference among the strains when tested towards both nisin compounds (Figure 8, nisin A). More differences were observed among the strains and countries with ALTA (Figure 9) and FARGO (data not shown), and even more with the CpV1 (Figure 10) and SF644 (data not shown) supernatants. Only representative results are shown. This variability in sensitivity to the antimicrobial compounds tested did not facilitate the choice of representative strains for the *Lm* cocktail. However, it was decided that choosing a strain originating from each country and having an average sensitivity to all compounds tested would be appropriate. This led to the selection of 5 strains of 4 different biotypes: IS 2-1, FR 3-1, PO 9-1, NL 9-1 and DK 4-2.



**Figure 9.** Sensitivity of various *L. monocytogenes* strains to ALTA™ 2341



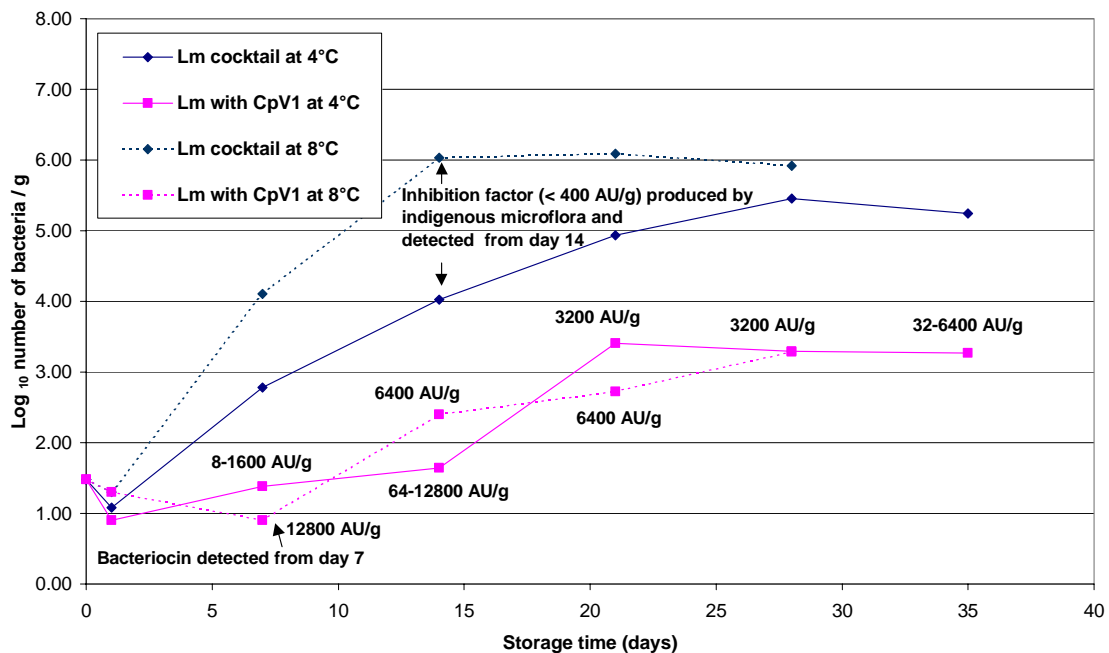
**Figure 10.** Sensitivity of various *L. monocytogenes* strains to CpV1 supernatant



## Challenge studies - Part B

Commercial CSS was inoculated with a *L. monocytogenes* cocktail and the inhibitory effect of various antimicrobial factors was evaluated, either separately (Tasks 3C & D) or in combination (Task 3E) at 4 and 8°C. As this trial encroaches 3 tasks, the results belonging to each task are presented separately.

TVC, LAB and *Listeria* counts were followed throughout storage of CSS for all treatments. Assessment of the initial quality of the raw material indicated that a higher TVC (log 4.4/g) was obtained as compared to the trial in part A (log 3/g). Similarly, the LAB count of the commercial CSS was higher for the latter trial (log 3.5 as opposed to log 1-2/g). This high proportion of LAB initially in the CSS contributed to their steady proliferation in the controls, i.e. the treatments inoculated only with the *L. monocytogenes* cocktail. After 2 weeks of storage, the LAB microflora had reached log 7-8/g in the controls. At this point, an inhibition factor was detected on the surface of the CSS dices, as confirmed when tested against *L. monocytogenes* P1 using the agar spot method. However, such antilisterial activity was not recovered from the diluted samples, indicating an activity of less than 400 AU/g. This antilisterial activity produced by the indigenous microflora of the controls caused a slowing down of the *Listeria* cocktail from day 14, especially at 8°C as shown by Figure 11.



**Figure 11.** *Listeria* counts of diced and spiked, vacuum-packed cold-smoked salmon stored at 4°C (full line) and 8°C (dashed line). (Bacteriocin activity given as evaluated for CpV1 treatments by the agar spot method using *L. monocytogenes* P1.)

In the case of the CSS spiked with both the *Listeria* cocktail and *C. piscicola* V1 (CpV1) strain, the LAB counts represented more or less the TVC throughout storage (data not shown) due to the high inoculation level of the *Carnobacterium* strain (log 5.6/g). Bacteriocin was detected from day 7 at both storage temperatures, with greater and maximum activity at 8°C. On the other hand, maximum activity at the lower temperature was observed on day 14. This resulted in a bacteriostatic effect on the

*Listeria* cocktail lasting for 1 week at 8°C but 2 weeks at 4°C, followed by its slow growth coinciding with a decrease in bacteriocin activity, leading to a 2-log increase on day 28. It is noticeable that growth of the *Listeria* cocktail at 4°C had stabilised from the third week to the last sampling day (day 35), which could be explained by the constant bacteriocin amount detected during that period. Overall, it can be said that the *Listeria* inhibition obtained corresponded to a difference in counts by 2 and 2.5 log at 4 and 8°C, respectively.

Regarding the sensory quality of the spiked CSS, a slight spoilage smell was noticeable after 28 and 35 days at 4°C for the control, but after 21 and 28 days at 8°C. For the CpV1 treatments, a spoilage odour was detected after 35 and 28 days of storage at 4 and 8°C, respectively.

### **TASK 3D: Control using antimicrobial agents**

#### **Effect of acidification of CSS by organic acids on its sensory quality**

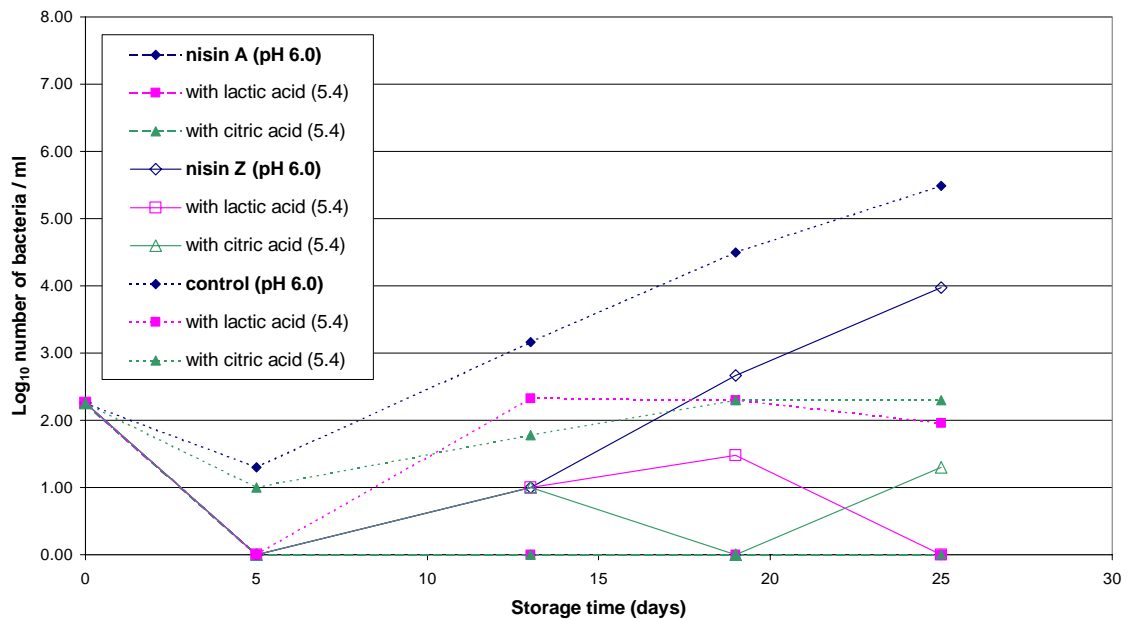
Organic acids could be used as antimicrobial agents, but it appeared necessary to assess their effect on the sensory quality of CSS in order to determine how much acidification was possible to reach in the product without causing quality defects. This was evaluated by acidifying a CSS paste with either lactic or citric acid down to pH 5.8, 5.6 and 5.4. A trained panel of 5 members was asked to comment on the appearance, colour, taste and smell of the samples when compared to a control, unacidified paste. Little or no differences were detected at pH 5.8 and 5.6 with either acid, while the colour was found to be lighter at pH 5.4 with a slightly sour aftertaste when using citric acid, but being even more sour and somewhat rancid with lactic acid. It was also pointed out that because of the smoky flavour and smell of newly produced CSS, it could be difficult to discern sourness caused by acidification at that early stage, which otherwise could be noticeable with further storage. Nevertheless, it appears that lowering the pH of CSS down to 5.6 is sensorically acceptable. Acidification with citric acid down to pH 5.4 brings some organoleptic changes, whereas use of lactic acid should be avoided.

#### **Use of nisin A, nisin Z and organic acids to inhibit *Listeria* in SCSFS at 4 & 8°C**

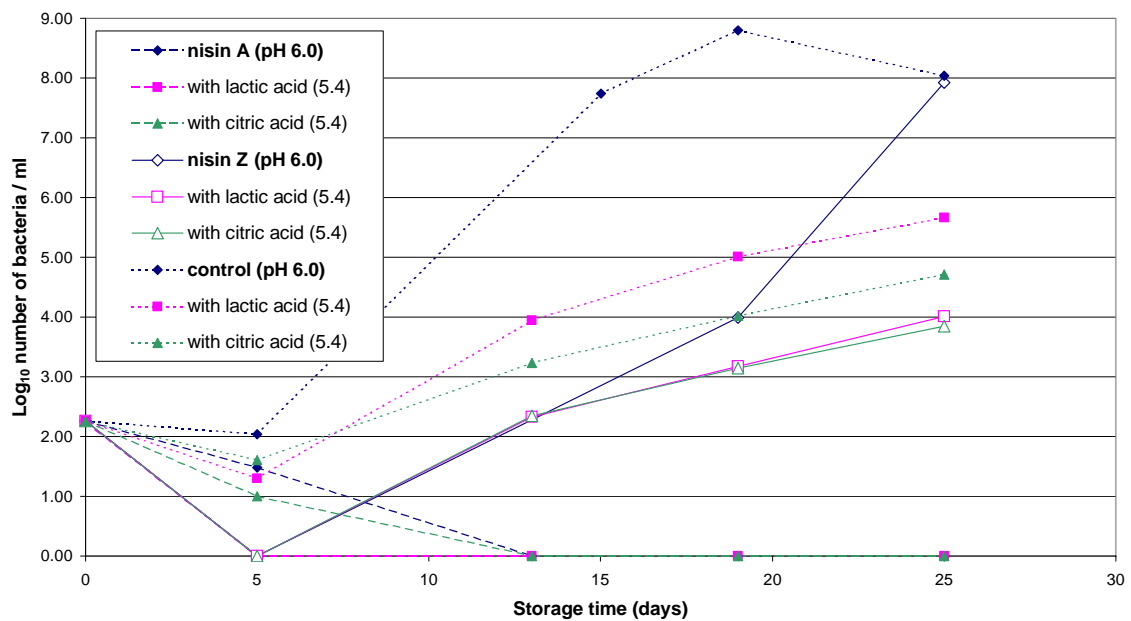
Nisin A is produced by some *Lc. lactis* strains and is the only bacteriocin that has been approved as a food additive in Europe. Nisin Z was produced by *Lc. lactis* SIK-83 and is a bacteriocin very similar to nisin, their difference lying in the substitution of one amino acid residue at position 27, i.e. asparagine instead of histidine. Nisin A and Z as well as organic acids (citric and lactic acids) were tested for their antilisterial activity in SCSFS at 4 and 8°C, either used separately or in combination to reach a pH of 5.4. From Figures 12 and 13, it can be seen that nisin A (3333 IU/ml) clearly had a bactericidal effect towards *L. monocytogenes* P1 at both temperatures, independently of the pH (5.4 and 6.0), as *Listeria* counts were below detection level (10 CFU/ml) during the incubation period of 25 days.

On the other hand, nisin Z only had a bacteriostatic effect on the *Listeria* strain, which lasted for about 3 weeks at 8°C. A noticeable bacteriostatic effect of the organic acids on *Listeria* growth (acidified control treatments) was also observed and was found to last longer than that seen with nisin Z (pH 6.0). The acids had a similar effect at 4°C, while citric acid provided a slightly better inhibition than lactic acid at 8°C. However,

combination of nisin Z with either organic acid greatly repressed the growth of *L. monocytogenes* P1 at 4°C, with counts at or below the detection level. At 8°C, the effect was only bacteriostatic but was greater than that seen with nisin Z or the acids when used separately. It should be mentioned that addition of the nisin solutions to unacidified SCSFS did not influence its pH.



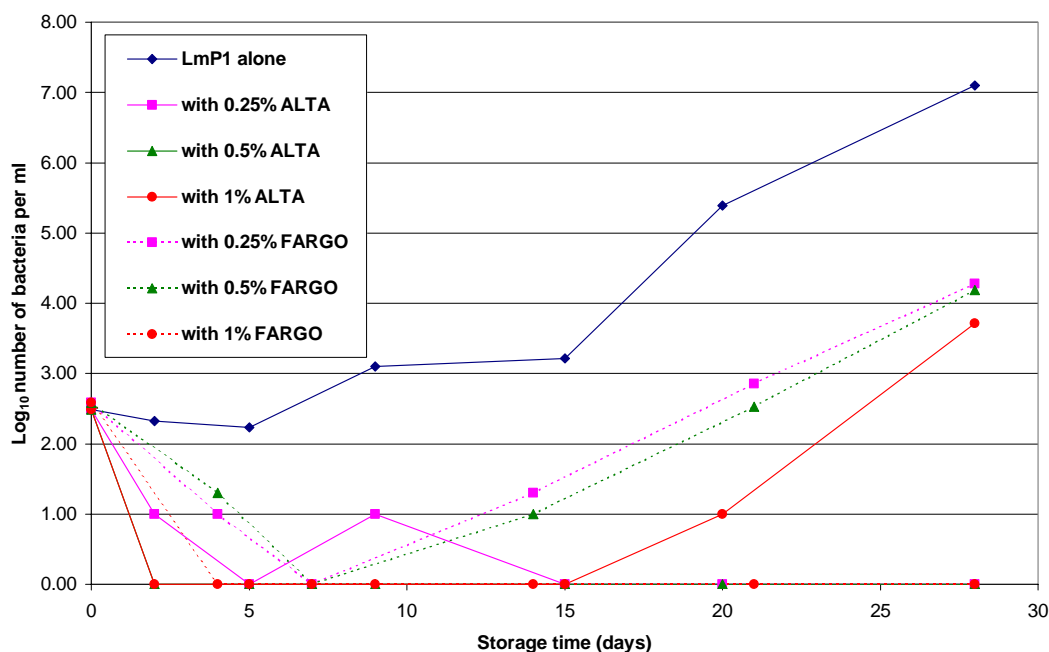
**Figure 12.** Effect of nisin A & Z and organic acids on the growth of *L. monocytogenes* P1 in SCSFS at 4°C



**Figure 13.** Effect of nisin A & Z and organic acids on the growth of *L. monocytogenes* P1 in SCSFS at 8°C

### Use of ALTA™ 2341 and FARGO™ 23 to inhibit *Listeria* in SCSFS at 4°C

ALTA™ 2341 is a crude fermentation product of lactic acid bacteria and contains pediocin and lactate. FARGO™ 23 contains in more the live culture. Both products were tested at different concentrations to determine the magnitude of the antilisterial effect achieved in SCSFS at 4°C. It should be mentioned that the addition of 1% ALTA solution to SCSFS did not influence its pH.



**Figure 14.** Effect of ALTA™ 2341 and FARGO™ 32 on growth of *L. monocytogenes* P1 in SCSFS at 4°C

Total inhibition of *L. monocytogenes* P1 was observed with 0.25 and 0.5% (w/v) ALTA and 1% FARGO for the 4-week incubation period (Figure 14), whereas recovery (delayed growth) of the *Listeria* strain occurred after about 2 weeks when using 1% ALTA and the lower concentrations of FARGO. This difference is difficult to explain as ALTA and FARGO are more or less the same, in spite of the live culture present in FARGO. In fact, this live culture did not grow under the incubation conditions of the study as its presence could be detected on NAP medium but with no further proliferation. Otherwise, the different methods of preparation of the solutions could be a probable explanation, as heating of the ALTA solution will cause its concentration and therefore making the comparison of the concentrations difficult. It is hence possible that comparison of the results of the 0.5% ALTA and 1% FARGO treatments is more appropriate. It could be speculated that the treatments would rank based on the increasing concentration of the compounds as follows: 0.25% FARGO – 0.5% FARGO – 0.25% ALTA – 0.5% ALTA – 1.0% FARGO and 1.0% ALTA.

This could mean that the lower concentrations (0.25 and 0.5% FARGO) are not sufficient to suppress growth of *Listeria* for the whole storage period, while higher concentrations are listericidal. However, the listericidal action of 1% ALTA did not last. This is not in line with the bacteriocin activity data obtained during the trial. According to Table 3, the bacteriocin activity resulting from 1% ALTA and 1%

FARGO was the same throughout storage, so that the antilisterial effect obtained should have been comparable. Moreover, different activity values were obtained among the listericidal treatments, which cannot be explained. Otherwise, a drop in activity was observed with increasing storage time for most treatments.

**Table 3.** Bacteriocin activity (AU/ml) measured for the various treatments by the agar spot method<sup>1</sup> throughout the incubation period in SCSFS at 4°C

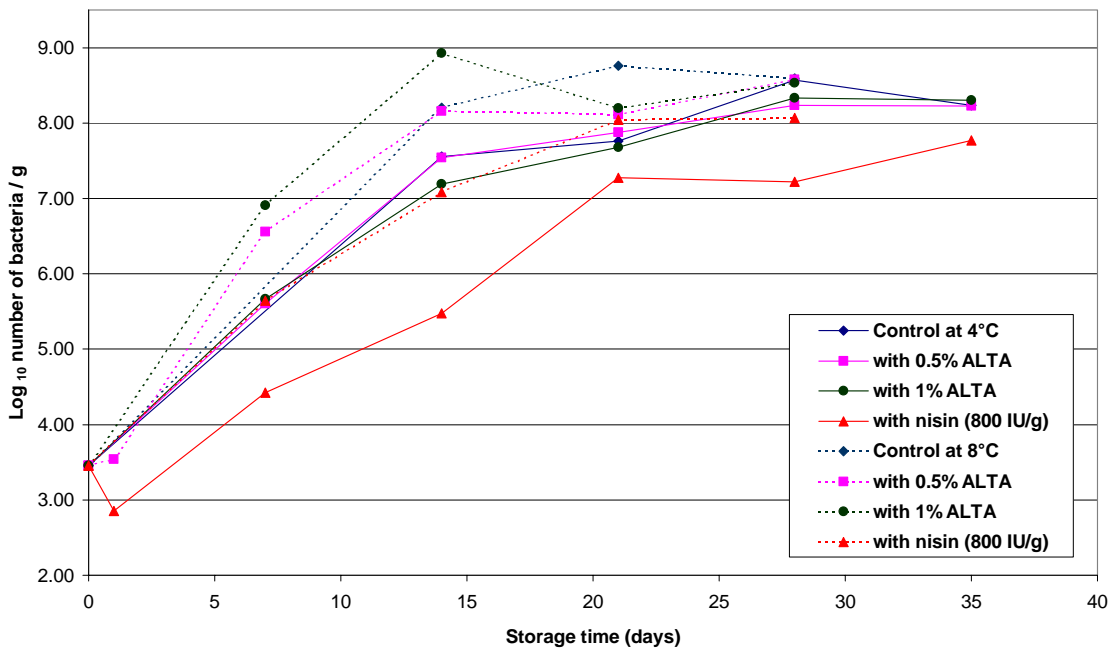
Storage time (week)	ALTA treatments (w/v)			FARGO treatments (w/v)		
	0.25%	0.5%	1%	0.25%	0.5%	1%
1	200	400	1600	100	800	1600
2	100	200	400	100	400	400
3	100	200	400	100	100	400
4	100	200	400	100	100	400

1: tested against *L. monocytogenes* P1

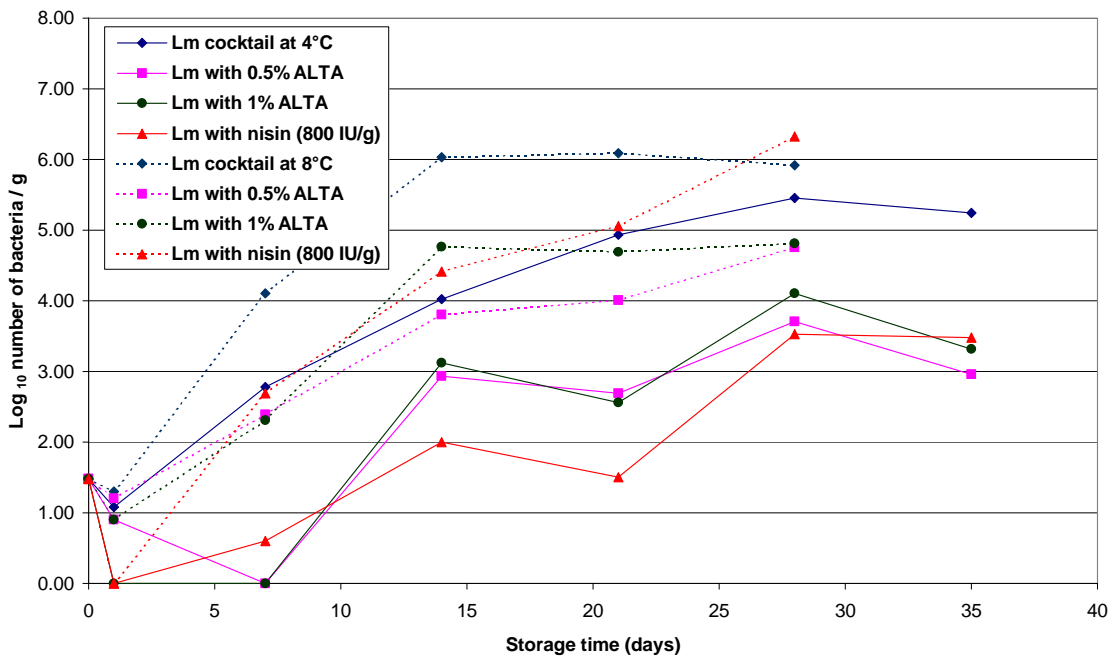
### Effect of ALTA™ 2341 and nisin A on growth of *L. monocytogenes* cocktail in CSS at 4 and 8°C

Commercial CSS was inoculated with a *L. monocytogenes* cocktail and the inhibitory effect of nisin A (800 IU/g) and ALTA™ 2341 (0.5 and 1% w/w) was tested at 4 and 8°C during a storage period of 35 and 28 days, respectively. TVC, LAB and *Listeria* counts were followed throughout storage of CSS for all treatments. The microflora of the control treatments proliferated steadily, reaching log 8/g after 14 days at 4°C but after 7 days at 8°C. The microflora developing in the ALTA treatments behaved similarly. In the nisin treatments, the natural microflora was delayed as it took 14 and 21 days at 4 and 8°C, respectively, to reach the counts of the other treatments (data not shown). Figure 15 shows the development of the LAB microflora as influenced by the different treatments at both temperatures. Again, similar results were obtained for the control and ALTA treatments at both temperatures, except for a slightly faster proliferation of the LAB microflora with the higher concentration of ALTA at 8°C. On the other hand, this microflora was slightly suppressed by nisin, lowering the counts by about 1 log throughout storage.

Figure 16 illustrates the effect of the compounds tested on growth of the *Listeria* cocktail at both temperatures. Nisin had a slightly better inhibitory effect than ALTA on *Listeria* during the first 3 weeks of storage at 4°C. Overall, only a mild bacteriostatic effect was obtained by either compound, leading to the proliferation of the *Listeria* cocktail at 4°C and an increase in counts of about 2 log from the inoculation level after 35 days of storage. At 8°C, a similar bacteriostatic effect was caused by the compounds during the first 3 weeks, though a slightly better effect was seen with 0.5% ALTA. With further storage, the bacteriostatic effect of both ALTA concentrations was maintained at one log below the control, while growth of *Listeria* continued in the nisin treated CSS to overpass the count of the control. It is noticeable that at 8°C, a levelling of the *Listeria* growth occurred from the second week for the control and ALTA treatments, but not for the nisin treatment. This difference could coincide with the findings relating to the LAB microflora (Figure 15) that developed steadily in the different treatments, but was delayed in the nisin treated CSS.



**Figure 15.** Effect of ALTA™ 2341 and nisin A on growth of LAB microflora (NAP, 30°C) in diced and spiked, vacuum-packed cold-smoked salmon stored at 4°C (full line) and 8°C (dashed line)



**Figure 16.** Effect of ALTA™ 2341 and nisin A on *Listeria* growth in diced and spiked, vacuum-packed cold-smoked salmon stored at 4°C (full line) and 8°C (dashed line)

It was previously reported (Task 3C) that the indigenous microflora of the controls produced a mild antilisterial factor, which was detected from the surface of the CSS dices. This antilisterial factor is believed to be produced by the LAB microflora and was also found from day 7 on the CSS treated with ALTA. This indicates that the unhindered growth of the indigenous LAB microflora in the control and ALTA treatments may have contributed to the bacteriostatic effect observed towards the *Listeria* cocktail in CSS, especially at 8°C. Due to the delayed growth of the LAB microflora in the nisin treated CSS, such an antilisterial factor was detected rather late (day 28) and did not seem to influence the *Listeria* load already accumulated at 8°C, but may have had some influence at 4°C where levelling of *Listeria* occurred during the 5<sup>th</sup> week of storage. It should be mentioned that the antilisterial factor detected in these various treatments was never detected from the diluted samples, but only on the surfaces of CSS dices, indicating an activity of less than 400 AU/g.

Based on the organoleptic assessment performed by the analysts at sampling, it can be said that the addition of ALTA and nisin did not contribute to the spoilage of the spiked CSS. In fact, they apparently retarded the loss in sensory quality (data not shown), especially in the 8°C treatments. Else, the addition of ALTA and nisin did not interfere much with the initial pH of the CSS as evaluated at the beginning of the trial. They actually caused a slight decrease in pH of about 0.05 unit.

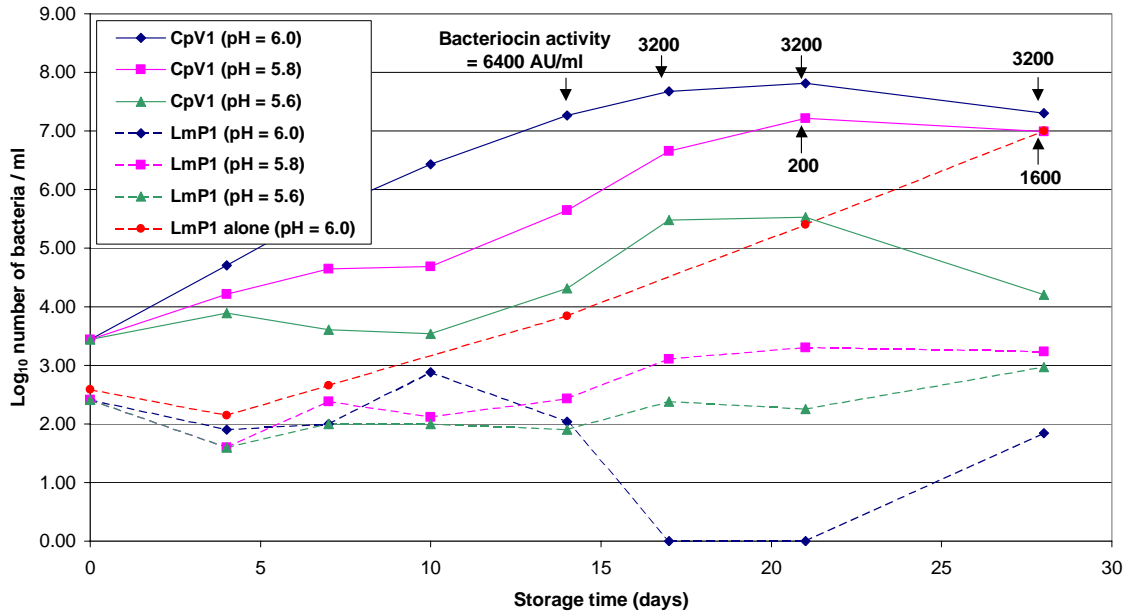
### **TASK 3E: Control using combinations of selected LAB and antimicrobial agents**

**Effect of pH on growth and bacteriocin production of *C. piscicola* strains in SCSFS at 4 and 8°C** - See following trial for summary of results.

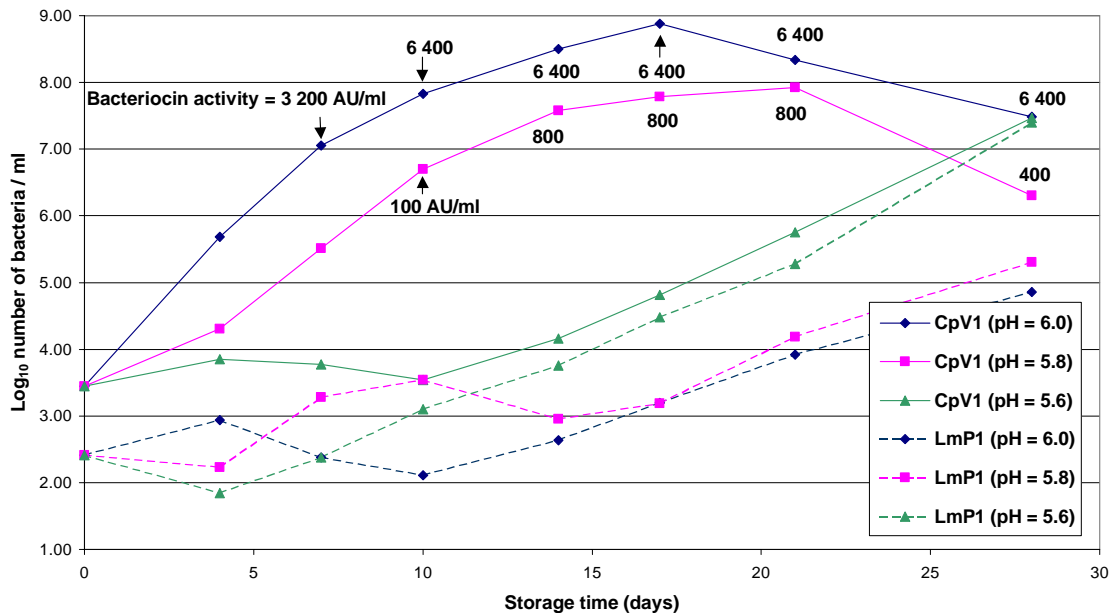
#### **Effect of pH (citric acid) on growth/bacteriocin production of *C. piscicola* V1 and its inhibition against *L. monocytogenes* P1 in SCSFS at 4 and 8°C**

A preliminary trial tested the effect of SCSFS acidification (5.2, 5.4 and 5.6) using either lactic or citric acid on the growth and bacteriocin production of *C. piscicola* V1 and SF644 at 4 and 8°C. Much acidification had a negative effect on both *Carnobacterium* strains, suppressing their growth and bacteriocin production, citric acid having generally a milder effect than lactic acid (data not shown). Based on these results, further testing was carried out to verify whether a slightly milder acidification of the SCSFS using citric acid in combination with *C. piscicola* V1 would allow for a better control of *Listeria* at 4 and 8°C.

Figures 17 and 18 demonstrate how growth of *C. piscicola* V1 and bacteriocin production were affected by the decreasing pH of SCSFS at 4 and 8°C. Even at a slightly lower pH (5.8), growth and bacteriocin production of CpV1 were diminished resulting in a lesser bacteriostatic effect on *Listeria* at 4°C, but at 8°C the antilisterial effect observed was similar to that at regular pH (6.0). As previously found, pH 5.6 was too acidic to allow for bacteriocin production of CpV1 and had a suppressing effect on its growth. However, a slightly better inhibition of *Listeria* at 4°C was seen at pH 5.6 than 5.8, probably due to the acidification itself.



**Figure 17.** Growth of *C. piscicola* V1 and *L. monocytogenes* P1 in SCSFS at different pH at 4°C (Bacteriocin activity given as evaluated for CpV1 treatments by the agar spot method using *L. monocytogenes* P1.)



**Figure 18.** Growth of *C. piscicola* V1 and *L. monocytogenes* P1 in SCSFS at different pH at 8°C



**Effect of ALTA™ 2341 and FARGO™ 23 on growth/bacteriocin production of *C. piscicola* strains and their inhibition against *L. monocytogenes* P1 in SCSFS at 4 and 8°C**

The antilisterial action provided by the *C. piscicola* strains V1 and SF644 has been previously demonstrated in SCSFS (Figures 1 and 2) and CSS (Figures 7 and 11) trials. However, their antilisterial action was found to be diminished in CSS. On the other hand, ALTA and FARGO were found to be listericidal at some of the concentrations tested in SCSFS (Figure 14). Though, the use of ALTA in CSS only provided a slight bacteriostatic effect towards the *Listeria* cocktail assessed (Figure 16). This led to evaluate whether a combined system consisting of either ALTA or FARGO in combination to *C. piscicola* V1 or SF644 would provide a better control of *L. monocytogenes* P1.

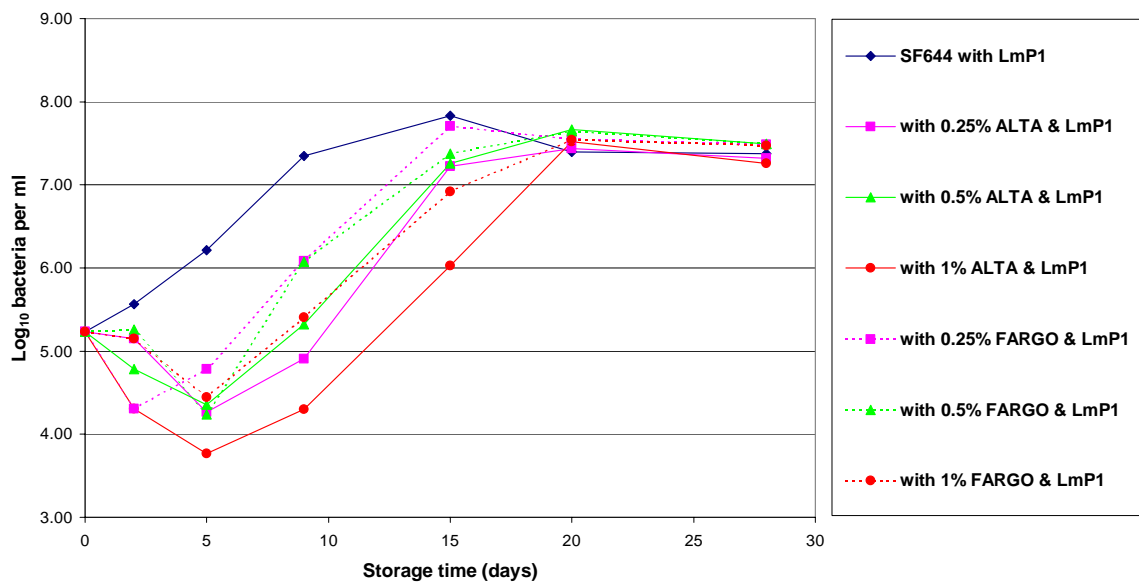
Table 4 shows the importance of the *Listeria* inhibition obtained in SCSFS at 4 and 8°C due to the presence of *C. piscicola* strains alone or in combination with ALTA or FARGO at 3 concentrations. The inhibition seen with CpV1 alone at 8°C was better than expected as in previous trials where its bacteriostatic effect lasted for only 2 weeks. This difference lies certainly in the level of inoculation of *Listeria*, being ca 1.5 log higher in earlier trials. Else, combining ALTA or FARGO to CpV1 was not apparently better than CpV1 alone. However, the use of either compound with SF644 contributed to the total inhibition of *Listeria*, which was not achieved with SF644 alone. All concentrations (0.25, 0.5 and 1% w/v) were listericidal. Interestingly, ALTA and FARGO delayed the growth of SF644 at both temperatures, with ALTA having a slightly stronger inhibitory effect at 4°C (Figure 19). Despite this effect, bacteriocin production of SF644 occurred earlier at 4°C (before day 15) and at lower cell counts when combined with either compound, as well being enhanced with increasing storage time at both temperatures (Figures 20 and 21). Stationary phase of SF644 was reached on days 15-20 at 4°C with no further bacteriocin production for SF644 alone, while increased production was observed with further storage due to the compounds. At 8°C, stationary phase was attained on day 9 and further bacteriocin production occurred till day 15 for all treatments. FARGO was slightly more stimulating on bacteriocin production of SF644 than ALTA, as observed with increasing concentration.

**Table 4.** Antilisterial effect obtained when using *C. piscicola* strains with or without ALTA™ 2341 or FARGO™ 23 in SCSFS at 4 and 8°C

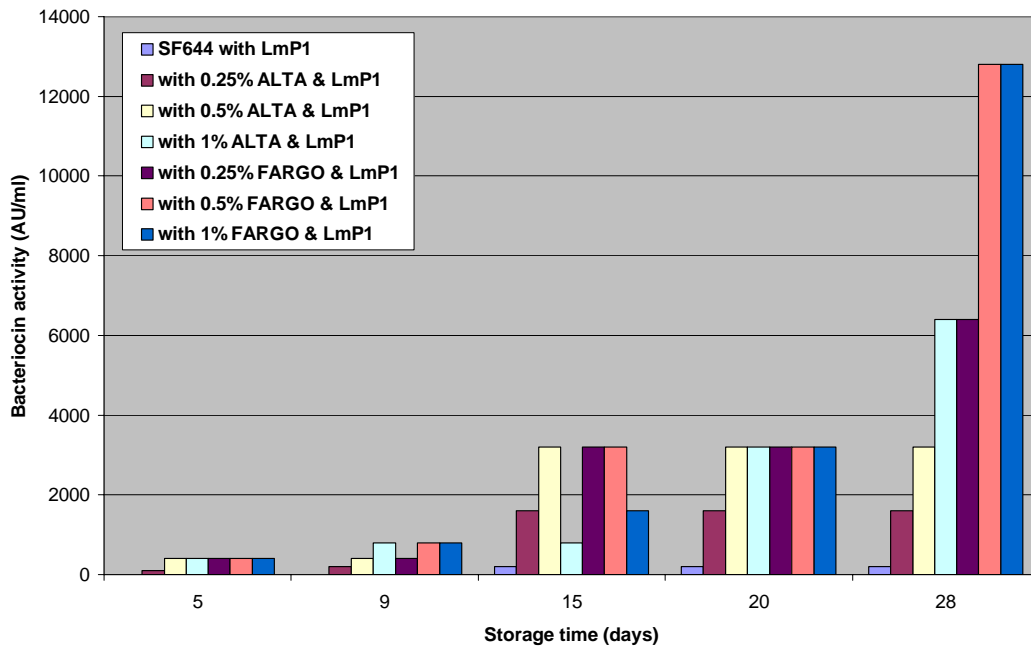
Inhibition of LmP1* with: and		CpV1		SF644	
		at 4°C	at 8°C	at 4°C	at 8°C
ALTA™ 2341	0% w/v	+++	+++	+	++
	0.25 %	+++	+++	+++	+++
	0.5 %	+++	+++	+++	+++
	1.0 %	+++	+++	+++	+++
FARGO™ 23	0% w/v	+++	+++	+	++
	0.25 %	+++	+++	+++	+++
	0.5 %	+++	+++	+++	+++
	1.0 %	+++	+++	+++	+++

+: slight inhibition (somehow bacteriostatic), ++: inoculation level maintained (bacteriostatic), +++: total inhibition (bactericidal); \*: inoculation level of *Lm* P1= log 2.5/ml

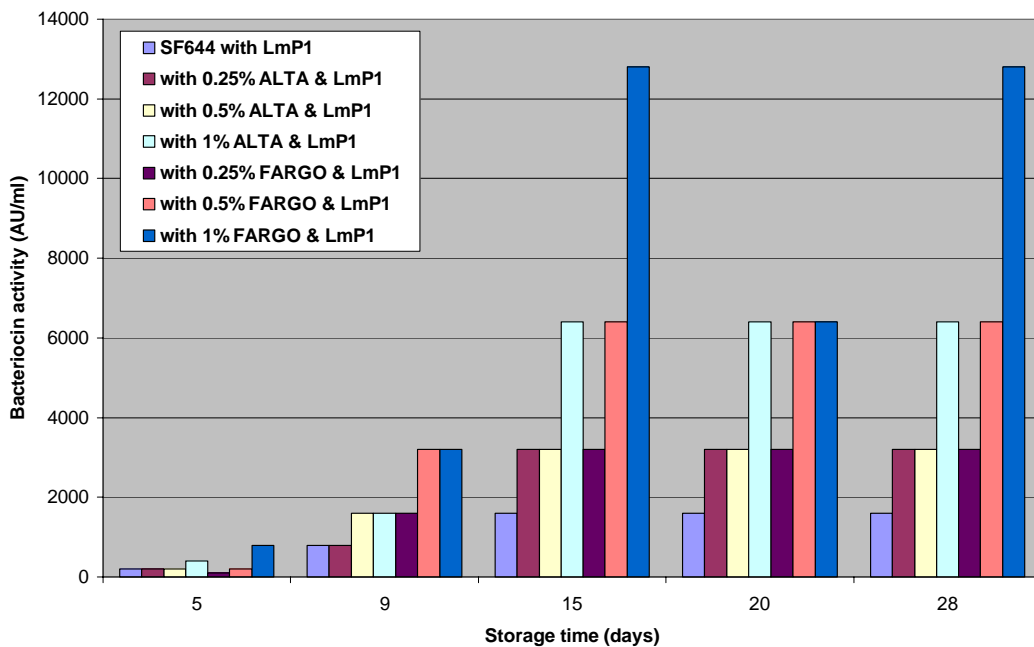
Bacteriocin production of CpV1 was also stimulated by the presence of ALTA and FARGO at both temperatures. However, growth of CpV1 was not influenced by the compounds (data not shown). At 4°C, an early enhanced production was observed on day 5 with the added compounds while no further enhancement took place with storage (Figure 22). Maximum bacteriocin production was reached for all treatments on day 15, parallelly to the stationary phase. At 8°C, an early enhanced production was also observed (day 2), as well as increased production later on during storage (Figure 23). Stationary phase of CpV1 was reached on day 5 with no further bacteriocin production for CpV1 alone, while increased production was observed with ALTA and FARGO treatments. It should be pointed out that bacteriocin production has been generally found to occur at cell counts of log 7-7.5 per ml for SF644 but log 6-6.5 for CpV1. Addition of ALTA or FARGO allowed for bacteriocin production at lower counts at 4°C for SF644 (log 4-5/ml) and at 8°C for SF644 and CpV1 (log 5.5-6/ml).



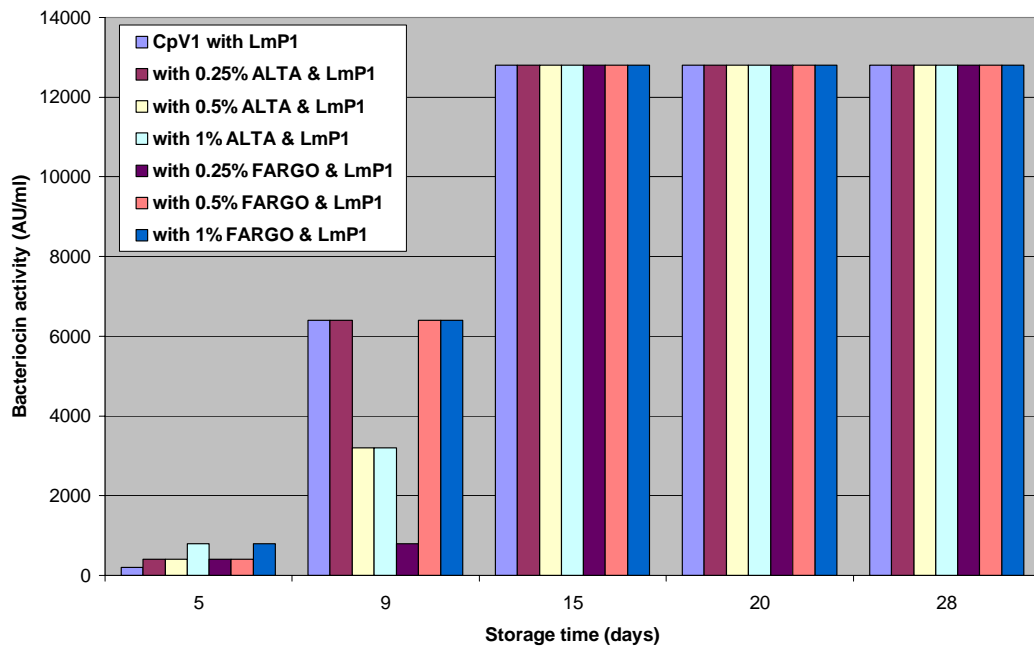
**Figure 19.** Growth of *C. piscicola* SF644 co-inoculated with *L. monocytogenes* P1 in SCSFS at 4°C in presence of different concentrations of ALTA™ 2341 or FARGO™ 23



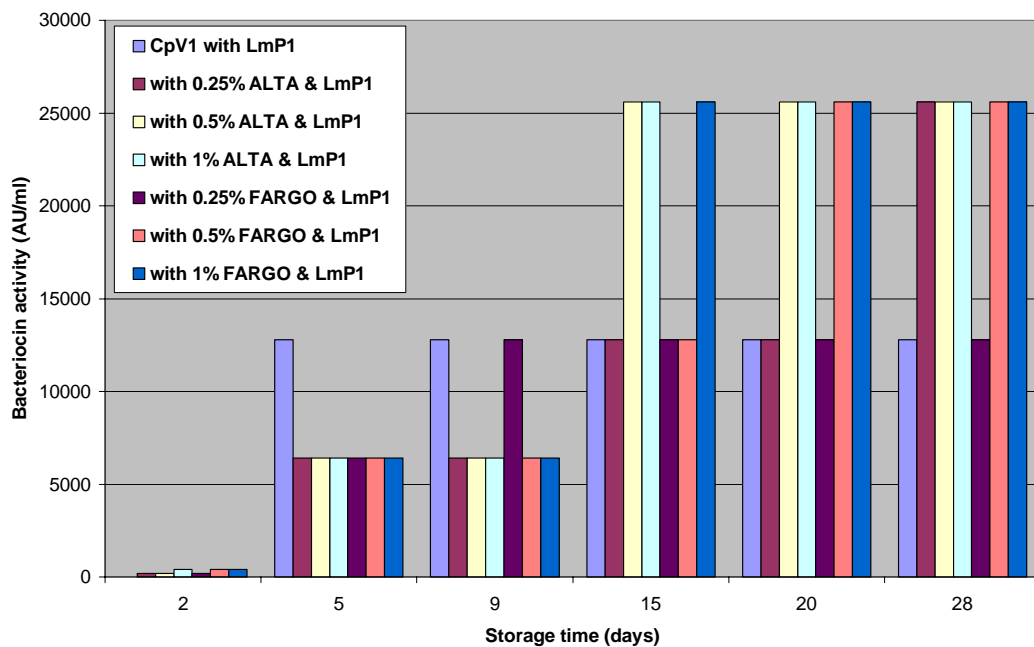
**Figure 20.** Bacteriocin production of *C. piscicola* SF644 co-inoculated with *L. monocytogenes* P1 in SCSFS at 4°C in presence of different concentrations of ALTA™ 2341 or FARGO™ 23



**Figure 21.** Bacteriocin production of *C. piscicola* SF644 co-inoculated with *L. monocytogenes* P1 in SCSFS at 8°C in presence of different concentrations of ALTA™ 2341 or FARGO™ 23



**Figure 22.** Bacteriocin production of *C. piscicola* V1 co-inoculated with *L. monocytogenes* P1 in SCSFS at 4°C in presence of different concentrations of ALTA™ 2341 or FARGO™ 23

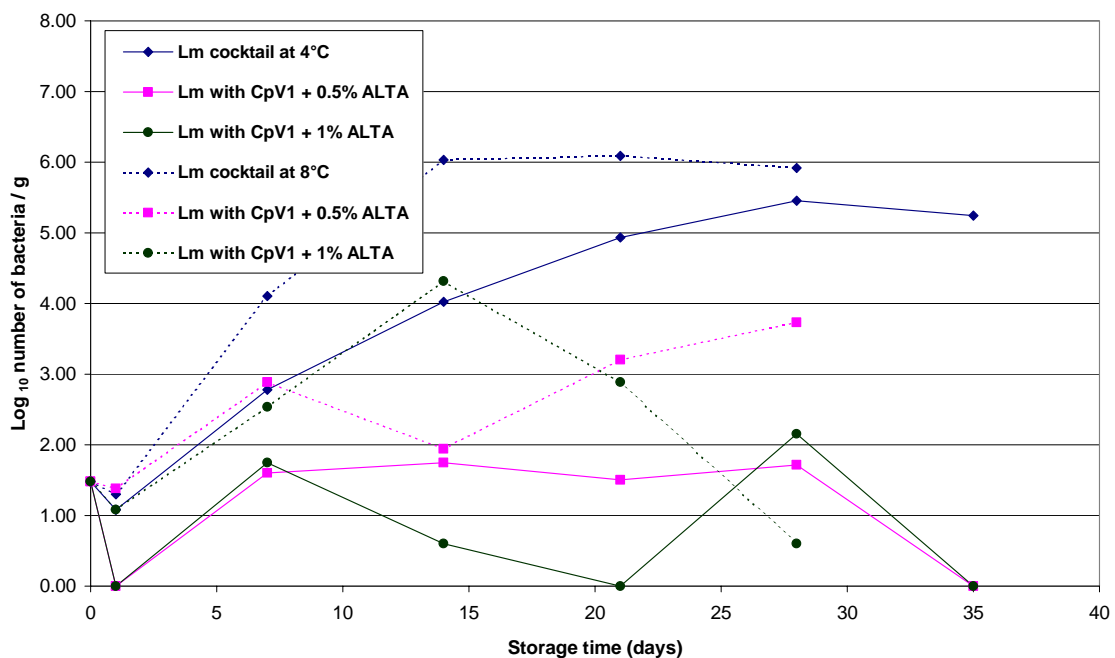


**Figure 23.** Bacteriocin production of *C. piscicola* V1 co-inoculated with *L. monocytogenes* P1 in SCSFS at 8°C in presence of different concentrations of ALTA™ 2341 or FARGO™ 23

**Effect of ALTA™ 2341 on growth/bacteriocin production/antilisterial action of *C. piscicola* V1 against *L. monocytogenes* cocktail in CSS at 4 and 8°C**

Commercial CSS was spiked with a *L. monocytogenes* cocktail and the inhibitory effect of a combined system, including *C. piscicola* V1 and 0.5% or 1% (w/w) ALTA™ 2341, was tested at 4 and 8°C during a storage period of 35 and 28 days, respectively. TVC, LAB and *Listeria* counts were followed throughout storage. The microflora of the controls treatments proliferated steadily, reaching log 8/g after 14 days at 4°C but after 7 days at 8°C. The microflora of the combined treatments was in fact dominated by the CpV1 load added to the CSS (data not shown).

Figure 24 illustrates the effect of the combined systems tested on growth of the *Listeria* cocktail at both temperatures, while Table 5 compares the bacteriocin production of CpV1 as influenced by its combination with ALTA. The combination of CpV1 and 0.5% ALTA had a strong bacteriostatic effect on the *Listeria* cocktail at 4°C which was maintained for 4 weeks, but on day 35 the cell count dropped below the detection level. This effect coincided with the amount of bacteriocin detected, being highest (12800 AU/g) on day 28 followed by a drop down to 3200 AU/g on day 35. Moreover, this combination provided a better control of *Listeria* than CpV1 (Task 3C, Challenge studies - Part B) or ALTA (Task 3D) did when used separately. The use of 0.5% ALTA apparently contributed to a higher bacteriocin production of CpV1 and for a longer period. At 8°C, only a mild bacteriostatic effect was seen for the same combined system, similarly to the results obtained when using CpV1 alone and with a comparable amount of bacteriocin produced. The importance of the effect produced by 0.5% ALTA at that storage temperature is therefore questionable.



**Figure 24.** Effect of combined use of ALTA™ 2341 and *C. piscicola* V1 on *Listeria* growth in diced and spiked, vacuum-packed cold-smoked salmon stored at 4°C (full line) and 8°C (dashed line)

When the combined system included 1% ALTA, the effect became listericidal for most samples analysed throughout storage at 4°C. Again, this combination provided a better control of *Listeria* than ALTA or CpV1 did alone. At the higher temperature, the effect was not apparent until the completion of the second week, after which a drop in cell counts took place to reach the detection level on day 28. The amount of bacteriocin detected for CpV1 alone or when combined with 1% ALTA was comparable at either temperature, indicating that the increased inhibitory effect observed for that combined system is not due to enhanced bacteriocin production but more likely to a synergistic effect of ALTA (1% w/w) and CpV1. In fact, it was usually difficult to compare the bacteriocin activity measured and the antilisterial effect observed, as higher activity did not necessarily mean greater effect as shown in Table 5.

**Table 5.** Bacteriocin activity (AU/g) detected in the various CpV1 treatments as measured by the agar spot method<sup>1</sup> throughout the storage period

Storage time (days)	CpV1 <sup>2</sup>		+ 0.5% ALTA		+ 1% ALTA	
	4°C	8°C	4°C	8°C	4°C	8°C
7	8-1600	12800	800	12800	8-1600	12800
14	64-12800	6400	6400	12800	6400	6400
21	6400	3200	64-12800	3200	6400	1600
28	3200	3200	12800	3200	6400	32-6400
35	32-6400		3200		32-6400	

1: tested against *L. monocytogenes* P1; 2: data collected in Task 3C (Challenge studies - Part B)

## Chapter 4 Discussion

Consumers' demand for commercially processed foods with low levels or no traditional preservatives is well-known. This has led to a search for safe and food-grade preservatives of biological origin (biopreservatives). Antibacterial peptides or bacteriocins produced by many strains of lactic acid bacteria (LAB) have been shown to inhibit food-borne pathogens. Growth of bacteriocin-producing LAB in food products stored at chilled temperatures is of particular interest and could be an alternative way of producing a safer food supply, especially when the use of preservatives should be restricted and/or current preservative methods are insufficient.

Cold-smoked fish (CSF) is a ready-to-eat commodity which has been found to be contaminated with *L. monocytogenes*. This pathogen is able to grow at refrigerated temperatures and its control is therefore essential to ensure the safety of consumers. Unfortunately, the current technological parameters involved in CSF processing have been shown to be unsuccessful in inhibiting the growth of this bacterium (Hudson & Mott, 1993; Guyer & Jemmi, 1991; Rørvik *et al.*, 1991). It is therefore necessary to define means to control *Listeria* growth. Various studies (Eijsink *et al.*, 1998; Jack *et al.*, 1996; Stiles, 1996; Nettles & Barefoot, 1993) have demonstrated the antilisterial effect of several bacteriocins. However, the legislation prohibits their use, nisin being the only approved bacteriocin but with restricted use.

*Listeria* proliferation could be controlled by the direct use of bacteriocin-producing LAB in the product, although this implies their ability to grow and produce bacteriocin under the product's conditions. Such cultures have been called protective cultures. Their origin is also of major importance, if they have to be legislatively approved for use, as well as their inability to cause spoilage of the products. All these criteria are in fact limitations for the use of LAB in chilled fishery products. Though, there have been reports (Nilsson *et al.*, 1999; Pilet *et al.*, 1995) on the antilisterial activity of *Carnobacterium* strains that were isolated from fish. One of these strains (Pilet *et al.*, 1995), *C. piscicola* V1 (CpV1), as well as *C. piscicola* SF644 from cold-smoked salmon, other previously owned strains (*C. piscicola* UI49, *Lc. lactis* SIK-83 and *Lb. bavaricus* MI401) and 3 commercial strains (ALC 01, Fishflor® and N-8) were tested in this study for their potential use as protective cultures in cold-smoked salmon.

Preliminary trials were conducted in a simulated cold-smoked fish system (SCSFS). At low temperatures, only *C. piscicola* V1 and SF644 could grow properly and suppress *Listeria* proliferation. The inhibitory action produced by *C. piscicola* SF644 was confirmed to be of proteinaceous nature (bacteriocin). Bacteriocin production of CpV1 has been found to occur early at the exponential phase but to reach a maximum production at the beginning of the stationary phase when grown at 20°C (Pilet *et al.*, 1995). This agrees with our findings for CpV1 at 8°C, while bacteriocin production at 4°C was only detectable in the stationary phase. Similar results were obtained for *C. piscicola* SF644. However, bacteriocin production of CpV1 was found to be greater than that of SF644 at both temperatures. The higher incubation temperature brought about an earlier bacteriocin production for both strains, but did not influence the amount (titer) produced. *C. piscicola* V1 has been found to produce 2 bacteriocins, piscicocins V1a and V1b, the former one being approximately 100 times more active than V1b (Bhugaloo-Vial *et al.*, 1996).

Both *Carnobacterium* strains were bacteriostatic to *L. innocua* P1, the effect lasting for about 2 weeks at 4°C but 6-9 days at 8°C, after which slow growth occurred. Interestingly, *L. monocytogenes* P1 was found to be more sensitive than *L. innocua* P1 to CpV1 which had a bactericidal effect at 4°C for the whole incubation period (31 days), but was bacteriostatic at 8°C. Otherwise, monocultures of *Listeria* strains were shown to proliferate steadily in SCSFS at 4°C, but much faster at 8°C.

The ability of these *Carnobacterium* strains to inhibit or delay *Listeria* growth at low temperatures in a smoked salmon extract containing 4% salt (SCSFS) indicated their possible use as protective cultures. However, it has been found (Paludan-Müller *et al.*, 1998; Leroi *et al.*, 1998) that *Carnobacterium piscicola* actually dominates the microflora of cold-smoked salmon later during storage. The *C. piscicola* strains could therefore be involved in the spoilage process of this product. Although these recent studies showed the contrary, it was felt necessary to verify whether their addition at high levels ( $10^4$  CFU/g) to newly processed cold-smoked salmon would accelerate the spoilage process. Sensory evaluation (smell, appearance and texture) of the inoculated products compared to a control (uninoculated cold-smoked salmon) confirmed that these strains did not promote spoilage. However after 4 weeks of storage at 8°C, the CpV1 treatment was found to be significantly worse than the control, but both were deemed spoiled. Despite this difference noticed by the sensory panel, comparable results were obtained among the samples when analysed by headspace analysis using

an electronic nose. The possibility that CpV1 may contribute to the spoilage of cold-smoked salmon at higher temperature is interesting because it would bring a certain margin of safety to the product, by spoiling it before the outgrowth of *Listeria*.

A challenge study of cold-smoked salmon, inoculated with *C. piscicola* strains and *L. monocytogenes* P1 and stored at 4 and 8°C, demonstrated the ability of the *C. piscicola* strains tested, especially CpV1, to grow in such a product at low temperatures and to delay *Listeria* proliferation in spite of its rather high inoculation level (log 2.5/g). A bactericidal effect on *L. monocytogenes* P1 was observed during trials in smoked salmon extract (SCSFS) with CpV1 at 4°C for the whole incubation period (31 days), whereas *Listeria* increased by one log in the product itself. At 8°C, only a slight bacteriostatic effect was obtained in CSS allowing for a 2-log *Listeria* increase. This apparent loss in biological activity for CpV1 agreed with the bacteriocin activity results obtained during the trials in SCSFS and in the product, i.e. where about 8 times less activity was detected in the product at 8°C (titer of 12 800 vs. 1600 AU/ml, when tested against *L. monocytogenes* P1).

In fact, similar losses of activity have been reported by other workers (Nilsson *et al.*, 1999). Schillinger *et al.* (1991) detected a much higher activity of sakacin A in meat juice than in pasteurized minced meat as produced by the bacteriocin-producing strain *Lb. sake*, suggesting a limited diffusion of the bacteriocin in the latter environment. Also, Scott & Taylor (1981) found nisin to be less effective in cooked meat medium containing particles than in Trypticase peptone yeast glucose broth or Brain heart infusion broth, suggesting binding of nisin to meat particles. Binding of the bacteriocin to food is a well-known phenomenon, which can lead to its inactivation, as well as the possible presence of indigenous proteases. Other intrinsic factors and diffusibility of the bacteriocin into the food structure may also play a role (Blom *et al.*, 1997). Eventhough bacteriocin activity could not be measured at 4°C, an inhibitory factor was detected on the packaging film and/or on the CSS from day 18. This inhibition could be explained by the presence/activity of *C. piscicola* strains and/or their bacteriocin on the packaging material and the CSS. It is also possible that the extraction method achieved by simply stomaching the CSS pieces in a diluent was not proper, leading to an underestimation of the actual bacteriocin production.

Moreover, CpV1 was found to be more efficient than SF644 in delaying the growth of *L. monocytogenes* P1 in CSS. Other workers (Nilsson *et al.*, 1999; Campos *et al.*, 1997; Mathieu *et al.*, 1994; Buchanan & Klawitter, 1992) reported the potential growth of *C. piscicola* strains, their bacteriocin production and enhanced inhibitory activity against *L. monocytogenes* at low temperatures. *Listeria* was otherwise found to proliferate well and at a similar rate when inoculated alone into SCSFS and on the surface of CSS. CpV1 appears to be a promising protective culture, but did not provide a total control of high levels (log 2.5/g) of *L. monocytogenes* P1 at chilled temperatures in cold-smoked salmon. CpV1 has been reported (Pilet *et al.*, 1995) to have a large spectrum of activity against other LAB. This could imply a certain ease or ability to compete with the microflora establishing in vacuum-packed cold-smoked salmon during storage.

In the search of a better inhibitory system, various antimicrobial compounds were assessed in SCSFS at low temperatures. Nisin A (nisaplin) was listericidal at 4 and 8°C while nisin Z, its variant, only provided a short bacteriostatic effect towards *L.*



*monocytogenes* P1. Growth of *L. monocytogenes* P1 was reduced in SCSFS acidified by organic acids (pH 5.4), citric acid being more efficient than lactic acid at 8°C. In fact, the bacteriostatic effect observed for the organic acids lasted longer than that seen with nisin Z. Thomas & Wimpenny (1996) investigated the effect of combined factors on *Listeria* inhibition and found that at 4% NaCl and below pH 5.8, growth decreased, but this was tested at 20°C. Moreover, they reported that in the presence of nisin, salt and pH tolerance of *Listeria* was diminished. In our trials, acidification of the nisin Z treatment to pH 5.4, using either lactic or citric acid, led to a better control of the pathogen at both temperatures than when using the antimicrobial compounds separately. Increased antibacterial activity of bacteriocins at low pH values is common (Stiles, 1996; Jack *et al.*, 1995) and has been attributed to their increase in net charge at low pH, which might facilitate translocation of bacteriocins through the cell wall.

Acidification by organic acids can be useful in biopreservation, but it should not affect the sensory quality of the food products. Sensory evaluation of an acidified CSS paste revealed that it was possible to lower the pH down to 5.6, using either citric or lactic acid, without interfering with its quality. However at pH 5.4, noticeable organoleptic changes occurred, being worst with lactic acid as it caused sourness and rancidity. The use of citric acid to acidify CSS would imply modifications of the processing steps, probably including the acid and/or its salt at the brining step. The technical feasibility of this modification was not tested in this study, but would need to be thoroughly assessed to ensure for instance proper salt uptake of the muscle.

ALTA™ 2341 (ALTA) is an all-natural, multi-functional food ingredient based on traditional lactic acid bacteria. It contains natural metabolites, including organic acids and the bacteriocin pediocin found in typical, fermented meat products. Since its characterization in 1987, there has been considerable scientific literature published supporting the effectiveness of pediocin against Gram-positive bacteria, including *L. monocytogenes*. ALTA can serve as an effective barrier to help control the development of *Listeria* (Szabo & Cahill, 1999; Szabo & Cahill, 1998; Glass *et al.*, 1995). FARGO™ 23 (FARGO) includes the same metabolites as for ALTA, but contains in more the live culture producing pediocin. In France, it is added to raw milk intended for raw milk cheese production.

In this study, 3 concentration levels (0.25, 0.5 and 1% w/v) were tested for either compound in SCSFS at 4°C and their antilisterial effect evaluated. At the lower concentrations (0.25 and 0.5%) of FARGO, *Listeria* was suppressed for 1-2 weeks after which steady growth occurred. The other levels of FARGO and ALTA were listericidal throughout storage (28 days), with the exception of 1% ALTA for which the effect lasted only for 3 weeks. These findings did not agree with the bacteriocin activity measured in the treatments. It is therefore difficult to explain the lesser effect of 1% ALTA, but this observation was reproducible. Otherwise, a drop in activity was observed with increasing incubation (storage) time, indicating that the compounds were being "used up" or inactivated with time. Similar results were obtained by Szabo & Cahill (1998) when assessing the antilisterial effect of 0.1 and 1% ALTA in a broth system. A bacteriostatic effect was seen at 4°C for 10-12 days, followed by a faster growth of the *Listeria* population at the higher ALTA level under 100% N<sup>2</sup> or air. Comparable results were found at 12°C. No explanation was suggested. The question is whether an excess amount of ALTA could stimulate some protective behaviour and

lead to the development of tolerance or resistance and eventual growth of some *Listeria* cells. If this is the case, the ALTA concentration selected should be in accordance with the expected contamination of the product. Interestingly, Ming & Daeschel (1993) reported fundamental changes in bacterial membrane structure and function of the *Listeria* strain studied in the presence of nisin, probably as a resistance response.

In the light of the results, further testing was done in SCSFS where the use of combined biopreservatives to suppress *Listeria* growth was evaluated. It was aimed at finding a combined system which would stimulate the bacteriocin production of *C. piscicola* strains and/or act synergistically. Acidification by organic acids (lactic and citric acid) had a negative effect on growth and bacteriocin production of both *C. piscicola* strains, leading to a reduced antilisterial effect, even at pH 5.8. Similarly, it was reported by Pilet *et al.* (1995) that the bacteriocin production of CpV1 doubled in pH 6.5 MRS-regulated cultures as opposed to a non-regulated medium, where the pH is expected to drop.

It has been speculated that the enhancement of LAB bacteriocin activity could be achieved by various strategies: (i) the addition of chelating agents (Stevens *et al.*, 1991), (ii) protein engineering to enhance solubility and stability (Rollema *et al.*, 1995), and (iii) the use of combinations of different LAB bacteriocins (Hanlin *et al.*, 1993). In a study by Mulet-Powell *et al.* (1998), it was pointed out that pediocin was a good candidate for use as a biopreservative, as it produced synergistic effects in combination with other bacteriocins. Hanlin *et al.* (1993) reported a greater killing action towards LAB and *Listeria* when combining pediocin AcH and nisin. Moreover, the chance of the development of resistant strains should be reduced. In our study, growth and bacteriocin production of the *C. piscicola* strains (CpV1 and SF644) in presence of ALTA or FARGO was evaluated as well as their effect on *L. monocytogenes* P1 proliferation in SCSFS at low temperatures. The combination with either compound was profitable to SF644, rendering their effect listericidal at both temperatures, the lowest concentration (0.25%) being just as effective as 1%. It is noticeable that 0.25 and 0.5% FARGO as well as 1% ALTA only delayed the growth of *L. monocytogenes* P1 when tested alone. This indicated that the listericidal effect obtained when combined to SF644 was the result of synergism between the strain and either compound. The effect was not as evident for CpV1 as it was found to be already listericidal when used alone. However, previous trials had demonstrated a lesser antilisterial effect of CpV1 at 8°C probably due to the higher *Listeria* inoculation level. The addition of the compounds did not affect the growth of CpV1 as it did for SF644. However, the compounds stimulated bacteriocin production for both strains, with earlier detection of bacteriocin and generally at lower cell counts. Also, enhanced production was observed as storage progressed, except for CpV1 at 4°C. The concentration (0.25, 0.5 and 1% w/v) of the compounds was apparently directly related to the bacteriocin production of SF644 and inversely to its growth, with FARGO being a better stimulator. No such effects were noticed for CpV1. Overall, nisin A, ALTA and FARGO produced an antilisterial effect in the simulated CSS system and had to be tested in the product itself for confirmation.

Based on these findings, a challenge study was conducted to evaluate the use of nisin A (800 IU/g), ALTA (0.5 and 1% w/w) as well as the combination of ALTA and CpV1 to control the growth of a *L. monocytogenes* cocktail in CSS, as compared to

the use of CpV1 alone at 4 and 8°C. *Listeria* proliferation in the controls was found to be suppressed (8°C) or slowed down (4°C) from day 14, coinciding with a high level of an indigenous LAB microflora and the detection of an inhibition factor of less than 400 AU/g. This was in fact a good example of a naturally occurring inhibitory microflora in food products. The addition of CpV1 (log 5.6/g) to *Listeria* contaminated CSS caused a short bacteriostatic effect on the *Listeria* load and allowed for some growth. Interestingly, the antilisterial effect seen at both temperatures was comparable with regard to the *Listeria* counts. In other words, their growth rate was similar, as opposed to a higher rate usually seen at 8°C in SCSFS experiments (Figures 1 and 2) and in the previous challenge study (Figure 7). This could be explained by the higher titer of CpV1 bacteriocin found at 8°C on day 7, being more inhibitory as seen by the lag phase (Figure 11). Despite the use of more tolerant *Listeria* strains in this trial as opposed to the sensitive strain *L. monocytogenes* P1 used earlier, the inhibition obtained was similar to that in the previous challenge study in CSS (Figure 7). In fact, bacteriocin production of CpV1 was greater in this second CSS trial (Figure 11) than in the previous one, where a maximum of 1600 AU/g was measured at 8°C on day 18. Also, it was detected at 4°C, in contrast to the earlier trial. This could be due to a better adaptation of the CpV1 culture in this trial as only a very short lag phase occurred at 4°C while none at 8°C, as opposed to longer lag phases observed in the earlier challenge study, especially at 4°C (data not shown). Another possibility could be that the competitive LAB microflora already established in the product stimulated CpV1 to produce more bacteriocin, as a means to dominate. This explanation would clarify the differences in bacteriocin titers observed at 4 and 8°C, as at the higher temperature a more rapid, hence threatening, indigenous LAB microflora is expected to grow, stimulating CpV1 bacteriocin production. Sip *et al.* (1998) tested the hypothesis that in the presence of bacteriocin-sensitive organisms, the bacteriocin production by an aggressive strain would be stimulated. They observed a significantly enhanced divercin production by *C. divergens* AS7 in the presence of *C. piscicola*. This means that the antilisterial effect obtained using CpV1 could depend on the indigenous microflora developing in CSS and therefore, could have been much lesser in the presence of a different background microflora, i.e. with a lower initial LAB load, as seen in the first CSS trial.

The use of the antimicrobial compounds, either ALTA or nisin A, in spiked CSS retarded the growth of the *Listeria* cocktail. The antilisterial effect of 0.5% ALTA was not lesser than that with the 1% concentration. Nisin A (800 UI/g) was found to have more effect than ALTA during the first 3 weeks of storage at 4°C, while its effect was apparently lost during late storage at 8°C. Szabo & Cahill (1999) obtained a better bacteriostatic effect on a *Listeria* cocktail using either 1% ALTA or nisin (400 IU/g) in CSS at 4°C, whereas Nilsson *et al.* (1997) reported a slight delay with nisin (500 and 1000 IU/g). These differences are certainly related to the different sensitivity of the *L. monocytogenes* strains tested. In our study, a sudden levelling of *Listeria* growth was observed from day 14 at 8°C in the ALTA treatments as reported for the controls, but was not seen with the nisin treatment. This inhibitory effect was attributed to the development of a competitive microflora producing an antilisterial factor. TVC and LAB counts of ALTA treatments compared well to those of the controls, while counts were lower in the nisin treatments. Nilsson *et al.* (1997) also reported lower LAB counts in nisin-treated cold-smoked salmon stored at 5°C. Based on smelling of the samples, nisin and ALTA did not apparently promote spoilage of CSS. This agrees with the study of Szabo & Cahill (1999). In fact, our evaluation

suggests that it may have contributed to a slight shelf life extension at 8°C. Though, a complete sensory evaluation involving tasting is necessary to confirm these findings. Nevertheless, nisin had a negative effect on the natural microflora of CSS which was revealed to be self-protective, while ALTA did not interfere with its development. The elimination of natural competitors in food is disadvantageous and the use of an antimicrobial compound causing such an effect is questionable, unless its addition leads to a much better control of the pathogen of concern.

The use of a combined system, including CpV1 and 0.5% or 1% ALTA, in spiked CSS provided a total control of *Listeria* as it suppressed the proliferation of the cocktail at 4°C, *Listeria* counts being at or below the detection level (8 CFU/g). At 8°C, the addition of 0.5% ALTA did not enhance the inhibitory effect of CpV1. On the other hand, a sudden drop in *Listeria* occurred following day 14 in the CSS treated with the combined system including the higher ALTA concentration. On day 28, *Listeria* count was at the limit of detection. Enhancement of bacteriocin production of CpV1 in CSS due to the addition of ALTA, as reported in SCSFS trials, was only evidenced at 4°C with 0.5% ALTA. A comparison of the antilisterial effect obtained towards the *Listeria* cocktail with the amount (titer) of bacteriocin measured emphasized some incoherences, especially among the 8°C treatments. For instance, similar titers were obtained with respect to storage time, but a greater antilisterial effect was observed with 1% ALTA in the combined system. Similarly, the 1% ALTA/CpV1 treatment at 4°C showed comparable or lesser titers than the 0.5% one, despite a similar or even better antilisterial effect. This suggests that the increased inhibitory effect observed for the 1% ALTA/CpV1 system was not due to enhanced bacteriocin production but rather to a synergistic effect of both counterparts.

The selection of *L. monocytogenes* strains for the preparation of the cocktail was based on a study assessing the sensitivity of 30 strains, isolated from CSS products from 5 European countries, towards 6 antimicrobial compounds (nisin A and Z, ALTA, FARGO, CpV1 and SF644 supernatants). The results were compared to the sensitivity of the Portuguese strain, *L. monocytogenes* P1, used during the project. The *Lm* strains had been biotyped by partner 01 and belonged to 8 different biotypes. It was found that sensitivity to the compounds was more related to the origin of the strains than to their biotype. Portuguese strains were the most sensitive, *L. monocytogenes* P1 being very sensitive to all compounds, while Danish strains were the most tolerant. Though, variations in strain sensitivity/tolerance to the compounds were found. For instance, there were least variations for the nisin compounds, but most for the CpV1 and SF644 supernatants. Hanlin *et al.* (1993) suggested that the differences in sensitivity and resistance of a strain to different bacteriocins, even though the bacteriocins were adsorbed on the cells, could be that gram-positive bacterial cells have different but specific surface receptors for different bacteriocins. The mechanism of action of the different compounds tested has not been clarified yet, but recent publications have contributed to additional information. The LAB bacteriocins are currently grouped into 4 classes, I-IV (Klaenhammer, 1993). Classes I and II consist of small, membrane-active, heat-stable peptides. Nisin compounds belong to the class I, while the other compounds tested are included in class IIa, a subgroup comprising pediocin and pediocin-like substances characterised by their antilisterial activity. The bacteriocin of SF644 has not been characterised yet, but it is likely to belong to class IIa, parallelly to CpV1 bacteriocin.

Bacteriocins have been shown to form poration complexes in target cell membranes (Abee, 1995; Jack *et al.*, 1995; Montville *et al.*, 1995; Klaenhammer, 1993). Class IIa bacteriocins have a highly conserved N-terminal consensus sequence which is believed to be involved in the recognition of a receptor site on *Listeria* (Fimland *et al.*, 1996). But recently, it was shown that a protein receptor is not always essential for the action of class IIa bacteriocins. For instance, pediocin PA-1 was found to cause CF efflux from complex lipid vesicles derived from *L. monocytogenes* (Chen *et al.*, 1997). Also, it has been suggested that the lipid composition of target membrane could modulate the action of pediocin, as it does for nisin. Pediocin PA-1 is the only known class IIa bacteriocin with two disulfide bonds and has the broadest range of activity (Montville & Chen, 1998). In fact, the antimicrobial range of class II bacteriocins seems to relate to their disulfide bond content (Jack *et al.*, 1995). Nisin does not require a protein receptor for pore formation since it dissipates the proton motive force and causes carboxyfluorescein (CF) efflux from lipid vesicles which lack membrane proteins (Breukink *et al.*, 1997; Winkowski *et al.*, 1996; Garcera *et al.*, 1993; Gao *et al.*, 1991). Montville & Chen (1998) found that the C-terminus of nisin was responsible for the initial interaction of nisin with the target membrane and that the anionic lipid content of target membranes was the major determinant for sensitivity. On the other hand, it was suggested by Bhugaloo-Vial *et al.* (1996) that piscicocin VIa, produced by CpV1, had a receptor-dependent antagonistic activity, similarly to other bacteriocins of its class (IIa). The concurrent production of a second bacteriocin (VIb) may contribute to larger differences in sensitivity among target organisms. These characteristics may render CpV1 bacteriocin more specific to some strains and may explain the large variations in sensitivity observed among the strains. Nevertheless, due to the complexity involved no one explanation can be proposed for the variations in strain sensitivity observed towards the compounds tested. Also, this project did not intend to study the mechanisms of action of bacteriocins

Although, time did not allow for an assessment of the *Listeria* survivors in CSS trials, i.e. whether the growing cells became just tolerant or developed true resistance to the antimicrobial systems tested, it can be said that partner 04 did not find any resistant cells following trials in SCSFS and CSS (Duffes, 1999).

Finally, it can be concluded that CpV1 can be used as a protective culture to delay *Listeria* proliferation in CSS. However, the antilisterial effect obtained will depend on factors such as presence of an indigenous LAB microflora and the importance of its load, the *Listeria* contamination level and its sensitivity to the CpV1 bacteriocin as well as the temperature profile of the product during storage. The use of the commercial antimicrobial compounds ALTA and nisin A did not provide a better inhibition than that seen with CpV1, especially at 8°C. In fact, the use of nisin A should be avoided if the product is to be temperature-abused (> 4-5°C) which is likely to occur in the course of its shelf life. Total control of *Listeria* in CSS was only achieved at 4°C by combining ALTA (0.5 or 1% w/w) and CpV1.

Alternative combined systems could be used/tested in CSS: nisin or ALTA combined with a CO<sub>2</sub>-rich environment (Szabo & Cahill, 1999; Nilsson *et al.*, 1997); as well as inducibility of a bacteriocin producer by the addition of bacteriocin-sensitive cells or autoclaved cells to the product (Sip *et al.*, 1998). Nevertheless, our findings demonstrated that due to the intrinsic and extrinsic parameters of CSS, the use of a

combined antimicrobial system appears to be necessary to ensure a proper control of *Listeria* in such products.

## Chapter 5      References

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