

Project Report
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**SPOILAGE AND SAFETY
OF COLD-SMOKED FISH**
TOPIC 2: Contamination with
Listeria monocytogenes

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Ágríp á íslensku:		<p>Markmið verkefnisins var þrjúþætt og skiptist það í 3 meginverkefni:</p> <ol style="list-style-type: none"> 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; 2. að ákvarða tíðni og smitleiðir <i>Listeria monocytogenes</i> í kaldreyktum fiski til að hægt verði að lágmarka áhættuna á smiti; 3. að finna leiðir (náttúrulega rotvörn) til að halda <i>Listeria</i> í skefjum og tryggja þannig geymsluþol og öryggi vörunnar. <p>Rf tók einungis þátt í seinni verkefnum, en í þessari skýrslu er fjallað um tíðni og smitleiðir <i>Listeria monocytogenes</i> í kaldreyktum laxi og í vinnslu á Íslandi.</p>	
Lykilorð á íslensku:		<i>kaldreyktur lax -Listeria - smitleiðir - tíðni</i>	
Summary in English:		<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"> 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 <i>Listeria monocytogenes</i> 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 <i>L. monocytogenes</i> in cold-smoked fish products. <p>IFL participated in the 2 latter topics. This report discusses the incidence and contamination routes of <i>Listeria monocytogenes</i> in cold-smoked salmon and processing areas in Iceland.</p>	
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FAIR CT96-1207

Spoilage and Safety of Cold-Smoked Fish

TOPIC 2: Contamination with *Listeria monocytogenes*

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Summary

In the course of 11 visits accomplished during the project, 4 smokehouses and a salmon farm were surveyed. *Listeria* incidence in the fish and environmental samples obtained from salmon farming was 11.9%, with 23.1% of the positive samples identified as *L. monocytogenes*. The prevalence of *Listeria* spp. in smokehouses was evaluated by the examination of various products and environmental samples (n=519) and found to be 16.2%. The overall frequency of *L. monocytogenes* was 70.2% among the positive samples. About 24% (19/78 samples) of the raw material sampled was found to be contaminated by *Listeria* spp., 73.7% of which was *L. monocytogenes*. However, *Listeria* was present at very low levels (2.9% or 3/102) in finished products (newly processed and packaged). This indicated the probable antibacterial effect of the cold-smoking process used and the implementation of good manufacturing practices following this processing step. Other sites of contamination included the brine, drains/walls/floor, working tools and aprons. Current cleaning procedures did not totally eliminate *Listeria* contamination in the processing environment. Following cleaning, 2 sites of *Listeria* contamination were common among the smoking plants and included the drains/walls/floor (20.7% or 12/58) and some working tools (7.0% or 7/100). These percentages increased to 36.9% (24/65) and 19.4% (14/72), respectively, after production had run for 2h. Raw material contamination may have played a major role in increasing the frequency of *Listeria* throughout the smoking plants during production due to an increased chance of cross-contamination. Storage trials of 6 lots of cold-smoked salmon (n=66) at 4 and 8°C revealed only few positive samples (8.9% *L. monocytogenes*), suggesting the sporadic incidence of this bacterium and a low contamination level in the finished products.

Three *Listeria* detection methods were compared by testing fish and environmental samples obtained during 8 surveys conducted at salmon smokehouses in Iceland. Two of the methods were conventional protocols, one being used at the Icelandic Fisheries Laboratories (IFL) and based on the USDA 2-step enrichment method, while the other was the ISO protocol commonly used in Europe. The third method, Dynabeads[®] anti-*Listeria* from Dynal A.S., was based on an immunomagnetic separation (IMS) of *Listeria* cells present following the pre-enrichment step of the conventional methods. Dynabeads[®] anti-*Listeria* method (Dynal) was slightly more rapid than the conventional protocols, allowing detection after 3 days instead of 5. The comparison of the 2 conventional protocols indicated a close agreement (98.6% or 70/71), excluding differences probably due to sampling as confirmed by the Dynal method. This IMS method was found to perform similarly well as the 2 others, with an agreement of 97.8% (131/134) with the IFL protocol, while a 97.2% (69/71) concordance was seen with the ISO protocol. The selective agar media used throughout the study gave similar results when comparing the conventional methods, but the use of two different selective media was found to be essential for the Dynal method to avoid false negative results. Selection among *Listeria* species was not favoured specifically by any of the methods tested.

Different *Listeria* detection and quantification methods were compared in this study. Based on a trial of artificially contaminated minced, cold-smoked salmon with *L. monocytogenes*, direct plating on Oxford formulation medium (LOX) from Oxoid

and a MPN method, developed by ASEPT (Laval, France) using 96-well microtiter plates for each dilution with Fraser broth, were found to be proper for enumeration of *Listeria* at a contamination level as low as 30-50 CFU/g. Other media (LCA: Lachica, 1990a; and Palcam Listeria Selective agar (PALC) from Merck) used for direct plating were similarly good at higher contamination levels (10^{3-4} CFU/g), but showed higher variation at the lower levels, perhaps influenced by the background flora. Enhanced Haemolytic Agar (Beumer *et al.*, 1996) did not give good results because of the background flora.

The efficiency of 4 cleaning and disinfecting agents used by the industry in solution were evaluated with regard to their effect on the reduction of a *L. monocytogenes* strain isolated from a conveyer belt in a surveyed plant and in the presence of an interfering substance. The strain used was characterised as biotype 2 and serotype 1/2. Biotyping of 139 Icelandic *L. monocytogenes* isolates showed that 96.4% of the strains belonged to this group. From this experiment only preliminary results are presented because of some problems we experienced during these tests.

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Chapter 1 Introduction

This FAIR project on the spoilage and safety of cold-smoked fish aims 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. establish the efficiency 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 have 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

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

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

During the project, the following work was carried out by participant 05 (IFL) in topic 2.

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

*Comparison of methods for isolation/detection of *Listeria* spp.: conventional, direct plating and rapid techniques

* Evaluation of methods for quantitative analysis of *L. monocytogenes*: MPN methods, direct plating on selective media

* Visits to smoking plants and an aquaculture farm: sampling throughout production line during production

* Sampling of the raw material (salmon) and final cold-smoked products by producers

- * Frequency of contamination of raw fish, the production environments and products at different production stages with *Listeria* spp.
- * Incidence of *Listeria* spp. during storage trials of cold-smoked salmon at 4 and 8°C

TASK 2C: Sanitation procedures & *Listeria*

- * Efficiency of sanitation procedures and working practices in smoking plants on the occurrence of *Listeria* spp. were evaluated by sampling throughout production line after cleaning/sanitation. Eleven surveys were performed to evaluate the efficiency of the sanitation procedures.
- * During sampling the plants were audited regarding good manufacturing practices (GMP) and internal control. The results from the visits to the plants were evaluated with regard to the cleaning procedure performed in surveyed plants.
- * Chemicals used for cleaning and disinfection were tested for efficiency regarding reduction of *Listeria monocytogenes* isolated from surveyed plants. These tests were performed in suspension with an interfering substance according to the French standard NF T 72-170. In this study, simulated cold-smoked fish system (SCSFS) was used as the interference substance. This was the first time that we took part in this kind of experiments. This must be taken into account when the results are evaluated. They give us some ideas, on what further research should be based on. But from this work we have obtained an important experience and encouragement to keep on going with further research regarding cleaning and sanitation. In fact, no methods have been used or developed in Iceland to assess the efficiency of cleaning and disinfecting agents to act upon the bacterial load of concern. The IFL aims to develop such an expertise and is planning to do further research in this field.

Chapter 2 Materials and Methods

Tasks 2A & 2C: Isolation of Listeria spp./ Sanitation procedures & Listeria spp.

Media and ingredients: The following media and ingredients were used: D/E Neutralising broth, Bacto Agar, Peptone, Yeast Extract (YE), Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) from Difco; Lab-Lemco powder, Maximum Recovery Diluent (MRD) and Listeria selective agar base-Oxford formulation supplemented with SR140E (LOX) from Oxoid; lithium chloride (LiCl), sodium chloride (NaCl) and Palcam Listeria selective agar base with added Fraser Listeria supplement (Palcam) from Merck; UVM modified *Listeria* enrichment broth from BBL Becton Dickinson Co.; acriflavin and ferric ammonium citrate from Sigma; Dynabeads[®] anti-Listeria from Dynal A.S.

Fraser's secondary enrichment broth was made of UVM broth supplemented with 0.3% LiCl, distributed into 10-ml portions, autoclaved (121°C for 15 min) and supplemented with acriflavin and ferric ammonium citrate to reach a final concentration of 0.00125% and 0.05%, respectively. TSB-YE and TSA-YE were prepared by adding 0.6% YE to TSB and TSA, respectively. Horse Blood Agar was composed of Lab-Lemco powder (10g), Peptone (10g), NaCl (5g), Bacto Agar (12g) and 1000 ml deionized water, autoclaved and supplemented with 50 ml of horse blood (Keldur, Iceland) prior to pouring. Enhanced Haemolysis Agar (EHA) was composed of TSA-EHA (Difco), 4-methylumbelliferyl- β -D-glucoside (Mu β G, Sigma), LiCl, Palcam supplement, spingomyelinase (Sigma) and sheep blood (Keldur). Lithium chloride-ceftazidime agar (LCA) was composed of BHI (brain heart infusion, Difco), LiCl and ceftazidime pentahydrate (Sigma).

Listeria detection and quantitative methods

The presence of *Listeria* spp. was assessed by three methods for comparison.

(i) *IFL-conventional Listeria detection method*, used at the Icelandic Fisheries Laboratories (IFL), is based on the USDA, FDA and others (Vanderzant & Splittstoesser, 1992). The first enrichment broth is UVM modified Listeria broth from BBL (30°C for 24 hours in air). From the first step 0.1 ml is inoculated into Fraser broth (above broth supplemented with acriflavin, LiCl and FeNH₄ citrate) (35°C for 26 \pm 2 h). Growth from black tubes is streaked onto Oxford agar (LOX) and Palcam (35°C, 48 h).

(ii) *ISO-method* (BS EN ISO 11290-1: 1997): a 2-step enrichment method followed by plating positive tubes onto LOX and Palcam media.

(iii) Dynabeads[®] anti-Listeria is designed for a rapid and selective enrichment of *Listeria* directly from pre-enriched samples using immunomagnetic separation (IMS). Dynabeads[®] anti-Listeria were incubated with a 1-ml aliquot of the pre-enriched sample (24h), in half-Fraser for ISO-method and UVM for IFL method, and the instructions given by Dynal A.S. were followed. Finally, the bead-bacteria complexes resulting from this immunomagnetic separation were plated onto LOX and Palcam media.

For quantitative analysis, 6 different methods were evaluated by:

a) Direct plating on

- Oxford formulation medium from Oxoid (LOX)
- Lithium chloride-ceftazidime agar LCA: Lachica (1990a)
- Palcam Listeria Selective agar (PALC)
- Enhanced Haemolytic Agar (EHA): Beumer *et al.* (1996)

b) Most probable number methods

- MPN method with diluted samples inoculated into 3x3 tubes containing UVM broth. The first three tubes were of double strength and incubated at 30°C for 24 hours. Then 0.1 ml from each tube was inoculated into Fraser broth and incubated at 35°C for 24 and 48 hours. Tubes showing black colour were streaked on LOX and PALC. The most probable number was found in MPN tables.
- MPN method, developed by ASEPT (Laval, France) using 96-well microtiter plates for each dilution with Fraser broth. Fifty µl of the first dilution were transferred to each tube of the first micro-titration plate. The same volume of two consecutive decimal dilutions was transferred to the other two micro-titration plates. For each dilution, positive wells, i.e. wells in which there was bacterial growth, were counted. Three values were obtained corresponding to a triplet for one count. This triplet was used to calculate the most probable number in 50 µl. The most probable number was the solution of the next equation (Maul, 1982):

$$\sum_{i=1}^{i=k} (n_i - p_i) q_i = \sum_{i=1}^{i=k} \frac{p_i q_i e^{-u q_i}}{1 - e^{-u q_i}}$$

u : microbial concentration : MPN

k : number of dilutions taken into account : in this study k = 3

n_i : number of wells inoculated for each dilution : in this study n_i = 96

p_i : number of wells giving a positive result

q_i : volume of inoculum inoculated (50 µl)

The swab samples were blended with 10 ml (or 50 ml for 2 or more swabs) of pre-enrichment broth shaken for 30s and incubated aerobically at 30°C for 24h. The succeeding day, 0.1 ml of the enrichment culture was pipetted into 10 ml of secondary enrichment broth and incubated at 35°C for 24 and 48h. A loopful of secondary enrichment culture from positive (black) tubes after 48h of incubation was streaked onto LOX and Palcam media and incubated at 35°C for 48h.

Whole fish were analysed by cutting and mincing the skin and belly flaps, weighing 25 g in a sterile bag, stomaching with 225 g of enrichment broth for 2 min and incubating at 30°C for 24h. The analysis was then continued as described above. Two raw fillets were considered as one sample and minced aseptically. The finished product samples were homogenised in a sterile Waring blender. Subsequently, 25 g of homogenised samples were weighed into a stomacher bag, 225 g of enrichment broth added, blended for 2 min and the analysis continued. Brine samples (25 ml) were enriched in broth (225ml). The farm water samples were filtered (0.22 µm, Millipore) and the filters enriched in 225 ml of enrichment broth.

Listeria isolation and identification

Five presumptive colonies were chosen from selective plates and streaked onto TSA-YE (35°C, 24h). The following tests were done to confirm the presence of *Listeria*: Gram staining (Hucker), catalase (3% H₂O₂) and motility (hanging-drop of cultures grown in TSB-YE, 22°C for 24 h). Gram positive, catalase positive and motile strains were identified by the API Listeria System (bioMérieux SA) and the hemolytic ability of strains tested on Horse Blood Agar (35°C, 24h).

Sampling at smokehouses. Salmon and environmental samples were obtained from processing plants during the 8 surveys performed. Samples included raw salmon, swabbing of equipments and other surfaces throughout the processing areas, brines, products collected after various stages of processing and finished products. All samples were taken aseptically, put into sterile containers or bags, kept cooled and analysed within 24 hours. The swab samples were taken using hydrophobic cotton swabs wet in D/E Neutralising broth. Swabs were streaked over an area of 2 x 50 cm² (where possible). During the 4 first visits, 3 whole salmons were sampled from the lot to be processed. During the succeeding visits, whole salmon (skin, gills and belly cavity) was swabbed using one swab per fish, unifying 2-3 swabs per sample. This sampling method allowed for a greater number of fish to be analysed.

Swab samples were taken at 15-20 places in the environment after cleaning just before operation started and again after two hours of processing. Ten to fifteen samples of raw material and products were collected from the reception area of the factories, along the processing line to the final production stage. In total, 50-55 samples were taken during each visit.

Sampling at a salmon farm. Salmon and environmental samples were collected during the 3 visits to a salmon farm. Seawater and water samples (100 or 500 ml) were collected in sterile bottles. Sampling of fish was done by swabbing of 2 -10 fish, which was counted as one sample. Swabbing of fish as a whole and separate swabbing of skin, gills, cloaca, belly or belly cavity (ungutted or gutted, respectively) was done. Environmental samples included swabs from the working room (equipment and tools), outside the housing, at the farming area (fjord) and at the barge area where bleeding was performed.

Incidence of *Listeria* spp. in cold-smoked salmon during storage at 4°C and 8°C.

The finished products from 6 lots were received newly produced or frozen, which were thawed overnight at 2°C prior to the storage trials. The presence of *Listeria* spp. was assessed at the start of the experiment (day 0) and each lot stored at 4 and/or 8°C for few weeks and analysed at 1 or 2 occasions. A total of 66 samples were examined.

The products of the 6 lots were as follows:

A: sliced products (85 g)	D: sliced products (50 g)
B: pieces of unskinned fillets (200 g)	E: end of tail, unskinned (100 g)
C: unskinned fillets (400-600 g)	F: end of tail, unskinned (100 g)

Lots D and E originated from the same producer and were packed on the same day. Quantitative analysis of *Listeria* in lots A, B and C was performed by directly plating 1 ml onto 2 LOX plates from the stomached UVM-sample mixtures and from samples (25 g) diluted into MRD (100 g) and stomached for 1 min. A MPN method (3 x 3 tubes) based on the IFL 2-step enrichment *Listeria* method was also used at the last sampling days (day 21 at 8°C and day 35 at 4°C).

Hygienic and management factors

Survey methods were based upon GMP and hygiene audit techniques together with bacteriological sampling of raw material, products, and process environment. Information on various factors related to hygiene, management and production facilities was collected during the surveys. An example of the information is shown in Table 1. This was done in co-operation with the quality manager of each plant. It should be mentioned that none of the plants surveyed were originally built for this kind of production.

Table 1. An example of the information gathered while sampling

general information	type of production
	water supplier
	HACCP
production facilities	cross-contamination
	state of repair
information about employees	hygiene
	use of working clothes
manufacturing routines	origin and handling of raw material
	salting procedure
	use of slicing
	routines for hand hygiene and footwear disinfection
	cleaning and disinfection routines

Efficiency of cleaning and disinfecting agents

The efficiency of 4 cleaning or disinfecting agents used by the industry in solution was evaluated with regard to their effect on the reduction of a *L. monocytogenes* strain isolated from a conveyer belt in a surveyed plant and tested in presence of an interfering substance. The strain used was characterised as biotype 2 and serotype 1/2. Biotyping of 139 Icelandic *L. monocytogenes* isolates, as performed by partner 01, showed that 96.4% of the strains belonged to this group.

The method used was based on the French standard NF T 72-170. The investigation of the activity was performed *in vitro* under defined conditions and in the presence of an interfering substance. The interfering substance used was made from smoked salmon extract (SCSFS) and the temperatures used for the test were 4 and 20°C. *Listeria* strains were cultured in TSB-YE at 35°C for 48 hours. The concentration of the test suspension should have been 1-3 x 10⁸ cells/ml. The use of a neutralising agent (NA) terminated the activity of the chemicals. The neutralising agent was chosen in co-operation with ASEPT (Laval, France) which performed the test with different neutralising agents. The NA contained 1% lecithin, 6% Tween 80, 1% sodium thiosulfate and 0.5% L-histidine. After inactivation the test suspension was plated onto TSA-YE and incubated at 35°C for 48 hours.

The active agents and the concentration of the compounds tested as in ready-to-use solutions are shown in Table 2. The disinfectants and cleaning agents were diluted to

the most commonly used concentration and two other concentrations. From this experiment only preliminary results are presented because of some problems we experienced during these tests.

Table 2. The antimicrobial compounds tested and their concentrations

Product	Active agent	Recommended concentration	Concentrations tested
A:Sóttþreinsir	Quaternary ammonium	1/100	1/50 - 1/100 - 1/200
B:FPC	Strong alkaline (NaOH)	1/50	1/10 - 1/50 - 1/100
C:Sítrussápa	D-limonen	1/20	1/10 - 1/20 - 1/30
D:Klór	Sodium hypochlorite	100 ppm	50 - 100 - 200 ppm

Chapter 3 Results

2A.1) Analytical methods

Comparison of *Listeria* detection methods

Following 8 surveys conducted at cold-smoked salmon processing plants, three *Listeria* detection methods, the IFL and ISO protocols as well as an IMS detection (Dynal) method, were compared by testing 71 naturally contaminated samples, including raw material, finished products and processing environment (Table 3). The IFL and ISO methods were found to be equivalent, except for 6 samples (6/71 or 4.5%). At 4 occasions, *L. monocytogenes* was detected by the ISO-method and usually confirmed by the Dynal (ISO) method, but not by the IFL protocol. The samples involved included fish intestine, a finished product and 2 environmental samples taken following cleaning. These 4 cases do not disqualify the IFL protocol for detection of *L. monocytogenes*, but rather exemplifies a difference due to sampling. This is shown by the results obtained by the Dynal method when applied following pre-enrichment respective to each method (UVM for IFL and half-Fraser for ISO). The Dynal (IFL) method using an aliquot from the UVM broth also gave negative answers for these samples, indicating the possibility that no *Listeria* cells were initially transferred to the UVM broth from the samples. Similarly, 2 environmental samples (during processing) were found to be contaminated by *L. monocytogenes* as detected by the IFL method, but not by the ISO-method. This difference is explained by the fact that 2 separate swabs for each sampling site were used and the possibility of finding the bacterium on only one of them, due to its low contamination level, is certainly inevitable.

The Dynal method (Table 3) was almost just as sensitive as the previously mentioned protocols. Interestingly, one cold-smoked salmon sample (1/71 or 1.4%) containing *L. monocytogenes* as detected by the ISO-method, was not found to be positive by the Dynal (ISO) method. This was also observed for the IFL and Dynal (IFL) methods. On the other hand, an environmental sample taken during processing was found to be positive for *Listeria* spp. by both Dynal and ISO methods, but *L. monocytogenes* present could only be isolated from the selective agar media plated for the Dynal method, while all five typical colonies isolated from the ISO method were identified as *L. innocua*.

Table 3. Comparison of three *Listeria* detection methods (n = 71)

Sample type	n	IFL-method		Dynal (IFL)		ISO-method		Dynal (ISO)	
		Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.
Fish intestines	3	3		3		2	1 (<i>Lm</i>)	2	1 (<i>Lm</i>)
Fish fillets	5	5		5		5		5	
Finished products	52	51	1 (<i>Lm</i>)	52		50	2 (<i>Lm</i>)	51	1 (<i>Lm</i>)
Environment - after cleaning ¹	4	3	1 (<i>Lm</i>)	3	1 (<i>Lm</i>)	1	3 (<i>Lm</i>)	1	3 (<i>Lm</i>)
Environment - during process. ²	7		7 (<i>Lm</i>)		7 (<i>Lm</i>)	1	6(5 <i>Lm</i> + 1 <i>Li</i>)	1	6(5 <i>Lm</i> + 1 <i>Lm</i> / <i>Li</i>)

1: Environmental swabbing of floor/drains following cleaning

2: Environmental swabbing of floor/drains/forklift during processing

Lm: *L. monocytogenes*, *Li*: *L. innocua*

Table 4. Comparison of two *Listeria* detection methods (n = 134)

Sample type	N	IFL-method		Dynal (IFL)	
		Neg.	Pos.	Neg.	Pos.
Whole salmon	14	11	3 (<i>Lm</i>)	11	3 (<i>Lm</i>)
Fish intestines	3	3		3	
Fish fillets	6	6		6	
Finished products	81	80	1 (<i>Lm</i>)	81	
Brine (used)	3	2	1 (<i>Lm</i>)	2	1 (<i>Li</i>)
<i>Environment: after cleaning</i>					
Floor	5	3	2 (1 <i>Lm</i> + 1 <i>Ls</i> / <i>Liv</i>)	3	2 (1 <i>Lm</i> + 1 <i>Ls</i> / <i>Liv</i>)
Drains	6	6		6	
<i>Environment: in process</i>					
Forklift (swab)	2	1	1 (<i>Lm</i>)	1	1 (<i>Lm</i>)
Floor	7	3	4 (<i>Lm</i>)	3	4 (<i>Lm</i>)
Drains	7	2	5 (4 <i>Lm</i> + 1 <i>Lw</i> / <i>Li</i>)	2	5 (4 <i>Lm</i> + 1 <i>Ls</i>)

Lm: *L. monocytogenes*; *Li*: *L. innocua*; *Ls*: *L. seeligeri*; *Liv*: *L. ivanovii*; *Lw*: *L. welshimeri*

Table 4 summarises the results obtained for all samples (n=134) tested by the IFL and Dynal (IFL) methods. Based on these findings, only 2.2% (3/134) of the samples examined gave different results. This means that 97.8% (131/134) of the results obtained with the Dynal (IFL) method were in accordance with the IFL protocol, while a 97.2% (69/71) agreement was seen for the Dynal (ISO) method when compared to the ISO protocol (Table 3).

The selective agar media used, LOX and Palcam, gave similar results throughout the study when comparing the IFL and ISO protocols, i.e. typical colonies grew on both media and the same *Listeria* species were isolated. However, LOX medium was preferred because of its ease of detection of typical colonies. Interestingly, the Dynal (IFL) method did at 4 occasions (floor and drain samples following cleaning and

during production) recover typical colonies only from the Palcam plates (data not shown). This was also observed once with the Dynal (ISO) method. This exemplifies the importance of using two different selective agar media for the detection of *Listeria* spp. with the Dynal method.

The detection methods were also assessed for their selectivity towards specific *Listeria* species. Based on Tables 3 and 4, most *Listeria* positive samples detected by the methods assayed showed comparative results, i.e. usually the same *Listeria* spp. were isolated, indicating no specific selection of *Listeria* spp. At only 3 occasions, with 2 samples (1.5%) for Dynal (IFL) and 1 sample (1.4%) for Dynal (ISO) methods, did the isolated colonies vary as confirmed by the API identification results (Tables 3 and 4, respectively).

Methods for quantitative analysis of *Listeria monocytogenes*

Minced smoked salmon was artificially contaminated with *L. monocytogenes* cultures at ca. 10^1 , 10^2 , 10^3 and 10^4 CFU/g. The cell concentration of the pure culture was 4.9×10^8 /ml after 72 h at 35°C. The results of the comparative study on quantitative methods for the enumeration of *L. monocytogenes* are shown in Table 5. Unfortunately, the dilution in 3-tube MPN method was not sufficient for the samples with higher cell concentrations and MPN counts could therefore not be evaluated in these cases. However, this method has been used at the IFL with good results for naturally contaminated samples. *Listeria* counts obtained on Oxford agar, Palcam agar and MPN 96-wells corresponded mostly to the expected values. For the MPN 96-well method, two media (Palcam and Fraser broth) were tested. Palcam was not found to be selective as all the wells became black. This broth has been used by ASEPT for the enumeration of *Listeria* in cheese and gave good results. But with cold-smoked salmon, Fraser broth proved to be better. On the other hand, LCA medium was more problematic as some difficulties were encountered when trying to identify typical *Listeria* colonies by the Henry's illumination method. Higher counts were obtained on LCA medium for samples 3 and 4, perhaps because of interference of the background flora at these low *L. monocytogenes* levels. EHA agar plates were also tested but the results were not useful. All kinds of colonies were found to grow on this medium, on which the haemolytic reaction was ascertained with difficulty. To confirm the presence of *L. monocytogenes*, 5 suspect colonies were isolated and identified as described previously. All tested colonies were identified to be *L. monocytogenes*, except those isolated from the EHA agar plates. The Dynal method, involving immunomagnetic separation of *Listeria* spp., was also tested on these samples and *L. monocytogenes* was detected in all cases.

Table 5. Comparison of methods for quantitative analysis of *L. monocytogenes* from spiked, minced cold-smoked salmon

Sample	MPN-3 tubes (log MPN/g)	MPN 96-wells (log CFU/g)	LOX (log CFU/g)	PALC log CFU/g)	LCA (log CFU/g)
1	NA	4.49	4.48	NA	4.48
2	NA	4.00	3.32	3.26	3.38
3	2.00	2.70	2.36	2.79	2.90
4	2.00	1.68	1.48	1.18	2.00

NA: not available

Overall, similar counts were seen between most of the methods tested (MPN 96-wells, LOX, PALC and LCA media), especially at the higher levels of *Listeria*

contamination. This is reflected by the mean count and the corresponding standard deviation calculated for each cell concentration level tested (Table 6). It should be pointed out that both the standard deviation and the coefficient of variability (C.V.) decreased with an increasing level of *L. monocytogenes*, suggesting less variation among the media assessed in presence of higher concentrations of *L. monocytogenes*.

Table 6. Statistical evaluation of the media assessed for quantitative analysis of *L. monocytogenes* from spiked, minced cold-smoked salmon

Sample	Expected cell concentration (log CFU/g)	Mean cell concentration (log CFU/g)	Standard deviation (log CFU/g)	C.V. (%)
1	4.69	4.48	0.01	0.2
2	3.69	3.49	0.34	9.9
3	2.69	2.58	0.35	13.8
4	1.69	1.68	0.36	21.7

2A.2) and 2C.1) Prevalence of *Listeria* in the environment of cold-smoked salmon processing and salmon farming

A total of eight visits were done from January 1997 to July 1998 to four cold-smoked salmon processing plants in Iceland. The frequency of *Listeria* contamination of farmed salmon (fresh, whole and gutted), production environments and products at different production stages was evaluated. This was accomplished by sampling throughout the production line following cleaning and during processing.

Among the 519 samples obtained from the smoking plants following cleaning and during processing, 16.2% were found to be *Listeria* positive. The overall frequency of *L. monocytogenes* was 70.2% among the positive samples. About 24% (19/78) of the raw material sampled was found to be contaminated by *Listeria*, 73.7% of which was identified as *L. monocytogenes* (Table 7). On the other hand, *Listeria* was detected from very few finished products tested (2.9% or 3/102) that had been newly processed and packaged. Other sites of contamination included the brine (30.0%), drains/walls/floor (29.3%), working tools (12.2%) and aprons (13.3%). The contaminated working tools comprised forklifts, washing tubs, packaging tables, skinning and packaging machines, knives, conveyer belts and a hatch. It should be specified that following cleaning, 2 sites of *Listeria* contamination were common among the smoking plants and included the drains/walls/floor (20.7%) and some working tools (7.0%). These percentages increased to 36.9 and 19.4%, respectively, after production had run for 2h (Table 8). The overall occurrence of *Listeria* in cold-smoked salmon processing environments following cleaning was found to be 10.6% (19/180), as opposed to 26.0% (43/165) during processing. Raw material contamination may have played a major role in increasing the frequency of *Listeria* incidence throughout the smoking plant during production due to an increased chance of cross-contamination.

The incidence of *Listeria* in the fish and environmental samples obtained during the 3 visits to the salmon farm (Table 9) was 11.9% (13/109), where 23.1% of positive samples contained *L. monocytogenes* which was isolated from the swabs of ungutted fish while the other samples were contaminated by *L. innocua* detected from the waste

water, an intestine tub and the drain/floor in the work room, outside the housing where the forklift circulated and at the farming area (feeding carriage with bird droppings, forklift and seawater around farming area).

Table 7. Incidence of *Listeria* spp. in raw material and cold-smoked salmon

Samples	<i>Listeria</i> +	<i>L. monocytogenes</i>	Other <i>Listeria</i> spp.
Raw material (n = 78)	24.4%	73.7%	<i>L. seeligeri</i> (21.0%) <i>L. innocua</i> (5.3%)
Semi-finished / finished products (n = 102)	2.9%	33.3%	<i>L. seeligeri/ivanovii</i>

Table 8. Frequency and sites of *Listeria* contamination in smoking plants

Sites	After cleaning			During processing		
	%	n	% <i>L.m.</i>	%	n	% <i>L.m.</i>
Drains / walls / floor	20.7	12/58	66.7	36.9	24/65	66.7
Working tools	7.0	7/100	57.1	19.4	14/72	78.6
Kiln	0	0/4				
Brine				30.0	3/10	100
Aprons	0	0/8		28.6	2/7	100
Hands / gloves	0	0/10		0	0/11	
Overall frequency	10.6	19/180	63.2	26.1	43/165	74.4

L.m. = *Listeria monocytogenes*

Table 9. Frequency and sites of *Listeria* contamination in salmon farm

Samples	<i>Listeria</i> + (%)	<i>Listeria</i> species
River discharging to sea (fjord) (n = 5)	0	
Farming area: seawater (n = 7)	14.3	<i>L. innocua</i>
Ungutted fish (n = 33)	9.1	<i>L. monocytogenes</i>
Gutted fish (n = 18)	0	
Working room (n = 23)	26.1	<i>L. innocua</i>
Outside housing (n = 2)	50	<i>L. innocua</i>
Farming area (n = 10)	20	<i>L. innocua</i>
Barge and tools (n = 11)	0	

Incidence of *Listeria* spp. in cold-smoked salmon during storage at 4 and 8°C

Because of the low *Listeria* incidence in newly produced (packaged) cold-smoked salmon despite the presence of this bacterium in the processing environment, storage trials of 6 lots were conducted. This was done to verify whether this low level was due to unsuccessful recovery of injured/stressed cells in newly processed products or to its absence in the finished products. The results are presented in Table 10. *Listeria* spp. was detected in 2 samples of one of the lots (9.5%) upon receipt (day 0) and was identified as *L. seeligeri/ivanovii*. At the last sampling day, *Listeria* spp. were detected in 3 other lots, bringing the incidence up to 17.2%, 60% of which being *L. monocytogenes*. However, the overall incidence of *L. monocytogenes* during storage was 8.9%. Interestingly, sliced products (lots A and D) were found to be less contaminated by *Listeria* spp. (5.3%) than fillets (whole or piece, 17.1%). These results show a tendency for *Listeria* to grow during storage. But it is noticeable that in 2 of the lots stored at 8°C, *Listeria* spp. were only detected initially or early during storage (day 7), not at the last sampling day, indicating its sporadic incidence and/or a low contamination level.

Table 10. Incidence of *Listeria* spp. in 6 lots of cold-smoked salmon during storage at 4 and 8°C

Temp. (°C)	Lot	Incidence of <i>Listeria</i> spp. during storage on each sampling day (+/n)					Incidence per lot	
		day 0*	day 7	d20-21	day 28	day 35	n	%
4	A	0/3		0/3		0/3	9	0
	B	0/3		0/3		0/3	9	0
	C	0/2		0/2		2/3 (<i>Lm</i>)	7	28.6
8	A	0/3	0/3	0/3			9	0
	B	0/3	0/3	0/3			9	0
	C	0/2	1/2 (<i>Lm</i>)	0/2			6	16.7
	D	0/5			1/5 (<i>Ls</i>)		10	10
	E	0/5			2/5 (<i>Ls, Lm</i>)		10	20
	F	2/3 (<i>Ls/Liv</i>)			0/2		5	40
Overall incidence of <i>L. monocytogenes</i>						day 0:	0%	
						during storage:	8.9%	

* newly produced or may have been kept frozen prior to storage trial

Lm: *L. monocytogenes*; *Ls*: *L. seeligeri*; *Liv*: *L. ivanovii*

Direct plating onto LOX medium, from either the UVM or MRD mixtures prepared, was assessed as an enumeration method for *Listeria* spp. in lots A, B and C. MPN method was tested on the last sampling day only. None or very few black colonies were isolated on LOX medium plated from the UVM mixtures and were not found to be typical at all for *Listeria* spp. Probably due to the selectivity of UVM broth, no black colonies were obtained from the 3 samples found to be contaminated by *Listeria* spp. during storage by the conventional detection method. On the other hand, LOX medium plated from MRD mixtures usually became black due to the lack of selectivity of the diluent and the predominance of the microflora developing during storage. At only one occasion (day 7), typical countable colonies (30 CFU/g) were seen concurrently to the detection of *L. monocytogenes* in this sample by the conventional method. Otherwise, it was very difficult to make use of this method to assess the level of contamination in these products. Lot C was found to be positive for

L. monocytogenes at the last sampling day (4°C), for which the contamination level was 0.37 MPN/g.

Hygienic and management factors

The processing of cold-smoked salmon includes several steps. Thawed or fresh fish are filleted manually and rinsed in cold water. The fillets are then salted in brine for 1-4 hours. After brining (brining bath and injection), the fillets are stored overnight to equilibrate before smoking. Prior to smoking the fillets are dried for 1-4 hours. After smoking the fillets are stored overnight before skinning, slicing and packaging. The final product is either stored below 4°C for 3-4 weeks, which is the storage time for unfrozen product or it is frozen down to -18°C. The time-temperature conditions during production and the flow-line are shown in Table 11.

Table 11. Time/temperature conditions during production of cold-smoked salmon in four plants.

Process flow	Time (hours)	Temperature (°C)	
		Fish	Ambient
Raw material	1-7 days	0-2.8	0-4
Raw material - frozen	Thawed overnight	0.2	0-4
After filleting		6	12
Brine**	1-4	2-5 (brine)	12-14
Equilibration 1	12-24	4	0-4
Smoking	Drying 1-4 Smoking 4-8.5		20-25*
Equilibration 2	12-24		0-4
Skinning / Slicing			12-16
Packaging			12-16
Storage - Chilling	3-4 weeks		0-4
Freezing			<-18

* Influenced by the size of the fillets. **Brine 18°

The ambient temperature in the morning before the operation started was 9-10°C. The fish had starved from 7-21 days, depending on the water temperature at the fish farm.

The information gathered while sampling indicates that there was a lack of effective control procedures throughout the production of the smoked fish. As mentioned before, the plants surveyed were not originally built for the production of cold-smoked salmon. The processing facilities were difficult to clean, due to little space and bad design. Also, the process was spread over 3 rooms, where rotation of people was constant during the day without any footwear disinfection between different areas. Therefore the risk of cross-contamination was high. Moreover cross-contamination could occur due to flow of air from raw material to finished products. Cleaning during production was not performed on routine basis but the plants were cleaned and disinfected at the end of the day in all plants, except in one plant which was only disinfected at the end of the week.

The steps of cleaning and sanitation involved 5 steps:

- rinsing with cold water
- foam-cleaning with strong alkaline cleaning agent
- rinsing with cold water
- disinfection with quaternary ammonium compounds (where the sanitizer stayed on the equipment overnight)

- rinsing before operation started

The results from the surveys showed that *L. monocytogenes* was detected in the environment and from raw material, therefore hygienic precautions at different steps in the process are most important to prevent the colonisation and spread of *L. monocytogenes*. Nevertheless, low incidence of *L. monocytogenes* was found in the Icelandic finished products.

Efficiency of cleaning and disinfecting agents

The antibacterial effect of 4 cleaning or disinfecting agents on a *L. monocytogenes* strain isolated from a conveyor belt in a surveyed plant was studied. The antibacterial activity was evaluated in the presence of an interfering substance (SCSFS). Preliminary results are presented in Table 12. For the four chemicals presented, the test was only effective at 20°C. If the test is accepted then N, n'1 and n'2 should be equivalent. The test was rejected in all cases at 4°C. None of the four cleaning or disinfecting agents was effective in achieving a 5-log reduction in suspension test in the presence of organic matter. From this test we can not conclude whether it is the neutralising agent or the interfering substance that inhibited the activity of the chemicals. Also, it should be mentioned that this standard method was developed for hard water. However, the Icelandic water is rather soft and it may have had some effect on the results.

Table 12. Antibacterial activity of cleaning or disinfecting products as determined by the French standard NF T 72-170.

(The product is bactericidal in a concentration when $n < \frac{n'2}{10}$, 5-log reduction)

Test temperature (°C)	Product	Active agent	N	n'	n'1	n'2	n			pH	
							Concentration (C) in contact with the bacteria			C max	C min
							1/50	1/100	1/200		
20	A (sanitiser)	quatarnary ammonium cpd	360	800	350	100	350	++++	++++	6.2	6.2
4	A		360	750	550	<100	+++++	++++	+++++	6.2	6.2
							Concentration (C) in contact with the bacteria				
							1/10	1/50	1/100		
20	B (cleaner)	NaOH	227	152	180	132	308	++++	++++	11.8	7.6
4	B		227	111	18	17	++++	++++	++++	11.8	7.3
							Concentration (C) in contact with the bacteria				
							1/10	1/20	1/30		
20	C (cleaner)	D-limonen	135	166	149	146	++++	++++	++++	na	na
4	C		135	168	104	151	++++	++++	++++	na	na
							Concentration (C) in contact with the bacteria				
							50 ppm	100 ppm	200 ppm		
20	D (sanitiser)	Sodium hypochlorite	280	250	200	100	++++	++++	++++	6.1	6.2
4	D		280	<100	<100	<100	++++	++++	++++	6.1	6.2

N: inoculum control; n': neutralising test; n'1: interfering substance reference; n'2: neutralising agent reference; n: test

N, n'1 and n'2 should be equivalent and $n' > 0.5 n'2$ for verification.

na – not available

++++ >300 colonies

Chapter 4 Discussion

Comparison of *Listeria* detection methods and quantitative methods

Listeria monocytogenes has been found in a variety of fishery products, both raw and ready-to-eat (Ben Embarek, 1994; Noah *et al.*, 1991; Weagant *et al.*, 1988), including cold-smoked salmon (Rørvik *et al.*, 1991 & 1995; Jemmi, 1990). Different selective detection media have been developed, some of which are based on an iron-esculin system which implies the development of a black color around the colonies. Oxford formulation from Oxoid (*Listeria* selective medium, LOX) and Palcam from Merck are examples of such selective media. Different results have been obtained on the superiority of certain selective media (Cortis *et al.*, 1989; Lee & McClain, 1987; Van Netten *et al.*, 1989). This existing controversy relies heavily on the type of food products being analysed as well as the bacterial load and microflora present. For instance, foods highly contaminated with bacteria have a better *Listeria* recovery with more selective media, whereas processed foods with a lesser bacterial background and possibly injured *Listeria* may need a less selective medium to recover the *Listeria* (Dillon *et al.*, 1992). This leads to the conclusion that the use of more than one selective medium may be a wise choice.

Similarly, different *Listeria* detection protocols have been followed and include pre-enrichment and enrichment steps which are meant to facilitate the recovery of injured *Listeria* cells. The method used at the Icelandic Fisheries Laboratories (IFL) includes the use of UVM modified *Listeria* broth in the pre-enrichment step with low levels of selective agents (no lithium chloride). This low initial selectivity may be favorable to the growth of *L. monocytogenes* and other *Listeria* spp. The use of more selective agents is disputable at this stage. Since this study is part of a European project, the ISO protocol (BS EN ISO 11290-1: 1997) was compared to the IFL method. Similar results were obtained with an agreement of 98.6% (70/71) between the 2 methods, taking into account some of the differences probably due to sampling as previously explained. However, the ISO-method failed to detect *L. monocytogenes* in one environmental sample when compared to the Dynal (ISO) method.

Detection of *Listeria* involving conventional methods requires 5 days. The need for more rapid detection techniques has brought about the development of new methods. Immunomagnetic separation using Dynabeads[®] anti-*Listeria* gives the possibility of concentrating *Listeria* following pre-enrichment and apply it by direct plating onto selective media to make its detection possible after 3 days from the start of the analysis. It is a rather cheap method which does not require expensive equipment, but can just be used parallel to currently used conventional methods. This method was compared to the IFL and ISO protocols and after the examination of 134 and 71 samples, respectively, it was concluded that the methods were close to be equivalent. At only 2 occasions (1.5%) did the Dynal (IFL) method failed to detect *L. monocytogenes*. Also, the Dynal (ISO) method failed to detect *L. monocytogenes* once and similarly did the ISO-method failed to detect *L. monocytogenes* in one sample found to be positive by the Dynal (ISO) method. The use of both selective agar media, LOX and Palcam, was shown to be necessary when using the Dynal method as some environmental samples were not found to be positive for *Listeria* spp. on LOX but did on Palcam. However, such discrepancy was not encountered when comparing the IFL and ISO protocols alone. Lastly, the three detection methods assessed did not appear to have a selectivity towards specific *Listeria* species. It should be mentioned that the

Dynal method has been claimed by the fabricant to be 10 times more sensitive for *L. monocytogenes* than other *Listeria* spp., which was not seen among the samples examined.

Prevalence of *Listeria* in cold-smoked salmon processing and salmon farming and evaluation of sanitation procedures

In the course of the 11 visits accomplished, four cold-smoked salmon processing plants and one salmon farm were surveyed and the prevalence of *L. monocytogenes* and other *Listeria* spp. evaluated by the examination of various products and environmental samples. *L. monocytogenes* was most often isolated from the smokehouses. The current cleaning procedures were not found to eliminate *Listeria* totally, as a contamination level of 10.6% (6.7% for *L. monocytogenes*) was seen following cleaning, mainly due to drains/walls/floor. *Listeria* incidence in the processing environment increased to 26% (19.4% for *L.m.*) during production, probably due to the introduction of raw material contaminated with *Listeria* spp. (24.4%: 17.9% for *L.m.*) and cross-contamination from the drains/walls/floor.

Similar findings have been reported in the literature. Rørvik *et al.* (1995) found 29% of the environmental samples and 17% of the raw material to be contaminated by *L. monocytogenes*, while Guyer & Jemmi (1990) reported a slightly higher level of contaminated raw material (28.6%). In a recent study, Rørvik *et al.* (1997) surveyed 40 salmon smokehouses and found that 33% of the plants were contaminated by *L. monocytogenes*, mostly due to drain contamination (63%). In their risk factor evaluation, they found that job rotation (people moving around the plant) was the strongest risk factor for isolation of *L. monocytogenes* from the smoked salmon, whereas drain contamination was considered as a sensitive predictor. The status of repair of the processing equipment, i.e. worn and/or torn, was also an important parameter in contributing to *Listeria* contamination since it reduced the efficiency of the cleaning and disinfection procedures. Therefore, well-maintained facilities showed a preventive effect.

Despite the presence of *L. monocytogenes* in the Icelandic smokehouses, this bacterium was isolated from very few finished products (1%) following packaging, whereas other *Listeria* species were isolated as well (1.9%). The prevalence of *L. monocytogenes* in such products has been reported to range from similarly 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). Dillon *et al.* (1994) also pointed out some seasonal variations, where for instance *L. monocytogenes* was not detected in such products during early summer months (May-July). Cortesi *et al.* (1997) reported findings, which indicated an uneven distribution of *Listeria* in cold-smoked salmon. This agrees with our storage trials conducted at different temperatures where a sporadic incidence of *Listeria* spp. was shown to occur along with a low level of contamination. However, a Danish study (Jørgensen & Huss, 1998) on the prevalence of *L. monocytogenes* in cold-smoked fish showed high initial levels with a slight increase at the end of shelf life following storage at 5°C. These results illustrated serious processing difficulties probably due to poor manufacturing practices and/or to insufficient cleaning procedures. However, further trials of cold-smoked salmon originating from various Danish smokehouses indicated that it was possible to produce such products with low prevalence of this bacterium. Nevertheless, growth of *Listeria* during refrigeration of cold-smoked products has been reported (Guyer & Jemmi, 1991; Rørvik *et al.*, 1991; Hudson & Mott, 1993),

independently of the inoculum level. 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 low *Listeria* incidence found in our study suggests that the cold-smoking process has some effect on *Listeria* spp. found on the raw material and/or the processing equipment. Jemmi & Keusch (1994) investigated three fishfarms and fish-smoking plants and their results demonstrated that samples taken after smoking, before packaging, did not contain *Listeria* spp. although the raw material was contaminated. Rørvik *et al.* (1995) similarly found that even though *Listeria* contaminate the product at the different steps of the processing, none was detected following smoking but with further processing (trimming, slicing and packaging) recontamination occurred. The antibacterial properties of the smoke components may explain the inhibition of *Listeria* since the temperature used in cold-smoking ranges from 24 to 27°C and is therefore not lethal to *L. monocytogenes*. In a study by Eklund *et al.* (1994), the application of smoke was shown to have a bactericidal effect on *Listeria* cells when they remained on the surface areas of the fillets but were not introduced into the flesh by injection of contaminated brine. Messina *et al.* (1988) reported that liquid smoke has an antimicrobial effect on *L. monocytogenes*, the effect varying with the phenol content. On the other hand, Guyer & Jemmy (1991) reported that cold-smoking had no significant effect on *Listeria* viability in inoculated salmon. The same trend was observed in a similar study on the smoking process where the organisms grew well at 4°C (Rørvik *et al.*, 1991) and remained viable at -20°C (Dillon & Patel, 1993).

Ben Embarek & Huss (1993) concluded that the use of selective agar for a direct plate count of injured *L. monocytogenes* cells was not appropriate. Our results are in agreement with these findings. Moreover, it can be said that apart from the physiological state of the cells in the product, the inherent microflora developing is an important parameter influencing the growth/detection of the *Listeria* cells on the selective media used. Either too much or too little selectivity of the diluents was observed in this study when plating directly from UVM broth or MRD, respectively. In cold-smoked fish, *Listeria* can be expected to be slightly injured or stressed, especially if the contamination had occurred early in the process. It is well possible that cells originating from post-processing contamination will proliferate more easily in the finished products under refrigerated storage. This suggestion is not in agreement with the studies of experimentally contaminated smoked salmon of Guyer & Jemmi (1991), where higher *Listeria* contamination levels (10^{2-4} MPN/g) were found to be maintained during fabrication and increased during storage. However, they also reported that the lower level of inoculation (10 MPN/g) did not lead to an increase in *Listeria* during storage. Lastly, it should be pointed out that the hygienic quality of the finished product may play an important role in the development of *Listeria* cells, as reported by Rørvik *et al.* (1991).

Ben Embarek (1994) emphasised the need for studies to confirm the presence of *L. monocytogenes* on live fish. The salmon farm surveyed in our study had a low contamination level with 2.8% *L. monocytogenes* and 9.1% *L. innocua*. Interestingly, newly slaughtered, ungutted salmon (3/33) were the only source of *L. monocytogenes* contamination, whereas gutted samples (0/18) were not found to be contaminated. *L. innocua* was found in environmental samples. Rørvik *et al.* (1995) reported sporadic *Listeria* contamination of slaughterhouses. Due to the ubiquitous nature of *Listeria* spp., it is probably impossible to eliminate its presence in salmon farming

environment. Though, farming areas should be carefully examined and any possible contamination sources controlled. It should be kept in mind that any pollution sign might increase the risk of a higher content of organic material, which could contribute to *Listeria* proliferation, especially in the seawater surrounding the farming area.

Sources of *Listeria* contamination were not determined in this study. But the results showed that contamination route for *L. monocytogenes* can be through the raw material or the processing environment. Hence, hygienic precautions at all the different steps of the process are extremely important to prevent recontamination of the product during all stages of processing. Also, means of elimination or reduction of *L. monocytogenes* on the outside surface of frozen or fresh fish before filleting could be an additional step to prevent further contamination.

The methods used in this project for biotyping (serotyping and resistance/sensitivity to tetracycline, cadmium and arsenic profiling) of the isolates did not help us to trace the dissemination of the organism. Strain identification (typing) using molecular techniques, e.g. Pulsed-Field Gel Electrophoresis (PFGE), is recommended to trace *Listeria* contamination in the processing environment. This is currently being done in a national project run at the IFL. Such knowledge should contribute to a better, improved cleaning programme, hence targeting specific contamination sites and facilitating corrective actions.

In all the plants, the cleaning procedure was not effective. Pieces of fish were observed on some equipments, e.g. on the skinning machine. The drains were not cleaned properly and they were most of the time heavily loaded with fish pieces. Sanitizers work best when conditions are right regarding the temperature, surface and the strength or concentration. As stated by Marriot (1997), the sanitizers work better against *L. monocytogenes* on nonporous than porous surfaces. Also sanitizers have to be more concentrated to destroy *L. monocytogenes* at chilled temperature (4°C) than at room temperature (20°C). The temperature used for cleaning and sanitation in surveyed plants was near 4°C.

Good manufacturing practices for smoked fish products are essential and would help to reduce the contamination in the processing environment. It is a requirement by law that food producers have appropriate internal control based on HACCP. Such control can not be effective unless it covers the product, process and plant specification. Employees must be trained to understand the problem, the potential sources and the control of *L. monocytogenes*. This need to be more than normal training in good hygienic practices (GHP). Also, to verify *L. monocytogenes* control, plants should implement an environmental monitoring programme for *Listeria* species. Sampling points and frequency should be determined based on knowledge of their specific operation and the control that has been put in place.

Chapter 5 References

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