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*BSc Thesis*

**Tracking *Listeria monocytogenes* in a Salmon Processing Plant on the Faroe Islands**

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## Abstract

*Listeria monocytogenes* is a natural environmental bacterium and a serious pathogen causing the disease listeriosis in humans. Salmon processing plants have frequently been found to be contaminated with *L. monocytogenes*. However little is known about where in the production pathway contamination of *L. monocytogenes* occurs.

To establish where in the production pathway contamination of *L. monocytogenes* occurred, we examined one salmon producing plant for *L. monocytogenes* on the Faroe Islands. This was done by taking seawater samples both by the marine aquacultures and at the harvesting sites. Swab samples were also taken in the harvesting and processing plants, and from fish from both plants. Positive *L. monocytogenes* samples were found both in the seawater and in the processing plant. We found positive samples all way through the production pathway. We used multilocus sequence typing (MLST) of 6 genes to determine the genetic link between the different *L. monocytogenes* isolates from seawater and the processing plant. Findings indicate that the main source of *L. monocytogenes* contamination could be the seawater coming into the plant via the fish.

## Samandráttur

*Listeria monocytogenes* er ein natúrlig umhvørvis bakteria og ein sjúkueldandi bakteria, ið elvir til álvarsligu sjúkuna listeriosu hjá menniskjum. Virkir ið viðgera laks hava javnan verið dálkaði við *L. monocytogenes*. Kortini veit man lítið um hvaðani í framleiðslu leiðini dálkingin av *L. monocytogenes* fer fram.

Vit hava kannað eitt virkið í Føroyum fyri *L. monocytogenes*, fyri at staðfesta hvaðani í framleiðsluleiðini dálking av *L. monocytogenes* fer fram. Hettar varð gjørt við at taka sjógv sýni bæði við aliringarnar og við tøkuringin. Svabara sýnir vóru eisini tikin á blóðgistøðini, á virkinum og frá fiski frá báðum støðunum. Positiv *L. monocytogenes* sýni vóru funnin bæði í sjónum og á virkinum. Vit funnu sostatt positiv sýni gjøgnum alla framleiðsluleiðina.

Vit brúktu “multilocus sequence typing” (MLST) av 6 genum fyri at avgera arva liðin millum tær ávísu *L. monocytogenes*. MLST vísti tættan arva lið millum *L. monocytogenes* isolerað frá sjógvi og á virkinum, ið bendir á at *L. monocytogenes* dálking kundi komi frá sjónum og inn á virkið við fiskinum.

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# 1 Introduction

## 1.1 Background

*Listeria monocytogenes* was first described by Murray et al. (1926) in 1924. The organism was isolated from rabbits and guinea pigs.

*L. monocytogenes* is a Gram-positive bacterium that occurs widely in both agricultural (soil, vegetation, silage, faecal material, sewage, and water), aquacultural, and food processing environments (Fenlon 1999). *L. monocytogenes* is a transitory resident of the intestinal tract in humans, with 2 to 10% of the general population being carriers of the microorganism without any apparent health consequences (Farber & Peterkin 1991 and CAC 2007).

*L. monocytogenes* is an opportunistic pathogen which can cause the bacterial infection listeriosis. The disease primarily manifests as sepsis, meningitis or materno-fetal infections (Anonymous 2010). The likelihood that *L. monocytogenes* can establish a systemic\* infection is dependent on a number of factors, including the number of microorganisms consumed, host susceptibility, and virulence of the specific isolate ingested. All strains of *L. monocytogenes* appear to be pathogenic (FAO/WHO 2004). Listeriosis is most prevalent in people in certain risk groups, which include the elderly, newborns, pregnant women and immunocompromised people (Lundén 2004).

Listeriosis is mainly observed in industrialized countries. It is not known whether these differences in incidence rates between developed and less developed countries reflect geographical differences, differences in food habits and food storage, or differences in diagnosis and reporting practices (FAO/WHO 2004). The total incidence of invasive listeriosis is estimated to be 2-10 per million population per annum in the countries where data are available (FAO 1999). Because of the high mortality rate some countries have introduced a zero-tolerance policy in ready-to-eat products.

Due to the remote location of the Faroe Islands, the Faroese people have for centuries had to rely on the ocean for food and their livelihoods. Even today, fish represents more than 96% of the total export. Farmed salmon represents around one third of all export from the Faroe Islands. No matter where you go on the Faroe Islands, the Atlantic Ocean is always close at

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\* An infection in which the pathogen is distributed throughout the body rather than concentrated in one area. *L. monocytogenes* multiplies intracellularly within the macrophages.

hand. The Faroe Islands have 1298 km (807 miles) of coastline, and the farthest away from the ocean one can get is 5 km (3 miles).

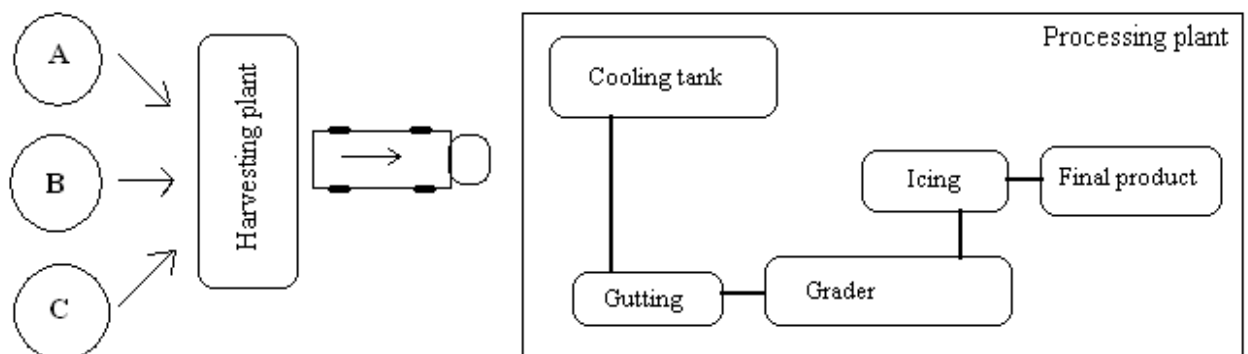
The Faroe Islands are not a part of the EU. However, the Faroe Islands have free trade treaties in place with the EU with regard to the free movement of goods between the EU and the Faroe Islands (FFFA). This means that salmon production in the Faroe Islands complies with all EU regulations and directives. In fact, the EU is the Faroe Islands' biggest trading partner.

## 1.2 Problem Statement

The general purpose of this thesis was to examine potential occurrence of *Listeria monocytogenes* in a Faroese salmon processing plant, including its processing pathway from the marine aquaculture environment to the finished product. If any *L. monocytogenes* was found in the processing plant, it was of interest to examine whether the bacteria came from contaminated seawater or if there was an “in house culture” causing *L. monocytogenes* contamination. This could be obtained by comparing with old *L. monocytogenes* positive samples from the processing plant (2009) with the potential new samples. We would also investigate whether *L. monocytogenes* came from abdominal cavity of the fish to indicate if precautions should be taken after the fish had been eviscerated.

## 1.3 Experimental Design

Figure 1 shows how the processing takes place.



**Figure 1:** Schematic design of the harvesting and processing plants. A, B, and C represent the fjords where the marine aquacultures are situated.

### **1.3.1 Description of the Processing Plant**

The processing plant has marine aquacultures situated in different places on the Faroe Islands. Prior to harvesting the marine aquacultures (marine salmon grow-out cages) are transferred to the location of the on-shore harvesting plant (the harvesting site) and the fish is pumped from the seawater into the harvesting plant. Inside the harvesting plant, the fish is stunned and transported on a conveyor belt to a road tanker. In the road tanker the fish is exsanguinated (bleeding out). The road tanker then transports the fish to the processing plant. When inside the processing plant the fish first reaches a large cooling tank, thereafter the fish is eviscerated and transported into the next cooling tank. A conveyor belt then transports the fish to quality check where the fish is sorted and graded according to weight. Thereafter the fish is packed and iced, before it is stored and then sold.

While we were taking samples the harvesting plant was situated in fjord A and the processing plant in fjord C. The fish was therefore transported in a road tanker from fjord A, after stunning to fjord C, where it was processed further.

### **1.3.2 Sampling Coverage**

All seawater samples were taken from three harvesting sites, located in different fjords (A, B, and C) on the Faroe Islands. Reference samples were taken by the marine aquacultures (surface water just beside the aquacultures). The harvesting and processing plants were also examined. We wanted to establish if the *L. monocytogenes* found in the processing plant came from the seawater or if there was another source of contamination. We also examined two old *L. monocytogenes* positive samples taken from the processing plant, to examine if the processing plant might have an “in house” *L. monocytogenes* culture.

Appendix 1 shows where the samples were taken and when. In the beginning of this project we had several *L. monocytogenes* positive seawater samples taken by the processing plant we examined. We examined seawater samples over a period of six months, one sample every month from October 2010 to March 2011. Sampling inside the processing plant was made over a period over three days.

Day 1 – 6 March 2011: Samples were taken at the harvesting (samples 1-4) and processing plant (5-15) before production started, to see if the cleaning and disinfection had been efficient enough.

Day 2 – 7 March 2011: Samples were taken about two hours after production started. Both in the harvesting (samples 16-17) and in the production plant (samples 23-30) Five swabs were taken from fish (samples 18-22 and 31-35). Meat samples from fish chops were taken in the production plant (samples 36-38). Seawater was sampled, both by the marine aquaculture (reference sample), at the harvesting site by the shore where the fish are pumped into the harvesting plant located in fjord A, and of the seawater that is cleaned and then cooled and pumped into the road tanker.

Day 3 – 14 April 2011: About five weeks later we decided to take more swab samples in the processing plant two hours after production. 14 more swab samples were taken (samples 39-52), including five swabs from fish (samples 47-51).

All samples were tested for *L. monocytogenes* and if positive, PCR products from six *L. monocytogenes* genes were sequenced to determine the bacterial strain.

## 2 Theories

### 2.1 *Listeria monocytogenes* and Listeriosis

#### 2.1.1 *Listeria monocytogenes*

*Listeria monocytogenes* was first described by Murray et al. (1926) in 1924. The organism was isolated from rabbits and guinea pigs. Animals infected with *L. monocytogenes* were observed to have caught monocytosis. This organism was originally named *Bacterium monocytogenes*, and was a few years later reported to be a human pathogen (Lundén 2004).

Currently the genus *Listeria* contains six species: *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria welshimeri*, *Listeria innocua*, *Listeria seeligeri* and *Listeria grayi* (Rocourt 1999 and Hitchins 2011). *L. monocytogenes* can be divided into 13 serotypes according to somatic (O) and flagellar (H) antigens (Lundén 2004 and Lund 2008). Table 1 summarizes the 13 serotypes.

**Table 1:** Serotypes of *Listeria monocytogenes* (Farber & Peterkin 1991, and Hitchins 2011)

Serotypes
1/2a, 1/2b, 1/2c
3a, 3b, 3c
4a, 4ab, 4b, 4c, 4d, 4e, 7

*Listeria monocytogenes* is aerobic, microaerophilic, facultative anaerobic, non-spore forming gram-positive rod and the causative agent of listeriosis (Rocourt 1999). Moreover, due to peritrichous flagella *L. monocytogenes* is motile at 20-25 °C but not at 37 °C (Hof 1996, Gründling et al. 2004, and Lund 2008).

The bacterium causes  $\beta$ -haemolysis on blood agar (i.e. the bursting of red blood cells due to bacterial-induced holes in their membranes), with narrow zone of haemolysis around the colonies (Farber & Peterkin 1991, Hof 1996, and NMKL 2007). As shown in Table 2, *L. monocytogenes* is a psychro- and halotolerant organism (Vogel et al. 2001) that grows in a temperature range from less than 0°C to about 45°C. Its optimal growth is at 30°C to 37°C (Vogel 2002). *L. monocytogenes* can grow in a NaCl concentration up to 10 % and survive 20% NaCl concentrations (Wilhelm 2003). Furthermore *L. monocytogenes* can grow in pH

ranging from 4 to 9, but optimally between pH 7-8 (Huss et al. 2000, Vogel 2002, and Wilhelm 2003).

**Table 2:** Growth limits for *L. monocytogenes* (FAO 1999)

<b>Environmental Factor</b>	<b>Lower Limit</b>	<b>Upper Limit</b>
Temperature (°C)	-0.3 to + 3	~ 45
Salt (% NaCl, water phase)	< 0.5	12-13
pH (HCl as acidulant)	4.2 – 4.3	9.4 – 9.5

### 2.1.2 Listeriosis

Listeriosis is a bacterial infection caused by the bacterium *Listeria monocytogenes*. Listeriosis is a relatively rare food infection that mostly infects elderly, newborns, pregnant women and immunocompromised people, which include people with organ transplants, alcoholism, diabetes and human immunodeficiency virus (HIV).

Manifestations of listeriosis include but are not limited to bacteremia, septicemia, meningitis, encephalitis, miscarriage, neonatal disease, premature birth and stillbirth (CAC 2007). Most healthy individuals are either unaffected by *L. monocytogenes* or experience only flu-like symptoms. The symptoms of listeriosis usually last about 7 to 10 days. The most common symptoms are fever, vomiting and muscle aches. If the infection spreads to the nervous system it can cause meningitis, an infection of the covering (protective membranes) of the brain and spinal cord, known collectively as the meninges.

The occurrence of listeriosis is very low in humans. No cases have been reported in Iceland or on the Faroe Islands since 1997, but in the other Nordic countries from 4 to 8 cases per million inhabitants have been reported annually (Gudbjörnsdóttir 2004). However, the mortality rate is between 20-30% (FAO 1999, Rocort et al. 2001, Lundén 2004, and FAO/WHO 2004). Pregnant women are much more likely to be infected than the rest of the population. Infected pregnant women may have only mild, flu-like symptoms. However, infection in a pregnant woman can cross the placenta and gain access to the fetus, leading to abortion, stillbirth or delivery of an acutely ill baby (Lund 2008).

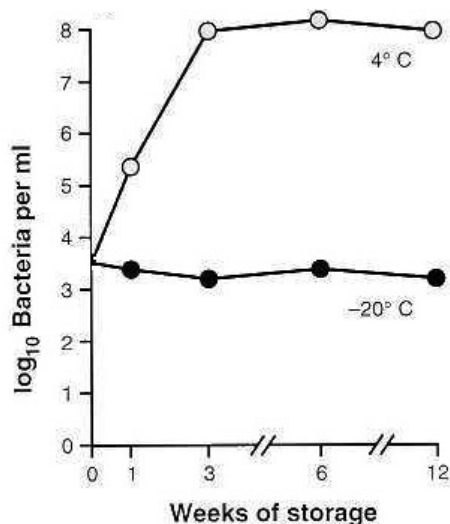
Incubation period for listeriosis can vary much, from a few days to three months (CAC 2007).

From the reported number of *Listeria* in contaminated food responsible for epidemic and sporadic food-borne cases, there is little evidence that a very low number of *L. monocytogenes* in food could cause listeriosis (Rocourt et al. 2000). Even if research indicate that low level of *L. monocytogenes* does not lead to infection, several countries have established a zero-tolerance, which means that no *L. monocytogenes* must be detected in a 25g sample (FAO 1999 and Vogel 2002).

### 2.1.3 Listeriosis Caused by Food

*Listeria monocytogenes* has been isolated from foods such as raw vegetables, raw and pasteurized fluid milk, cheeses (particularly soft-ripened varieties), ice cream, butter, fermented raw-meat sausages, raw and cooked poultry, raw and processed meats (all types) and raw, preserved and smoked fish (CAC 2007). Even when *L. monocytogenes* is initially present at a low level in contaminated food, the microorganism may multiply during storage in foods that support growth, even at refrigeration temperatures.

*L. monocytogenes* is of particular concern in ready-to-eat products in which the bacterium can multiply. Such foods, or their ingredients, may have received a listericidal treatment (e.g. pasteurization of milk for cheese production) or cooking, but subsequent processing (e.g. fermentation and ripening of cheeses, slicing and packaging of cooked meats) may allow a risk of contamination (Lund 2008). The occurrences of listeriosis over the last 15 years indicates that some products display greater risk than others. Ready-to-eat products kept at refrigerator temperature over a longer period of time, are among the products that display a greater risk, because *L. monocytogenes* has had opportunity to reproduce and reach infective doses (Rocourt et al. 2001) as illustrated in Figure 2.



**Figure 2.** Multiplication of *Listeria monocytogenes* in broth at low temperatures (Hof 1996)

Modern processing and packaging techniques have enabled production of foods with extended shelf-lives, and therefore made it difficult to control the *L. monocytogenes* levels. Besides the amount of *L. monocytogenes* present in food the infectious dose of a food-borne pathogen depends on a number of variables including the condition of the host, the virulence of the strain, the type and amount of food consumed, and the concentration of the pathogen in the food (Rocourt et al. 2000).

#### **2.1.4 Trade Problems**

There is no international agreement concerning the *Listeria monocytogenes* problem. Some countries e.g. USA, Russia, and Italia have established a zero-tolerance policy in ready-to-eat products (Shank et al. 1996, Onischenko 2001, Wilhelm 2003, and Chen et al. 2007). The European Commission recommended that it should be an objective to keep the concentration of *L. monocytogenes* in food below 100 colony forming units (cfu) per gram and to reduce the fraction of foods with a concentration above 100 cfu of *L. monocytogenes* per gram significantly (EC 1999). Since 2006, the EU Regulation on microbiological criteria has been in force. The Regulation distinguishes between products supporting growth of *L. monocytogenes* and products not supporting growth. All ready-to-eat (RTE) products are covered and the maximum limit value at the end of shelf-life is 100 cfu/g (EC 1999, EC 2005, and Anonymous 2010). The Faroe Islands have no strict policy regarding *L. monocytogenes*. Other countries have a *L. monocytogenes* tolerance on less than 100 cfu/g in some provisions. Regarding fish and fishery products, the dose-response has been estimated by combining data on the incidence of listeriosis in Germany with data on the levels of *L. monocytogenes* in smoked-fish in that country (FAO 1999). It was necessary to make many assumptions, but all were chosen to result in conservative estimates. Epidemiological and food survey data were combined, using a predictive modeling approach, to estimate a dose-response relationship for *L. monocytogenes* levels and incidence of listeriosis. Two methods were used to model and calculate the dose-response relationship. Both methods gave a similar result. Using that approach, for the estimated immunocompromised sub-population of Germany (20% of the population), the model predicts a 1 in 59 million chance of infection from consumption of a 50g serving of fish containing 100 cfu/g (FAO 1999).

Several studies show that 10-60% of newly manufactured cold-smoked salmon is contaminated with *L. monocytogenes* (FAO 1999). Therefore *L. monocytogenes* detection

causes great trade problems, in the countries that have established a zero-tolerance policy. According to Vogel et al. (2001) it is impossible to produce cold-smoked salmon that is 100% free of *L. monocytogenes*.

Salmon from the Faroe Islands is sold almost worldwide. The salmon can be sold to smokehouses for further processing or it can be sold unprocessed to ordinary citizens through a fishmonger or fish market.

## **2.2 Appearance of *Listeria monocytogenes* in Food, Especially Fish**

*Listeria monocytogenes* is found naturally in many kinds of raw foods e.g. vegetables, milk, meat and fish (Wilhelm 2003 and Autio 2003). Products, which do not receive any heat treatment by the consumer i.e. ready-to-eat products, like cheese, meat and fish delicatessen may contain high levels of *L. monocytogenes*. High levels of *L. monocytogenes* compromise a great health risk for the people in the risk group. Ready-to-eat products include any food (including beverages) that is normally consumed in its raw state, or any food handled processed, mixed, cooked or otherwise prepared into a form in which it is normally consumed without further processing (CAC 1999).

From the reported numbers of *L. monocytogenes* in contaminated food responsible for epidemic and sporadic foodborne cases, there is little evidence that a very low number of *L. monocytogenes* in food causes listeriosis (FAO 1999). It is considered that the vast majority of cases have resulted from consumption of high numbers of the bacterium, and foods where the level of the pathogen was higher than 100 cfu/g (FAO/WHO 2004). Seafood has been classified according to risk for listeriosis as seen in Table 3.

**Table 3:** Seafood classified according to risk for listeriosis (Rocourt et al. 2000).

<b>High risk</b>	<b>Low risk</b>
Molluscs (including fresh and frozen mussels), clams, oysters (in shell or shucked)	Heat-processed (sterilised, packed in sealed containers).
Raw fish	Fresh frozen fish and crustaceans.
Lightly preserved fish products (i.e. NaCl <6% (w/w) in water phase, pH >5.0). This group includes salted, marinated, fermented, cold-smoked- and gravad fish.	Semi-preserved fish, i.e. NaCl >6% (w/w) in water phase, or pH <5.0, preservatives (sorbate, benzoate, NO <sub>2</sub> may be added). This group includes salted and/or marinated fish, caviar and fermented fish.
Mildly heat-treated (pasteurized, cooked, hot smoked) fish products and crustaceans (including pre-cooked, breaded fillets).	Dried, dry-salted and smoke-dried fish.

While a number of fish products are obviously more risky than the average foods, outbreaks with high numbers of cases have not yet been observed and case-control studies have not identified these products as major risk factors. In addition, production factories processing fish and fishery products are usually smaller than factories processing dairy or meat products, thus resulting in a smaller number of products contaminated by various strains in the market. This could explain why large outbreaks have not yet been observed and why case-control studies have not identified these products as major risk factors (Rocourt et al. 2000). Table 4 shows documented cases of foodborne listeriosis from fish products.

**Table 4:** Main documented cases of foodborne listeriosis associated with fish and fishery products (FAO 1999)

Location / year	No. cases	Clinical forms <sup>1</sup>	No. deaths	Food	cfu/g	Serotype
USA- 1989	2 <sup>2</sup>	2 <sup>2</sup>	0	Shrimp	NK	4b
Italy - 1989	1	0/1	0	Fish	NK	4b
Australia - 1991	2	0/2	0	Smoked mussels	1 x 10 <sup>7</sup>	NK
New-Zealand 1992	4	4/0	0	Smoked mussels	NK	1/2b
Canada - 1996	2	0/2	NK	Imitation crab meat	2 x 10 <sup>9</sup>	1/2b
Sweden - 1994/95	8	3/8	2	gravad/cold-smoked rainbow trout	<100-6200	4b

<sup>1</sup>: number of pregnancy related cases / number of non-pregnancy related cases;

<sup>2</sup>: not known if this was the total number of cases

NK: not known

The preservation parameters for all the lightly preserved fish products fall within limits of *L. monocytogenes* growth. *L. monocytogenes* can namely grow at low temperatures (>0 °C), at a wide pH range (4-9) and in up to 10 % NaCl concentrations. Studies have shown that *L. monocytogenes* only has limited growth in naturally infected fish (Huss et al. 2000).

### 2.3 Occurrence of *Listeria monocytogenes* in Seawater

*Listeria monocytogenes* is present in freshwater and in seawater near the coastline. Fecal contamination from animals and run-off from land may be a significant source of *L. monocytogenes* to shellfish and fish. However, unpolluted seawater and ground- or spring water is free from this organism, and has not been detected in fish from such locations (Huss et al. 1995).

There only exist few studies of abundance of *L. monocytogenes* in seawater when it comes to marine aquaculture of salmon. Ben Embarek et al. (1997) took samples of seawater and examined them for *Listeria*. Rørvik et al. (1995) and Wilhelm (2003) sampled seawater near the slaughterhouse and near shore respectively, and examined it for *Listeria* (see Table 5).

**Table 5:** Seawater examined for *Listeria* and *Listeria monocytogenes*

Sample site	Number of samples taken	Number of positive samples for <i>Listeria</i> (%)	Number of positive samples for <i>Listeria monocytogenes</i> (%)	References
Seawater taken near a slaughter-house, Norway	33	12 (36)	3 (9)	Rørvik et al. 1995
Seawater taken by aquaculture, Norway	8	0	-	Ben Embarek et al. 1997
Seawater near shore, where the fish is before harvesting, factory A, Faroe Islands	6	5 (83)	2 (33)	Wilhelm 2003
Seawater near shore, where the fish is before harvesting, factory B, Faroe Islands	5	5 (100)	0 (0)	Wilhelm 2003

The lack of *Listeria monocytogenes* in unpolluted seawater is confirmed in Table 5 as no *Listeria* is found by Ben Embarek et al. (1997) in the samples taken by the aquaculture, situated in a low populated area outside Bergen, Norway. In contrast, Rørvik et al. (1995) and Wilhelm (2003) found *L. monocytogenes* in seawater outside the slaughterhouse, which presumably was contaminated.

According to Ben Embarek et al. (1997) no studies of the natural occurrence of *Listeria* in seawater has been published.

In the seafood processing plants, drains are often found contaminated with *L. monocytogenes*. However, the drains are not considered to be a source of the contamination, but rather an indicator of *L. monocytogenes* contamination of a processing area (Lundén 2004).

## **2.4 Occurrence and Tracking of *Listeria monocytogenes* in Production**

The subtyping of *Listeria monocytogenes* isolates offers an approach for investigating the relatedness of isolates and identifying and tracing the sources of epidemics. Subtyping has been successfully used in identifying and tracing epidemics of *L. monocytogenes* caused by food, and to determine which food caused the epidemic. If *L. monocytogenes* is found in finished products, all steps in production can be examined, from raw material and through processing environment to determine the origin of the contamination. Both phenotyping and molecular typing methods have been applied in the subtyping of *L. monocytogenes* isolates. *L. monocytogenes* contamination studies have been performed to identify contamination routes and sites in food processing plants and to gain information on how to prevent product contamination (Rørvik et al. 1995).

Traditional typing methods like serotyping do not give sufficient information when it comes to epidemics of *L. monocytogenes*. Most listeriosis cases are associated with a restricted number of serotypes: 1/2a (15-25%); 1/2b (10-35%); 1/2c (0-4%); 3 (1-2%); 4b (37-64%) and 4 not b (0-6%) (Farber & Peterkin 1991) but none of the present methods have consistently identified strains that are nonpathogenic or less virulent (FAO/WHO 2004). The numbers clearly show that serotypes 1/2a, 1/2b, and 4b have been associated most frequently with listeriosis in humans (Lund 2008).

## **2.5 Persistency and Control of *Listeria monocytogenes***

*Listeria monocytogenes* can be found in contaminated seawater and in seafood plants (Rørvik et al. 1995). The bacteria can form biofilm (Faber & Peterkin 1991). This can explain how many studies have shown persistent *L. monocytogenes* in the production environment for years (Vogel et al. 2001).

Cleaning and disinfection is therefore a vital part of the control of *L. monocytogenes* in the food industries. Cleaning and disinfection are two complementary processes, whereas none of them can get a good result on its own (Wilhelm 2003). There are often problems with *L. monocytogenes* in equipment that is difficult to dismount when cleaning and disinfecting (Borlaug 2005). If the cleaning and disinfection are not efficient enough *L. monocytogenes* may establish biofilm. Biofilm is an aggregate of microorganisms that adhere to each other on a surface. These adherent cells are embedded within a self-produced matrix of extracellular

polymeric substance (EPS). If biofilm is established in a food processing plant, it creates an “in house culture” of *L. monocytogenes* and this continues to contaminate the food produced.

Some studies indicate that *L. monocytogenes* can be found on the skin and in the abdominal cavity of fish (Huss et al. 2000). Uncontaminated water can be used to clean fish for *L. monocytogenes*, but that results in much water in the drains, and if cleaning with high-pressure washer *L. monocytogenes* is spread over the whole factory (Borlaug 2005).

## **3 Methods**

We examined one salmon producing company for *Listeria monocytogenes*. Samples were taken at the harvesting plant and at the processing plant. Samples were taken of raw material, seawater, production equipment and manufactured goods. Samples of the production equipment were taken after cleaning and disinfection, and at least 2 hours after production. Figure 1 shows how the processing is designed.

### **3.1 Samples**

All samples taken inside the harvesting plant and processing plant were taken as swab samples. The water samples were taken with 9 litres sterile cans. Fish chops were sampled at the processing plant and examined for *Listeria monocytogenes*.

#### **3.1.1 Materials**

Swab samples were taken with bio-spo<sup>TM</sup> (Environmental Sampling Products, Solar Biological Inc.). After using the swab cloth, it was returned to the sterile bag that it came from. When samples were taken of the cleaned equipment, the swabs were soaked in neutralizing buffer (Difco 8902823y5).

Gloves were used when the samples were taken. The samples were stored at 5 °C, until further processing in the laboratory.

#### **3.1.2 Samples: Production Equipment, Raw Material, Manufactured Goods and Seawater Samples**

The samples of the clean production equipment were taken one day before the factory started production. Samples taken during production were taken at least 2 hours after production started. Swab samples were taken of fish after stunning in the harvesting plant and of finished produced fish in the processing plant. The 9 litres seawater samples were taken of surface water over a period of 6 months, from October 2010 to March 2011.

### **3.2 *Listeria monocytogenes* Identification**

Identification of *Listeria monocytogenes* was made according to NMKL method number 136/4<sup>th</sup> ed. 2007 as modified and routinely used by the Faroese Food and Veterinary Agency microbiology laboratory. The laboratory is accredited by Danak, registration number 303. Identification of *L. monocytogenes* was performed using TaqMan *Listeria monocytogenes* Detection Kit (01/2006).

The DNA sequence procedure was performed according to Zhang et al. (2004) and Chen et al. (2007) as described in paragraph 3.3.

#### **3.2.1 *Listeria monocytogenes* Enrichment**

We added between 195g – 205g Half-Fraser broth to the sterile bag with the swab cloth. Then the bags with the swab cloth and Half-Fraser broth were incubated at 30 °C for 24±4 hours. Two samples were taken from each of the three fish chops. One sample consisted of 25g of meat with scales and the other samples was 25g of meat from the abdominal cavity. 225g of Half-Fraser broth was added to the samples, and then the samples were stomached (an apparatus homogenizing the sample, transferring microbes into the broth) (AIE Laboratoire Group, MIX 2) for 30 seconds, before incubation at 30 °C for 24±3 hours.

The seawater samples were filtered and the filter paper (Millipore, EZ-Pak® Membrane Filters, white gridded 0,45 µm, 47 mm) was put in a sterile bag. 100 mL of Half-Fraser broth was added to the samples and the samples were stomached for 30 seconds. Then the samples were incubated at 30 °C for 24±3 hours.

After incubation 0.1 mL of both the fish and water samples were added to 10 mL Fraser broth, and incubated at 37 °C for another 48±4 hours.

#### **3.2.2 *Listeria monocytogenes* Isolation**

From the swab Half-Fraser broth samples 1.0 mL of the sample was taken and DNA from the *L. monocytogenes* was purified with DNeasy mini kit, and 50 µl were taken and plated on RAPID'*L.mono*<sup>TM</sup> plates.

The fish and seawater samples were plated on two selective media, as stated in the NMKL method. Oxoid's Chromogenic *Listeria* Agar (OCLA) (Oxoid Ltd., Cambridge, England) and PALCAM agar (Oxoid Ltd.; described by Van Netten et al., 1989) and incubated at 37 °C for 24±3 hours. The positive samples from OCLA and PALCAM were plated on blood agar and

incubated at 37 °C for 24±3 hours. All the positive samples were plated on RAPID'*L.mono*<sup>TM</sup> plates.

From all the positive samples a colony was purified with DNeasy mini kit.

OCLA is a medium selective for *Listeria*, because of the inclusion of lithium chloride, polymyxin B, nalidixic acid and amphotericin to inhibit other bacteria and fungi and chromogenic due to bacterial  $\beta$ -glucosidase activity staining the colonies and phospholipase activity causing an opaque white halo around the colonies (Oxoid Ltd.).

PALCAM is also a selective medium, through the presence of lithium chloride, ceftazidime, polymyxin B, and acriflavine hydrochloride, which suppress growth of most non-*Listeria* spp., and chromogenic based on esculin hydrolysis and mannitol fermentation. All *Listeria* spp. hydrolyze esculin as evidenced by a blackening of the medium. Mannitol and the pH indicator, phenol red, have been added to differentiate mannitol-fermenting strains of these species from *Listeria*. Mannitol fermentation is demonstrated by a colour change of the medium from red to yellow due to the production of acids (Oxoid Ltd.).

### **3.2.3 *Listeria monocytogenes* Identification**

The presumptive identification of *L. monocytogenes* in the seawater and fish samples was made on OCLA and PALCAM plates. The positive samples were then plated on blood agar. If there was haemolysis ( $\beta$ -haemolysis) on blood agar, colonies were taken and tested using AccuProbe (AccuProbe Culture Identification Reagent Kit, Gen-Probe Incorporated, San Diego, CA).

The swab samples were tested for *L. monocytogenes* using the *Listeria monocytogenes* Real-Time PCR detection kit (Applied Biosystems, 7500 Fast Real-Time PCR System) and later also plated on RAPID'*L.mono*<sup>TM</sup> plates.

### **3.3 Multilocus Sequence Typing (MLST) of *Listeria monocytogenes***

To determine the genetic relationship between the different *L. monocytogenes* isolates six virulence genes (*prfA*, *inlA*, *inlB*, *inlC*, *lisR*, and *clpP*) were amplified by PCR on a ABI 2700 (Applied Biosystems) with the HotStart Taq polymerase according to manufactures recommendations (Qiagen) using the primers designed by Zhang et al. (2004) and Chen et al.

(2007) and listed in Appendix 2. After amplification the PCR fragments of approximately 400 to 500 bp were visualized by agarose gel electrophoresis (Owl Separation Systems Inc., Portsmouth, USA). After agarose gel electrophoresis the PCR products were purified with the Jetquick kit according to the manufacturer's protocol (PCR Product Purification Spin Kit/250, Löhne, Germany). Purified PCR products were then sequenced using the BigDye™ terminator sequencing kit (Applied Biosystems) according to the manufacturer's recommendations. After purification the sequencing products were run on the ABI-3100 sequencing instrument (ABI Prism® 3100-*Avant*). Only forward primers were used in DNA sequencing. Sequence analysis was performed using ABI Sequencing Analysis software (V.5.1).

### **3.3.1 Phylogenetic Analysis using ClustalX and TreeView**

To visualise the relationship between the *L. monocytogenes* found in the different samples, the sequences from the six genes from each sample were concatenated (lined up after one another). The concatenated sequences were aligned with ClustalX (standard settings, v. 1.81), and shown graphically using TreeView (Win 32, v. 1.6.6). This essentially visualises the number of differences found between the different concatenated sequences. To get a statistical estimate of the reliability of some grouping, bootstrapping was used. Bootstrapping is a method in which one takes a subsample of the sites in an alignment and creates trees based on those subsamples. That process is iterated multiple times and the results are compiled to allow an estimate of the reliability of a particular grouping.

## 4 Results

There were taken 24 seawater samples over a period of 6 months, of which 16 were *Listeria monocytogenes* positive. Two samples of cooled water from the road tanker were also taken. None of them were positive (see Table 6).

**Table 6:** Seawater samples

Sample site (fjord)	Number of samples taken	Number of <i>L. monocytogenes</i> positive samples	% <i>L. monocytogenes</i> positive samples	Sample number (Resembling serotype)
Harvesting site A	9	6	66.7	4A (1/2a), 5A (4b), 7A (?), 8A (1/2a), 9A (1/2a), and the December sample
Harvesting site B	4	2	50	2B (4b) and the December sample
Harvesting site C	6	4	66.7	1C (1/2a), 3C (1/2a), 6C (4b), and the December sample
Ref. by marine aquacultures A, B and C	3 (5*)	3 (3*)	100 (60)	Ref. A, Ref. B, and Ref. C
<b>Total</b>	24	15	62.5	-

\*Two more reference samples were taken when visiting harvesting site A. None of them were *L. monocytogenes* positive.

1. See Appendix 1
2. The December sample tested positive, but was not further investigated (see text).

Table 6 includes three seawater samples taken in December (one positive from each fjord). Even though the samples tested positive for *L. monocytogenes*, they were not saved and processed further, due to a misunderstanding. The samples are therefore not included in further results i.e. Figure 4 and Figure 5.

We visited the harvesting plant over two days; the first visit was after cleaning, and the second in production. All samples at the harvesting plant were negative. We also took samples of 25 fish (five fish per swab cloth) just before they went into the road tanker, which also were *L. monocytogenes* negative.

Samples at the processing plant were taken three times. At the first visit the samples indicated that the processing plant was clean. There were taken 11 samples which all were negative.

At the second and third visit the processing plant was in production. Both times *L. monocytogenes* was detected, with 14.4% and 12.5% positive samples respectively (Table 7).

Five swab samples were taken of 25 fish (five fish per swab cloth) when they had been through the production line. Here one sample (20 %) was positive for *L. monocytogenes*.

Three fish chop samples were also taken, which all were negative for *L. monocytogenes*.

**Table 7:** Swab samples

Sample	Place	Total samples	Positive	Name (Resembling serotype)
<b>Day 1:</b> Before production				
1-4	Harvesting plant	4	0 (0%)	
5-15	Processing plant	11	0 (0%)	
<b>Day 2:</b> During production				
16-17	Harvesting plant	2	0 (0%)	
18-22	Fish samples – Harvesting plant	5	0 (0%)	
23-30	Processing plant	8	1 (14.4%)	S24 (1/2a)
31-35*	Fish samples – Processing plant	5	0 (0%)	
<b>Day 3:</b> During production				
39-47	Processing plant	9	1 (12.5%)	S41 (1/2a)
48-52	Fish samples – Processing plant	5	1 (20%)	S51 (1/2a)

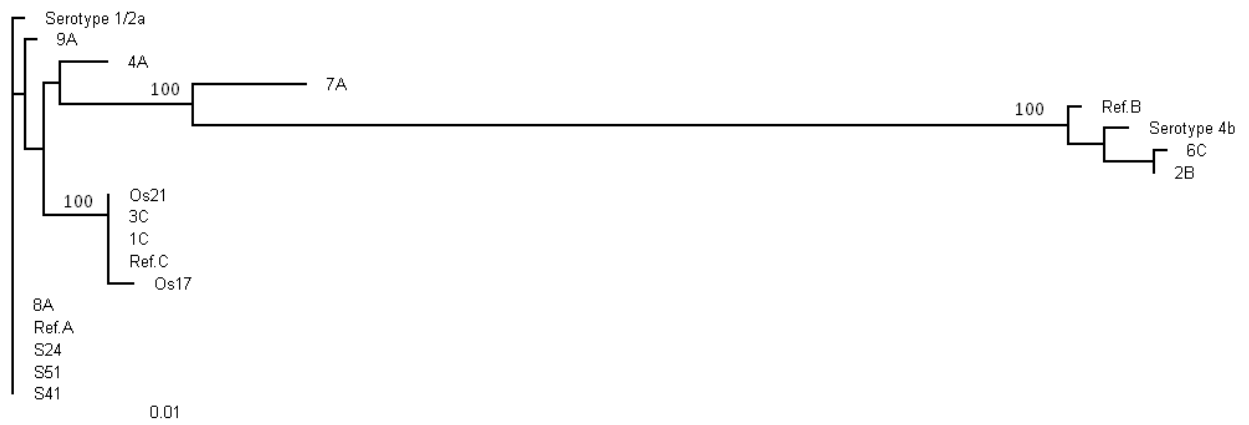
\*Samples 36 to 38 are fish chops from the processing plant (not swab samples).

During our work, we observed that sample 6C (a sample from fjord C) was lacking haemolysis on blood agar, but was positive both on OCLA and in AccuProbe. Then we plated the sample on RAPID'*L.mono*<sup>TM</sup> plate, where *L. monocytogenes* should form blue colonies. However, the bacteria formed white colonies (Figure 3). We therefore sequenced PCR products from the six virulence genes, but the sequences were consistent with *L. monocytogenes*.



**Figure 3:** RAPID'*L.mono*<sup>TM</sup> plates. Left plate showing sample 6C with white colonies and right plate with blue colonies, as they should be if they are *L. monocytogenes*.

When all the samples were sequenced, and we combined all the genes from the same samples, they were aligned using ClustalX and genetic distance was shown graphically using TreeView. The only gene fragment that we did not manage to amplify by PCR was *inlA* for sample 5A. So we did two TreeView images one using all the genes, where we had to exclude sample 5A (Figure 4) and one using all the samples but excluded gene *inlA* (Figure 5). Two old swab samples from 2009 (from the same processing plant) were included for comparison. The greater the horizontal distance between two samples is in the tree, the greater is the genetic difference between the samples.



**Figure 4:** All samples except 5A. All six gene fragments combined (*prfA*, *inlA*, *inlB*, *inlC*, *lisR* and *clpP* - approximately 2500 pb). Sample Ref. A, Ref. B, and Ref. C are reference samples sampled at the aquaculture site. Other seawater samples are sampled at the harvesting site. Note that sample 6C is the “white” *L. monocytogenes* described above. S stands for swab sample, Os for old swab samples, and the rest are seawater samples. The letter behind the seawater samples shows at which harvesting site the sample is taken (A, B, or C). The number 100 is a statistical value, telling us that the samples on each side belong to two different groups.



**Figure 5:** All samples. With the sequence from five gene fragments (*prfA*, *inlB*, *inlC*, *lisR* and *clpP* – approximately 2100 pb). Sample Ref. A, Ref. B, and Ref. C are reference samples sampled at the aquaculture site. S stands for swab sample, Os for old swab samples, and the rest are seawater samples. The letter behind the seawater samples shows at which harvesting site the sample is taken (A, B, or C). The number 100 is a statistical value, telling us that the samples on each side belong to two different groups.

Figure 4 and Figure 5 show unrooted bootstrapped trees. The trees tell us only about phylogenetic relationships, they tell us nothing about the directions of evolution – the order of descent. The bootstrapping shows that Ref. B, 5A, 6C, and 2B belong to a group that is different from the other main group which includes the rest of the samples.

## 5 Discussion

Literature shows that seawater near the coastline might be contaminated with *Listeria monocytogenes* (Lund 2008). This is consistent with our results. We have found clear evidence of *L. monocytogenes* in the seawater, both at the aquaculture sites and the harvesting sites in fjord A, B, and C. This means that *L. monocytogenes* can be found in several fjords on the Faroe Islands. In the beginning of this project we did not expect to find *L. monocytogenes* in our reference samples. This did not turn out as expected. The first three reference samples were all positive. Furthermore 62.5 % of all seawater samples taken over a period of 6 months were *L. monocytogenes* positive. Even though the Faroese fjords are perfect for aquacultural production as they provide exceptional biological conditions, excellent circulation of fresh seawater according to FFFA, *L. monocytogenes* still challenges/provokes Faroese salmon production. Marine aquacultures in fjord A, B, and C are situated in low density inhabited fjords on the Faroe Islands. Even though the marine aquacultures are placed as far out in the fjords as possible, *L. monocytogenes* was found in three of five reference samples. This might be because of the many sheep and rivers on the Faroe Islands. Sheep can carry *L. monocytogenes* on their wool or in their faeces and the rivers then transport the bacteria to the sea (NVI 2011). Multiplication of the bacterium in poorly prepared silage can cause listeriosis in sheep, that subsequently feed on the silage (Lund 2008). With *L. monocytogenes* being an environmental bacterium also found in soil, the bacterium can then enter the river water through soil or as a result of faecal contamination and then contaminate the costal seawater. The organism has been reported, sometimes in relatively high numbers in river water (Lund 2008). However this might not be the only contamination, resulting in finding *L. monocytogenes* in seawater. The feed could also be an contamination source of *L. monocytogenes* by the marine aquacultures, although this seems less likely than the environmental contamination described above. The salmon feed was not examined for *L. monocytogenes*.

We swabbed 50 fish (5 fish per swab cloth) at the end of the production and 25 fish (5 fish per swab cloth) at the harvesting plant after they were stunned. Only one of the samples of the final product was positive. The quantitative levels of *L. monocytogenes* in the freshly produced products is of interest and must be known before a proper risk assessment can be made (Huss et al. 2000). We did not perform quantitative tests. However, the three meat

samples were all negative for *L. monocytogenes* – both at the surface and in the abdominal region.

According to Wilhelm (2003) and Ben Embarek et al. (1997) there is no problem with *L. monocytogenes* in the marine aquacultures on the Faroe Islands and in Norway respectively. The problem first arises when the fish is transported to the harvesting plant. However, our results are in strong contrast.

Even though marine aquacultures may be placed in unpolluted (i.e. *L. monocytogenes* free seawater) contamination may take place long before the raw fish reaches the processing plant. *L. monocytogenes* has been found in the slime, skin and belly cavity of salmon (Huss et al. 2000).

Salmon from Faroe Islands is sold worldwide and must therefore satisfy certain standards, when it comes to *L. monocytogenes*. All fish sold to Russia must be *L. monocytogenes* free, as Russia has a zero-tolerance policy when it comes to *L. monocytogenes* (Onischenko 2001). The Russian zero-tolerance policy includes only 25g fish samples not swab samples. However inside the processing plant and harvesting plant altogether 49 swab samples were taken and only 3 were *L. monocytogenes* positive. That corresponds to 6.1%. Two of the three positive samples were taken from the processing equipment during processing and one was taken from finished produced fish. Altogether we swabbed 75 fish (5 fish per swab sample) and found only one sample containing *L. monocytogenes*.

There has not been any clear agreement of where the contamination comes from in the final product. According to Suihko et al. (2002) the occurrence of *L. monocytogenes* in even the most hygienic food processing conditions is difficult to prevent totally. Eklund et al. (Huss et al. 2000) proposed that the primary source of contamination was the raw fish material coming into the plant. In contrast Rørvik et al. (1995) concluded from their work that raw fish material was not the source of contamination for the final product (i.e. cold smoked-salmon). Our MLST data may suggest an origin of the *L. monocytogenes* found in the production plant (S24 and S41) and the final product (S51). These samples are identical to two of our seawater samples (Figures 4 and 5), one being a reference sample from the marine aquaculture and the other from harvesting site A. Thus, it is likely that these samples have the same origin of the detected *L. monocytogenes*. This information and our TreeView results indicate that *L. monocytogenes* is brought in to the plant with the seawater. A previous paper reported isolation of *Listeria* from the water column, but not from the surface of the fish after feeding

the fish contaminated feed (Bremer et al. 1994). We did not swab the fish before the marine aquaculture was moved to the harvesting site, but as the fish at the harvesting plant was negative, it seems unlikely that it would be positive at the aquaculture site.

The old samples (Os21 and Os17) were taken in 2009 as a part of the internal control of the production plant. Both samples were taken from the drains. They are almost identical with only two base pair difference. Two other *L. monocytogenes* positive samples found in fjord C were also identical, one seawater sample is from the harvesting site and the other from the marine aquaculture.

In 2009 the production plant received salmon from the harvesting site and marine aquaculture in location C. Even though our seawater samples were taken a little over a year after, we found an identical *L. monocytogenes* in the seawater in fjord C. The fact that we two times found the same *L. monocytogenes* in harvesting site and marine aquaculture in fjord A and C, could possibly indicate that the aquaculture is placed too close inhabited area, or that sheep or soil continually contaminate the seawater.

It is tempting to suggest that the old samples (Os17 and Os21) also had their bacteria originating from the seawater. This would then mean that an identical *L. monocytogenes* still is in the seawater over a year later.

According to Hsu et al. (2005) in the presence of natural microflora, *L. monocytogenes* died off rapidly in seawater within 36 hours at room temperature. When held at or less than 11 °C, *L. monocytogenes* lost viability throughout storage but was still detectable after more than 6 days of incubation. In the absence of natural microflora, *L. monocytogenes* grew better than it did in the presence of natural microflora (Hsu et al. 2005), but it seems unlikely that *L. monocytogenes* would be able to survive for one year or more in seawater. This probably means that there is the same source still contaminating the fjord.

If we look at all our seawater samples, there are a few samples that differ from each other; these are the once resembling serotype 4b. In these two fjords (A and C) both serotype 1/2a and 4b of *L. monocytogenes* are found (Figure 5). In fjord B we only found two positive samples, which both resemble serotype 4b, but with a difference of nine base pairs.

Figure 3 shows two RAPID<sup>™</sup> *L.mono*<sup>™</sup> plates, one with white colonies and one with blue colonies. According to the manufacturer, blue colonies are *L. monocytogenes* whereas white colonies are other *Listeria* spp. Still, our sequencing suggested that also the white colonies

were *L. monocytogenes*. When we discovered our rather peculiar result, we took new colonies from blood agar and plated them on a new RAPID'*L. mono*<sup>TM</sup> plate, but the result was the same. The bacterium had no haemolysis on blood agar, but tested positive on OCLA and in AccuProbe for *L. monocytogenes*.

According to Bauwens et al. (2003) the synthesis of listeriolysin O, a haemolysin, and phosphatidylinositol-specific phospholipase C (PIPLC) distinguishes pathogenic *L. monocytogenes* and *L. ivanovii* strains from the non-pathogenic species. The recently commercialised selective and differential medium, RAPID'*L. mono*<sup>TM</sup> enable the isolation of *Listeria* spp. and presumptive identification of *L. monocytogenes* and *L. ivanovii* based on their PIPLC production (Bauwens et al. 2003). When our white colonies are *L. monocytogenes* one of the following options may explain why. (i) The phosphatidylinositol-specific phospholipase C (PIPLC) has mutated and is therefore not active. (ii) The regulatory parts of the gene has mutated, resulting in non-expression of the enzyme. (iii) The bacterium has several phospholipase C genes and enzymes and does not express the specific PIPLC gene. A search in a genome database (<http://cmr.jcvi.org/tigr-scripts/CMR/CMrHomePage.cgi>) containing four variants of *L. monocytogenes* (1/2a F6854, 4b F2365, 4gb H7858, EGD-e) suggesting the possibility of two phospholipase genes. (iiii) The bacterium is a *Listeria* type that has similar sequence in the genes sequenced, but is different when it comes to PIPLC.

The white *L. monocytogenes* variant was initially detected by the lack of haemolytic activity, however the bacterium tested positive on OCLA, in AccuProbe, and in Real-Time PCR. According to Lei et al. (1997) it is intriguing that serotype-associated antigenic and genetic features of one group of *L. monocytogenes* strains (serotype 4b, 4d, and 4e) are present in certain strains of a different species (*L. innocua*) but not in other *L. monocytogenes* serotypes (e.g. 1/2a, 1/2b, 3a, etc.). Our MLST results rely only on the six virulence primers and not on an entire genome sequencing. Similarities may be between the different genes and certain *Listeria* species. And therefore resulting in indicating that the white colonies were *L. monocytogenes*.

As OCLA, AccuProbe, Real-Time PCR, and MLST indicate that the white colonies were *L. monocytogenes*, we treated the sample as *L. monocytogenes* and not other *Listeria* species.

We compared our three *L. monocytogenes* positive samples with the two old samples, to see if there was an “in house culture” of *L. monocytogenes* contaminating the processing plant. It is not likely that the processing plant has an “in house culture”. Which is also shown in the bootstrapped tree (Figure 4 and Figure 5) where the old samples and the samples we took in the processing plant clearly belong to two different subgroups. However the old samples are identical to samples (1C, 3C, and Ref. C) taken in fjord C. If we only look at our *L. monocytogenes* positive samples (S24, S41, and S51) the samples are identical. Even if they are not found in the same place, it is probable that the bacteria come from the same contamination source. This contamination source is probably the seawater from harvesting site A, because it is identical to our three positive samples. The trees show that the three positive samples from the plant (S24, S41, and S51) were identical to the two seawater samples (Ref. A, and 8A) but slightly different from two other seawater samples from fjord A (4A and 9A). Somewhat speculatively, this might suggest that “S24/S41/S51/Ref. A/8A” variant is more common than the other variants in fjord A as S41 and S51 were sampled five weeks after S21. Alternatively, the bacteria could have originated from the same unfortunate contamination event.

We also wanted to exclude the possibility that *L. monocytogenes* polluted seawater was pumped into the road tanker. No *L. monocytogenes* was found in these samples. It therefore appears that the purification procedures for the road tanker seawater was efficient enough. There is no indication in our results that precautions must be made after the fish is eviscerated in our results. However it is difficult to make a definite conclusion of that matter when we only found three *L. monocytogenes* positive samples in the processing plant.

## 6 Conclusion

Our results suggest that the few positive *Listeria monocytogenes* samples found in the processing plant derived from the seawater at marine aquaculture A. Our three positive *L. monocytogenes* samples found in the processing plant have identical sequences of the PCR products from the six virulence genes (*prfA*, *inlA*, *inlB*, *inlC*, *lisR* and *clpP*). These five samples (S24, S41, S51, 8A, and Ref. A) vary with just one base pair from serotype 1/2a, indicating that the found *L. monocytogenes* probably are from serotype 1/2a. The sequences similarity of these samples with the two old samples also indicate that the *L. monocytogenes* found in the processing plant come from a common source, which probably is the seawater. Our results also show that the processing plant has a very good cleaning and disinfection program. None of the samples taken from the clean processing plant were positive and only three turned positive 2 hours after production started. We know from the seawater samples that *L. monocytogenes* is found in seawater on all locations of the plants marine aquacultures namely A, B, and C.

None of the samples taken in the harvesting plant were *L. monocytogenes* positive even though contaminated water was pumped into the plant with the fish. Although not known it might be because of a high number of microflora in the water over growing the *L. monocytogenes*.

## 7 Perspective

The harvesting plant was situated in fjord A, when we took our samples. It would be interesting to examine the processing plant again after the moving of the harvesting plant to fjord B, to see if possible *L. monocytogenes* findings would be similar to the ones we found in fjord A, or if we would find another type, more similar to the one found in fjord B in the seawater samples. Our samples suggest that the *L. monocytogenes* serotypes found in fjord A and B are different. Fjord B bacteria were most comparable to serotype 4b, whereas the *L. monocytogenes* found in fjord A were most likely serotype 1/2a.

In further tests rivers should also be examined to establish if they are a possible contamination source for the *L. monocytogenes* found in the seawater. Fish feed should also be examined for *L. monocytogenes* to rule out the feed as a contamination source to the marine aquacultures.

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## 9 Appendix

### Appendix 1:

#### Positive seawater samples:

<b>Location</b>	<b>Sample</b>	<b>Date</b>
Harvesting site C	1C	Oct. 2010
Harvesting site B	2B	Oct. 2010
Harvesting site C	3C	Nov. 2010
Harvesting site A	4A	Nov. 2010
Harvesting site A	5A	Jan. 2011
Harvesting site C	6C	Jan. 2011
Harvesting site A	7A	Jan. 2011
Harvesting site A	8A	Feb. 2011
Harvesting site A	9A	Mar. 2011
Near marine aquaculture A	Ref. A	Oct. 2010
Near marine aquaculture B	Ref. B	Nov. 2010
Near marine aquaculture C	Ref. C	Oct. 2010

<b>Harvesting plant before production/after cleaning</b>	
<b>S1</b>	Wessel where the fish enters the harvesting site
<b>S2</b>	Stunning machine
<b>S3</b>	Conveyor belt leading to road tanker
<b>S4</b>	Pipe leading the fish into the road tanker
<b>Processing plant before production/after cleaning</b>	
<b>S5</b>	Edge on the conveyor belt leading the fish to evisceration
<b>S6</b>	Small conveyor belt just before evisceration
<b>S7</b>	Inside the evisceration equipment
<b>S8</b>	Manuel gutting line
<b>S9</b>	Drain from flotation tank
<b>S10</b>	Beneath grader conveyor belt
<b>S11</b>	Keyboard by the weight
<b>S12</b>	Hose
<b>S13</b>	Drain in storage room
<b>S14</b>	Drain in grader room
<b>S15</b>	Drain in gutting room
<b>Harvesting plant 2 hours after production started</b>	
<b>S16</b>	Wessel where the fish enters the harvesting site
<b>S17</b>	Conveyor belt leading to road tanker
<b>S18</b>	5x fish sample
<b>S19</b>	5x fish sample
<b>S20</b>	5x fish sample
<b>S21</b>	5x fish sample
<b>S22</b>	5x fish sample

<b>Processing plant 2 hours after production started</b>	
<b>S23</b>	Edge on the conveyor belt leading the fish to evisceration
<b>S24</b>	Small conveyor belt just before evisceration
<b>S25</b>	Evisceration equipment
<b>S26</b>	Manuel gutting line
<b>S27</b>	Beneath the grader conveyor belt
<b>S28</b>	Hose
<b>S29</b>	Drain in grader room
<b>S30</b>	Drain in evisceration room
<b>S31</b>	5x fish sample
<b>S32</b>	5x fish sample
<b>S33</b>	5x fish sample
<b>S34</b>	5x fish sample
<b>S35</b>	5x fish sample
<b>S36</b>	Fish cutlet
<b>S37</b>	Fish cutlet
<b>S38</b>	Fish cutlet
<b>Processing plant 2 hours after production (five weeks after first visit)</b>	
<b>S39</b>	Edge on the conveyor belt leading the fish to evisceration
<b>S40</b>	Small conveyor belt just before evisceration
<b>S41</b>	Conveyor belt after evisceration
<b>S42</b>	Manuel gutting line
<b>S43</b>	Beneath grader conveyor belt
<b>S44 and S45</b>	Drain in evisceration room
<b>S46</b>	Drain in grader room
<b>S47</b>	5x fish sample
<b>S48</b>	5x fish sample
<b>S49</b>	5x fish sample
<b>S50</b>	5x fish sample
<b>S51</b>	5x fish sample
<b>S52</b>	Under the stairs in ice room

## Appendix 2: Primers specific for *Listeria monocytogenes* virulence genes

Gene	Primer	Sequence (5' - 3')
<i>prfA</i>	<i>prfA</i> -forward	AAC GGG ATA AAA CCA AAA CCA
	<i>prfA</i> -reverse	TGC GAT GCC ACT TGA ATA TC
<i>inlA</i>	<i>inlA</i> -forward	GCT TTC AGC TGG GCA TAA C
	<i>inlA</i> -reverse	ATT CAT TTA GTT CCG CCT GT
<i>inlB</i>	<i>inlB</i> -forward	CAT GGG AGA GTA ACC CAA CC
	<i>inlB</i> -reverse	GCG GTA ACC CCT TTG TCA TA
<i>inlC</i>	<i>inlC</i> -forward	CGG GAA TGC AAT TTT TCA CTA
	<i>inlC</i> -reverse	AAC CAT CTA CAT AAC TCC CAC CA
<i>lisR</i>	<i>lisR</i> -forward	CGG GGT AGA AGT TTG TCG TC
	<i>lisR</i> -reverse	ACG CAT CAC ATA CCC TGT CC
<i>clpP</i>	<i>clpP</i> -forward	CCA ACA GTA ATT GAA CAA ACT AGC C
	<i>clpP</i> -reverse	GAT CTG TAT CGC GAG CAA TG