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Listeria monocytogenes in Norwegian fish producing plants and preventative measures

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1 Introduction

L. monocytogenes is an important human pathogen that has received increased attention the recent years. Over the last 10-15 years there has been several disease outbreaks due to *L.monocytogenes* and the bacterium have been detected in many different foodstuffs. The increase of incidences is probably due to a change in eating habits towards more ready-to-eat products that often do not need heat treatment before consumption (Rocourt et al. 2000).

In Norway, ready-to-eat products from fish are particularly in focus, due to the increasing demand of smoked, cured, fermented and untreated fish products. These products have higher risk of contamination since it has to go through many steps before the product is finished. Due to the fact that *L. monocytogenes* can readily be isolated from smoked fish and because inoculation trials have demonstrated significant growth in such products, the risk of getting listeriosis from consuming these types of products must be taken seriously.

In 2000 the European Commission decided to put a marginal value to a maximum of 100 bacteria per gram food allowed (SANCO 594, 2000) as part of a worldwide program to reduce disease caused by *L.monocytogenes.*

The aim of this study is to summarize some of the literature regarding *L.monocytogenes* in Norwegian ready-to-eat products containing fish, and to provide an overview of the existing preventive measures.

2 Listeria monocytogenes - literature overview

2.1 History

L. monocytogenes was first described by E.G.D. Murray in 1926 after observing characteristic mononuclear leukocytosis in six cases of sudden death of young rabbits in 1925 (Murray et al., 1926). He first named it Bacterium monocytogenes. It was then renamed by Harvey Pirie in 1927 to *Listeria monocytogenes* (Harvey Pirie, 1940). The first confirmed isolation of the bacterium was made in 1929 from sheep and humans (Gray & Killinger, 1966).

2.2 Taxonomy

The taxonomy of *L. monocytogenes* has been problematic. It was previously in the family of Corynebacterica (Stuart & Pease, 1972). On the basis of DNA-DNA hybridization, Stuart & Welshimer (1974) suggested a new family named Listeriaceaeto. Today the genus Listeria

belongs to the clostridium subbranch togheter with Staphylococcus, Streptococcus, Lactobacillus and Brochothrix.

Listeria includes 10 species:

Listeria monocytogenes and *L. ivanovii* and eight non-pathogenic species: *L. innocua, L. seeliger, L. welshimeri, L. gray, L. marthii, L. rocourtiae, L. fleischmannii, L. weihenstephanensis* (Mraheil et al., 2013). *L. monocytogenes* is the only listeria spp that can cause disease in both humans and animals. Occasionally human infectins with *L. ivanovii* and *L.seeligeri* has been reported (Gilot & Content, 2002).

L. monocytogenes may be divided into 13 different serotypes, all of which may cause listeriosis. The majority of cases, however, is caused by serotypes 1/2a, 1/2b and 4b (Farber & Peterkin, 2000). All Listeria species are phenotypically very similar, but they can be separated using the following tests: Hemolysis, CAMP test and acid production from D-xylose and mannitol (Figure 1).

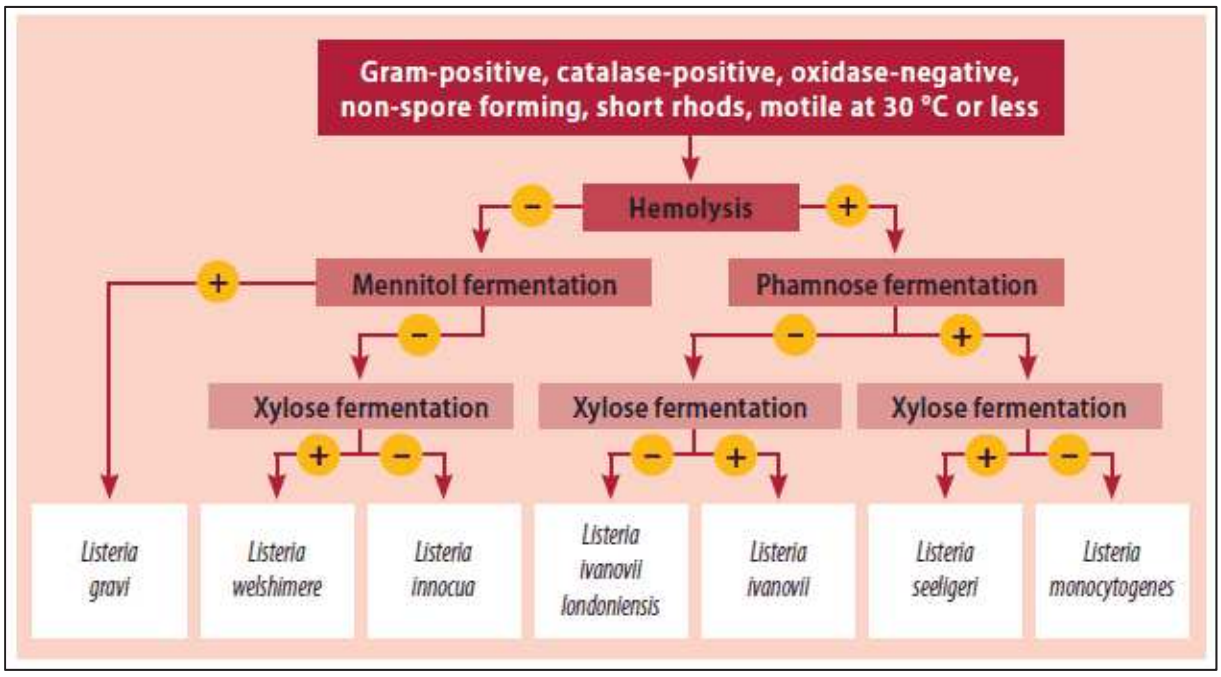

Figure 1: Schematic view of biochemical identification for Listeria spp. based on carbohydrate fermentation tests and hemolysis. From Dongyou (2008).

2.3 Microbiology

L. monocytogenes is a gram- positive, none spore-forming, intracellular facultative anaerobic rod, that grows best in temperatures between -0.4 °C and 50 °C (Jatisatienr & Busse, 1989;

Walker & Stringer, 1987). The bacterium can grow under both aerobic, mikroaerobe and anaerobic conditions and thrives in a reduced oxygen atmosphere in addition it can grow in pH between 4, 4 and 9, 6 while the optimum pH is 7, 0 (George & Lund, 1992).

When culturing *L.monocytogenes* it is able to grow in media containing 0-10% NaCl, and can survive at concentrations up to 30% (Seeliger & Jones, 1986). Survival at low pH and high salt concentration is highly temperature dependent, with 15°C as the optimum (Farber & Peterkin 1991). As a consequence *L.monocytogenes* is able to grow in many food products with extended shelf lives (Barakat & Harris, 1999; Rørvik et al., 1991)

L.monocytogenes is one of few foodborne pathogens that can grow at a water activity (aw) 0.93 (Farber et al., 1992). On nutrient agar the colonies are 0.2-0.8 mm in diameter, after 24 hours of incubation at 37 C° the colonies are slightly raised, smooth and punctiform. After 5-10 days, well-separated colonies at 5mm or more in diameter can be seen (Gray, 1957). It is catalase positive and oxidase negative and forms b-hemolysis on blood agar. The hemolysis act in synergy with the b-hemolysis of Staphylococcus aureus on sheep erythrocytes known as a CAMP reaction (Christie et al., 1944) (Figure 2).

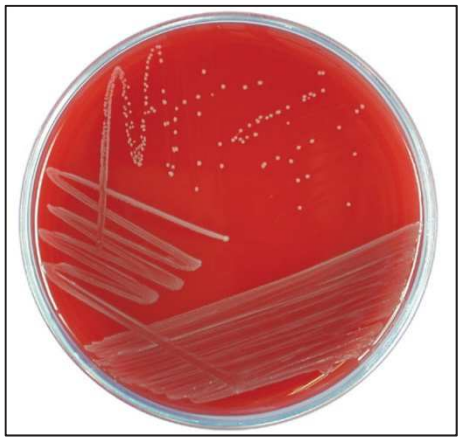

Figure 2: B-hemolysis on blood agar. Retrieved from http://parasites.czu.cz/food/_data/192.jpg, 22/11-2015.

L.monocytogenes has flagella that give it motility occurring only in narrow temperature range. Meaning if the bacteria grow between 20-25°C, flagella are produced, but at 37°C flagella are markedly reduced (Henry, 1933).

L.monocytogenes is widely present in plant, soil, water, sewage, slaughterhouse waste, silage, milk of mastitis cow, human and animal feces (McCarthy, 1990). Due to this all food that is

harvested from natural environments where *L.monocytogenes* can be found must be identified as a natural source of listeriosis in humans.

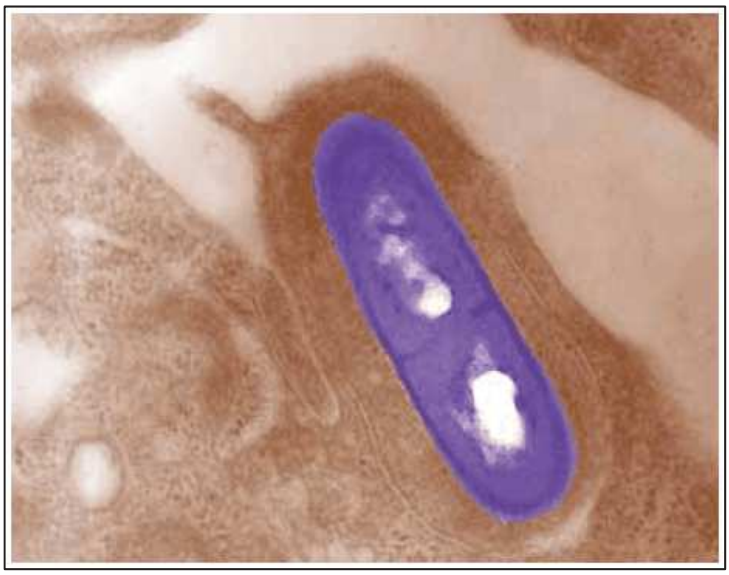

Figure 3: Electron micrograph of a Listeria L.monocytogenes bacterium in tissue. Retrieved from http://phil.cdc.gov/phil/details_linked.asp?pid=10828.

Particularly the ability of *L.monocytogenes* to grow and multiply at lower temperatures than other bacteria makes it special. This was demonstrated by Dortet et al. (2011), where both *L.monocytogenes* and Escherichia coli where cultured in a medium and refrigerated at 4 °C. The colonies of the two species were kept separate to prevent horizontal gene transfer. Then the amount of bacteria was measured every few weeks and a growth curve was determined (Figure 4).

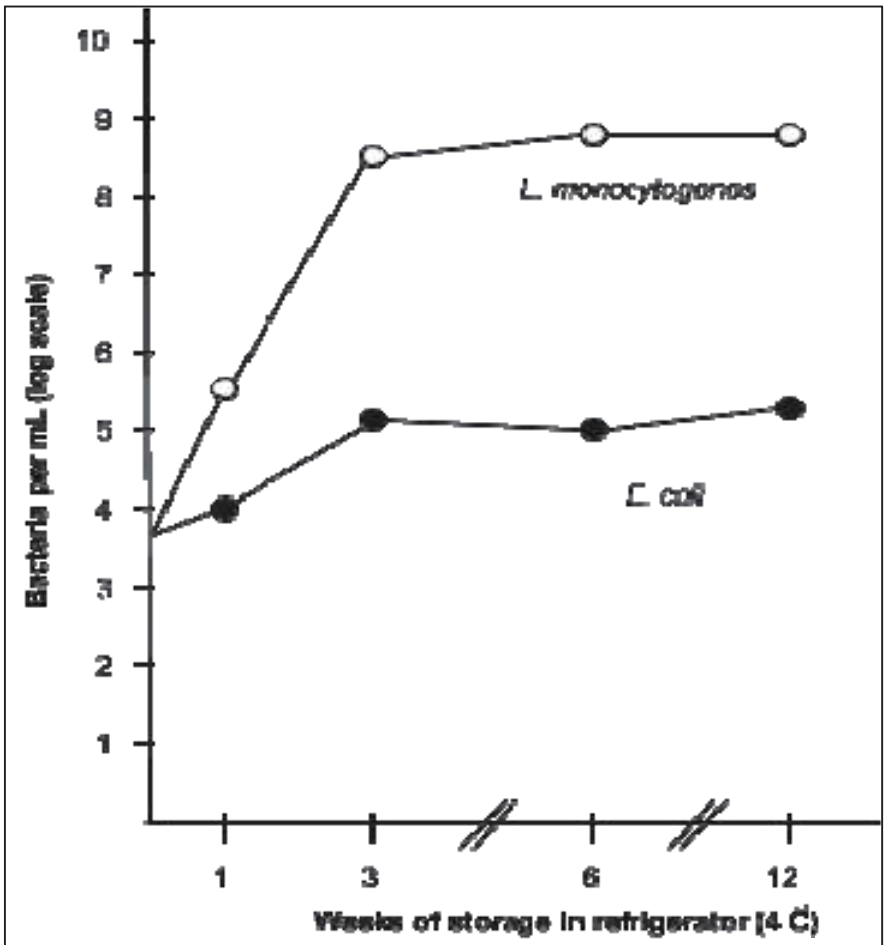

Figure 4: *L. monocytogenes* and *E. coli* bacteria growth curves. From Dortet, et al. (2011).

Another unique character of *L. monocytogenes* is the ability to produce and grow in biofilms (Herald & Zoottola, 1988; Frank & Koffi, 1990) that protect them against environmental stress. A biofilm is a mass of microorganism where cells stick to each other on a surface. Biofilms usually form over time due to *L.monocytogenes* ability to adhere to food-contact surfaces that have not properly been cleaned and disinfected. (Bresford et al., 2001; Frank & Koffi, 1990; Herald & Zoottola, 1988). For each individual food-processing plant, a limited number of clones of *L. monocytogenes* may become established and persist for years inside biofilms (Møretrø & Langsrud, 2004). *L. monocytogenes* is able to adhere to all materials commonly used in the food industry and form biofilms (Kim & Frank, 1995).

The formation of biofilm:

Initial attachment: The bacteria attach to surfaces and start multiplying.

Irreversible attachment: The biofilm grows through a process of cell division as the cells sticks to each other by the help of a self-produced matrix of extracellular polymeric substance. The extracellular polymeric substance will strengthen the bonds between the bacteria over time. In the end the attachment is irreversible.

Maturation: The biofilm develop into a organized resistant structure. New bacteria can now be released into the environment (Channaiah, 2014) (Figure 5).

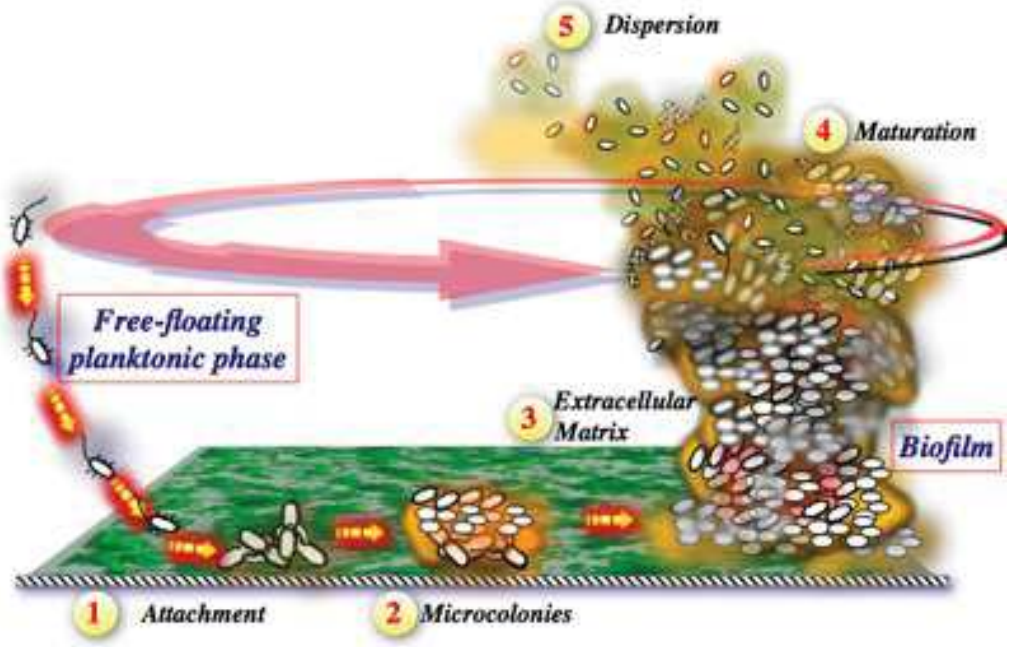

Figure 5: Biofilm formation. Retrieved from http://biomikro.vscht.cz/en/research/groups/rokoska/projects.php.

In each individual food-processing plant a limited number specific strains of *L.monocytogenes* may become established and persist for years. Persisted strains will first adhere to surfaces and eventually form biofilms (Herald & Zoottola, 1988; Frank & Koffi, 1990), this indicates that adherence to contact surfaces is important for the survival of L.monocytogenes in food processing environments (Møretrø & Langsrud, 2004).

In biofilms *L. monocytogenes* is significantly more resistant to disinfection than free living L. *monocytogenes* this is due to the fact that thick, complex biofilms are more difficult to remove than adherent single cells of *L. monocytogenes (*Møretrø & Langsrud, 2004).

2.4 Virulens

L.monocytogenes is an intracellular bacteria, virulence is associated with the ability of the bacteria to move into host cells by polymerization of the host cell actin found at one end of the bacteria. This helps is to propel trough the cytoplasm (Dussurget et al., 2004). This show that flagellar motility is rather used for the spreading in the environment than inside the host (O`Neil, 2006).

L. monocytogenes is an extremely invasive bacteria and is able to cross the intestinal (Marco, et al. 1997), transplacental (Gray & Killinger, 1966; Lecuit et al., 2004) and blood brain barriers (Uldry et al., 1993; Berche, 1995) of the host. The most common route of infection is by crossing intestinal barriers, particular through the payer's patches before entering the blood. The enzyme listeriolysin O and phospholipase C produced during exponential growth in the cell contribute to the lysis of the vacuole of the host cell (Portnoy et al., 1992). This makes the bacteria able to move from cell to cell without coming into contact with the body's immune system. This property also makes the bacterium more resistant to antibiotics (Watson et al., 1978; Armstrong, 1995).
Of the species in the genus Listeria is the only *L. monocytogenes* and in rare cases, *L. ivanovii* which is reported to be responsible microorganism associated with disease in humans (Lunestad, 1997).

2.5 Listeriosis in animals

L.monocytogenes is not host specific and has been isolated from feces of 37 different mammals, both domestic and wild (Gray & Killinger, 1966).
The reason animals is at high risk of being infected with *L. monocytogenes* is due to the ubiquitous nature of it leading to contamination of feed.

Listeriosis is cattle, three main forms:

- Meningoencephalitis: (Scott, 1994; Gibbons, 1970)

Occurs in 8-10% of clinical cases of listeriosis in adult ruminants. It is the most common manifesting type of symptom (Rebhun, 1987; Gray & Killinger, 1966). The infection is not as acute as seen in sheep and the recovery rate is up to 50%.

Affected cattle is anorectic, depressed, disorientated and often propel themselves into corners or leaning against objects. Facial paralysis often develops on the affected side.

- Abortion: (Osebold et al., 1960)

  The route of infection is mostly hematogenous but vaginal transmission also occurs. Abortion occurs mostly during the last trimester (Dennis, 1968).
- Mastitis: (Gitter et al., 1980)

  L. monocytogenes is one of several causes of bovine mastitis but not as frequent as Brucella spp, Mycobacterium bovis, Escherichia coli, Staphylococcus spp, Streptococcus spp.

Listeriosis in sheep, three main forms:

- Encephalitis: (Low & Renton, 1985)

  Also called circeling disease in sheep. Clinical signs are similar to those of cattle, but the diaseas is more peracute leading commonly to death in 4 to 48 hours. The clinical sign resembles to that of meningitis in cattle as mentioned previously.
- Placentitis:

  L.monocytogenes causes placentitis via the hematological route leading to infection of the foetus.

  Abortion typically occurs in the last trimester of gestation.
- Gastrointestinal septicemia:

  Peracute infections seen in lambs leading to diarrhea and death within 24 hours.

2.6 Listeriosis in humans: zoonosis and epidemiology

L. monocytogenes is knowed to cause listeriosis in humans. The first case of human liseriosis was reported in 1929 (Nyfeldt, 1929). Today, listeriosis is regarded as a food-borne disease of public health concern due to the approximate mortality rate of 30% ranging up to 75% in high risk groups (Jalali & Abedi, 2008; Mead et al., 1999; Low & Donachie, 1997).

Newborn, young children, elderly and immunocompromised are more susceptible to *L. monocytogenes* because of the immature or inefficient immune system. Rocourt & Brocsh (1992) found that 22% of the total cases of listeriosis occurred at age below 2 month and 31% of cases occurred in people older than 60 years. Also people with chronic diseases like diabetes mellitus, cardiovascular disease, neoplastic disease and other systemic disease are in higher risk of potentially getting listeriosis.

L. monocytogenes has been reported to be the most frequently isolated *Listeria* species associated with human disease (Janda & Abbott, 1999). A study in 1999 from The United States showed that 92,2% of the total 2518 outbreaks of *L.monocytogenes* resulted in hospitalization and accounted for 27,6% of the total foodborne deaths. (Mead et al.,1999).

The incidence of human listeriosis varies between countries but are showed to range from 4, 4 to 7, 4 million incidence globally (Lorber, 1997). Contaminated food of animal origin is the main cause of human listeriosis (Parihar, 2004).

Zoonosis caused by fish:

L.monocytogenes is sporadically isolated from a range of seafood including ready to eat products specially cold and hot smoked salmon, gravad salmon, shrimps, fermented fish and fish salads (Ben Embarek, 1994). The prevalence of *L. monocytogenes* in raw fish, as reported in published studies, is low, ranging from 0-1% to 10% (Johansson et al., 1999; Autio et al., 1999; Jemmi & Keusch 1994; Jinneman et al., 1999; Weagant et al., 1988).

After cold smoking, studies show an increase in the percentage of samples containing *L. monocytogenes*. The variation is high, ranging from 0 to 100% (Ben Embarek, 1994; Eklund et al., 1995; Dillon et al., 1994; Heinitz & Johnson 1998; Jørgensen & Huss 1998; Fonnesbech Vogel et al., 2001). In Danish samples from 10 smoke houses, the number of positive samples of finished product varied from 0 to 100%, whereas a survey of 6 United States smoke houses showed *L. monocytogenes* in the product ranging from 50 to 100% of the samples (Eklund et al., 1995). Heinitz & Johnson (1998) reported that an average of 17.5% of cold-smoked fish (291 samples) and 8.1% of hot-smoked fish (234 samples) from the United States contained *L. monocytogenes*, and 7.3% of 96 cold-smoked fish samples from 5 United States smoke houses were positive (Norton et al., 2000). In a study from Norway is was shown that 9% of samples from smoked salmon were positive (Rørvik & Yndestad, 1991)

2.7 Clinical signs and diagnosis

Healthy adults and children generally experience characteristic foodborne illness like vomiting, diarrhea and mild fever and will recover after a short period of time. However if infection occurs in certain populations such as pregnant women, elderly and immunosuppressed people they are prone to develop severe form of listeriosis potentially with lethal outcome. *L. monocytogenes* has developed efficient strategies to survive in the intestine and cross the intestinal, blood-brain and placental barriers leading to clinical features of the disease including gastroenteritis, septicemia, central nervous system infections, and mother-to-child infections. In systemic listeriosis it frequently invades the CNS leading to meningoencephalittis, cerebritis and brain abscess (Lorber, 1997).

Clinical signs in pregnant women:

Mild influenza symptoms and headache due to bacteremia, bloody vaginal discharge, gastroenteritis and endocarditis occurs in 10 % of pregnant women and affect the left side of the heart valve leading to an increased mortality up to 50% (Lorber, 1997).

Perinatal infection:

Cross infection from the infected mother to the fetus has been reported (Larsson et al., 1978). Clinical signs occur in the fetus or neonate within the 1st week after delivery and show symptoms like serious septicemia with respiratory signs, pneumona and purulent conjuctivits. If clinical signs develop in the utero the foetus may die and prognosis is poor (Farber & Peterkin, 1991).Post-partum infection is also possible (Lecuit et al., 2004) leading to late onset of listeriosis occurring within 2nd to 4th week of life and is manifested as meningitis (McLauchlin, 1992). The infant gets the infection from the mothers birth canal or due to post-partum cross infection.

Diagnosis of listeriosis is based on isolation of *L. monocytogenes* from blood or cerebrospinal fluid (Ryser & Marth, 1999). It has been found that isolation from feces is not suitable since humans can be healthy carries (Gray & Killinger, 1966).

2.8 Treatment

L. monocytogenes is susceptible to a wide range of antimicrobial treatments . Susceptibility testing of the most common antimicrobial agents against listeria was performed during 1955-1997 (Safdar & Armstrong, 2003). The drugs used were penicillin, ampicillin, erythromycin,

tetracycline and gentamycin. Ampicillin plus gentamycin is the standard therapy for systemic listeriosis and methoprim-sulfamethoxalone (TMP-SMX) is applied in patients with beta lacatam-intolerance.

Table 1: Antimicrobial resistance of *L. monocytogenes*. From Safdar & Armstrong (2003).

Antimicrobial agent	1955-1966		1970-1979		1991-1997	
	Susceptible	Restistant	Susceptible	Resistant	Susceptible	Resistant
Penicillin	17	1	39		24	0
Ampicillin	4	0	29	6	24	0
Erythromycin	17	1	41	0	24	0
Tetracycline	16	2	22	0	24	0
Chlormafenikol	14	4	9	1	22	2

It should also be empathized that according to an animal study it was demonstrated a 100-fold greater killing of *L. monocytogenes* following therapy with penicillin or ampicillin plus gentamicin (MacGowan et al., 1998; Moellering et al., 1972).

3 Listeriosis in Norway

Generally, Norway have had a low number of listeriosis incidences. Although 31 cases were registered in 2012, these are mainly sporadic cases. It is only registered a few cases where multiple people get infected with *L.monocytogenes* in Norway (NIPH, 2015). The Norwegian Institute of Public Health (NIPH) is a national competence institution for governmental authorities, the health service, the politicians, the media, the general public issues related to forensic science, prevention of communicable diseases and the prevention of harmful environmental influences. The NIPH help in improving the health of the population and it collaborates with the WHO, EU and universities. The vision of NIPH is to prevent disease. The main goals are to prepare for acute health threats, advise and provide services that improve public health, give an overview of the health of the population, factors influencing public health and to have knowledge about what cause common diseases.

NIPH cooperate with the Norwegian Surveillance System for Communicable Diseases (MSIS), where bacterias from humans are analyzed. All doctors in Norway are by law required to notify all notifiable diseases to the MSIS and the NIPH including *L.monocytogenes.*

Annually, 10-20 people in Norway get infected (MSIS 1998, 1999, 2000). In 2000 there were reports of 18 cases in Norway. The majority of these cases concerned either old or sick adult persons.

Cases of reported *L.monocytogenes* incidences in Norway the last 30 years are summarized in table 2. As this table show, there is a significant increase in reported cases the last 30 years.

Table 2: Nr of listeriosis diagnosed in the period 2010-2015 (MSIS 2010, 2011,2013,2014,2015) compared to cases in 1985-1990 (MSIS 1985,1986,1987,1988,1989,1990). MSIS - Norwegian Surveillance System for Communicable Diseases.

Year	Cases diagnosed	Year	Cases Diagnosed	Year	Cases diganosed
1985	6	1995	12	2005	14
1986	8	1996	15	2006	27
1987	13	1997	19	2007	50
1988	19	1998	10	2008	34
1989	9	1999	19	2009	31
1990	9	2000	18	2010	22
1991	8	2001	16	2011	21
1992	22	2002	21	2012	30
1993	8	2003	16	2013	21
1994	14	2004	23	2014	29
				2015	17

3.1 List of major outbreaks of listeriosis in Norway according to the Norwegian Institute of Public Health (NIPH):

Listeria in fermented trout 2013: NIPH received in December 2013 a warning from the FSA about fermented trout who had been on the market, and which had to be withdrawn because of the discovery of *L.monocytogenes*. Reference Laboratory at the NIPH received strains from

the fish to do serotyping. The Same type *L. monocytogenes* was detected in a total of three patients in different locations in Norway. Interview of patients and their relatives showed that all patients had eaten the current fish. It was published online where people who had eaten the product were asked to look for symptoms and contact a doctor in case of illness. All three patients had underlying disease (NIPH, 2015).

National outbreak in 2007: In all, 21 people were diagnosed during an outbreak of *L.monocytogenes* in 2007, 19 patients where infected, and 5 of them died (NIPH, 2015). The source was traced to an ecological camembert cheese produced at a dairy farm in Northern Trøndelag. The cheese was served at hospitals and was also sold at local markets in central Norway.

Ålesund in 2005: Three cases of listeriosis was reported at Ålesund hospital. The strains had identical DNA profiles, and the same strain was also found on topping cutter on the hospital's kitchen, but it was not determined which foods were the source of infection.

Trondheim area 1992: In 1992 it was announced 8 cases of listeriosis in Trøndelag. All patients were hospitalized in the Trøndelag region, 6 were older and / or immunocompromised patients, 1 younger diabetic and 1 newborn child. Dietary history was obtained in 4 of the patients and it was taken samples of food from one of the patient's fridge. Of these, 5 were positive for *L.monocytogenes*. The positive samples where from two different types of heat-treated meats from the same manufacturer. The findings resulted in withdrawal of all relevant food from the same producer.

4 Norwegian food safety authority

The Norwegian Food Safety Authority (NFSA) is a governmental body in Norway. NFSA is responsible to ensure through regulations and controls, that food and drinking water are as safe and as healthy as possible for consumers. They must also promote plant, fish and animal health all over the country. Another part of the NFSA work is to draft and provide information on legislation, perform risk-based inspections, monitor food safety as well as plant, fish and animal health and provide updates on developments. They are to give advice to the Ministry of Agriculture and Food and the Ministry of Fisheries and Coastal Affairs.
The mission of the NFSA is to promote:

- Safe, healthy food.
- Safe drinking water.

- Healthy plants, fish and animals.
- Excellence in animal welfare and respect for fish and animals.
- Environmentally friendly production.
- Health, quality and consumer interests throughout the food production chain.

They supervise all fish producing plants in Norway. In 2013 the Norwegian Ministry of Agriculture and Food instructed the NFSA to prioritize fish health. The result was increased inspections and surveillance regarding hygiene in fisheries and *L.monocytogenes* in ready-to eat-food.

Regulations regarding *L.monocytogenes* made by the NFSA:

Regulation (EC) 852/2004 specifies requirements for cleaning and maintenance of business premises and equipment. The regulations focus on preventive measures and require that the businesses should have an internal control system based on hazard analysis critical control point (HACCP) principles.
Regulation (EC) 2073/2005 specifies requirements for sampling. Samples shall be taken from both end products and of the environment. Results of the sampling shall be monitored and

Trends should be analyzed so that a possible negative development can be followed up with actions. The job of the supervisors is to do a systematic review of the processing plants HACCP including controls and routines. The supervisors should also look at all the documentation, have conversations with key people in the plant and runs inspections both announced and unannounced. The result of the revision is summarized in a report.

5 *Listeria monocytogenes* in the smoked salmon plant

L. monocytogenes differs from other food-borne pathogens in that it can survive and multiply in ready-to-eat products at refrigerated temperatures, the infective dose does not damage the food making it harder to detect.

The smoking process:

As part of the smoking process a freezing step is usually included, either the raw fish or the finished product is frozen. A study done by El-Kest et al. (1991) showed that by freezing the

product at between at -18 °C - 0.4 °C, reduction of *L. monocytogenes* was minimal. It is assumed that fatty fish offers a good protection against damages caused by freezing and this leads to marginal reduction of *L.monocytogenes.*

Salting have to be finished before the smoking of the fish takes place. Salt levels in the final product range between 3-10 %, normally ranging between 3,5-5% (Truelstrup Hansen et al., 1998) As salt levels lower that 30% NaCl does not kill the bacteria, this level have no inhibitory effect on the growth of *L.monocytogenes* (Peterson et al., 1993).
After salting the fish is smoked, either hot smoked or cold smoked. The hot smoked process, 60 °C for 30 min is usually sufficient to kill L.monocytogenes (Jemmi & Keusch, 1992). During cold smoking, 20-30 °C for 24 h, Eklund et al. (1995) showed that *L.monocytogenes* was able to grow during cold smoking.

In spring 2000 the European Commission selected *L. monocytogenes* as the first bacterial disease to be defined by a marginal value. This value is set be maximum 100 bacteria per gram of food (SANCO 594, 2000). According to the World Health Organization's (WHO) risk assessment report, listeriosis can be reduces significantly if margins as kept below 100 cfu/g at the point of consumption (WHO, 2004).

In recent years the consumption of ready to eat seafood have increased in Norway, but the low levels of *L.monocytogenes* detected (below 100 cfu/g) explains why only a few cases of listeriosis have developed. However levels higher than 100 cfu/g have been detected in time to time due to insufficient screenings and preventive measures.

Contamination patterns

In a study from 1990-1991, (Rørvik et al., 1991) the contamination pattern of *L.monocytogenes* in Norwegian salmon slaughterhouse were investigated. The examinations for *L. monocytogenes* and other *Listeria* spp. were carried out according to the methods of the Nordic Committee of Food Analysis. Altogether four hundred and seventy-five samples were collected on six occasions during a period of eight months. The plant included a salmon slaughterhouse with a smokehouse located in the same building. The slaughterhouse received salmon from transport boats (Rørvik et al., 1991). Contamination patterns of *L.monocytogenes* are shown in table 3.

Table 3: Listeria monocytogenes and other listeria spp. Contamination in water, fish and environmental sampled from a salmon slaughterhouse and smoked salmon processing plant in Norway (Rørvik et al., 1991).

Source of sample	No. of samples	No. of samples containing *L. monocytogenes* %	*Listeria spp* %
Water			
Seawater environment *	33	3 (9%)	12 (36%)
Seawater, slaughterhouse**	6	0	0
Fresh water	10	0	0
Ice	10	0	0
Slaughterhouse			
Slaughtered fish	50	0	1 (2%)
Processing environment	83	6 (7%)	17 (20%)
Smokehouse			
Fish before filleting	24	4(17%)	3(13%)%)
Fish during process	47	12(26%)	22 (47%)
Processing environment	147	42 (29	43 (29%)
Vacuumed-packed smoked salmon	65	7 (11%)	7 (11%)
Total	475	76 (16%)	105 (22%)

*seawater from outside the slaughterhouse, where transport boats pumped water into the fish tanks, as well as from the boats.

** seawater from deep water, away from the shore, used during the slaughterhouse process.

The study shows that from the total of 475 samples the overall frequency of *L. monocytogenes* was 16%. No listeria spp where found in seawater from the slaughterhouse or in the fresh water used in the plant. Of the 50 samples of slaughtered fish inside the slaughterhouse or stored in boxes none was positive for *L.monocytogenes.* The bacteria was most often detected

in samples from the smokehouse, where 29% of the environmental and 26% of the fish samples during processing where contaminated with the bacteria.

Contamination patterns of *L.monocytogenes* in the smokehouse are showed in table 4.

Table 4: Listeria monocytogenes and other listeria spp in fish samples taken in the smokehouse after each step of the processing (Rørvik et al., 2001).

Source of sample	No of samples	Nr of samples containing:	
		L.monocytogenes%	Other listeria spp%
Fish before filleting	24	4 (17%)	3(13%)
Fish after filleting	5	2 (40%)	3(13%)
Fish after smoking	8	0	0
Vacuumed packed fish	65	7(11%)	7(11%)

The fish was heavily contaminated after filleting and trimming, while no listeria spp where found in the fish after smoking. The study show that the environmental samples from the filleting department where most often contaminated. The high frequency of *L.monocytogenes* in the smokehouse compared to the frequency in the slaughterhouse plus the absence of listeria spp in newly slaughtered fish show that the problem is in the smokehouse (Rørvik et al., 1991).

The reason for this is most likely due to biofilm formation and establishment of a reservoir of *L.monocytogenes* in the premises. After further investigation and examination of the listeria spp with a two-step enrichment method and multilocus enzyme electrophoresis it was found that ET-6 was the predominant strain in the smokehouse. ET-6 is the most common ET in Norway and have been isolated from several sporadic cases (Kolstad et al., 1992).
The result of this investigation reinforce that the findings done by Boerling et al., (1991) showing that different *L.monocytogenes* ET from meat/fish and animal is different is reliable. This concludes that the infection of the fish is due to contamination of the environment, not by the fish itself (Boerling et al., 1991; Rørvik et al., 1991).

5.1 Listeria monocytogenes in Norwegian ready-to-eat fish products

The Norwegian Food Safety Organization (NFSA) completed a control program of ready-to-eat products in Norway from November 2013 to February 2014 (Sagen & Abbasi, 2014). The main purpose of this project was to map the presence of *L. monocytogenes* in ready-to-eat products in Norway, especially in regards to fish products. During the project period a total of 501 samples were analyzed including, fish and fish products. The samples were stored in unbroken consumer packaging by recommended temperature range up to expired date, and subsequently analyzed for *L.monocytogenes*.

It was detected *L. monocytogenes* in 21 of the 480 products (4.4%). It was found *L. monocytogenes* in 14 fish products, 4 meat products, 2 mayonnaise bases salads and one milk product. In 4 of the positive samples the amount of *L. monocytogenes* was higher than 100 cfu per grams. The highest content of *L. monocytogenes* was 6000 cfu/g and detected in a duck Paté. Another sample with high levels of *L. monocytogenes* was smoked salmon (2000 cfu / g), fermented trout (620 cfu/g) and pickle (140 cfu/g). (Sagen & Abassi, 2014). All the products were produced in Norway.

The majority of the positive samples from the fish group belonged to the group fermented fish (6 of 14 positive samples, 43%), smoked fish (4 of 14 positive samples, 29%), topping salmon (3 of 14 positive samples, 21%) and cured fish (1 of 14 positive samples, 7%).

From the positive fish and fish products 1,2% showed levels above 100 cfu/gram. All levels above 100 cfu/gram indicate a risk of listeriosis. Even though the incidence of listeriosis is low in Norway this report show us that we are not safe and implementing good preventive measures against *L.monocytogenes* is crucial.

Table 5. Table showing the distribution of positive samples in fish products. Adapted from Sagen & Abbasi (2014).

Fish and fish products	n	n positive	% positive
Fermented fish	22	6	27,3
Smoked salmon/ trout	47	4	8,5
Salmon topping	6	3	50,0
Cured salmon	8	1	12,5
Total	83	14	16,9

6 Preventive measures

Prevention is essential to keep *L.monocytogenes* at a minimal level, to do this it is necessary to have increased focus on Hazard Analysis Critical Control Point. This must be aimed at the entire production chain and include raw material producers, slaughterhouses and processing plants. Hazard Analysis Critical Control Point (HACCP) is a systematic approach to identify and control hazards regarding *L.monocytogenes.* The aim of the HACCP is to make it easier to identify where *L.monocytogenes* is most likely to be found and how plants can prevent it.
The Regulation on Microbiological Criteria for Foodstuffs (Commission Regulation, 2005) contains microbiological criteria for specific food/microorganism combinations and the implementing rules to be followed. These criteria should be used by food business operators when validating and verifying the correct function of their HACCP based procedures and other hygiene control measures.

The National Fisheries Institutes (NFI) in cooperation with the national food processors association have made specific criteria for proper HACCP in regards to controlling *L. monocytogenes* in the fish industry (NFI/NFPA, 2002). Nofima (www.nofima.no) is one of Europe's largest industry-oriented research institutes that conduct research and development for the aquaculture, fisheries and food industries in Norway. They offer solutions and guidance to aquaculture companies.

By using the guidelines from these institutions, I have summarized the most important preventions regarding *L. monocytogenes* in ready-to-eat fish producing plants. The following

sections are thus mostly based on NFI/NFPA (2002), Commission Regulation (2005), NFSA (2014) and Heir et al. (2015).

6.1 Routines normally connected to Listeria contamination

During the study of contamination patters of *L. monocytogenes* in smoked fish, Eklund et al. (1995) identified incoming products as the primary source of contamination. Later Autio et al. (1999), Rørvik et al. (1995) and Fonnesbech Vogel et al. (2001) showed that raw material rarely is responsible for contamination in the finished product. Instead they showed that the processing plant environment is responsible for most incidences of contamination in the finished product. According to this, the most important preventive measures are hygiene, controls and routines inside the processing plant (Heir et al., 2015). The potential reservoirs of L.monocytogenes in the premises are given in table 6.

Table 6: Potential reservoirs of L. monocytogenes in smoked seafood plants (Heir, et al. 2015).

Activity	Problems connected to increased risk of listeria contamination
Deviations from normal production	If the production is larger than normal, for example double shifts leading to less time cleaning.
Rebuilding of premises or production lines	Increased activity and transport in the production premises will increase the risk of infection
Too high temperature in the production facilities	Rebuilding, loss of maintenance in the premises and high outside temperature.
Used equipment from other facilities are installed	Listeria can survive for a long time in used equipment. Listeria may be introduced into the plant if equipment that previously used in other plants is used.
Violations of the hygiene zones	Particularly related to carts, wagons, trucks and personnel crossing from “unclean” to “clean” zones.
Infection from unclean surfaces to product	A splash from the floor during flushing might

	enter clean surfaces.
Holiday substitutes working in the production	Lack of training and understanding can contribute to the spread of Listeria.
Equipment	The lack of cleaning can cause contamination

6.2 Hazard Analysis Critical Control Point (HACCP)

6.2.1 Staff and visitors to the facility

All persons including staff, service people, maintenance personnel and visitors entering the manufacturing premises must replacing their footwear, wear protective clothing plus hairnet, make hand washing and wear gloves to prevent the introduction of listeria in the plant.

Good routines before entering the clean zones are essential to prevent the introduction of listeria. For visitors it is important that they have suitable protective clothing, this includes coveralls or coat, gloves and hairnet disposable. Footwear should be easy to clean and rubber boots should always be used and must be available for all staff and visitors. Shoe covers are easily damaged and are not suitable. All jewelry should be removed before entering the production site, since bacteria can easily accumulates here.

6.2.2 Environment and equipment

All of the employees and personnel working in the processing plant represent an potential source of *L. monocytogenes* and introduction of it. They can transfer *L. monocytogenes* from one area to another area of the plant due to contamination on their shoes, clothing, hands and unprotected body parts. They can also serve as a direct source of contamination if they are involved in the post-processing handling of products. It has been shown that 1-10% of healthy adults may be fecal carries of L.monocytogenes (Farber & Peterkin, 1991; Schuchat et al., 1992).

To effectively manage the risk of environmental contamination it is necessary to find where along the production flow the fish is more likely to become contaminated.

The greatest risk for product contamination is when the product comes in contact with contaminated surface. Areas can also serve as an indirect source of contamination since these areas can harbor the organism, and under specific conditions lead to contamination of contact surfaces or to the product. By keeping the presences of *L. monocytogenes* as low as possible in the environment this can reduce the risk of contamination of a product or a product contact

surface. The importance of these areas will vary depending on the facility, the processes, the temperature and humidity of the room and the product. Considerations should always be taken given of the potential of *L.monocytogenes* to be brought back into the clean environment. Cross contamination can be the result of traffic in the processing and packaging area e.g. by people or equipment like trolleys entering from the unclean areas into the clean area. This can also happen during equipment maintenance.

It is possible to have random isolated contamination with *L.monocytogenes* from the environment, but most likely contamination will occur after the bacteria have established itself in a biofilm. When this happens routine cleaning and sanitizing become ineffective. In places where *L.monocytogenes* is able to multiply itself and form biofilm it can work its way out of the biofilm and become deposited onto outer surfaces of equipment. As products moves over or touches the equipment, the contamination will be spread downstream to other areas along the production flow. Identifying where the niche/ biofilm of *L. monocytogenes* grows and eliminating the reservoirs can prevent this situation. Sites that have been identified as potential persistent reservoirs of *L. monocytogenes* in smoked salmon plants are drains, floor, slicers, walls, insulation inside walls or around pipes and cooling systems that have become wet, trucks and cleaning tools like brushes and sponges.

Control strategy

- Separate raw products from semi-finished and finished products to prevent cross contamination.
- Control the traffic flow between the raw ingredients and the production side of the plant. This is needed to avoid transfer of *L.monocytogenes* from the “dirty” side to the “clean” side.
- The equipment should be designed to facilitate cleaning and to eliminate sites where *L.monocytogenes* can persist in the environment. The equipment should also be maintained regularly and any new design should be reviewed before any new replacement is acquired.
- New equipment should be examined for dead ends, crevices and other areas that can serve as reservoirs for *L.monocytogenes*.
- Minimize the use of bolts, screws and threads in the equipment as they can serve as a reservoir, if unavoidable they should be removed for cleaning and sanitizing.

- Equipment that are previously used and cleaned may harbor pathogens. Such equipment even though visually clean should be disassembled and sanitized before putting back into the production.
- Trolleys or other transportation used to transport exposed ready-to-eat food must have cover guards over the wheels to prevent spraying from the wheels.
- Equipment that is damaged pitted or has cracks or other deformations should be replaced.
- The equipment should not be designed to collect water, and should be hollow to keep water draining it.
- A preventive maintenance program should be followed to minimize the potential for harborages of *L.monocytogenes* and to reduce the potential for contamination of equipment due to unscheduled repairs.
- When performing maintenance of equipment in the ready-to-eat area it may be necessary to apply trolleys only used in this are or to sanitize tools prior to use in this area. Maintenance personnel should wear clean covers that have not been used in the raw material area.
- If possible equipment fixed to the roof in the ready-to-eat area should be avoided. Dust and condensate can collect and fall into the product leading to contamination. If not possible to avoid, overhead pipes and fixtures must be cleaned and sanitized regularly.
- Footbaths should be installed containing stronger concentrations of sanitizers than would normally be used on equipments. Depth of the footbath must be 2 inches.

6.2.3 Personnel hygiene

To be able to have an effective *L.monocytogenes* control program it requires that employees understand their role and what the management expects. Control strategies are not likely to be effective if employees don't cooperate, or don't understand what they are expected to do.

It is the management's responsibility to make the HACCP as clear as possible for all employees involved in the plant. Procedures or behavior should be monitored and actions taken to reward compliance or penalize those who do not follow the regulations.

It is important that in addition to basic hygienic measures it should be established a hygiene training program regarding L.*monocytogenes* for all new employees.

Control strategies:

- All employees and visitors entering areas where exposed finished products are, have to wash and sanitize their hands and put on clean outer covers, hair covering, shoe covers and booths.
- All workers should at any time wear clean clothes, and as often as possible clean gloves.
- The use of gloves does not minimize the need for employees to wash and sanitize hands regularly.
- There should be one color of the smock for the raw side and another color of the smock for the ready to eat side to easily differentiate the “dirty” and the “clean” side.
- Disposable items should be discarded when leaving the work area and always be replaced by new clean clothes when returning.
- It is essential that workers in the ready-to-eat compartment understand that the purpose of wearing clean garments and other protective clothes is to protect the product from contamination and not to protect themselves from being dirty during work.
- Always if an unclean surface is touched, hands must be washed and sanitized before gloves are changed.
- Dirty clothes must not be stores in lockers.

A training program should be obligatory before working in the plant. This training should include basic knowledge about *L.monocytogenes,* basic hygienic measures and hand washing.

Important elements of the training:

- Basic information about *L.monocytogenes* including morphology, zoonosis, listeriosis, high risk groups, mortality rate, high risk groups and examples of plants that have to close etc.
- Personnel and bathroom hygiene.
- Hand washing procedures and hand washing requirements.
- Demonstration on how to wash hands properly (Figure 6)

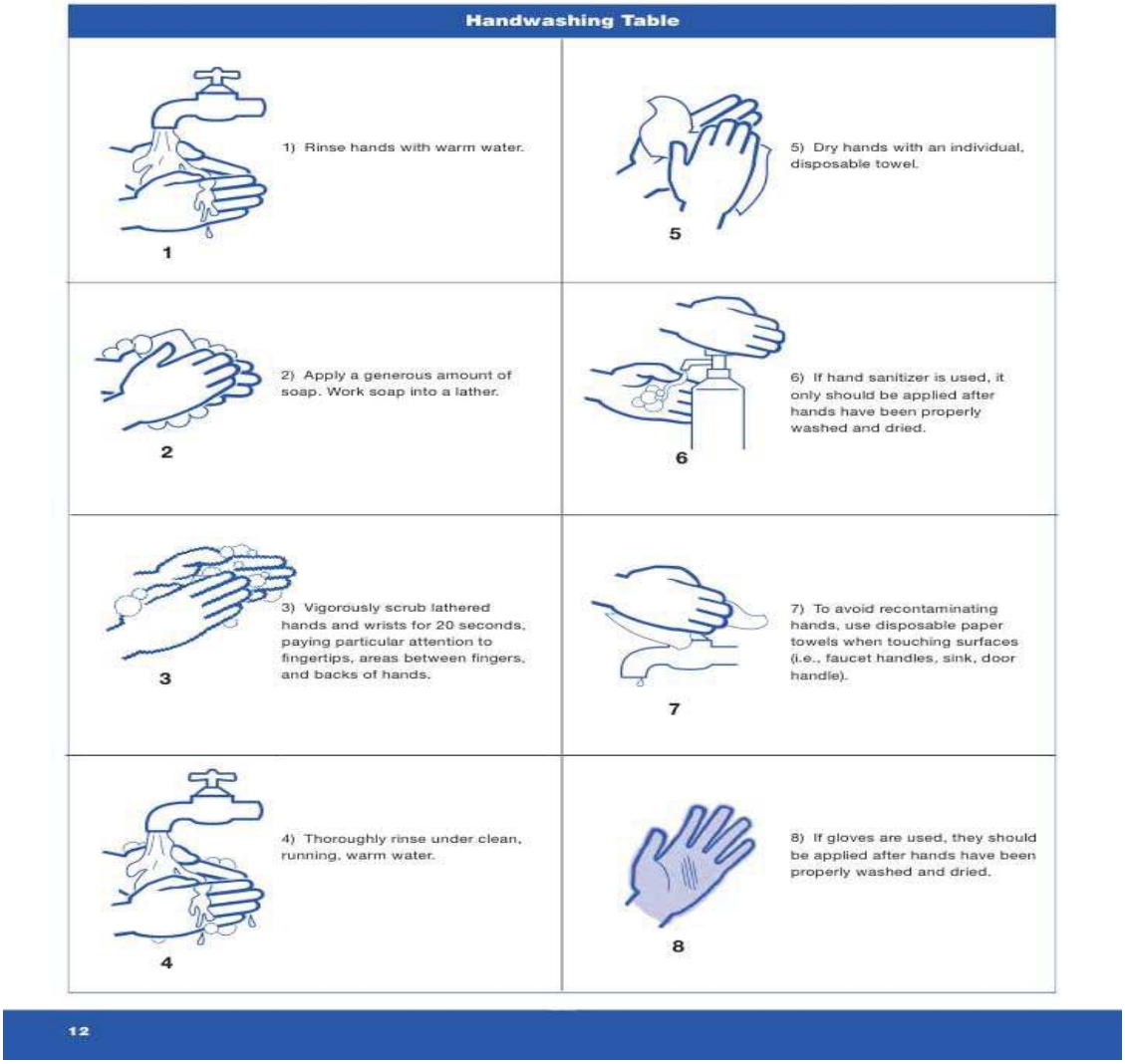

Figure 6: Hand wash protocol. From Cutter et al. (2006).

- Direct entry from the outside of the plant or from the exterior of the plant to the ready to-eat-area must be prohibited.
- Workers should not move from the ready-to eat area during the workday unless precautions are taken to make sure their movement does not cause contamination. These precautions may include changing garments, washing hands, changing clothes, gloves and boots before entering the ready-to-eat area.
- In case of need of maintenance personnel they should be carefully trained about their movements inside the facility to prevent cross contamination or transfer of *L.monocytogenes* from the "dirty" side to the "clean" side.

- Containers and trash barrels used in the ready-to-eat area must not be used elsewhere in the plant. They should also be labelled or have a color code to prevent confusion among employees. It is advised that they are be cleaned and sanitized daily.
- Standing water should be avoided, particularly in the read- to-eat area to prevent potential transfer of bacteria to products.
- Footwear should be changed between different zones and must always be replaced when changing from unclean to clean zone. Gloves should be changed after touching the surfaces which are not in direct contact with the salmon (e.g. Sinks and floors).

6.2.4 Modifications, repairs and maintenance

This type of work poses a risk of spreading disease. Avoid maintenance and repairs during production. Have procedures and training to ensure that all employees and external personnel (such as service personnel) comply with hygiene procedures in the plant and thus preventing cross contamination in the plant. Tools can be a source of infection and should be disinfected before and after use. Always perform adequate cleaning after repairs and maintenance

Removal of biofilm:

Bacteria in biofilms are comprised principally of fat, carbohydrates and proteins. The most commonly used cleaning agent to remove biofilms is chloralkali, based on a synergy between high pH (alkali) and hypochlorite. Lut is alkali multifunctional and will dissolve and break down organic matter, especially protein and fat. Kaliumlut (KOH) may be most effective for greasy dirt and sodium hydroxide solution (NaOH) for the protein-rich dirt.
The most commonly used disinfectants are based on surfactants (e.g. quaternary ammonium compounds), peracetic acid or hypochlorite. The best way to ensure proper sanitation is by switching between agents with different mechanism of action, for example, quaternary ammonium compounds and peracetic acid.

6.2.5 Monitoring of L.monocytogenes and sample taking

Monitoring of listeria status in the plants is essential to be able to detect where the problem is most likely to arise. A monitoring program must be used all the time to help determine what control measures is the most effective and what control measures that need to be modified. Regular testing can help improve the performance of the plant over time.

The management must be committed to use a monitoring program and all employees must understand their role in control measures and its importance. Using a record keeping system to record all the important parts concerning the daily routines including information like temperature, sanitation log etc. is a useful way to monitor some of the daily routines. By involving the employees in creating a food safety protocol like this they will understand the prevalence of *L.monocytogenes* and in addition increase their motivation to implement the control program. Another important aspect of the control program is to be able to detect potential sources of contamination. The environmental testing is an important part of identifying problem areas or locate contamination sources in the plant. It can also help to confirm that problem solving measures have been effective.

In a recent review of control strategies Tompkin (2002) identified two important factors to increase the effectiveness of a control program; the design of the environmental testing program, and the response to a positive finding. It also showed that sampling should be aggressive in its attempt to detect *L.monocytogenes* in the premises.

According to the national food processor association testing should include:

- Raw materials
- Non-food contact surfaces in the plant environment
- Finished products

Raw material

It is impossible to prevent the sporadic introduction of *L. monocytogenes* from raw material arriving at the plant. But if control measures reveals that certain suppliers often delivers salmon with *L. monocytogenes* the company should be notified and undergo revision. As a result the plant should not use this supplier anymore. Testing for *L.monocytogenes* in raw material helps the plant better understand the contamination sources. Samples should be taken before the salmon comes into direct contact with equipment and machines in the plant. For round/hole salmon samples should be taken from the sides and the gills and for gutted salmon samples should in addition be taken from the belly.

Non-food contact surfaces

L.monocytogenes as mention earlier can survive in biofilms inside the premises. Each plant should determine which environmental sites *L.monocytogenes* is most likely to be found and where to take samples based on the potential for finished product contamination.

When testing non-food contact surfaces this can help understand contamination patterns and to identify *L.monocytogenes* niches to evaluate effectiveness of sanitation.
If a contact surface frequently tests positive, the number of samples should be increased conversely when a surface repeatedly tests negative the sampling frequency might be reduced.

Control strategies:

- Weekly samples are recommended for wet areas; in dry-cleaned areas sampling can be less frequent.
- Samples should be taken at different times during the production e.g. one sample during operation and one sample at the end of the production prior to cleaning.
- Plants must always know what actions that has to be taken if *L.monocytogenes* is detected at frequencies exceeding the upper control limit. Due to the fact that positive samples are likely to be plant specific corrective actions will vary. The environment should always be intensively cleaned and re-tested.
- Plants should investigate to find out the reason for positive samples if any. Investigation should be conducted to identify possible equipment containing niches or biofilms.
- All plants must consider whether positive finding of *L.monocytogenes* on food contact surfaces should result in the need for product testing for listeria.
- When taking samples it should be used with force on the wiper passing it repeatedly back and forth across the sampling area. Avoid contamination when sampling by wearing sterile gloves, always change gloves between different sample areas.
- Several samples will increase the cost. It is possible to use pool sampled where more samples is analyzed as one.

<u>Finished product testing</u>

In fact finished product testing is not necessarily an important part of the control program. Many plants conduct product testing only when requested, or they can use testing of the finished product as a confirmation that sanitation and other control measures are effective.

6.2.6 Product labelling

As soon as possible after the product is finished it should be kept refrigerated or frozen. As mentioned earlier *L.monocytogenes* grows slower at refrigerated temperatures.

The label should contain information like “important must be kept refrigerated at below 3, 3 degrees or kept frozen”

Summary preventive measures:

The primary source of contamination is often due to biofilm/ niches of L.monocytogenes. When an effective control program is in pace the contamination will be line-specific. The contamination will flow downstream along the packaging line.

When seeking the source of a niche it is recommended to use separate samples not pool samples and every sample should be analyzed separately. Tear down suspected pieces of equipment or materials for cleaning and sanitation. Also place small parts in an owen and heat to 160 degrees.

Always consider the possibility of any employee to be involved in the contamination, refreshing training when it comes to food hygiene is necessary to prevent *L.monocytogenes* contamination.

7 New methods for reducing Listeria monocytogenes

In 2015 the Norwegian Nofirma reviewed new methods and technologies to potentially be used to reduce *L.monocytogenes* on salmon and salmon products. Selected methods were tested and evaluated for listeria reduction or growth inhibition on fresh and smoked salmon (Heir, et al. 2015).

The aim of the testing was to determine the effect of the methods and if it could be used to reduce listeria found on the salmon.

The tested method used:

- Exposure with ultraviolet light
- Desliming using H_2O_2 based compounds (freebac.mucosol)
- Treatment with tenside based compound (lauryl arginate)
- Treatment using organic acids salts (verdad N4 and opti. Form PPA plus)

The treatment was performed on fresh and/or smoked salmon. Approximately 70-90% reduction in listeria was proven after treatment with UVC, pulsed UV or lauric arginate. This treatment can be sprayed on the product during slicing of smoked salmon. Treatment of salmon with UVC or Pulsus probably has the greatest potential for application in late the process, e.g. during packaging. This would provide a reduced lever level in the process stage also leading to lower incidences of cross contamination. Killing germs with UVC or pulse can also be used to decrease germs on surfaces of equipment. The desliming treatment showed limited effect. Organic acid salts showed limited effect in this study.

8 Conclusion

The role of *L.monocytogenes* as a food-borne pathogen was first recognized following outbreaks of human listeriosis caused by the consumption of contaminated foods in North America and Europe during the mid-1980s. Later research have showed that the incidences of human listeriosis varies greatly between countries, with a total global number of incidences ranging from 4.4 to 7.4 million annually.

Listeriosis might be a fatal disease if infection occurs in immunocompromised, elderly or prenatal, due to the high mortality rate in this group of people I believe prevention and control of *L.monocytogenes* is extremely important. Norway have low incidence of listeriosis compared to other countries, but according to MSIS the incidences have increased in the last 30 years. In my mind the fish producing plants play an important role contributing to this increase. Ready–to-eat food containing fish have increasing popularity in Norway, something that leads to larger demand and higher production rate in processing plants. Due to this I believe a lot of the smaller plants do not have the capacity to handle this kind of increase in production, but due to economic reasons they still increase their production rate. The impact of this will lead to higher risk of contamination, negligence of hygiene, increased tear of equipments and less training programs for employees.

Scientists agree to the fact that by reducing the level of *L.monocytogenes* the food safety is improved. I believe *L.monocytogenes* must be considered a serious hazard in fish producing plants to be able to protect customers and the business itself. Understanding the source of the pathogen and the factors contributing to the risk of contamination this will minimize contamination of the finished product. By carrying out an HACCP plan and an effective control program this can be achieved. This will also reduce the economic losses connected to a possible outbreak of L.monocytogenes in the premises. It should be empathized that

L.monocytogenes is a naturally occurring bacteria and contamination seems to be impossible to avoid 100%. An effective HACCP and control program is the best defense to keep the levels of *L.monocytogenes* under 100 cfg/ gram food to avoid food borne disease.

9 Summary

In this study I have summarized the importance of *L.monocytogenes* in Norwegian fish production plants and the most important aspects of its preventive measures. In the last ten years the prevalence of *L.monocytogenes* in ready–to-eat food has increased. It is frequently isolated from seafood products like smoked salmon, curled salmon, cooked fish, fermented fish and shellfish products. *L. monocytogenes* is a common bacterium that survives extremely well in the processing plant environment, and it is the causative agent of the infectious disease listeriosis. Today listeriosis is regarded as a food-borne disease of public health concern due to the approximate mortality rate from 30% to 75% in high risk groups like pregnant, immunocompromised, elderly and newborns. Recent study has showed that the main source of contamination in food is from the environment, not the fish itself; this is due to the establishment of specific *L.monocytogenes* strains inside each individual plant. The ability of *L. monocytogenes* to grow and multiply inside biofilm is one of the major challenges in fish producing plants. By using an effective control program each specific plant can gather information on potential sources of *L. monocytogenes* to prevent establishment of bacterial niches/biofilms in the premises. The goal of the control program is to find if the organism is present, so that the potential for contamination of the finished product can be minimized and prevented.

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