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Exposure Assessment of Listeria Monocytogenes in Vacuum Packed Cold-smoked Salmon in the Republic of Ireland

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Exposure assessment of *Listeria monocytogenes* in vacuum packed cold-smoked salmon in the Republic of Ireland

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Abstract

Contamination of cold-smoked salmon with *Listeria monocytogenes,* a bacterium causing listeriosis, presents a risk to consumer health. The overall aim of this thesis was to investigate the prevalence and source of *L. monocytogenes* in different stages of vacuum packed cold-smoked salmon production chain/retail market and to develop a risk assessment model to quantitatively assess the risk of human listeriosis upon consumption of vacuum packed cold-smoked salmon. The study necessitated the identification of novel isolation techniques for the isolation and quantification of *L. monocytogenes* in vacuum packed cold-smoked salmon. The techniques currently used in isolation of *L. monocytogenes* from ready-to-eat food (EN/ISO 11290-01 and -02) were evaluated and were found to be 64 % effective in isolating *L. monocytogenes.* Use of 16S rRNA gene sequence analysis and molecular fingerprinting method multi-locus variable number tandem repeat analysis (MLVA) in combination with EN/ISO 11290-01 and -02 was found to be more effective (98 %) in quantification of *L. monocytogenes* in vacuum packed cold-smoked salmon.

The prevalence of *L. monocytogenes* in five brands of vacuum packed cold-smoked salmon (n = 120) marketed in different retail outlets in the Republic of Ireland was 21.60%.The prevalence of *L. monocytogenes* surveyed in a vacuum packed cold-smoked salmon factory (n = 444) was 24.54 %. The final product (vacuum packed cold-smoked salmon) was contaminated with three major types of *L. monocytogenes*; one type originating from the raw material and the others colonising the production line. To validate these routes of contamination, 60 raw salmon were tagged and sampled after each stage of processing, the results showed that the final product was contaminated with 3 strain types of *L. monocytogenes* isolated from raw, curing and filleting stages of cold-smoking respectively. The prevalence and tagging results indicate the current ubiquitous nature of *L. monocytogenes* in vacuum packed cold-smoked salmon

A product specific model was developed and validated under dynamic temperature conditions to predict the growth of *L. monocytogenes* in cold-smoked salmon taking into account the retail and consumer phases of the food pathways. The values of bias factor and accuracy factorof the modelwere close to unity, indicating good agreement between observations and predictions of the model.

Finally a quantitative Monte Carlo risk assessment model was developed to assess likely human exposure and the probability of human illness by *L. monocytogenes* on cold-smoked

salmon in Ireland. A surveillance study conducted at the retail level served as the starting point for the model with a mean prevalence of *L. monocytogenes* in vacuum packed cold-smoked salmon of 21.60 % and a mean count on contaminated vacuum packed cold-smoked salmon of 2.60 $\log_{10}$ CFU/g (95 % confidence interval 0.00 – 4.53 $\log_{10}$ CFU/g). The model predicted the annual log probability of illness by consuming contaminated vacuum packed cold-smoked salmon in a low risk and high risk population, with mean values -5.76 and -1.63, respectively (assuming weekly consumption). The model sensitivity analysis highlights the importance of reducing the initial contamination levels of *L. monocytogenes* on raw fish and the maintenance of proper storage conditions. Various 'what-if' scenarios were studied to assess the likely impact on the log probability of illness per serving. Careful control of consumer storage temperature and time were identified as the best strategies to decrease the probability of illness.

In conclusion, the results from this study indicated that sub-typing of the different strains using MLVA implicated a possible carryover of *L. monocytogenes* from the raw fish and in-house strain to the final product. Therefore, suitable processing parameters and pre-processing handling practices should be treated as important control measures to minimise the exposure to this pathogen. The product specific dynamic model developed in this study provides the sea food industry with a useful tool for effective management and optimization of product safety and may contribute to more realistic estimations of safety risks related to vacuum packed cold-smoked salmon. The results from the quantitative risk assessment developed in this thesis may help risk managers to make informed decisions with regard to possible control measures for *L. monocytogenes* in cold smoked salmon and therefore improve food safety.

Declaration page

I certify that this thesis which I now submit for examination for the award of Doctor of Philosophy, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for another award in any Institute.

The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

The Institute has permission to keep, to lend or to copy this thesis in whole or in part, on condition that any such use of the material of the thesis is duly acknowledged.

Signature _____ Date _____

To the memory of my mother (May’2010)

To my father

To my brother

To my husband

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2	General materials and methods	- Methods used in this study to detect, isolate and characterise *L. monocytogenes* from vacuum packed cold-smoked salmon - Inoculation of smoked salmon with *L. monocytogenes* - Statistical methods used in the evaluation of models applied in this study
3	Development of rapid and reliable method for detection of *L. monocytogenes*	- Various selective plating methods tested for the isolation of *L. monocytogenes*. - Evaluation of the plating methods using 16S rRNA method developed in this study.
4.	Prevalence of *L. monocytogenes* in vacuum packed cold-smoked salmon marketed in retail outlets in Dublin	- Prevalence of *L. monocytogenes* in cold-smoked salmon marketed in retail outlets in Dublin for a period of one year
5.	Contamination pathways of *L. monocytogenes* in an Irish cold-smoked salmon processing factory	- Factory was divided into four zones and samples from each zone were analysed for the presence of *L. monocytogenes* - The *L. monocytogenes* isolates were characterised using MLVA to study the similarities of the isolates from various zones - Contamination pathways of *L. monocytogenes* in vacuum cold-smoked salmon was established
6.	Tracking *L. monocytogenes* during different processing stages of vacuum packed cold-smoked salmon in a cold-smoked salmon processing factory	- 60 raw salmon were tagged and sampled after every critical step in the processing of vacuum packed cold-smoked salmon, to confirm the contamination pathways established in the previous Chapter (5)
7.	Growth modelling of *L. monocytogenes* in vacuum packed cold-smoked salmon at 4, 8, 12 and 16 °C.	- Determination of kinetic parameters of both *L. monocytogenes* (isolates of *L. monocytogenes* obtained from cold-smoked salmon Chapter 4) and native microflora at 4 °C and at abuse temperatures 8, 12 and 16 °C. - Illustration of the nature of growth relationship between native flora and *L. monocytogenes*
8.	Dynamic modelling of *L. monocytogenes* in vacuum packed cold-smoked salmon	- Development of a product specific growth model for vacuum packed cold-smoked salmon contaminated with *L. monocytogenes* covering retail and consumer storage temperature range.
9.	Risk assessment of *L. monocytogenes* in vacuum packed cold-smoked salmon	- Development of a quantitative risk assessment model of human listeriosis linked to the consumption of vacuum packed cold-smoked salmon.
10	General conclusions and recommendations for future work	- Conclusions, implications, limitations and recommendations for future work.

Chapter 1 Introduction and Review of Literature

1.1 *Listeria monocytogenes* as a food borne pathogen

Listeria monocytogenes, is an opportunistic psychrophilic bacterium which is widespread in the environment. *L. monocytogenes* was first reported in 1924 by Murray (Murray, *et al.*, 1924). It was isolated as a causative agent of monocytosis in rabbits and guinea pigs. It is a Gram positive bacterium and is part of the genus *Listeria* which has six identified species: *L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seeligeri and L. grayi* (Gandhi and Chikindas, 2007).

L. monocytogenes is an intracellular pathogen that causes a group of disease that are collectively termed as listeriosis. Since 1981, listeriosis is known to be an important bacterial food-borne disease (Mataragas, 2010). In recent years it has emerged as a significant cause of human infection in industrialised countries (Zunabovic, 2011). This is attributed to the emergence of a vulnerable immunocompromised population and the concomitant development of large-scale agro-industrial plants and refrigerated food (Lecuit, 2007).

1.2 Growth and survival characteristics

1.1.2 Growth characteristics

The growth of *L. monocytogenes* in food is dependent on the intrinsic characteristics of the substrate (such as, pH and water activity - a_w), the extrinsic characteristics (including, storage temperature and relative humidity) and processing techniques used in the production of food

(such as cooking and non-thermal processing; Hwang and Sheen, 2009). Some of the growth and survival limits for *L. monocytogenes* are shown in Table1.1.

Table 1-1 Growth and survival limits for *L. monocytogenes* (FSAI, 2007)

Parameter	Range	Optimal[d]	Can survive (But no growth)[e]
Temperature (°C)	-1.5 to 45	30 to 37	-18
pH[a]	4.2 to 9.5	7	3.3 to 4.2
Water Activity(a_w)	0.90 to >0.99	0.97	<0.90
Salt (%)	<0.5 to 12	N/A	≥20

[a]Hydrochloric acid as acidulant (inhibition is dependent on type of acid present)
[b]Sodium chloride as the humectant
[c]Percent sodium chloride, water phase
[d]When growth rate is highest
[e]Survival period will vary depending on nature of food and other factors
[f]A temperature of 70°C/2min is required for a 10^6reduction in numbers of *L. monocytogenes* cells
N/A Not Applicable

The primary factors that influence the growth of *L. monocytogenes* in food are temperature, pH and water activity (Zhao *et al.*, 2004). Additionally, it has also been demonstrated that previously stressed cells (e.g. exposure to sub-lethal heating before process heating) can be more resistant to additional stresses (Yoshida *et al.*, 2001). Although, *L. monocytogenes* has an optimum growth temperature of between 30 – 37 °C at neutral or slightly alkaline pH (i.e. pH ≥ 7) it can also grow at refrigerated temperatures < 5 °C (Table 1.1).

1.2.2 Survival characteristics

L. monocytogenes can survive in adverse environmental condition such as low temperatures and extreme pH by forming biofilms on surfaces.

A. Survival at low temperatures:

L. monocytogenes has the ability to grow over a wide range of temperatures (2–45 °C; Rocourt and Cossart, 1997). Survival at these temperatures takes place with changes in *L. monocytogenes* membrane composition. One of the main changes is an increase in the proportion of carbon chain $C_{15:0}$ at the expense of $C_{17:0.}$ Moreover, growth at low temperature results in an increase in unsaturated fatty acids (Beales, 2004) which would help in the survival of *L. monocytogenes* under low temperatures.

L. monocytogenes produces cold shock proteins (Csps) in response to cold acclimation which balances the growth at low temperature (Bayles *et al.*, 1996). They accumulate compatible solutes such as glycine, betaine and carnitine during refrigeration temperature. These solutes stimulate growth of cells subjected to cold stress. Under adverse environmental condition, transcription of genes is made possible by the association of alternative sigma factories with the core RNA polymerase (Becker *et al.*, 2000)

It possesses three small, highly homologous protein members of the cold shock protein (Csp) family CspA, CspB, and CspD. Cold stress induced by low temperature may inadvertently cross-protect cells against NaCl stress due to induction of cspA, cspD, and cspB gene expression. The hierarchies of their functional importance differ, depending on the environmental stress conditions: CspA>CspD>CspB in response to cold stress versus CspD>CspA/CspB in response to NaCl salt osmotic stress. The fact that Csps are promoting L. monocytogenes adaptation against both cold and NaCl stress has significant implications in view of practical food microbial control measures. The combined or sequential exposure of L.

monocytogenes cells to these two stresses in food environments might inadvertently induce cross-protection responses (Schmid *et al.,* 2009).

B. Survival under acid stress

L. monocytogenes encounters a low pH environment in acidic foods, during gastric passage and in the phagosome of the macrophage (Cotter and Hill, 2003). The pathogen responds to and survives in these low-pH environments by utilising a number of stress adaptation mechanisms. Exposure of *L. monocytogenes* to mild acidic pH of 5.5 (1 M lactic acid) induces the acid tolerance response (ATR), wherein the cells are resistant to severe acidic conditions (O'Driscoll *et al.,* 1996). *L. monoytogenes* develops acid tolerance upon exposure to sublethal acid conditions, a response that has been designated the ATR (acid tolerance response). The effectiveness of this response appears to be critically dependent upon two principal factors: (i) the pH of the adaptive exposure and (ii) the duration of the adaptive period (Davis *et al.,* 2006). The bacterium further utilises the glutamate decarboxylase (GAD) system to survive acid stress. The glutamate decarboxylase (GAD) acid resistance mechanism has been found to play a major role in the acid resistance of a relatively small number of bacteria. When the cell is exposed to low pH, the GAD system converts a molecule of extracellular glutamate to extracellular γ-aminobutyrate (GABA), while consuming an intracellular proton. The net effect is to reduce the proton concentration within the cell, thus alleviating acidification of the cytoplasm. In addition, γ-aminobutyrate is less acidic than glutamate, which contributes to an alkanization of the environment. A glutamate decarboxylase system protects Listeria monocytogenes in gastric fluid (Cotter, 2001).

C. Survival under osmotic stress

The use of salt to lower the water activity is one of the methods of food preservation used by the food industry; however, the ability of *Listeria* to adapt and survive in high concentrations of salt makes it difficult to control the pathogen in foods (Hill *et al.*, 2002). One of the mechanisms used by *Listeria* to tolerate salt stress is a change in its gene expression leading to an increased or decreased synthesis of various proteins (Duche *et al.,* 2002). Two general stress proteins (DnaK and Ctc) were identified among the salt shock proteins (SSP), (induced in *L. monocytogenes*. DnaK functions as a heat shock protein, stabilizing cellular proteins. Among the elevenstress acclimation proteins (Saps) identified, GbuA, which functions as an osmo-protectant transporter for glycine betaine was induced in response to salt stress.

D. Survival in biofilms

L. monocytogenes can grow as surface attached communities of cells embedded in an extra-cellular polysaccharide matrix known as a biofilm (White *et al.*, 2002). Biofilm growth is important because in this form the bacteria are more resistant to physical and chemical agents intended to kill the bacteria and are able to survive for extended periods with minimal nutrient supply (Somers and Wong, 2004). Surface biofilm, particularly in locations which are difficult to identify and clean, can act as a persistent source of food contamination through the release of *L. monocytogenes* from the biofilm (Chae and Schraft, 2000 and Borucki *et al.*, 2003).

E. Resistance to antimicrobial agents

Antimicrobial resistance in the food-borne pathogen *Listeria* is emerging in recent years (Sik *et al.,* 2006). Bacteria can acquire resistance by getting a copy of a gene encoding an altered protein or an enzyme like beta lactamase from other bacteria, even from those of a different species (Ayaz *et al.,* 2010). There are a number of ways to get a resistance gene:

- During transformation - in this process, akin to bacterial sex, microbes can join together and transfer DNA to each other.
- On a small, circular, extrachromosomal piece of DNA, called a plasmid - one plasmid can encode resistance to many different antibiotics.
- Through a transposon - transposons are "jumping genes," small pieces of DNA that can hop from DNA molecule to DNA molecule. Once in a chromosome or plasmid, they can be integrated stably.
- By scavenging DNA remnants from degraded dead bacteria.

Studies have shown that several species of *Listeria* isolated from humans or from food production or processing facilities are resistant to one or more antibiotics (Ayaz *et al.,* 2010 and Morvan et al., 2010). Morvan *et al.,* (2010) looked at 1001 isolates of *Listeria* from retail foods to determine their levels of resistance to eight antibiotics. About 10.9 % of the isolates were resistant to one or more antibiotics. Resistance to penicillin or tetracycline was the most common and there was no resistance to the antibiotics commonly used for treatment of listeriosis. However, this does not eliminate the possibility that resistance to antibiotics used for listeriosis treatment such as ampicillin and gentamycin cannot be acquired, since penicillin and ampicillin belong to the same family of beta-lactam antibiotics (Walsh *et al.,* 2001). In addition to antibiotic resistance, the emergence and spread of resistance among food borne

organisms to sanitizers and disinfectants used by the food industry are also becoming a concern (Sik *et al.,* 2006).

1.3 Virulence

The disease causing ability of *L. monocytogenes* is linked to the virulence or virulence-like genes on itschromosome. These virulence genes codefor surface and secreted proteins as well as other regulators which help the bacterium to adapt to diverse environments and express virulence traits. *L. monocytogenes* genome is approximately 3.0 Mb (Michel, 1992; Genbank/EMBL accession number AL591824) and information on its sequence can be found at The Institute for Genomic Research Table 1-2.

Table 1-2: Genomic characteristics of *L. monocytogenes* (Michel, 1992).

Size of the chromosome (kb)	2,944,528
G+C content (%)	39
G+C content of protein-coding genes (%)	38
Total number of protein-coding genes	2853

Other species such as *L. innocua* lack genes which are essential for virulence. For example, virulence genes that code for a surface protein and aid are invasion into host cells is present are *L. monocytogenes* but is absent in *L. innocua* (Kreft *et al.,* 1999).

1.4 Listeriosis

The term listeriosis is referred to a group of diseases caused by *L. monocytogenes* in both human and animals (McLauchlin, *et al.*, 2004). The illness is caused when the pathogenic strain of *L. monocytogenes* induces the virulence genes after lodging themselves in human cells. Human listeriosis is a potentially fatal food-borne infection which can cause abortion in pregnancy related cases (Smerdon *et al.*, 2001).

1.4.1 Nature of the disease

Two types of disease are associated with *L. monocytogenes*, non-invasive and invasive listeriosis (Smerdon *et al.*, 2001). Non-invasivelisteriosis (referred to as febrile listerial gastroenteritis) is the milder form of the disease. Symptoms include diarrhoea, fever, headache and myalgia (muscle pain; Goulet, 2001). Symptoms occur after a short incubation period. Outbreaks of this disease have generally involved the ingestion of high doses of *L. monocytogenes* by otherwise healthy individuals. Only limited information is available regarding the public health impact of this disease.

Invasive listeriosis affects 'high-risk people' (including pregnant women, cancer patients, AIDS patients, the elderly and the very young; Vazques-Borland *et al.*, 2001). This disease is characterised not only by the severity of the symptoms but also by a high mortality rate (Table 1-3).

Table 1-3: Symptoms of invasive listeriosis in human (FSAI 2007)

Symptoms	Incubation Period	Mortality Rate
❖ **Mild fever (with or without slight gastroenteritis or flu like symptoms)** ❖ **Myalgia** ❖ **Meningitis** ❖ **Septocaemia** ❖ **Spontaneous abortion**	1-90 days	20-30 %

Thirteen serotypes have been identified for *L. monocytogenes*. All of these may be associated with human listeriosis; however, most human infections are associated with the serotypes 1/2a, 1/2b or 4b (Corcoran *et al.*, 2006). Steps involved in the human listeriosis is represented in Figure (1.1)

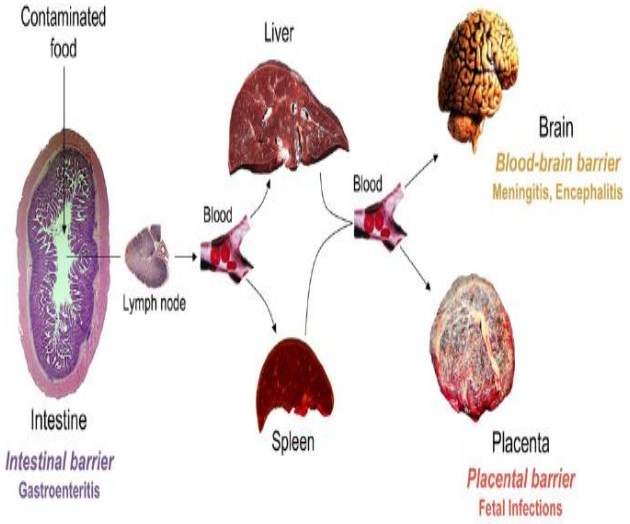


Figure 1-1: Successive steps of human listeriosis affecting the different organs in the human body (Leuit *et al.*, 2007)

1.4.2 Transmission route of listeriosis in food

The consumption of contaminated food is the main route of transmission of listeriosis accounting for 80-90 % of cases (WHO, 2004). Foods that are most often associated with human listeriosis include those which:

- Support the growth of *L. monocytogenes.*
- Have a long shelf life under refrigeration
- Are consumed without further listericidal treatments

1.4.3 Food-born outbreaks of listeriosis.

The first documented outbreaks of listeriosis in Europe and in the USA were due to milk products (Linnan *et al.,* 1988). From 1991 to 2002 a total of 18 outbreaks of invasive listeriosis, three cases of gastroenteritis listeriosis and one case of invasive and gastroenteritis listeriosis have been reported in nine different countries in Europe, with a total of 526 outbreak related cases (FSAI, 2007).

There have been number of outbreaks between 1991 and 2003 in Europe which have been tabulated in Table 1.4.Listeriosis outbreak according to food type and country are tabulated in Table 1.5.

Table 1-4 Listeriosis outbreak in Europe 1991-2002 (FSAI, 2007)

Outbreak Type	Number of Outbreaks	Associated Foods
Invasive	6	Processed Meat Product
	5	Cheese
	3	Processed Fish Product
	3	Undetermined
	1	Butter
Gastroenteritis	1	Rice Salad
	1	Corn Salad
	1	Undetermined
Invasive and Gastroenteritis	1	Frozen cream Cake

Table 1-5 Reported listeriosis outbreaks in Europe according to food type and country (Lunden *et al.,* 2004)

Year	Country	Product Type	Number of Cases (deaths)
1949-1957	Germany	Raw milk	about 100
1983-1987	Switzerland	Soft cheese	122 (33)
1986	Austria	Raw milk/vegetables	28 (5)
1989-1990	Denmark	Blue-mold cheese/hard cheese	26 (6)
1995	France	Soft cheese	37
1997	France	Soft cheese	14
1998-1999	Finland	Butter	25
2001	Sweden	Soft cheese	33

In 2005, there where twenty five reported cases of listeriosis in humans in the EU (Table 1.6**).**Overall, 1,439 cases were reported in the EU and 99.4 % of these were laboratory confirmed. Cases from France, Germany and the United Kingdom accounted for 65 % of all the confirmed cases. The overall incidence was estimated to 0.3 confirmed cases per 100,000 population similar to the incidence recorded in 2004 (0.3 per 100,000 population). The highest listeriosis cases were recorded in Denmark (0.9), Belgium (0.8) and Finland (0.7).

Table 1-6: Reported listeriosis cases in humans, 2001-2005 and incidence for confirmed cases (EFSA, 2006)

Country	2005		2004	2003	2002	2001
	Total cases	Confirmed cases	Total cases			
Austeria	9	9	19	8	16	9
Belgium	62	62	70	76	44	57
Cyprus	0	0	-	-	-	-
Czech Republic	15	15	16	-	-	-
Denmark	46	46	41	29	28	38
Estonia	2	2	2	-	-	-
Finland	36	36	35	41	20	28
France	221	221	236	220	218	187
Germany	510	510	296	256	240	217
Greece	-	-	3	0	5	3
Hungary	10	10	16	-	-	-
Ireland	12	11	11	6	6	7
Italy	51	51	25	0	-	31
Latvia	3	3	5	8	16	11
Lithuania	2	2	1	2	-	-
Luxembourg	0	0	-	-	-	-
Malta	0	0	-	-	-	-
The Netherlands	96	96	55	52	32	16
Poland	22	22	10	5	31	9
Portugal	-	-	38	-	-	-
Slovakia	5	5	8	6	7	6
Slovenia	0	0	1	6	-	-

Country	2005		2004	2003	2002	2001
	Total cases	Confirmed cases	Total cases			
Spain	68	68	100	52	49	57
Sweden	35	35	44	48	39	67
UK	223	223	232	255	158	167
EU-Total	1,427	1,426	1,264	1,070	909	910

According to Health Protection Surveillance Centre (HPSC), Ireland has seen an increase in listeriosis notification in 2010 (Figure 1.2). The increase is primarily among the pregnancy-related and neonatal cases (collectively called as pregnancy-associated cases) and represents a very significant increase in the proportion of cases that are pregnancy-associated over recent years. The increase in listeriosis was seen the ethnic minority-pregnancy related cases. This cause was related to the food which was imported from their home land (FSAI 2006).

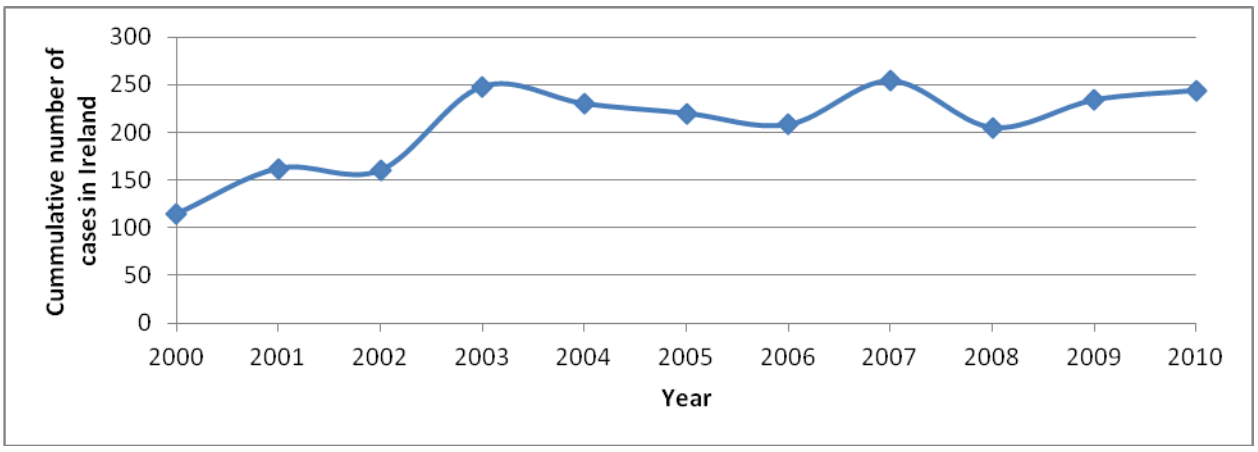


Figure 1-2 No. of reported cases of L. monocytogenes in the Republic of Ireland from 2000 to 2010 (HPSC 2010)

According to the European Food Safety Authority (EFSA, 2006), 54 % of the reported listeriosis cases in the EU occurred in individuals above 65 year. This proportion was similar to that observed in 2004 (51 %). In 2006, listeriosis in children less than four years accounted for 7 % of the cases. In 2005, 74 listeriosis cases were associated with pregnancy. These cases were reported in Germany (34 cases), France (37 cases) and Denmark (3 cases). The distribution of human listeriosis cases in the EU by age group for the year 2005 is illustrated in Figure (1.3).

L. monocytogenes serotypes for 244 cases reported in 2005 by the member states of the EU revealed that 48.7 % belonged to the 1/2a serotype and 30.3 % to the 4b serotype. Cases belonging to the serotypes 1/2, 1/2b, 4 and others accounted for 4.5 %, 13.5 %, 1.2 % and 1.6 %, respectively.

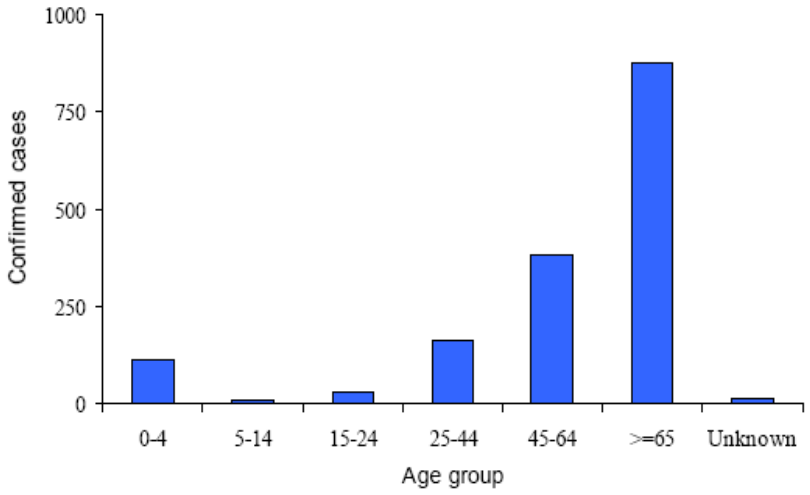


Figure 1-3: Distribution of human listeriosis case by age(EFSA, 2006).

There has been an steady increase in the pregnancy related lisetriosis in the UK in 2009, 19 pregnancies related death were reported in the UK in comparison to the 11 cases reported in 2008 (HSE, 2009). In the EU (2009-2010) outbreak involving 34 cases of invasive listeriosis: 25 outbreak cases originated

from seven of nine Austrian provinces. Eight of the 34 cases in this outbreak had a fatal outcome (Fretz *et al.,* 2010).

1.5 Ready to Eat Foods associated with listeriosis

Raw and minimally processed foods are typically sold to the consumer in a ready-to-use or ready-to-eat (RTE) form. These products generally do not contain preservatives or antimicrobial agents and rarely undergo any heat processing prior to consumption (Seymour, 2001). The increase in consumer demand for RTE food and the changes in commercial food production (such as minimal processing) are the major reasons for the increase in food borne illness in recent times (Christison *et al.*, 2008). Occurrence of *L. monocytogenes* in these foods has led to public health incidents, product recalls and large financial losses across the world (WHO, 2004). According to EFSA (2007), 100 CFU/g of *L. monocytogenes* in RTE food represents a very low risk of listeriosis for all population groups. In 2005, the community legislation of the member European Union laid down threshold for the presence of 2 log CFU/g of *L. monocytogenes* on a 25 g sample of RTE food products (directive 92/46/EEC). The number of ready-to-eat food samples tested throughout the EU in 2005 for *L. monocytogenes* by the food category and number of positive findings are presented in Figure (1.4).

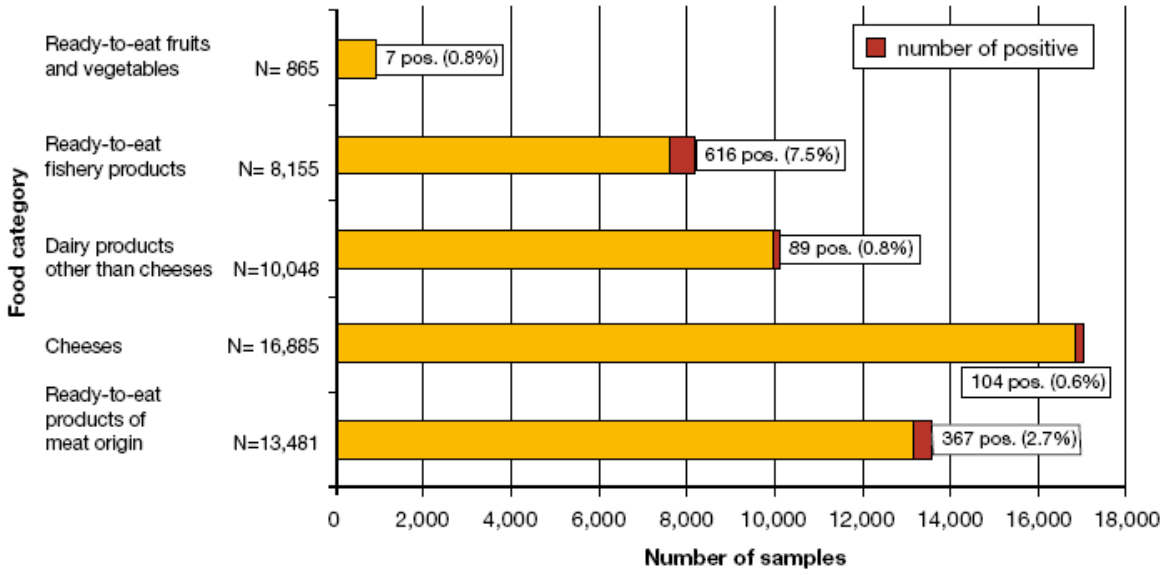


Figure 1-4: Number of food samples tested for *L. monocytogenes* by food category and number of positive findings (EFSA, 2006).

1.5.1 *L. monocytogenes* in seafood

L. monocytogenes is ubiquitous in nature and therefore aquatic creatures are also potential sources of the bacterium. There are several studies that have reported the presence of *L. monocytogenes* in seafood (Azevedo *et al.,* 2005, Miettinen and Wirtanen, 2006, Salihu *et al.*,2008 and Garrido *et al.,* 2010). *L. monocytogenes* has shorter generation times in seafood (pH 6.1– 7.6) than in meat and meat products (pH 5.1–6.2). This is partly due to the effect of pH on the growth of *L. monocytogenes*, which has higher growth rates at near neutral pH (Garrido, *et al.,* 2009). The contamination rate of seafood products with *L. monocytogenes* can vary from zero to 75 % (Embark, 1994; Jinneman *et al.*, 1999 and Miettinen and Wirtanen 2005). Initial levels of *L. monocytogenes* in seafood can be influenced by various factors such as origin (wild or farmed), season, fishing technique, processing factory, handling and storage conditions (Garrido, *et al.,* 2009). As L. *monocytogenes* is ubiquitous is its presence, they can contaminate

the seafood at any of the above mentioned stages and progress to the final preparation as they can withstand varying temperatures, pH and salt conditions.

One of the key factors that influences the presence of *L. monocytogenes* is the final seafood product is its preparation and the way it is consumed. For example Vacuum packed cold-smoked salmon are ready-to-eat sea food products that are cold-smoked below 20 ˚C for several hours and are normally purchased vacuum packed, with a shelf-life at refrigeration temperatures of a couple of weeks, and are consumed without any heat treatment. The salt content, pH and water activity values of vacuum packed cold-smoked salmon are normally within a range permitting the growth of *L. monocytogenes* (Rorvik *et al.*, 2000).The prevalence of *L. monocytogenes* in freshly produced vacuum packed cold-smoked fish is relatively high and is typically between 10 to 40 % (Azevedo *et al.,* 2005, Miettinen and Wirtanen 2006, Chitlapilly Dass *et al.,* 2010a and Chitlapilly Dass *et al.,* 2010b). The presence of the pathogenic microorganism *L. monocytogenes* in minimally processed refrigerated food products (vacuum packed cold-smoked salmon) and the ability of this microorganism to grow under storage conditions has made this product in the 'high-risk' category (Garrido *et al.,* 2009).

Vacuum packed cold-smoked salmon has a wide ranging consumption patterns in Europe and thus is of considerable economic importance for the seafood market, representing a 468 million Euro industry (Cardinal *et al.,* 2004). This is due to the health benefits associated with the consumption of this product. Salmon is rich in proteins and omega-3 which protects against heart disease by lowering blood triglycerides and preventing blood clotting (Stolyhwo, 2006 and Domingo, 2007). The product is also high in vitamin E and possess high antioxidant properties (Sallam, 2007). The product is also low in carbohydrates and thus used as a staple diet by the health conscious population. Ireland is one of the leading producers of vacuum packed cold-smoked

salmon and is also leading exporters of this product worldwide. The population that consumes fish and fish products in Ireland is about 66 % (IUNA, 2005), thus making it one of the product that is largely consumed.

Although, the health benefits associated with the consumption of the vacuum packed cold-smoked salmon exists, the potential of contamination of the food-born pathogen *L. monocytogenes*also prevails. Increased international trade of vacuum packed cold-smoked salmon coupled with an increase in susceptible populations, (especially the elderly (> 60 years) and immunocompromised individuals suffering underlying diseases), has made *L. monocytogenes* in vacuum packed cold-smoked salmona cause for concern (Lindquist and Westöö, 2000).

1.5.2 Vacuum packed cold-Smoked salmon product characteristics

Cold-smoked salmon is lightly preserved seafood, traditionally smoking was applied as a preservation method, but in recent years smoking is more important in terms of sensory value (Rorvik, 2000). Smoking of salmon may imply cold-smoking at below 20 ºC or hot-smoking at high temperatures (>60ºC). The production of vacuum packed cold-smoked salmon includes filleting, salting, drying, smoking, trimming and packaging (Figure 1-5). These processes involve a lot of handing by workers as well as the use of technically complex equipment (Jemmi and Keusch 1992). The fillets may be dry salted or brined in a bath or by injection. The salt content varies with the consumers' preference.This results in a product with 3–8 % water phase salt (WPS) corresponding to water activities of 0.950 -0.983 and a pH of 5.9 – 6.3. Contents of smoke components are equivalent to 6700 – 15,400 ppm of phenols and levels of water phase lactate (WPL) of 6700–15,400 ppm have been reported. Smoking may be

performed in a smoke oven or by using liquid smoke. The intensity of smoking also varies with customers' taste. Nitrite is not typically used but levels < 40 ppm have been found. At 5 ºC shelf life can be as short as 2 weeks or as long as 12 weeks. The difference shelf -life depend on the levels of nitrite used.Spoilage is primarily due to microbial activity and at 10 ºC the spoilage microflora has been dominated by lactic acid bacteria sometimes together with *Photobacterium phosphoreum* or *Enterobacteriaceae*. Lacticacid bacteria can reach their maximum population density rapidly and remain at this level up to 50 % of the products shelf life (Hansen *et al.*, 1998 and Jørgensen *et al.*, 2000). Storage temperature and water activity markedly influence the shelf life of cold smoked salmon and a synergistic effect of NaCl and smoke components on shelf life has been reported (Leroi *et al.*, 2000). Processing of cold smoked salmon includes no recognized critical control point for *L. monocytogenes* although cold smoking seems to reduce numbers of the bacteria, it is unlikely the product can be produced completely free of this pathogen (Gram, 2001). This bacterium can grow in naturally contaminated cold smoked salmon and this is of major importance for the human health risk (Jørgensen and Huss 1998 and Gram, 2001).

Figure 1-5: Schematic representation of different processing stages during the production of vacuum packed cold-smoked salmon.

1.5.3 Occurrence of *L. monocytogenes* in vacuum packed cold-smoked salmon and other seafood products

The contamination of seafood products with *L. monocytogenes* has been widely studied. Variation in rates of prevalence of *L. monocytogenes* can be found between different product types as well as by producers (Table 1.7). The prevalence of these organismsin freshly produced cold-smoked salmon range between 10- 75 % is relatively high and is typically between 10 to 40 % (Miettinen and Wirtanen 2006, Azevedo *et al.,* 2005, Fonnesbech-Vogel *et al.,* 2001, Rorvik *et al.,* 2000, Autio *et al.,* 1999). This high prevalence could be due to the low smoking temperature involved during the cold-salmon processing; as these conditions would be ideal for the proliferation of *L. monocytogenes* if the raw salmon harboured the pathogen or acquired the pathogen from the processing environment.

The contamination of hot-smoked fish products, including those reported only to be smoked ranged from 0 to 33 % (Gombas et al., 2003). The largest amount of *L. monocytogenes* cells found in hot-smoked fish product was $1.3x10^5$ CFU/g (Loncarevic *etal.,* 1996). Seafood salad *L. monocytogenes* contamination occurred at a rate between 4.7 to 27 % and the highest number of cells found was 100-1000 CFU/g (Gombas *et al.,* 2003). The contamination of cold-salted fish varied between 21 to 50 % (Gombas *et al.,* 2003) and the largest number of *L. monocytogenes* cells found was $3.4x10^3$ CFU/g (Loncarevic *et al.,* 1996). In Japan, contamination occurred in 10 % (7/67) of the studied Roe fish samples (Handa *et al.,* 2005). The overall *L. monocytogenes* contamination rate of seafood products was 7.8 % according to Table 1.7

Table 1-7:*L. monocytogenes* contamination in fish processing factories and comments on the contamination pattern (Miettinen *et al.*, 2005).

Product type, (Year of study)	**Sample types**	**No. of samples**	**% positive for *L. monocytogenes***	**Comments on *L. monocytogenes* contamination**
Vacuum packed	Raw fish	9	44	No regular cleaning and disinfection
Cold-smoked salmon,	Fish during processing	36	19	
(1994)	Final Product	16	6	
	Environment	113	32	
Vacuum packed	Raw fish	46	65	Primary source of *L. monocytogenes*
Cold-smoked salmon,	Fish during processing	85	33	external surface of fish
(1995)	Final Product	23	30	
	Environment	37	59	
	Fish processing are	89	71	
Vacuum packed	Drains		63	Risk factors: rotation during duties, finding of *L. monocytogenes*
Cold-smoked salmon,	Fish during processing		33	in drain
(1997)				
Vacuum packed	Raw fish	60	2	Two major contamination sites of final product, brining and slicing.
Cold-smoked fish,	Fish during processing	75	29	Eradication programme successful.

Product type, (Year of study)	**Sample types**	**No. of samples**	**% positive for *L. monocytogenes***	**Comments on *L. monocytogenes* contamination**
Vacuum packed Cold-smoked salmon (1999)	Final product	22	100	
	Environment	122	13	
	Brine	65	30	
	Personnel	6	67	
	Air	19	32	
	Environment, brine, products	94	0	
Vacuum packed Cold-smoked salmon, (2001)	Raw fish	18	0	One *L. monocytogenes* clone persisted over four years.
	Fish during processing	4	0	Slicing area associated with final product contamination
	Final product	128	47	
	Contact surface	50	40	
	Environment	96	17	
Vacuum packed Cold-smoked salmon, (2003)	Drains	128	63	Raw fish and processing environment had different *L. monocytogenes* population
	Other environment site	96	32	
	Food contact sites	32	3	
	Raw fish	187	3 4	

Product type, (Year of study)	Sample types	No. of samples	% positive for *L. monocytogenes*	Comments on *L. monocytogenes* contamination
Shrimp, salmon, cod,	Environment	309	10	Cleaning procedures insufficient
(2004)	Environment	214	20	
	Floors and drains	91	19	
	Floors and drains	75	27	
	Personnel	48	6	
	Brine	23	9	
	Raw material	74	14	
	Fish during processing	102	4	
	Final product	104	18	
Vacuum packed	Raw material	86	16	Raw material and environment
Cold-smoked salmon,	Brine	14	21	contamination source
(2005)	Final and unfinished proc	125	4	
	Environment	134	3	
	Environment	99	11	
	Floors and drains	68	18	
	Floors and drains	69	25	
	Personnel	48	6	

Vacuum packed cold-smoked salmon has been linked to outbreaks of listeriosis (Brett *et al.*, 1998 and Miettinen *et al.*, 1999), and has been categorized as a high risk food, as an estimated 6.2 cases of listeriosis occur per billion servings (FDA, 2003). *L. monocytogenes* can multiply in a wide temperature range and in high levels of NaCl and taken as individual processing steps, salting or smoking temperature are not believed to reduce numbers of *L. monocytogenes* (Cole *et al.*, 1990). Due to the low smoking temperature, *L. monocytogenes* could proliferate during the smoking process.

The amounts of *L. monocytogenes* found in different seafood products that have been suspected of causing listeriosis cases vary. In Quebec, Canada, a listeriosis outbreak was connected to vacuum packed cold-smoked salmon in 2009 (Harwig *et al.*, 2010). In Finland, a listeriosis outbreak was connected with the consumption of cold-smoked rainbow trout. The cold-smoked rainbow trout from the same lot and the same retail store was found to contain 1.9×10^5 CFU/g of *L. monocytogenes* (Miettinen *et al.*, 2006). In a case with smoked mussels, *L. monocytogenes* amounts of 1.6×10^7 CFU/g and 3.2 × 106 CFU/g occurred in the mussels from the patients' refrigerators (Mitchell 1991). High numbers of *L. monocytogenes* (2.1×10^9 CFU/g) were also found in imitation crab meat that caused a listeriosis outbreak (Farber *et al.*, 2000).

According to European Food Safety Agency (EFSA, 2006), fish and fishery products were the food categories with the highest proportion of *L. monocytogenes* positive samples as well as the highest proportions of samples with more than 100 CFU/g as shown in Table 1.7. The highest proportions of positive samples were reported by The Netherlands, Belgium, Austria and Sweden, all with a prevalence ranging from 10.8 % - 25.9 %. Furthermore, The Netherlands, Germany, Italy, Austria and Spain reported products containing the bacteria more than 100 CFU/g with rates between 0.9 - 3.5 % as shown in Table 1.8.

Table 1-8: *L. monocytogenes detected* in fishery productsSource: EFSA, 2005

Product **Country**	**Ready-to-eat fishery product (25 g)**	**No. of Samples**	**%** ***L. monocytogenes***	**> 100 CFU/G of** ***L. monocytogenes***
Fish				
Austria	Smoked	389	9	0
Belgium	Cold-smoked (processor)	145	15.9	-
Germany	Unspecified	2,481	9.4	22
Germany	Smoked	773	9.7	8
Ireland	Unspecified (retail)	36	0	-
Ireland	Smoked (processor)	61	1.6	-
Ireland	Smoked (retail)	26	0	-
Italy	Smoked	263	9.5	3
The Netherlands	Smoked	563	25.9	20
Norway	Unspecified (processor)	129	2.3	-
Other fishery product				
Austria	Unspecified	69	13	2
Austria	Raw fish product	33	9.1	1
Denmark	Unspecified	208	1.9	0
Estonia	Ready-to-eat (processor)	30	6.7	-
Spain	Unspecified	412	1.7	5
Ireland	Unspecified (retail)	303	0	-
Ireland	Unspecified (processor)	54	5.6	-
Italy	Unspecified	548	1.5	0
Slovakia	Unspecified	116	1.7	-
Sweden	Unspecified	37	10.8	-

1.5.4 Sources and routes of *L. monocytogenes* contamination in the seafood industry

The processing environment was found to be the major source of contamination for seafood products (Rørvik *et al.,* 1995, Autio *et al.,* 1999, Johansson *et al.,* 1999, Dauphin *et al.,* 2001, Fonnesbech Vogel *et al.,* 2001, Norton *et al.,* 2001, Lappi *et al.,* 2004, Thimothe *et al.,* 2004 and Gudmundsdóttir *et al.,* 2005). In case of lightly preserved seafood, slicing and brining was established as the major source of contamination (Autio *et al.,* 1999, Johansson *et al.,* 1999 and Fonnesbech Vogel *et al.,* 2001). In addition, potential sources of final product contamination were cross-contamination, job rotation, employee hygiene and food handling practices (Rørvik *et al.,* 1997 and Thimothe *et al.,* 2004). The raw materials have not always been reported as an important final product contaminant source (Johansson *et al.,* 1999, Dauphin *et al.,* 2001). The raw materials, however, have clearly been found to be a source of *L. monocytogenes* contamination of the final products in certain factories (Eklund *et al.,* 1995, Fonnesbech Vogel *et al.,* 2001 and Gudmundsdóttir *et al.,* 2005). Another observation was that all the factories had their own *L. monocytogenes* contamination patterns and different degrees of contamination at the process environment and on final products (, Lappi *et al.,* 2004) despite the use of similar raw materials (Thimothe *et al.,* 2004). This was particularly influenced by the factory design, structure and conditions as well as operational and sanitation procedures (Hoffman *et al.,* 2003, Thimothe *et al.,* 2004). Table 1.9 summarises the prevalence of *Listeria* in various types of seafood. In most of the fish processing factories, one or a few *L. monocytogenes* clones were found to persist for several months or years in the processing environment despite the normal washing regime (Johansson *et al.,* 1999, Fonnesbech Vogel *et al.,* 2001, Gudmundsdóttir *et al.,* 2005, and Miettinen and Wirtanen, 2006). In addition to persistent *L. monocytogenes* clones, sporadic *L. monocytogenes* clones were also found in the factories (Johansson *et al.,* 1999, Fonnesbech Vogel *et al.,* 2001, Lappi *et al.,* 2004).

Table 1-9: Prevalence of *Listeria* spp. and *L. monocytogenes* in live seafood, fresh seafood in retail markets and in fresh raw seafood material from processingfactories (Miettinen *et al.*, 2006)

Seafood type (Country) year	Sampling location	Specification	No. of samples	% of positive *L. monocytogenes*
Salmon (Norway) 1998	Live, farmed	Skin and belly cavity swabbed	10	0
Salmon (Norway) 2003	Processing factory	25 g Collar, tail and belly	81	21
Salmon (Norway) 2001	2 processing factory	25 g or skin scraping	215	7
Salmon (Norway) 2001	Processing factory	Skin swabbed	7	86
Salmon (Norway) 2005	Producer	25 g salmon	46	4
Salmon (Norway) 1995	Processing factory	25 g salmon	50	4
Salmon (UK) 2001	Commercial outlet	25 g flesh and skin	5	0
Salmon (UK) 2001	Processing factory	Skin swab	61	0
Salmon (USA) 2001	2 processing factory	25 g Collar, tail and belly	19	88
Salmon (USA) 2003	Freezer warehouse	Slime layer	50	30
Salmon (USA) 1995	Freezer warehouse	Skin	48	21
Salmon (USA) 1995	Freezer warehouse	Flesh under skin	26	0
Salmon (USA)1995	Freezer warehouse	Belly-cavity lining	22	0
Salmon (USA)1995	Freezer	Head, warehouse	140	4
Salmon (USA)1995	Freezer warehouse	Tail	27	10
Salmon (USA)1995	Freezer	Flesh	45	26

Seafood type (Country) year	Sampling location	Specification	No. of samples	% of positive *L. monocytogenes*
	warehouse			
Salmon (Chile) 2003	Commercial outlet	Skin and surface swab	45	22
Salmon (Portugal) 1998	Producer	Flesh and Skin	74	11
Different fish species (Japan) 1998	Municipal fish market	10 g fish	25	12
Different fish species (Portugal) 2004	Producers, retail stores	25g fish	17	18
Oyesters, mussels, cockles (France) 1998	Live, collected on shore	25 g oysters, mussels, cockles	120	9
Shrimps (USA) 1991	Live, Collected	25 g shrimp	74	11
Shrimp (All over the world) 1994	Imported to USA, fresh and frozen	25 g shrimp	205	4
Crawfish (USA) 2002	2 processing factories	25 g crawfish	78	4
Crawfish (USA) 2004	2 processing factories	25 g crawfish	179	8
Different Shelfish (India) 1996	Fish market, processing factories	25 g shelfish	36	12
Shelfish (Middle East) 2006	Live	25 g shelfish	15	33

1.6 Isolation and identification of *L. monocytogenes* in cold-smoked salmon.

Historically, it has been challenging to isolate *Listeria* from food or other samples and this explains why it remained unnoticed as a major food pathogen until recently. In early studies it was noted that *Listeria* is able to grow at low temperatures and this feature has been used to isolate these bacteria from clinical samples by incubation for prolonged periods at 4ºC on agar plates until the formation of visible colonies. This method of isolation takes up to several weeks and usually does not allow for the isolation of injured *Listeria* cells, which will not survive and grow under stress.

Tests considered for approval by regulatory agencies must be able to detect one *Listeria* organism per 25 g of food. Generally, this sensitivity can only be achieved by using enrichment methods in which the organism is allowed to grow to a detectable level of 10^2 $CFUml^{-1}$. *Listeria* cells are slow growing and can be rapidly out-grown by competitors, and hence bacteriostatic agents, such as acriflavin and nalidixic acid that specifically act to suppress competing microflora, have been introduced into enrichment media or selective agar (Welshimer, 1981). These two agents are incorporated into all standard methods used to isolate *Listeria* from food and environmental samples. In the food industry, such standard culture procedures are used as reference methods for regulatory purposes and for validation of new technologies. These methods are sensitive, but often time, consuming and may take 5–6 days before the result is available.

In Ireland, the culture reference method used by the FSAI is the EN/ISO 11290-01 and -02 FSAI, 2007. This method requires enrichment of a 25 g food sample in a selective broth, designed to slow the growth of competing organisms, prior to plating onto selective agar and biochemical identification of typical colonies.

The ISO 11290 method has a two stage enrichment process: the food sample is first enriched in half Fraser broth for 24 h, and then an aliquot is transferred to full strength Fraser broth for

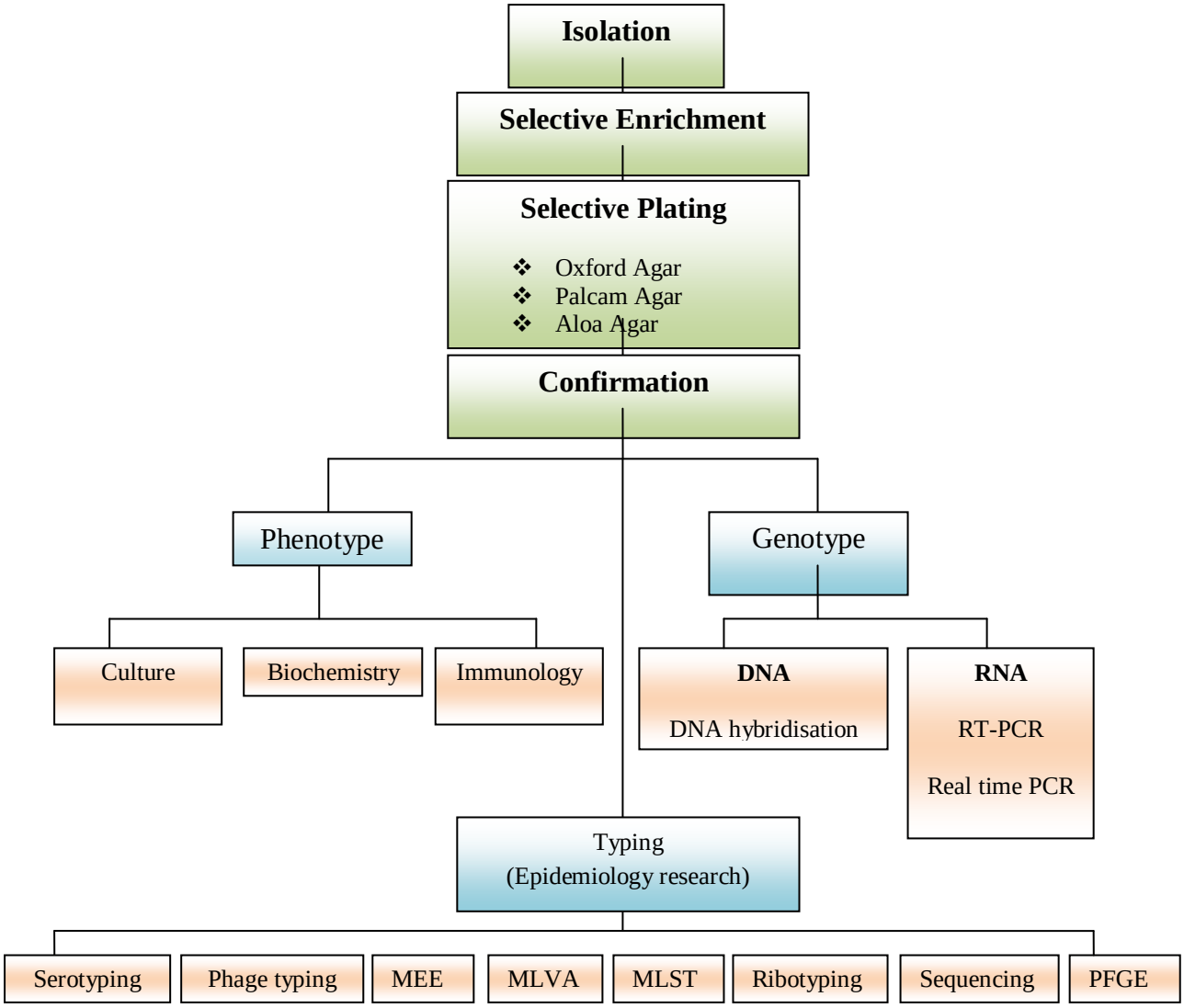
further enrichment. Fraser broth also contains the selective agents' acriflavin and naladixic acid as well as esculin, which allows detection of β-glucosidase activity by *Listeria*, causing a blackening of the medium. Both the primary and secondary enriched broth are plated on Oxford and Palcam agars.

Following the isolation method, the confirmation of presumptive *L. monocytogenes* colonies on the selective media according to the ISO 11290 is performed with Gram-staining, catalase reaction, motility at 25 °C, β-haemolysis test, fermentation of rhamnose and xylose and CAMP-test. These current isolation and identification methods for detection of *Listeria* in foods are laborious and time consuming; the three to six day period needed to determine whether a particular food sample is free of *L. monocytogenes* is unacceptable to large sectors of the food industry that deal with highly perishable products (Capita *et al.,* 2001).

Oxford and Palcam, the most widely used media does not differentiate between *Listeria* spp. Non-pathogenic species of *Listeria* cannot be excluded when selecting suspected colonies for confirmation (Gasanov and Hansbro, 2005). Furthermore, it is well known that, during enrichment in a selective broth, *L. monocytogenes* can be overgrown by faster growing *L. innocua* and, as a consequence, may be missed when picking only five colonies from the isolation for confirmation (standard for ISO 11290 method).

According to Capita *et al.,* (2001), the differential system of the Palcam and Oxford media showed significant number of *Bacillus* spp and *Enterococcus* spp growth on both the media, resulting in false positive results. The *Enterococcus* spp and *Bacillus* spp also utilise esculin and may have a similar appearance (Gasanov and Hansbro, 2005). Despite these challenges, classical cultivation techniques still remain the official methods used although many rapid molecular methods exist. Molecular screening using Polymerase Chain Reaction (PCR) is one of the most promising techniques for rapid detection of microorganisms in food. This

technique provides increased sensitivity for detection and therefore enhances the likelihood of detecting bacterial pathogens (Gasanovand Hansbro, 2005). PCR and DNA hybridisation method have become a feasible alternative to cultural and serological techniques. The major advantage that molecular techniques offer over conventional methods is that these are based on differences within the genome and do not rely on the expression of certain antigenic factors or enzymes to facilitate identification. They are extremely accurate, reliable and can be performed within a short time frame (Gasanov and Hansbro, 2005). An overview of the isolation and identification techniques is given inFigure 1.6.



Key: RT-PCR: Reverse transcriptase, MEE: Multilocus enzyme electrophorosis, MLVA: Multi Locus Variable number tandem repeat analysis, MLST: Multi Locus Sequence typing and PFGE: Pluse field gel electrophorosis

Figure 1-6: Overview of the isolation and identification techniques of *L. monocytogenes*

1.6.1. Molecular typing method

Molecular detection techniques are based on DNA hybridization, PCR, restriction enzyme analysis or direct sequencing of specific genes or loci (Gasanov, 2005). Direct sequencing of specific genes or loci is the most accurate way of comparing genetic differences or similarities. Due to the reliability of these techniques, methods that give arelatively accurate reflection of genetic variation as well as a high sample throughput in a rapid timeframe were

developed. These methods are aimed at establishing the degree of allelic variation of particular genes, which then forms the basis of measuring genetic relatedness of *Listeria* strains. Allelic variations can be measured as variations in the length of DNA fragments that can be generated, either by restriction digests or PCR, or as a change in conformation due to sequence differences conformational polymorphism (O'Connor *et al.,* 2000). Conformational polymorphism is generally considered to be most suitable for the detection of mutations in short stretches of DNA (O'Connor et al., 2000).

In order to interpret data with greater accuracy, electrophoretic techniques were developed to allow better resolution of DNA fragments, such as pulse-field gel electrophoresis (PFGE), which is primarily used in conjunction with restriction enzyme (endonuclease) digests of DNA (Rudy *et al.,* 2003).

Denaturing gradient gel electrophoresis (DGGE) or capillary electrophoresis (CE) are electrophoretic techniques that are used in conjunction with single strand conformational polymorphism (SSCP) analysis to detect single nucleotide variations. Some are well-established techniques such as ribotyping, macrorestriction digests, PFGE, PCR-restriction fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD), are used routinely.Other techniques such as SSCP, multilocus sequence typing (MLST) and multilocus variable number tandem repeat analysis (MLVA) are currently becoming established as typing techniques for *L. monocytogenes* and show great promise. However, they are not used routinely and not many field data exist for these methods (Gasanov *et al.,* 2005).

A. Sequencing 16S rRNA analysis

As direct sequencing of the entire bacterial genome is expensive and time consuming, partial sequencing of specific regions in the whole genomic region is carried out regularly as a part

of microorganism identification. The 16S rRNA gene is the most extensively sequenced ribosomal RNA gene. 16S rRNA genes contain stretches of highly conserved regions but also regions that are variable. Primers designed to target conserved DNA sequences can be used to analyze a wide variety of different organisms using PCR amplification with subsequent sequencing of the PCR product whilst highly diverse regions can be used to sub-type strains (Wang *et al.,* 1993). The use of 16S rRNA as a distinct signature for a bacterial species has become the method of choice for identifying and differentiating microorganisms when no other easily specifiednucleic acid sequence uniquely defines the desired target (Gasanov *et al.,* 2004). Differences between the species were observed, in 16S rRNA gene sequence analysis even between the closely related *L. monocytogenes* and *L. innocua*. Two sequence differences found within the V9 region were used to develop species-specific nucleic acid probes for *L. monocytogenes*, and their efficacy has been demonstrated in a hybridization assay. The ability to determine the relationship between different bacterial isolates from the same species is extremely important in tracking the source of a food-borne infection and identifying problematic reservoirs (Czajka *et al.,* 1993).

The other alternative to 16S rRNA gene sequence analysis would be to analysis RNA polymerase B subunit gene rpo B and Recombinase A (rec A). These are considered as highly conserved alternatives to 16s rRNA (Holmes, 2004).

B. Subtyping using Multiple-Locus Variable number of tandem repeat Analysis (MLVA)

Multiple-Locus Variable number of tandem repeat Analysis (MLVA) was developed as a new generation protocol to subtype food-borne pathogens including *Salmonella* (Lindstedt *et al.,* 2003), *Staphylococcus aureus* and *Escherichia coli* O157 (Lindstedt *et al.,* 2003). MLVA

is a PCR-based method that can be used to discriminate amongst different strains of a bacterium and can therefore infer genetic relationships amongst the various subtypes. This approach is based on the detection of the number of tandem repeats (TRs) at a specific locus in the genome of a microorganism. The complete TR is amplified and sized using a conventional agarose gel. These can vary as a consequence of DNA polymerase enzyme slippage, during replication and these differences can be detected using PCR primers designed to adhere to the flanking regions (Keim *et al.*, 2000).

How TRs are selected:

- Serotypes 4b (F2365) & 1/2a (EDG-e) used in the design of the MLVA technique.
- Both serotypes - among the most prevalent in food-borne listeriosis cases
- cover 2 main genomic regions of *L. monocytogenes*
- (i.e. serotype 1/2a is genomic division I and serotype 4b genomic division II).

Process of how primers are designed based on the TRs

1. Sequences were downloaded from the GenBank website (www.ncbi.nlm.nih.gov/).

2. Submitted into Tandem Repeats Finder Programme (http://tandem.bu.edu/),

- identifies,

- locates,

- displays TRs in DNA sequences (Benson, 1999).

3. Suitable repeats- selected based on their location, period size & copy number.

1.7 Risk Analysis

Increased international trade and expansion of the population of the most susceptible to this food-borne disease, especially the elderly (> 60 years) and immunocompromised individuals suffering underlying diseases, has marked *L. monocytogenes* as an important food-born pathogen (Garrido *et al.*, 2009). Therefore, it is necessary to provide sufficient information to consumers regarding risk of consumption of cold-smoked salmon contaminated with *L. monocytogenes*. Risk assessment is the scientific process of determining the relationship between exposure to a given pathogen under a defined set of conditions and the likelihood of an adverse health effect or disease (Pouillot *et al.*, 2007).

Quantitative Microbiological Risk Assessment (QMRA) can help in to obtain the necessary information regarding the severity of a health disturbance because it is based on knowledge concerning exposure to the pathogen and an individual's response (Mataragas, *et al.*, 2010). In addition to assessment of probable severity of harm caused by a specific hazard, other economical factors must be considered; such as product recall cost, loss of costumers and potential costs of compensation. For this reason, governments and industries have begun to focus their attention on safe food practises to minimise risk to public health (Cassin *et al.*, 2008). With the increasing trend of listeriosis in Ireland (FSAI, 2008) and listeriosis being high among the other zoonotic diseases, especially among the vulnerable population, it is important to develop a risk assessment profile for this pathogen. In addition, the expansion of population susceptible to this food-borne disease, especially the elderly and immunocompromised suffering underlying diseases, has led to the control of *L. monocytogenes* being one of the goals set by governments and food industries throughout the world (Painter and Slutsker, 2007).

Microbiological risk assessments consist of four essential steps; hazard identification, hazard characterisation, exposure assessment and risk characterisation (Cornu and Beaufort, 2005). The hazard identification is the process of identification of the biological, chemical and

physical agents capable of causing adverse health effects and which may be present in a particular food or group of foods. The hazard characterization is the qualitative and/or quantitative evaluation of the adverse health effect associated with the hazard, the relationship between exposure levels (dose) and frequency of illness. The exposure assessment is the qualitative and/or quantitative evaluation of the likely intake of the hazard, as well as exposure to other sources (if relevant). Finally, the risk characterisation is the qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of the known or potential adverse health effects based on hazard identification, hazard characterisation and exposure assessment (Rocourt *et al.,* 2001).

1.7.1 Hazard Identification

The high prevalence of *L. monocytogenes* in vacuum packed cold-smoked salmon, together with the high mortality rate from listeriosis, suggests that *L. monocytogenes* represents an important hazard to human health (Autio *et al.,* 1999, Rorvik *et al.,* 2000, Azevedo *et al.,* 2005, Miettinen and Wirtanen 2006, Pouillot *et al.,* 2007, Lenhart *et al.,* 2008 and Garrido *et al.,* 2010).

1.7.2 Hazard Characterisation

Serotyping distinguishes 13 different serotypes of *L. monocytogenes*, but cases of human listeriosis are caused mainly by only three serotypes (4b, 1/2a and 1/2b). Most human epidemics and a great percentage of the sporadic cases have been caused by serotype 4b for a reason not yet understood. In contrast, serotype 1/2a strains seem to be more often recovered from food (Lindqvist *et al.,* 2008). For example, 64 % of the clinical isolates occurring in the Ireland are serotype 4b, whereas only 4 – 6 % of the isolates found in the environment are of this serotype (Murphy *et al.,* 2007). No studies have been able to explain why a serotype 4b accounts for most cases of human listeriosis and at the same time it is seldom found in foods. Thus, as extensive investigations have failed to find systematic differences in virulence

between food isolates that have not been implicated in listeriosis, and clinical isolates, all *L. monocytogenes* strains should be considered pathogenic (Murphy *et al.,* 2007). However, analyses accompanying epidemiological investigations have indicated that foods implicated in both sporadic cases of listeriosis and outbreaks, typically had elevated levels of the pathogen. Furthermore, foods which have been implicated in human outbreaks of listeriosis, have always have been foods which are known to support growth of the pathogen. In addition, most people regularly ingest foods containing low numbers of *L. monocytogenes* without becoming ill and there are little suggestions that an accumulative effect is significant. From animal experiments it is know that *Listeria* infections are dose dependent and that the infectious dose is rather high, above 10^5 in different models for intragastrial inoculation (Schlech *et al.,* 1993). However, it is not known exactly how to extrapolate these data to humans. New approaches using dose-response models based on probability distributions have been introduced (Farber *et al.,* 1996 and Buchanan *et al.,* 1997). These dose-response models could be incorporated in the risk analysis and the exposure to *L. monocytogenes* could be estimated.

1.7.3 Exposure Assessment

In the last few years, several surveys have provided knowledge of both the qualitative and quantitative presence of *L. monocytogenes* in vacuum packed cold-smoked- salmon (Buchanan, 1997, Lindqvist and Westoo, 2000, Cornu and Beaufort 2005, Pouillot *et al.,* 2007, Lenhart *et al.,* 2008 and Garrido *et al.,* 2010).

The exposure assessment describes the pathways through which a pathogen population is introduced, distributed and altered in the production, distribution and consumption of food (WHO, 2006). The result desired from the exposure assessment is the prevalence, concentration and, if possible, virulence of the pathogen in foods at the point that they are consumed and the level of consumption of the food by the population of interest. The key

desired output of the exposure assessment is prevalence, concentration and, if possible, physiological state of *L. monocytogenes* in foods at the point of consumption (Cornu *et al.*, 2006). In the case of *L monocytogenes*, although the final numbers ingested by consumers are usually not known, an estimate can be derived based on models of the effect of physical processes and conditions that the food undergoes through the 'farm-to-fork' chain. Such estimates are based on predictive microbiology models. The models are parameterized by data from studies carried out on products shelf life and their ingredients at different stages in the production-to-consumption chain. These models can be used by the food industry and the government to minimize and estimate food poisoning (Cornu *et al.*, 2006).

1.7.4 Risk Characterisation

Characterizing the risk associated with *L. monocytogenes* in foods involves aconsideration of all the information gathered in the hazard identification, hazardcharacterization and exposure assessment steps. It can be helpful in determining thecause of the risk and in providing managers with background information to carry out risk management. This information can be combined to assess various outputs, i.e., the annual incidence of listeriosis, the impact of time and temperature abuse on probability of illness and the effectiveness of various exposure reduction strategies (Lindqvist and Westoo, 2000). Although the quantitativerisk assessment approach is preferred over the qualitative approach, it is notyet clear whether the former approach is possible and/or appropriate for characterizingthe risks associated with food-borne bacterial pathogens (Garrido *et al.*, 2010).

An important measure of the level of risk to listeriosis through exposure from aspecified food source is the annual incidence rate, i.e. actual annual number ofhuman listeriosis cases. The incidence of listeriosis depends on the proportion of thepopulation exposed to *L. monocytogenes* (prevalence), the level of exposure of eachindividual at risk, and their individual tolerances (Cornu and Beaufort, 2005).The incidence of listeriosis can be

interpreted as the product of the annualincidence of exposure and the average probability of illness. The incidence of exposure is a combination of the prevalence of virulent *L. monocytogenes*strains in the total annual servings ofthe specified food and thenumber of servings per capita (Pouillot *et al.*, 2007).

1.8 Microbial-growth modelling

Risk assessments typically rely on microbial growth models to characterise growth of the microorganism under environmental factors controlling the response of microorganism in food. Microbial models are mathematical expressions that describe the number of microorganisms in a given food product or system, as a function of relevant intrinsic or extrinsic variables (temperature, pH, a_W and salt content; Marks, 2008). They quantify populations of organisms, or probabilities of the presence of organisms.

Microbial models can be classified as primary, secondary or tertiary (Cornu *et al.,* 2006). A few combined models have been constructed to describe changes in a microbial population subjected to conditions that vary from the growth to inactivation ranges (Ross *et al.*, 2005). The use of mathematical modelling techniques for safety and risk prediction in the food supply is strongly supported by the incidence of food-borne outbreaks worldwide (Marks, 2008)

1.8.1 Primary models

Primary models describe the evolution (i.e. growth, inactivation or survival) of microorganisms as a function of time (Baranyi *et al.,* 1993**).** The most frequently used primary growth models are the modified Gompertz and Baranyi (Baranyi *et al.*, 1993) equations, the first being a sigmoidal relationship and the second being based in part on the concept that the rate of bacterial growth is controlled by the rate of a “bottleneck”

biochemical reaction. The Gompertz equation has been extensively used by researchers to fit a wide variety of growth curves for different microorganism. The Gompertz function has also been applied to growth curves based on turbidity data, mixed cultures of *Pseudomonas* spp. and *Listeria* spp.; *Lactobaciilluis curvatus*; spoilage of vegetables, beer, and meat; and germination and growth of *Clostridium botulinum*. There are, however, some limitations associated with the use of the Gompertz function. The Gompertz rate (μ_{max}) is always the maximum rate and occurs at the arbitrary point of inflection, thus the generation time can be underestimated by as much as 13 %. In addition, since the slope of the function cannot be zero, the lower asymptote must be lower than the inoculum level, giving a negative (lag phase) for some data sets.

The Baranyi model has been used extensively to model microbial growth. The model has been facilitated by the availability of two programs: DMFit, an Excel add-in and MicroFit, a stand-alone fitting program by the Institute of Food Research (IFR) in the U.K. One of the advantages of the Baranyi model is that it is readily available as a series of differential equations that allow modelling in a dynamic environment, generally resulting from non-isothermal temperature profiles (Marks 2008).

Xanthiakos and co-workes (2006) found that the Gompertz model seems to be influenced more by the quality of the data set than is the Baranyi model. They also concluded that the Baranyi model provided the best fit for the majority of their data and gave reasonably precise estimates of the lag time. Another study also found that the Gompertz equation can overestimate the model parameters, which could bias the comparison with a different model (Zwietering *et al.*, 1994).

1.8.2 Secondary modelling

There is significant information about the impact of individual variables (particularly temperature) on the growth and inactivation of bacteria. For example, several reviews have summarised significant heat resistance for various pathogens, as influenced by food material, temperature and pH (Dalgaard, 1998). Numerous studies have evaluated the effect of environmental factors such as temperature, oxygen and level of mixed microbial population in the food. However, the most important factor for controlling microbial growth in the production and distribution chain of chilled foods is temperature (Garrido *et al.,* 2010). Although microbial growth occurs from about -8 to +90ºC, the growth rate of most microbes of significance to food poisoning decreases above 35 – 40ºC. Within the practical distribution range for refrigerated products (0–35ºC), temperature affects the duration of the lag phase, the rate of growth, and the final cell numbers. Food-poisoning bacteria can multiply within a temperature range from about 0 – 50ºC; however, refrigerated storage will favour gram-negative bacteria and psychrotrophic pathogens, whereas higher storage temperature may favour mesophilic food-born spoilage microorganisms (Cornu *et al.,* 2006). It is presumed that the temperature will also affect the multiplication rate below the detection limit. Two models, Arrhenius and Ratkowsky's equation (square root model), have been widely used to investigate the temperature dependence of the growth of *L. monocytogenes*.

A.Arrhenius model

Based on thermodynamic considerations, the Arrhenius model has had success in describing the temperature dependence of many chemical reactions related to shelf life of food. Since the replication of the gene during cell division is a chemical process, it seems logical that the growth rate would follow the Arrhenius law for a certain temperature range.

Arrhenius model is:

$\mu_{max} = A \times \exp(-E_a / RT)$ ---------- Equation 1-1

Where T is the temperature (K), E_a is the activation energy (KJ/mol). A is a pre-exponential factor (log CFU/g^{-}days^{-1}) and R is the gas constant 8.31 (KJ; Kmol)$^{-1)}$. The activation energy E_a is considered as the sensitivity of the microorganism to temperature change. The Arrhenius model could also be used to model the temperature dependence in both of the lag phases, the lower one of which would be the most critical phase for prediction of safe shelf life under variable temperature conditions, where the initial microbial load is below detectability as would be expected for pathogens. To make the Arrhenius plot, the inverse of the lag time is used; however, the fit is usually not as good as for the growth rate data from the exponential phase (Dalgaard, 1998).

B. Ratkowsky's equation

Ratkowsky and co-workers (1982), proposed an empirical equation for the temperature dependence of microbial growth up to the optimum temperature as:

$$\sqrt{\mu_{\max}} = b\,(T - T_{min}) \text{..... Equation 1-2}$$

Where b is a constant, T (°C) is the storage temperature and $T_{min}$is the theoretical minimum temperature for growth of the organism.$T_{min}$was estimated by extrapolation of the regression line to $\mu_{max} = 0$. McMeekin and co- workers (1988), suggested that the non-applicability of the Arrhenius model for modelling the temperature-dependence of microbial growth is a result of the change in E_a value with temperature. They related the activation energy to the Ratkowsky equation by: $E_a = 2RT^2/ (T - T_{min})$. Thus, the change of E_a for a given organism is greater for a low value of T_{min} (5<T<30 ˚C). However, this correction may not be fully correct since the pre-exponential factor (k_0) in the Arrhenius equation may also be changing with temperature. It should be noted that Fu and co-workers (1991), demonstrated with *Pseudomonas fragi* growth in a simulated milk system, that if enough data points are collected over time (i.e. 15 or more), and at least five temperatures are used, there is a good

fit for both the Arrhenius model and the Ratkowsky equation. This study demonstrates the benefit of using many data points to fit simple equations, rather than using over manipulated empirical equations.

1.8.3 Tertiary modeling

Tertiary model is defined as the integration of primary and secondary models with a user-friendly interface (Whiting, 1995). Tertiary modelling tools which are freely available and are widely used are ComBase (www.combase.cc), available from the Food Standards Agency and the Institute of Food Research and Seafood spoilage and pathogen modelling (SSSP-DTU Aqua National Institute of Aquatic Resources Denmark), the Pathogen Modeling Program (PMP, v.7.0) developed by the USDA—Agricultural Research Service (USDA 2003).

ComBase Predictor is an on-line tool for predicting the response of pathogens and spoilage microorganisms to key environmental factors (temperature, pH and salt concentration, etc) characterising the food environment. The majority of its models predict the growth/survival of food-borne pathogens as a function of temperature, pH and salt concentration, but in some cases the effect of an additional fourth environmental factor, such as the concentration of carbon dioxide or organic acids is modelled, too. ComBase Predictor can simultaneously produce predictions for up to four microorganisms. It is also capable of predicting the bacterial response to dynamic temperature environments (Baranyi and Roberts, 1994).

The Seafood Spoilage and Safety Predictor (SSSP) software predicts shelf-life and growth of bacteria in different fresh and lightly preserved seafoods e.g. the effect of product temperature profiles recorded over time by data loggers. The SSSP software is developed specifically to predict shelf-life and safety of seafood. SSSP can predict growth of *L.*

monocytogenes in vacuum packed cold-smoked salmon for both constant and changing storage temperatures (Mejlholm and Dalgaard, 2009).

The PMP is a package of models that can be used to predict the growth and inactivation of foodborne bacteria, primarily pathogens, under various environmental conditions. These predictions are specific to certain bacterial strains and specific environments (e.g., culture media, food, etc.) that were used to generate the models. The accuracy of these predictions cannot be guaranteed for other bacterial strains and/or environments, without proper validation studies. Since the early 1990s, the PMP has been distributed in various forms, ranging from spreadsheets to stand-alone software, and most recently online. We recommend that you bookmark this webpage and revisit it on a periodic basis since we are continuously adding new models and other enhancements (Koutsomanis, 2010).

Aims of the study

L. monocytogenes differs from most known food-borne pathogens in that it is ubiquitous, resistant to diverse environmental conditions including low pH and high NaCl concentrations, and is microaerophilic and psychrophilic. Given the high rate of mortality associated with *L. monocytogenes* and with an increase in the reported cases of listerisos in Ireland, there is a need to investigate the occurrence of this bacterium. The lack of knowledge in the route of contamination in which the bacterium enters a seafood processing plant, its tenacity to survive the industrial environment, its ability to grow at very low temperatures and to survive in cold-smoked salmon for prolonged periods under adverse conditions have made this bacterium a very important topic for research. Considering the above points, there is a need to developa risk assessment based study specific for Ireland, as there has been no previous study to estimate the risk associated with the consumption of vacuum packed cold-smoked salmon for consumers in different risk population in Ireland (immunocompromised and immunocompetent population).

Therefore the overall aim of the present thesis was to investigate the prevalence and source of *L. monocytogenes* in different stages of vacuum packed cold-smoked salmon production chain /retail market and to develop a risk assessment model. This will enable a closer analysis of the source and contamination pathways of *Listeria* in Irish vacuum packed cold smoked salmon and facilitate the identification of possible control measures.

In order to achieve the specific aim above the following objectives were identified:

- Assessment of isolation methods for *L. monocytogenes* in vacuum packed cold-smoked salmon
- To determine the prevalence of *L. monocytogenes* in a vacuum packed cold-smoked salmon marketed in the retail outlets in Dublin, Ireland.

- To investigate the source and ecology of *L. monocytogenes* in vacuum packed cold-smoked salmon processing factory.
- To study the possible *L. monocytogenes* contamination pathways by tagging raw salmon along the cold-smoking processing stages in a cold-smoked salmon processing factory.
- To develop a product-specific growth model of *L. monocytogenes* (inoculated studies) in vacuum packed cold-smoked salmon and validation under dynamic temperature conditions.
- To establish a quantitative microbial risk assessment of *L. monocytogenes* in vacuum packed cold-smoked salmon in Dublin, Ireland.

Chapter 2 General Materials and Methods

2.1 Sampling

A. Prevalence of *L. monocytogenes* in retail vacuum packed cold-smoked salmon

Vacuum packed pre-sliced cold-smoked salmon (250 g) were purchased from 5 retail establishments in Dublin (2007-2008). The five brands used in this study were coded as brand A through to E. The samples were returned to the laboratory under refrigerated conditions within 30 min of purchase and the samples were processed immediately upon arrival in the lab. Two samples from each brand were tested every month for a period of one year giving a total of 24 samples of each brand. The samples represented a shelf life period of 18-20 days and were sampled in a range from 7 days up to 1 day prior to the expiry date. For all the samples, refrigeration temperatures were maintained at 4 ($\pm$ 1) °C.

The brand A, was produced in an Eastern European country, brands B, D, and E were produced by a common Dublin processor and brand C was produced in the UK. The Dublin and UK processors procured raw fillets from the same slaughter house in the UK.

B. Determination of contamination pathways of *L. monocytogenes* in vacuum packed cold-smoked salmon

A total of 12 surveys (a monthly survey) were conducted in a smoked salmon production plant located in Dublin. In order to investigate the persistence of contamination during different seasons the plant was surveyed from winter (Sep 2008 to April 2009) to summer (May 2009 to Aug 2009).

The processing plant was divided into four zones (Figure 2.1) based on the contact and proximity to the product (FASI, 2008). A total of 444 samples from four zones were sampled

during the 12 month period. Thirty seven samples were examined each month and the samples were collected from the same place to maintain consistency throughout the survey.

The survey sampling was carried out in three different ways depending on the nature of the sample. Samples from the equipment were swabbed (conveyor belt, slicer, smoking tray, deboning pin, tables, vacuum packer, cold and frozen unit, filleting board and skinner), production environment (floor, drain, walls and switch), personal (gloves, aprons, boots, head protection and sanitizers) and the surrounding environment (waste collection point, service area, rubbish collection area, waste disposal site and traffic area). Each site was swabbed 3 times using sterile cotton swab moistened with 0.1 % peptone water and the swabs were pooled as one sample. The swabs were exposed for 60 seconds to the swabbing environment, the swabs where exposed to the same area during the 12 month survey. The samples were transported under refrigerated conditions to the laboratory for further processing.

Samples of salmon were taken after each critical processing step (raw salmon, cured salmon, cold-smoked salmon and finally vacuum packed cold-smoked salmon). In all cases 50 g of the samples were taken and sealed in a sterile Petri-plate and transported under refrigeration condition to the laboratory for further processing.

Liquid samples (drain, fish wash, brine and ice) aliquots of 25 ml were taken in sterile bottles and transported under refrigeration conditions to the laboratory for further processing.

All the samples were collected after 1 to 1 ½ h of production run of the processing plant.

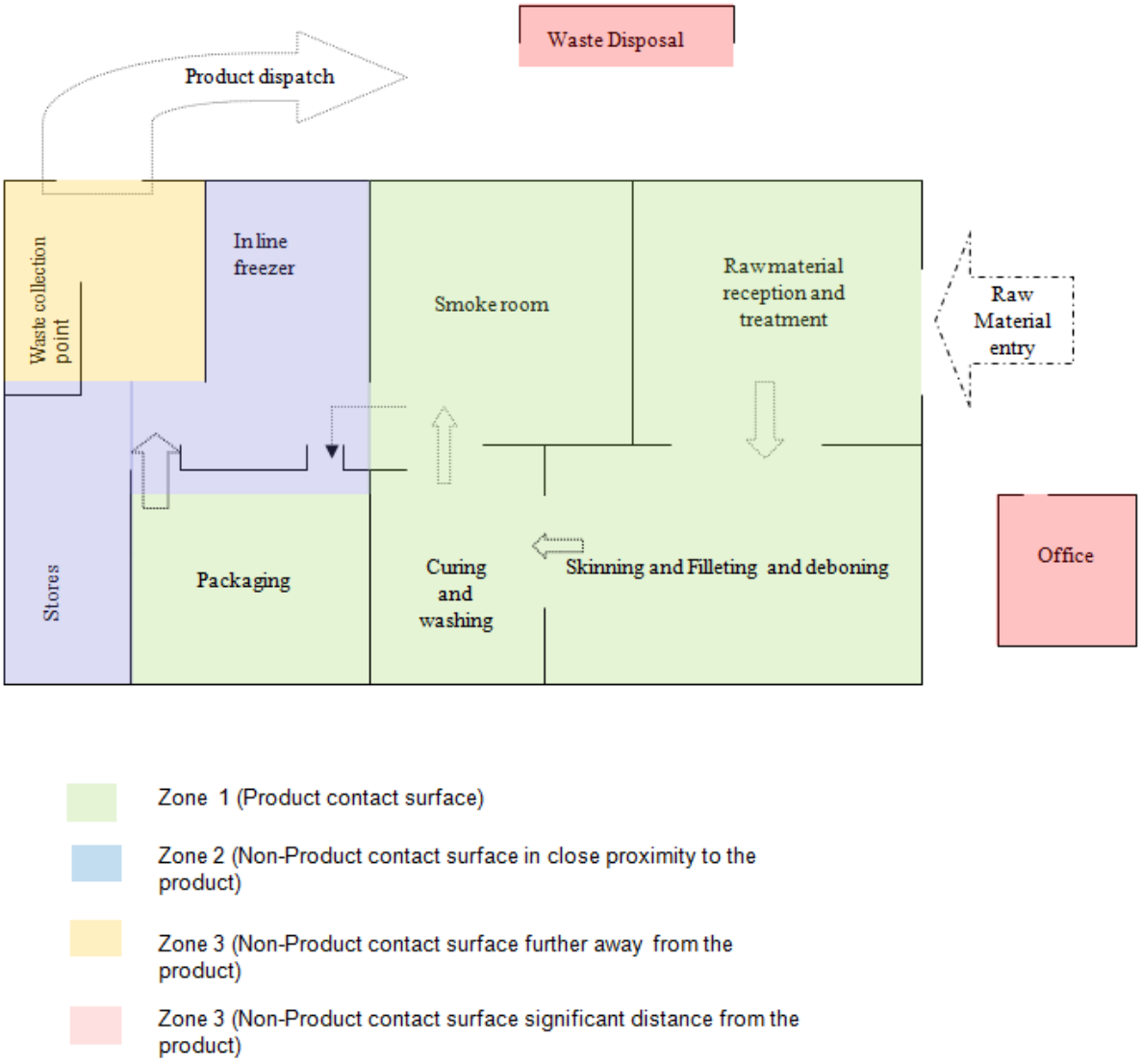


Figure 2-1: Schematic representation of the cold-smoked salmon processing factory floor plan representing the four zones.

C. Confirmation of the contamination pathway of *L. monocytogenes* by tracking the cold-smoked-salmon through its various processing stages

A total of 12 surveys were undertaken from Dec 2008 – Nov 2009 in a cold-smoked salmon processing factory located in Dublin. A total of 60 raw salmon were tracked through the production line. Raw fish analysed were tagged with a wire string and passed through the various intermittent stages of processing. Samples containing 25 gm of fish mass were

collected from each critical processing step (raw, filleted, cured, cold-smoked, sliced and final vacuum packed cold-smoked salmon).

Each month during the study period, 5 raw salmon were tracked through the different stages of smoked-salmon processing (Figure 2.2). The samples were collected after 1 ½ h of production run of the factory and were transported within 30 min to the lab for further processing. The samples were collected in a sterile Petri-plate and were transported under refrigerated conditions to the lab for further analysis.

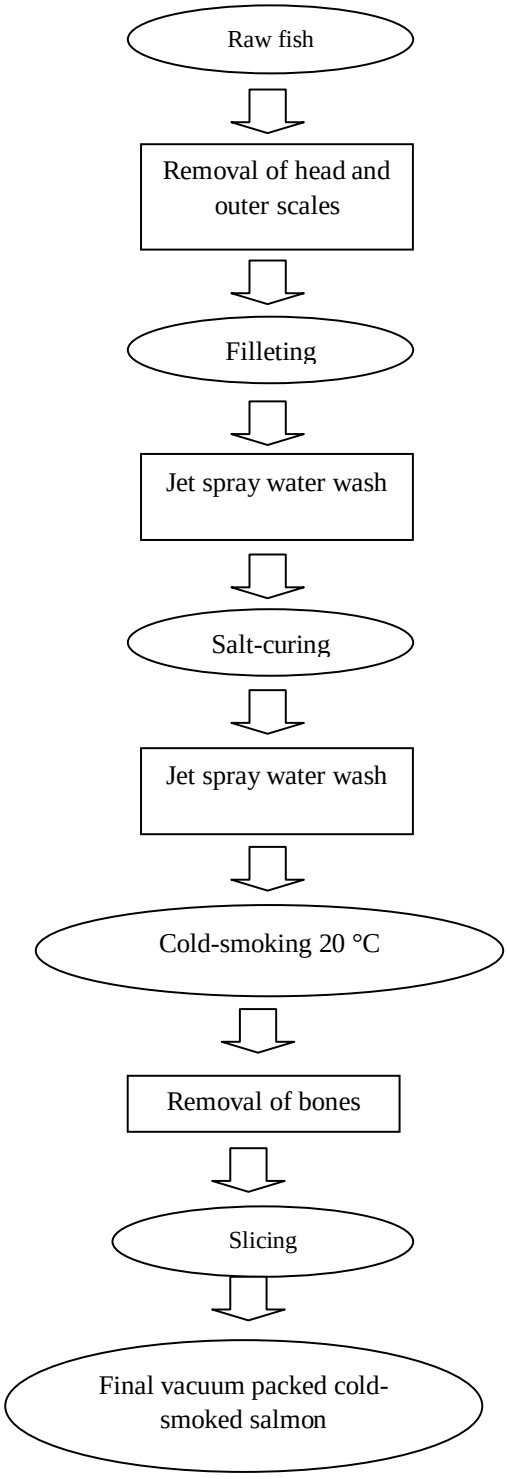


Figure 2-2: Schematic representation of different processing stages during the production of vacuum packed cold-smoked salmon. The stages represented in oval sketch were taken for sampling.

2.2 Detection and enumeration of *L. monocytogenes*

Detection of *L. monocytogenes* was carried out as recommended in the ISO 11290 -01 and 02. According to the procedure; 25 g of each sample was homogenised with 225 ml of buffered *Listeria* enrichment broth base (Oxoid, Hampshire, England) in a stomacher Lab-Blender 400 (Seward Medical, London, UK) for 2 min. The homogenate was incubated at 30 ℃ for 1 h to resuscitate stressed microorganisms. For the enumeration of *L. monocytogenes*, a volume of 0.1 ml from each homogenate was directly spread on Palcam agar (Oxoid, Hampshire, England) plates in triplicat and incubated at 37 ℃ for 24 - 48 h. From each plate, 5 colonies presumed to be *Listeria* spp. were streaked onto tryptic soy agar plates (Scharlau – Chemie on Palcam) and incubated at 30 ℃ for 24 h (Appendix 1: Colony morphology of *L. monocytogenes*). Colonies which showed typical appearance (Appendix 1) of *L. monocytogenes* on tryptic soy agar (TSA) were taken further for Gram's staining, catalase and oxidase test (Appendix 2). Isolates which were rod shaped and Gram positive and showed positive for catalase and negative for oxidase were tested for haemolytic activity and CAMP tests on sheep blood agar for *L. monocytogenes* confirmation.

2.3 Native flora estimation

The cold-smoked samples were analysed for the estimation of the background flora. Enumeration was carried out by transferring 25 gm of smoked salmon aseptically into a stomacher bag and blending with 225 ml of ¼strength Ringer's solution in a stomacher (Lab blend 400, Seward Medical, London, UK) for 60 s at medium speed. One ml of the homogenate was taken and decimal dilution was prepared with ¼ Ringer's solution and 0.1 ml of the diluted sample was spread on duplicates plate count agar (Scharlau – Chemie, Barcelona, Spain) for background flora estimation (total bacterial population on cold-smoked

salmon, this study dint look into any specific bacterial community in the native flora estimation) at 24 °C for 48 h. Data from the plate count were log transformed.

2.4. DNA extraction

The bacterial isolates for presumptive *Listeria* spp. were inoculated into 10 ml tryptic-soya-broth (Scharlau– Chemie, Barcelona) and incubated at 37 °C for 24 h. The overnight cultures were centrifuged at 13,000 g for 2 min and the pellets were recovered. The pellets were used to obtain the genomic DNA using Promega Wizard Genomic DNA purification kit (Promega, Madison, WI) according to the manufacturers' recommendations.

A. 16S rRNA gene sequence analysis

L. monocytogenes isolates, representatives of the positive samples, were subjected to 16S rRNA gene sequence analysis. The DNA was extracted as in section 2.4. The 16S rRNA gene was amplified using 27 f and 1525 r primers (Lane, 1991). The final primer concentration in the reaction mixture was 0.4 µM. The oligo nucleotide sequences of the primers and PCR conditions are tabulated in Table 2.1 and Table 2.2.

The identification strategy included detection of *L. monocytogenes* specific signature sequence (sequencing carried out at Geneius lab, Newcastle University, UK), which was obtained by alignment of all 16S rRNA gene sequences (obtained from National Centre for Biotechnology-**NCBI**) of the genus *Listeria* using CLUSTAL W (alignment setting for CLUSTAL W: gap opening penalty of 10 and gap extension penalty of 0.2) and selection of *L. monocytogenes* specific sequence. The oligonucleotide sequence obtained (**AGTACAAAGGGTCGCGAAGCCGCGAGGTGGAGCTAATCCCATAACT)** was checked *in silico* byBasic Local Alignment Search Tool (**BLAST**) against the sequences on

Ribosomal Datatabase Project (**RDP**) and (**NCBI**) databases. The sequencing reactions were carried out at the 3' end as a single directional read spanning about 500 bps. The sequences which showed the presence of the signature nucleotide were identified as *L. monocytogenes*. The rest of the strains were assigned to taxa based on BLAST hits of the partial sequence.

Table 2-1: Oligo nucleotide sequence of primers for *L. monocytogenes*

Primer	Sequence
27f	AGAGTTTGATCMTGGCTCAG
1525r	AAGGAGGTGWTCCARCC

Table 2-2:PCR conditions for the amplification of *L. monocytogenes*isolates

PCR (condition)	Temperature (°C)	Time(min)	
Initial denaturation	95	10	
Denaturation	95	1	
Annealing	55	1	35 cycles
Extension	72	1	
Final Extension	72	10	

B. MLVA typing

L. monocytogenes isolates representative of the positive samples were analysed for MLVA. The DNA was extracted as in section 2.4. Amplification of the DNA was carried out by six primers set as in Table 2.3 (Murphy *et al.,* 2007). Primer sets were synthesized commercially by MWG-Biotech AG (Ebersberg, Germany). The final primer concentration in the reaction mixture was 0.4 µM. GoTaq Hotstart polymerase master mix (Promega, Madison, WI) was

used for all the reactions. All amplifications were performed using thermal cycler (G-Strom GS-1, Gene technology Ltd, UK). The PCR conditions are mentioned in Table 2.4.

Amplified products (5 µl, approximately 100 ng) were resolved by electrophoresis through horizontal 2 % (w/v) agarose gels at 80 V for approximately 2 h, in (1x) TBE buffer containing 0.5 µg/ml ethidium bromide and the results visualised and photographed in a Gel Documentation system (UVP laboratory products, UK). Equal concentrations of molecular weight marker (100bp DNA Ladder, Sigma, Germany) were included in all gels to facilitate the sizing of the amplified DNA fragment Index.

Table 2-3: MLVA Primer sets (Murphy *et al.*, 2007)

Primer	Sequence 5′ to 3′	Size [mer]	Tm °C	% GC	Amplicon (bp)	TR Sequence	TR	Copy no.	Diversity Index (%)
LM-TR 1	-GGC GGA AAA TGG GAA GC-	17							
	-TGC GAT GGT TTG GAC TGT TG-	20	52.5	36	*652 [695]	-TAAAACCTA-	9	4.8	77.8
LM-TR 2	-CCT AGA ACA AAT CCG CCA CCA T-	22							
	-TCG CCA TTG TAA ACA TCC CCT ATT-	24	52.7	32.9	*569 [613]	-TATTTTTATTTAAAAATG-	18	2.4	50.5
LM-TR 3	-GCG TGT ATT AGA TGC GGT TGA G-	22							
	-GCA TTC CAC TAT CCC CTG TTT T-	22	53.1	38.5	*423 [548]	-CCGGTAGAT-	9	13.9	86.5
LM-TR 4	-TCC GAA AAA GAC GAA GAA GTA GCA-	24							
	-TGG AAC GAC GGA CGA AAT AAT AAT-	24	52.5	34.2	*450 [478]	-GAAGAACCAAAA-	12	2.3	68.2
LM-TR 5	-GTT TAT GCG AAT GGC GAG AT-	20							
	-CTG GCT TCA TAG GAT TTA CTG GAT-	24	52.2	41.4	*203 [390]	-GTAGATCCG-	9	20.9	86.6
LM-TR 6	-AAA AGC AGC GCC ACT AAC G-	19							
	-TAA AAA TCC CAA TAA CAC TCC TGA-	24	51.9	39.7	*232 [268]	-CCAGACCCAACA-	12	3	81.5

Table 2-4: PCR conditions for the amplification of *L. monocytogenes* isolates

PCR (condition)	Temperature (°C)	Time(min)	
Initial denaturation	95	10	
Denaturation	95	1	
Annealing Lm1 to Lm5	55	1	25 cycles
Annealing Lm 6*	52	1	
Extension	72	1	
Final Extension	72	10	

*** Primer Lm 6, the PCR conditions were similar to Lm 1 to LM 5 but the annealing temperature was 52 °C. This was carried out as separate PCR reaction**

2.5 MLVA data analysis

The sizes of the amplicons and number of tandem repeats were calculated using Quantity One Software (Version 3.2, Biorad, Hercules, CA). The allele strings were imported into a BIONUMERICS software package (version 4.5; Applied Maths, Sint-Martems-Latem, Belgium). A cluster analysis used the categorical coefficient and the neighbour joining algorithm. The use of categorical parameters implies that character states were considered unordered. The same weight was given to a large or small number of differences in the number of repeats at each locus. The genetic diversity at each TR was calculated in BIONUMERICS software package using Simpson's index.

2.6 Inoculation of vacuum packed cold-smoked salmon

Fifty grams quantities of vacuum packed cold-smoked salmon were obtained from a smoked salmon processor in Dublin. A strain cocktail of *L. monocytogenes* was prepared by combining each of the 3 strains of *L. monocytogenes* isolated from smoked salmon

(Chitlapilly Dass, 2010a). These mixtures of bacteria were cultured overnight in sterile tubes containing 10 ml tryptic soy broth at 30 °C. After the incubation, the culture tubes were centrifuged at 3000 x g at 4 °C for 30 min. The pellets were washed with sterile ¼ Rin er's solution (Oxoid Ltd, UK) and centrifuged for 30 min at 3000 x g at 4 °C and the resulting pellets were resuspended in ¼ Ringer's solution to a final volume of 5 ml. The cell level in the resulting composition was 6 log CFU/ml, as assessed by McFarland's standard 1 (BioMérieux, Marcy-l'Etoile, France).

To obtain a 1 log CFU/ml, 200 µl of the appropriate diluted culture were added to 50 gm of cold-smoked salmon sample. To achieve uniform distribution, the inoculum was spread on the surface of the smoked salmon samples using sterile spatula and finally the cold-smoked salmon sample was re-vacuumed packed (Multivac, MSC, Ireland). A total of 18 samples along with 18 duplicates were inoculated.The process of inoculation and re-vacuum packing of the smoked salmon samples took 10 min per sample.

2.7 Microbial analysis for inoculated studies

The cold-smoked samples were analysed everyday for 18 days (shelf-life as provided by the manufacturer). Enumeration was done by transferring 25 gm of smoked salmon aseptically into a stomacher bag and blending with 225 ml of ¼ Ringer's solution in a stomacher (Lab blend 400, Seward Medical, London, UK) for 60 s at medium speed. One ml of the homogenate was taken and decimal dilutions were prepared with ¼ Ringer's solution. Aliquots of 0.1 ml sample of three appropriate dilutions were spread plated in duplicate on Palcam (Scharlau – Chemie, Barcelona, Spain) for *Listeria* and incubated for 48 h at 30 °C and on plate count agar (Scharlau – Chemie, Barcelona, Spain) for native flora estimation at 24 °C for 48 h. Data from the plate counts were log transformed.

2.8. Physicochemical estimation

The pH of cold-smoked salmon was measured using an Orion pH meter (Beverly, Mass., U.S.A.). The water activity (a_w) of cold-smoked salmon was measured using an AquaLab Model CX2 water activity meter (Decagon Devices, Pullman,Wash., U.S.A.). The pH and $a_w$was measured for each day of the cold-smoked salmon sampling for the inoculated study. The samples were recorded as replicates of five per sample and the mean was noted as final value.

2.9. Microbial modelling

A. Primary modelling

Microbial counts obtained from the microbiological analysis for shelf life were fitted using DMfit Excel Add-In software (www.ifr.bbsrc.ac.uk) which is based on the model of Baranyi *et al.*, (1993) and resulted in the generation of growth parameters; maximum growth rate (μ_{max}), lag phase (λ) and maximum population density (MPD).

B. Secondary modelling

Square root model was used to estimate the maximum specific growth rate at isothermal conditions as a function of temperature (Ratkowsky *et al.*, 1983)

$$\sqrt{\mu_{max}} = b\,(T - T_{min}) \quad \text{.....Equation 2-1}$$

Where, b is a constant, T (°C) the temperature and T_{min} is the theoretical minimum temperature for growth of the organism, estimated by extrapolation of the regression line to $\mu_{max} = 0$.

C. Tertiary modelling (predictive software)

The growth parameters — maximum growth rate (μ_{max}), lag phase (λ) and maximum population density (MPD) from the inoculated studies were compared with predictive softwares including Growth Predictor Combase (www.combase.cc) and the Seafood Spoilage and Safety predictor (SSSP) v. 3.1. Both programs can provide predictions for microbial growth under fluctuating temperature conditions. In SSSP growth predictor, temperature fluctuations recorded from data loggers can be uploaded and the predictions are made on experiments based on sliced vacuum packed cold-smoked salmon in contrast to Combase where the predictions are made on synthetic media. Comparison of the prediction was based on the bias factor (B_f), the accuracy factor (A_f) and the goodness of fit (GoF) given in equation 2.2, 2.3 and 2.4 respectively.

$$\text{Bias factor } (B_f) = 10^{\frac{1}{n}\sum \log_{10} \frac{(\text{predicted value})}{(\text{observed value})}} \quad \text{Equation 2-2}$$

$$\text{Accuracy factor } (A_f) = 10^{\frac{1}{n}\sum \left(\log_{10} \frac{(\text{predicted value})}{(\text{observed value})}\right)} \quad \text{Equation 2-3}$$

$$\text{Goodness of fit (GoF)} = \sum \frac{(\text{observed value} - \text{predicted value})^2}{\text{predicted value}} \quad \text{Equation 2-4}$$

2.10 Risk assessment model

Quantitative microbial risk assessment of *L. monocytogenes* contamination in cold-smoked salmon was performed by running the @Risk add-on package (Palisade Software, Newfield, NY, USA) on the various input parameters as summarised in Table 2.5 and the Monte Carlo simulation were performed using Latin hypercube random sampling. The simulation was performed for 10,000 iterations.

Table 2.5 Quantitative microbial risk assessment of *L. monocytogenes* in vacuum packed cold-smoked salmon model inputs

Description	***Units***	***Variable***	***Formula/model/values***
Prevalence	Percentage	Prev	Beta(26+1,120-26+1)
Initial contamination level	log CFU/g		Cumulative (x:s) Table 2
Time from retail fridge to consumer fridge	Hours	T1	Uniform(30,180)/24
Temperature consumer transport	ºC	Tp1	Uniform(4,8)
Temperature consumer storage	ºC	Tp2	FSAI, 2001 Table 3
Maximum storage time in consumer fridge	Days	T_{max}	30
Minimum storage time in consumer fridge	Days	T_{min}	21
Consumer storage time	Days	T2	Uniform(T_{min}:T_{max})
(mCurv)		m	1
nCurv		v	10
Bacterial adaptation		h_o	$\mu_{max} \times \lambda$
Growth constant		q_o	1/exp(h_0)-1
Concentration of L. *monocytogenes* after consumer travel from retail to consumer storage	logCFU/g	y(T1)	Baranyi and Roberts model 1994 Equation (1)
Concentration of L. monocytogenes after consumer storage	logCFU/g	Y(T2)	Baranyi and Roberts model 1994. Same as Equation (1), with change in parameters $Y_{01,}$ $Y_{max1,}$ t1and μ_{max1} replaced by new parameter $Y_{02,}$ $Y_{max2,}$ t2and μ_{max2}
Serving size	g	S	Normal(35,26,Truncate(0,))

Chapter 3 Development of rapid and reliable method for detection of *Listeria monocytogenes* in vacuum packed cold-smoked salmon and to assess the efficiency of the isolation methods EN/ISO 11290-01 and -02.

3.1 Summary

As a part of a quantitative risk assessment of *Listeria monocytogenes* in cold-smoked salmon, this study was undertaken to identify a rapid and reliable protocol for the detection and quantification of *L. monocytogenes* in vacuum packed cold-smoked salmon.

The objectives of this study were to evaluate the prevalence of *L. monocytogenes* in vacuum packed cold smoked salmon in Ireland and to assess the efficiency of the isolation methods EN/ISO 11290-01 and -02. Sixty vacuum packed cold-smoked salmon were examined for the presence of *L. monocytogenes*; the detections were based on the EN/ISO 11290-01 and -02 protocols. 13 out of 60 samples were positive for *L. monocytogenes*. From 112 colonies presenting typical *L. monocytogenes* characteristics on Palcam and Oxford agar, only 64 % (Palcam) and 55.7 % (Oxford) were confirmed as *L. monocytogenes*. The confirmations were based on the 16S rRNA gene sequence analysis. Selection of Palcam and Oxford agar over ALOA agar for identification was based on the recovery rates of standard laboratory strains of *L. monocytogenes*. This study emphasises the need to design and develop good selective isolation protocols for *L. monocytogenes* to facilitate rapid screening of food samples in relation to food safety.

Some parts of the results from this chapter were accepted for publication in the Journal of Food control and also presented as poster at the 21st International ICFMH Symposium-FoodMicrobiology 2008 (Page: 212 - 213)

Keywords: *L. monocytogenes*, Oxford, Palcam, 16S rRNA gene sequence analysis.

3.2 Introduction

A number of selective media have been developed for the detection of *L. monocytogenes*on food and used over the years with varying degrees of success (Roche *et al.*, 2009). The standard microbiological methods (EN/ISO 11290-01 and -02) routinely used for the isolation of *L. monocytogenes* in foodstuffs or other materials, usually require two enrichment steps in liquid media and further isolation on solid selective media. For the identification of the colonies on the selective media, the typical practice requires that five of the suspected colonies from the selective media are confirmed through phenotypical characteristics (e.g. Gram stain, haemolysis and carbohydrate fermentation). The time required for the detection and identification of *L. monocytogenes* in each food sample can take up-to 7-8 days (Barocci *et al*., 2008).

Specific compounds added to the media for selective isolation of *L. monocytogenes* do not necessarily suppress the growth of other microbes or help in the differentiation within the *Listeria* spp. (Captia *et al.*, 2007).Growth rates during enrichment vary among *Listeria*species due to interactions with food matrices, production of inhibitors by the organism (monocins, bacteriophages), or the competing background flora (Besse *et al.*, 2005).Poor detection of low-virulence strains of *L. monocytogenes* have been reported in selective media such as; Palcam, ALOA, Rapid' L. mono and Oxford agar (Roche *et al.*, 2009 and Gracieux *et al.*, 2003). Despite these challenges, classical cultivation techniques still remain the gold standard for detection of *Listeria* in food.

In this study, 16S rRNA gene sequence analysis has been used in confirming the isolates obtained from the selective media as *L. monocytogenes*. 16S rRNA genes contain stretches of highly conserved regions and regions that are variable. Primers designed to target conserved DNA sequences can be used to analyze a wide variety of different organisms using PCR amplification with subsequent sequencing of the PCR product; whilst highly diverse regions

can be used to sub-type strains (Wang *et al.,* 1993). The use of 16S rRNA as a distinct signature for a bacterial species has become the method of choice for identifying and differentiating microorganisms when no other easily specified nucleic acid sequence uniquely defines the desired target (Gasanov *et al.*, 2004). The genus *Listeria* comprises of five species: *L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri,* and *L. seeligeri,* off which *L. monocytogenes and L. innocua* are closely related and are difficult to differentiae by cultural methods. 16S rRNA gene sequence analysis can differentiate between the closely related *L. monocytogenes*and *L. innocua*. Two sequence differences found within the V9 region were used to develop species specific nucleic acid probes for *L. monocytogenes,* and their efficacy has been demonstrated in a hybridization assay. The ability to determine the relationship between different bacterial isolates from the same species is extremely important in tracking the source of a food-borne infection and identifying problematic reservoirs (Czajka *et al.,* 1993).

The aim of this study was to develop a rapid method for the identification of *L. monocytogenes* in vacuum packed cold-smoked salmon, and to confirm the isolates from the selective media (Palcam and Oxford) as *L. monocytogenes* using 16S rRNA gene sequence analysis thereby testing the efficiency of EN/ISO 11290-01 and -02 .

3.3 Materials and methods

3.3.1 Selection of selective media

Eight standard *Listeria* strains (Table 3.1); 3 virulent strains, 2 non virulent strain and 3 low virulence strains of *L. monocytogenes*preserved in 80 % glycerol stock solution were used. The strains belonged to serotypes 1/2a, 1/2c, 1/2b and 4b. The strains were cultivated at 37

°C for 18 h in 10 ml Nutrient broth (Difco Laboratories, Detroit, MI, USA), and 10ml cultures of each strain were transferred aseptically to a 50ml centrifuge tube and were vortexed for 10s to ensure a homogenous cocktail. The cocktail of *L. monocytogenes* was prepared by combining the individual cells after centrifugation at 3000 x g for 30 min at 4 °C. The resulting pellet was washed twice with sterile saline (0.85%), and suspended in saline to a final concentration of approximately 10^9CFU/ml of the stock cocktail inoculums. 0.1ml of cocktail was inoculated on ALOA, Palcam and Oxford media and incubated for 48 hrs and observed for typical colonies.

Table 3-1: *L. monocytogenes* used for selection of isolation media

L. monocytogenes	**Strain type**	**Source**
Virulence	ATCC12567	bacterial culture collection lab
	ATCC27126	Newcastle university,UK
	NTCC2187	
Low-Virulence	NTCC2765	
	NTCC6754	
	NTCC7658	
Non-Virulence	ATCC3654	
	ATCC6896	

3.3.2 Sampling

A total of 60 samples of vacuum packed cold-smoked salmon were collected from a retail outlet in Dublin. Approximately 10 samples were tested per month for a period of 6 months; the samples represented a shelf-life period of 18-20 days and were sampled in a range from 7

days before to 1 day over the expiry date. The samples were transported back to the laboratory within 30 min of purchase for further analysis. For all the samples, refrigeration temperatures (0 to 4 °C) were suggested by the producers, the samples were stored in a cooling incubator and the temperature was maintained at ±4 °C.

3.3.3 Detection and Enumeration of *L. monocytogenes*

Refer to section 2.1 (Chapter 2, Materials and Methods).

3.3.4 16S rRNA gene sequence analysis

Refer to section 2.2(Chapter 2, Materials and Methods)

3.4 Results and discussions

3.4.1 Selection of the isolation media

The performance of each of the examined plating media with regard to the isolation of *L. monocytogenes* from different *L. monocytogenes* strains is shown in Table 3.2. It can be observed that Palcam (97.2 %) and Oxford (95 %) showed similar results for *L. monocytogenes*isolation. ALOA media showed only 93 % of isolation rates of the eight strains of *L. monocytogenes* with varying degrees of virulence.

Low virulence strains of *L. monocytogenes* were late growers on Palcam and Oxford, typical *L. monocytogenes* colonies appeared only after 3 to 4 days of incubation and in case of ALOA after 4 days. On the bases of these observations, Palcam and Oxford media were used in the study to determine the prevalence of *L. monocytogenes* in vacuum packed cold-smoked salmon.

3.4.2 Occurrence of *L. monocytogenes* in vacuum packed cold-smoked salmon

Out of the 60 packs tested, 13 packs were positive for *L. monocytogenes*, indicating 21.6 % prevalence. The levels of *L. monocytogenes* in the positive samples were found to be < 10 CFU/g in 98 % of the cases which is in compliance with the food safety criteria provided for RTE foods able to support the growth of *L. monocytogenes* (Commission Regulation EC N 1441/2007). The prevalence in RTE food samples containing > 100 cfu/g was in agreement with those reported in the Scientific Committee on Veterinary Measure relating to public health for Europe (SCVPH, 1999) and in Ireland the prevalence for *L. monocytogenes* was 26.1 % (Chitlapilly Dass *et al.,* 2010).

3.4.3 Collection of the presumptive *Listeria* spp.

One hundred and twelve isolates which showed typical *L. monocytogenes* colony type were isolated from both Palcam and Oxford agar. About 60 % of the total isolates showed 100 % identity to *L. monocytogenes*. The non- *L. monocytogenes*isolates that were identified by means of partial sequencing were as follows: 23 % *Enterococcus faecalis*, 6 % *Listeria seeligeri*, 4 % *Enterococcus faecium*, 4 % *Bacillus* spp. and 3 % *Serratia* spp

Table 3-2: *L. monocytogenes* and non-*Listeria* isolates on Palcam and Oxford**.**

Selective agar	Total no. of isolates	*L. monocytogenes*	Non-*Listeria* isolates
Palcam	42	27	15
Oxford	70	39	31

3.4.4 16S rRNA gene sequence analysis

Of the members of the genus *Listeria, L. monocytogenes* is a major food pathogen and hence it is necessary to accurately discriminate it from related taxa. All Listeria spp. fall into closely related lineages (Andrighelto et al., 2009). A signature sequence specific to *L. monocytogenes* in the 16S rRNA gene (Figure. 3.1) was developed to discriminate between other species of Listeria as well as from other genera. This was achieved by collection of all complete 16S rRNA gene sequences of Listeria spp. available on RDP II database. The sequences were aligned using Clustal W with a gap opening penalty of 10 and gap extension penalty 0.2. A L. monocytogenes unique 49bp region of the 16S rRNA gene (Figure 3.1) was identified. In this signature sequence positions 1, 34 and 48 (highlighted) are diagnostic for *L. monocytogenes* amongst *Listeria* species and the whole sequence is diagnostic for *L. monocytogenes* when checked in-silico. This has been confirmed by aligning all the *L. monocytogenes* sequence in NCBI and RDP databases and performing BLAST. Figure 3.2 is a graphical representation of the samples that were sequenced for the identification of *L. monocytogenes*. The rest of the strains that were non *L. monocytogenes* which grew on both Palcam and Oxford were assigned to taxa based on BLAST hits of the partial sequence. As expected, L. innocua is closely related to *L. monocytogenes*, the gradient of differences is only 2 of 1,281 bp in the 16S rRNA that absolutely differ between the two species. Although difficult to quantify, given the small number of differences, the 16S rRNA sequences successfully differentiated the two species.

GTACAAAGGGTCGCGAAGCCGCGAGGTGGAGC AATCCCATAAAAC A

Figure 3--0-1: Signature sequences of *L. monocytogenes*

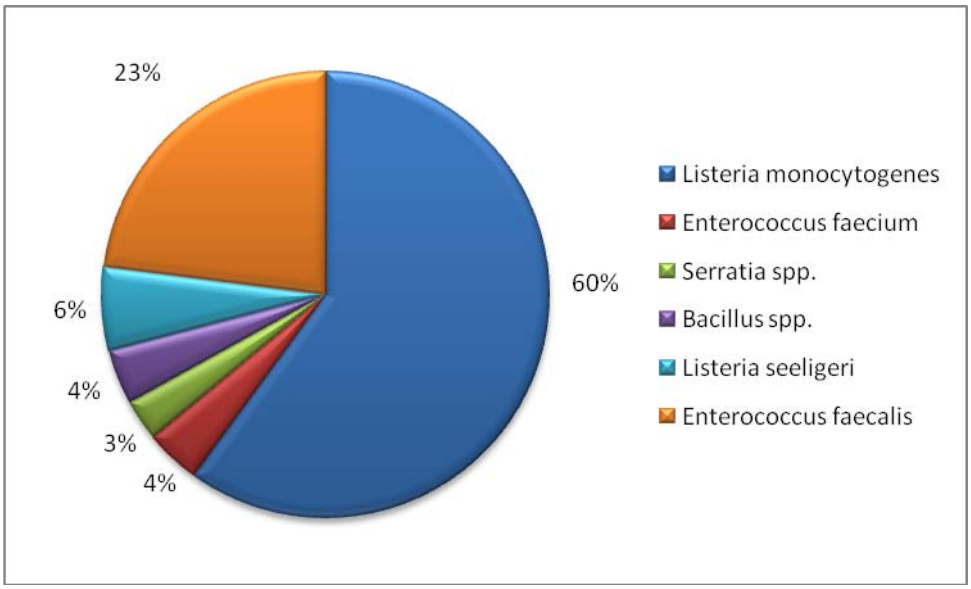


Figure 3-0-2: Isolates confirmed by 16S rRNA gene sequence analysis

According to Oh *et al.*, (2008) 16S rRNA is deemed to be reliable since it is based on the conserved region amplification. Another advantage of 16S rRNA is its sensitivity, which is critical factor for any screening method.

Direct sequencing of DNA is the most accurate method of evaluating genetic relationships of organisms, however it is expensive and time consuming. Since the introduction of PCR the amplification of even minute amounts of target DNA is possible and hence sequencing has become a more applicable tool. The 16S RNA gene is the most extensively sequenced RNA gene (Lane et al., 1991). The ability to accurately discriminate *L. monocytogenes* from *L. innocua* and other *Listeria* spp. is of importance to identify reservoirs of *L. monocytogenes*.

3.4.5. Comparative performance of the isolation media

From the 112 isolates, 70 isolates were from Oxford and 42 of the isolates were from Palcam. Out of the 70 isolates of the Oxford agar, 55.7 % showed 100 % identity to *L. monocytogenes*

by 16S rRNA gene sequence analysis. On the other hand 42 isolates of the Palcam agar analysed by 16S rRNA gene sequence analysis showed 64 % positive for *L. monocytogenes* with 100 % similarity.

Figure (3.3 and 3.4) shows the distribution of taxa other than *L. monocytogenes* in both Palcam and Oxford. *Enterococcus faecalis* and *L. seeligeri* showed false positive results on Palcam media and *E. faecalis*, *L. seeligeri*, *E. faecium*, *Serratia* spp. and *Bacillus* spp. showed false positive results on Oxford media.

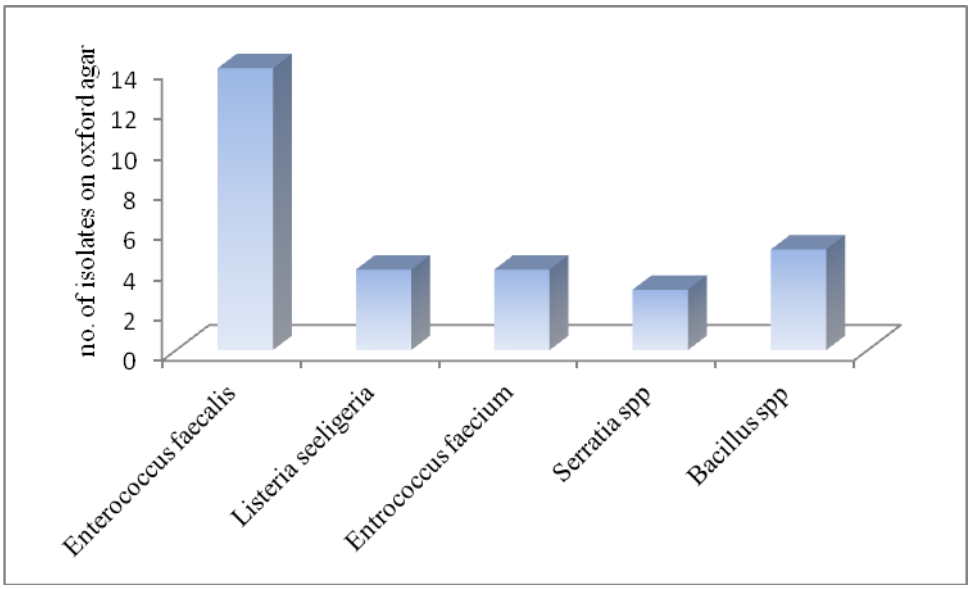


Figure 3-0-3: Non-*L. monocytogenes* isolates on Oxford agar

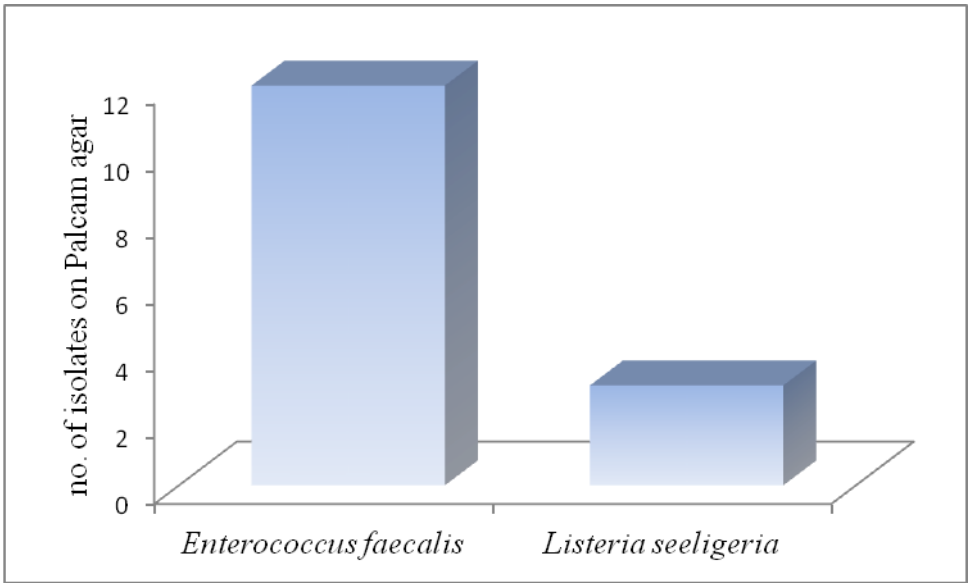


Figure 3-0-4: Non-*L. monocytogenes* isolates on Palcam agar

According to Capita *et al.*, (2001), the differential system of the Palcam and Oxford media showed a significant number of *Bacillus* spp. and *Enterococcus* spp. growth on both types of media, resulting in false positive results. This is consistent with the results found in this study. The potential origins of this problem lie in the fact that *Enterococcus* spp. and *Bacillus* spp. utilise esculin (which is the selective component in Oxford agar), may have a similar appearance (Gasanov *et al.*, 2005). Embarek *et al.* (1997) reported growth of Gram-positive, motile, rod-shaped bacteria with black *Listeria* – like colonies on Oxford agar. This is in line with results obtained in this work, as Oxford agar showed about 43 % interference with background food flora of smoked salmon. According to Velho (2005), in 10 out of the 33 (cold-smoked salmon isolates) *L. monocytogenes* presumptive positive colonies were confirmed positive for *L. seeligeri*. These results were also in line with the present study which showed 6 % of *L. seeligeri* growth on Oxford and Palcam agar (Fig 3.4).

Non- *L. monocytogenes* colonies which showed similar colonies to that of *L. monocytogenes* could have been missed during the initial identification procedure (Gram straing, catalase, oxidase and CAMP test). This is because 5 representative colonies from the *Listeria* positive plates are sub-cultured on a TSA plate for identification, these are in accordance with the ISO 11290 -01 and -02 method employed in identification. These results reflect a gap in the representative sampling methods for identification of *L. monocytogenes*, given that most of the selective media support the growth of other microorganisms which adapt to the specific ingredients added in the selective media (Velho *et al.,* 2005).

The true prevalence of *L. monocytogenes*could therefore be different than that found using cultural methods. These media may not support recovery of the organism if significant levels of other microorganisms are present (Curiale *et al.,* 1997). According to Waldroup (1996), the presence of any *Listeria* spp. or other microorganisms which mimics *L. monocytogenes* on selective medium would lead to the conclusion that *L. monocytogenes*could be present. As previously reported by Johansson *et al.,* (1999), these results demonstrate the need for more specific media for the detection of *L. monocytogenes* in foods and suggests that molecular techniques might be a viable alternative. Designing of specific media could be based on several specific traits of the bacteria, for example carbon ulitilisation, specific genetic trait in addition to antibiotic specificity (to eliminate other bacteria and select specific bacteria).

In modern food microbiology, a general shift to genetic methods is inevitable. Therefore research activities are focused on comparative studies of various alternative methods with known strategies for the detection, identification and characterisation of *Listeria* spp. revealing sufficient and highly resolved information contents. Determining bacterial phenotypes by characterizing colony morphology or biochemical alignment is not satisfactory for the clarification of the diversity between closely related strains. Current bacterial typing methods are based on DNA banding patterns, DNA sequencing and DNA hybridization.

However, no single method has universal application capacities. Based on the way of looking at a problem or situation, the choice of the genotypic method is highly dependent on the evolution of the marker.An important criterion for choosing suitable methods is the data exchange capacity and quality of data for storage and inter-laboratory comparison. The increase of listeriosis cases in the European Union may not only be seen as the cause for needing and developing rapid and reliable methods for the food industry and official control bodies. This increase may also have several other reasons such as changes of surveillance systems, improved rates of reporting, changes of consumption behaviour and growing life expectancy

3.5 Conclusions

The decision to find "the most suitable" method for the detection and tracing of *L. monocytogenes* in food processing is difficult as the information should not only relate to the presence of the pathogen, but also to use information about transmission routes and types of strains correlating to raw products, mid-products, end-products and product environment. Once the information is obtained by a proven method, it should be further integrated into effective prevention systems and HACCP programs, thus to be able to better evaluate risk profiles (e.g., production steps or areas).

16S rRNA gene sequencing could identify the stressed and non-cultivable *Listerial* strains. Phenotypic typing methods may be replaced by molecular tests, which reflect genetic relationships between isolates and which are more accurate. Currently, these new methods are mainly used in research but their considerable potential for routine testing in the future cannot be overlooked.

The method of detection of *L. monocytogenes* from food by 16S rRNA gene sequence analysis is rapid and accurate when compared to cultural methods, but it is cost intensive

when compared to the ISO 11290-01 and -02 methods for detection. A drawback of this method is the requirement of specialised equipment, such as an automated DNA sequencer, to carry out the analysis.

Significant shortcomings in the application of selective media for screening *L. monocytogenes*in food products have been highlighted in this study. Competitive bacteria, which should have been inhibited by enrichment, grew on Palcam and Oxford. With bacterial adaptation to different environments causing similarities in phenotype, as well as resistance to ingredients in enrichment and selective media, the transition from conventional methods of detection to genetic methods should be carried out (Gouws *et al.*, 2005). The application of cultural and serological methods in routine surveillance is of limited value because of their poor discriminatory power. Methods of higher discriminatory power, including the molecular methods such as PCR and DNA hybridisation tests, have been developed to differentiate between strains and identify sub-types of *L. monocytogenes* serotypes. This study further emphasises the design and development of good selective isolation protocols for *L. monocytogenes*to facilitate rapid screening of food products. 16S rRNA gene sequence analysis proved to be more reliable and time saving method in this study.

Chapter 4 Prevalence of *Listeria monocytogenes* in retail vacuum packed cold-smoked salmon marketed in the Republic of Ireland

4.1 Summary

Prevalence of *Listeria monocytogenes* in vacuum packed cold-smoked salmon marketed in the retail outlets in Republic of Ireland was established in this study. The findings from this study forms a part of quantitative risk assessment. The novel method of identification using 16SrRNA discussed in the previous chapter and Multi Locus Variable Number Tandem Repeat Analysis (MLVA) were used to identify and subtype *L. monocytogenes*.

The prevalence of *L. monocytogenes* in five brands (A, B, C, D and E) of vacuum packed pre-sliced cold-smoked salmon (n = 120) marketed in different retail outlets in the Republic of Ireland was investigated. The prevalence of *L. monocytogenes*in the cold-smoked salmon was 21.6 %. The *L. monocytogenes* strains isolated from the prevalence studies were typed by using MLVA; this method confirmed the type division amongst the *L. monocytogenes* isolates. A minimum spanning tree (MST) showed that isolates from brand A clustered together. Few isolates from brand B, C, D and E showed genetic relatedness while others were spread throughout the cladogram. The genetic distinction among subtypes may be processor specific whilst the similarities among the subtypes support the hypothesis of well adapted clones of *L. monocytogenes* in the fish industry. The results highlight the need for food safety control at both pre-processing and processing stages as there is no bactericidal process involved to eliminate any pathogen during the processing of cold-smoked salmon.

Some of the results from this chapter have been accepted and published in Journal of Food Safety (Page: 212).

Keywords: *L. monocytogenes*; Prevalence; Smoked salmon; 16S rRNA gene sequencing; MLVA

4.2 Introduction

*L. monocytogenes*contamination has been associated with unpasteurised milk, soft cheese, ice cream, raw vegetables, fermented raw-meat sausages, raw and cooked poultry, raw meats (all types), cold cuts, and raw and smoked fish (Hamon *et al.*, 2006, Czuprynski, 2005). The prevalence of these organismsin freshly produced cold-smoked fish is relatively high and is typically between 10 to 40 % (Miettinen and Wirtanen 2006, Azevedo *et al.*, 2005, Fonnesbech-Vogel *et al.,* 2001, Rorvik *et al.,* 2000, Autio *et al.*, 1999). This high prevalence could be due to the low smoking temperature involved during the cold-salmon processing; as these conditions would be ideal for the proliferation of *L. monocytogenes*if the raw salmon harboured the pathogen or acquired the pathogen from the processing environment. Under favourable conditions of storage time and temperature, *L. monocytogenes*may exceed the legal limit of 100 cfu/g (FSAI, 2008).

L. monocytogenes contamination in cold-smoked salmon depends on several factors such as raw materials, working habits and the presence of surface persistent *L. monocytogenes*. MLVA is a PCR-based method that subtypes organisms by determining the number of a variable number of tandem repeat (VNTR) found in multiple regions in the bacterial genome (Lindstedt *et al.,* 2008). This method allows for very sensitive subtype discrimination beyond that achieved by Pulse Field Gel Electrophorisis (PFGE) which is the golden standard in molecular identification (Foley *et al.,* 2009; Miya *et al.,* 2008 and Murphy *et al.,* 2007). MLVA has been applied to food-borne disease surveillance and outbreak detection (Torpdahl *et al.,* 2006) and is gaining acceptance as a robust and rapid method for genotyping bacterial isolates (Lindstedt *et al.,* 2008 and Sperry *et al.,* 2008). The characterisation of *L. monocytognes* using such molecular subtyping methods is very useful tool for

epidemiological studies and for research on the distribution and transmission of the pathogen along the food chain (Garrido *et al.,* 2009).

Despite the great amount of data available on *L. monocytogenes* at the retail level there are only a few studies which determine the occurrence by brand, thus limiting the possibility of identifying potential common contamination sources. It is necessary to increase the available data on the presence and levels of the pathogen within factory-packaged foods at the point of sale as the technological and hygienic measures of each manufacture could have different impacts on the prevalence of the pathogen (Garrido *et al.*, 2009).

The aims of the present study were to (a) investigate the prevalence of *L. monocytogenes* in five brands of vacuum packed cold-smoked salmon in the Republic of Ireland; (b) confirm the presumptive *L. monocytogenes*isolates from the prevalence study by 16S rRNA gene sequence analysis; (c) sub-typing of the confirmed *L. monocytogenes* isolates by MLVA which will categorise the subtypes and provide possible indicators of pathogen origin.

4.3 Materials and methods

4.3.1 Sampling

Vacuum packed pre-sliced cold-smoked salmon (250 g) were purchased from 5 retail establishments in Dublin (2007-2008). The five brands used in this study were coded as brand A through to E. Two samples from each brand were tested every month for a period of one year giving a total of 24 samples of each brand. The samples were returned to the laboratory under refrigerated conditions within 30 min of purchase and the samples were processed immediately upon arrival in the lab. The samples represented a shelf life period of 18-20 days and were sampled in a range from 7 days up to 1 day prior to the expiry date. For all the samples, refrigeration temperatures were maintained at$\pm$ 4 °C.

The brands B, D, and E were produced by a common Dublin processor, while brand C was produced in the UK and brand A produced in an Eastern European country. The Dublin and UK processors procured raw fillets of salmon under refrigeration conditions, with the raw material coming from the same slaughter house in the UK.

4.3.2 Detection and Enumeration of *L. monocytogenes*

Refer section 2.1 (Chapter 2: Materials and Method)

4.3.3 16S rRNA gene sequence analysis

L. monocytogenes isolates (n = 61), representative of the positive samples, were subjected to 16S rRNA gene sequence analysis. Refer section 2.2 (Chapter 2: Materials and Method).

4. 3.4 MLVA typing

Refer section 2.3 (Chapter 2: Materials and Method).

4.3.5 Data Analysis

A dendrogram was constructed by the unweightedpair group method using average linkages (UPGMA), available in the PHYLIP package, to represent the genetic relationships of the MLVA profiles. The minimum spanning tree (MST) was constructed using the Bionumerics software (version 4.5; Applied Maths, Sint-Martems-Latem, Belgium).

4.4 Results and Discussion

4.4.1 Prevalence

A total of one hundred and twenty cold-smoked salmon from five bands (A, B, C, D and E) were tested for the presence of *L. monocytogenes*, 26 samples (21.6 %) tested positive. Brand A and brand D showed the highest percentage of positive samples (Table 4.1).

Table 4-1: *L. monocytogenes* in cold-smoked salmon: Occurrence by brands and contamination levels.

Brand (n = 24 in each brand)	**No. of positive samples**	**No of positive samples by colony count (CFU/g)**		
		<10	**>10-100**	**>100-1000**
A	8	2	5	1
B	5	1	4	-
C	4	-	3	1
D	6	-	6	-
E	3	1	2	-
Total (n=120)	26 (21.6 %)	4 (3.3 %)	20 (16.6%)	2 (1.6%)

In this study an estimation of prevalence and molecular characterisation of *L. monocytogenes*in vacuum packed cold-smoked salmon marketed in the Republic of Ireland was provided. There is a lack of research in this area with only one previous study conducted in Ireland in retail outlets in 2001 where a prevalence of 13 % in smoked salmon was found (FSAI 2001). With an increase in listeriosis incidence across Europe and with the high consumption of cold smoked salmon there is a need for vigilance in monitoring of the pathogen (FSAI, 2008).

Five brands of vacuum packed cold-smoked salmon were tested for the presence of *L. monocytogenes*. The prevalence estimated in this study (21.6 %) is higher when compared to the study conducted on cold-smoked salmon in Ireland in 2001 (FSAI 2001) and these results are in agreement with increasing trends seen elsewhere in Europe (Gudbjornsdottir *et al.,* 2004, Mclaughlin *et al.,* 1993). Several investigators have reported even higher levels (78 %)

in smoked fish (Azevedo *et al.*, 2005, Eklund et al 1995, Hudson *et al.*, 1992) and 77 % in cold smoked salmon in New Zealand (Fletcher *et al.,* 1994). As the processing of cold-smoked salmon does not involve any bactericidal step to eliminate *L. monocytogenes*, the contamination at the raw fish level (or the raw fish acquiring the pathogen along the production line) will have a significant positive relationship with the presence of this organism in the finished product.

4.4.2 16S rRNA gene sequence analysis

Collections of 61 isolates (Table 4.2) from the *L. monocytogenes* positive Palcam plates were set for 16S rRNA gene sequence analysis. A signature sequence specific to *L. monocytogenes* in the 16S rRNA gene was identified to discriminate between other species of*Listeria*. *L. monocytogenes* was identified at the 49 bp region of the 16S rRNA gene. The bases at positions 1, 34 and 48 were diagnostic for *L. monocytogenes* amongst *Listeria* species (Figure. 4.1).

GTACAAAGGGTCGCGAAGCCGCGAGGTGGAGC AATCCCATAAAAC A

Figure 4-1: Signature sequence of *L. monocytogenes* diagnostic for *L. monocytogenes*

The sequencing reactions (sequencing carried out at Geneius laboratories ltd., Newcastle University, UK) were carried out at the 3' end using 1525R primer as a single directional read spanning about 500 bps. The sequences which showed the presence of the signature nucleotide were identified as *L. monocytogenes*.

Table 4-2: Number of *L. monocytogenes* isolates originated from each brand

Brand	No. of isolates
A	15
B	19
C	13
D	8
E	5

In this study *L. monocytogenes* has been discriminated from the non *L. monocytogenes* by 16S rRNA gene sequence analysis isolated from Palcam. Of the members of the genus *Listeria,L. monocytogenes*is a major food pathogen and hence it is necessary to accurately discriminate it from related taxa as all *Listeria* spp. fall into closely related lineages (Andrighelto *et al.,* 2009). *L. monocytogenes* was identified by the bases at positions 1, 34 and 48 of the 16S rRNA signature sequence. 16S rRNA gene sequencing could identify the stressed and non-cultivable *Listerial* strains. The oligonucleotide sequence used as a signature sequence showed promising results *in silico* as well as with the isolates checked in our study, this could be used as a molecular marker to rapidly identify *L. monocytogenes* strains. According to Oh *et al.,* (2008), 16S rRNA is deemed to be reliable since it is based on the conserved region amplification. Another advantage of 16S rRNA is its sensitivity, which is critical factor for any screening method.

4.4.3 MLVA typing

MLVA was used to subtype 61 *L. monocytogenes* isolates which were characterised by 16S rRNA gene sequence analysis. The TR containing amplicons were produced for all isolates at

all loci. Diversity indices were calculated for the TR locus in each case using Simpson's index and these values ranged from 68 to 87 % as shown in Table 4.3. The number of alleles identified in the isolates in this collection, ranged from 1 to 26.

Table 4-3: Simpson Index for MLVA of *L. monocytogenes*

Primer	Simpson's Index (%)	Amplicon (bp)
LM-TR 1	77.8	695
LM-TR2	50.5	569
LM-TR3	86.5	548
LM-TR4	86.5	478
LM-TR5	68.2	390
LM-TR6	81.5	268

The MLV technique discriminated between the 61 isolates from the 5 brands tested. From the MLVA profiles a Minimum Spanning Tree (MST) was constructed which was rooted to the samples with the highest number of related samples to it (Figure. 4.2). All the other samples were derived from this node thereby highlighting the genetic relationships that existed amongst these isolates. The gel representing the MLVA types is presented as Appendix 3.

Isolates from brand A originated from one node there by demonstrating genetic similarities amongst all the isolates based on its origin. Isolates from brands B, C, D and E created a number of sub-clusters, thereby demonstrating a genetic distinction between these isolates. There were 12 distinct MLVA profiles for (B, C, D and E). The sub-cluster were not distinct for any one brand, but were mixture of all four brands (B, C, D and E), showing some degree of relatedness amongst some isolates in the four brands.

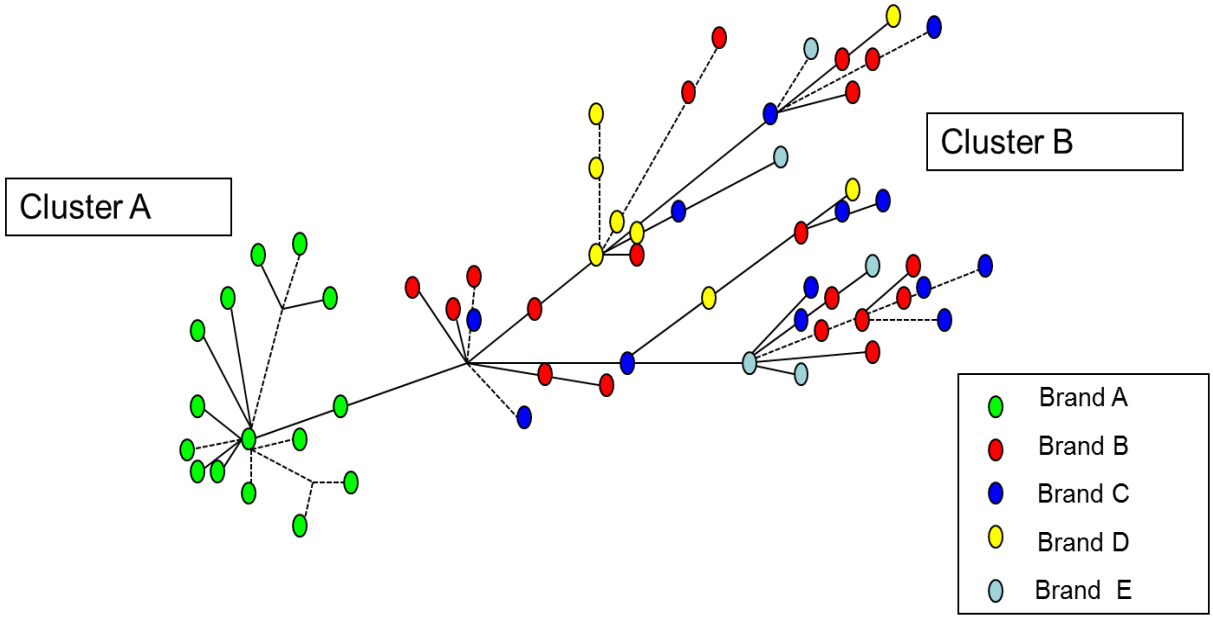


Figure 4-2 :MST constructed using highest number of single locus variants (SLVs) as the priority rule with no creation of hypothetical types.

Among the molecular subtyping method MLVA was used because of its excellent discriminatory power and reproducibility (Murphy *et al.*, 2007). Separate clustering of brand A could be due to the geographical location of the production area as it was imported into Ireland from an Eastern European country, thus demonstrating a genetic distinction between other subtypes (brand B, C, D and E) based on their origin. These results are in line with the survey conducted by Wulff *et al.*, (2006) which concluded that one group of genetically similar *L. monocytogenes* strains frequently dominates and persists in several fish, slaughter and smokehouses present in the same geographical location. These results support the hypotheses of the presence of a well adapted clone in the fish processing industry.

The isolates from B, C, D and E formed several sub-clusters with no distinct cluster for any one brand. The similarities amongst the isolates in the sub-cluster could be linked to the

slaughter house from where the processors acquired the raw salmon fillets, as the slaughter house was common for the two processors of brand B, C, D and E. Data presented in this thesis, supports previous works on contamination patterns of *L. monocytogenes* in cold-smoked salmon (Corcoran *et al.*, 2007 and Murphy *et al.*, 2007 and Wulff *et al.*, 2006), which leads to the hypothesis that the contamination could be from a few strains which may have been introduced with the raw material, and found a niche in the processing factory, from where they are constantly shed during the processing, thus contaminating the products. If the raw fillets carried *L. monocytogenes* into the processing factory, the *L. monocytogenes* could have undergone modification to adapt to the new environment and cold-smoking conditions specific to each processor. As cold-smoking is done below 20 °C and there is no other bactericidal step involved in eliminating the pathogen, there is a possibility that *L. monocytogenes* could have survived though the processing stage. In some samples multiple strains of *L. monocytogenes* were isolated, this supports the hypothesis that the product does not constitute a particular microenvironment in which only one strain survive.

Contamination of food products and the environment by *L. monocytogenes* is a serious threat to those involved in food processing, catering and retailing (FSAI, 2005). It is difficult to produce cold-smoked salmon totally *Listeria*-free. It is therefore essential to implement a food safety programme at the pre processing and processing environment along with new disinfection strategies to control the persistent *L. monocytogenes* strains which would have adapted to the current cleaning strategies. These combined food safety strategies, at different levels (raw fish, pre-processing environment and processing factory) will help in controlling the human exposure of this harmful pathogen.

The ability to carry out epidemiological investigations to determine the primary source of bacterial contamination is important so that preventive measures can be implemented to reduce *L. monocytogenes* prevalence and protect human health. It is demonstrated in this

study that MLVA is easy to perform and relatively fast, inexpensive and can be conveniently incorporated into any molecular laboratory without the need to acquire additional expensive equipment. Molecular methods such as PFGE take up to a week to complete, whereas MLVA results can be available within a day (Murphy *et al.*, 2007).

4.5 Conclusions

The results obtained from this study demonstrate a relatively high prevalence of *L. monocytogenes* in vacuum packed cold-smoked salmon in the Republic of Ireland (21.6 %) when compared to the previous study in Ireland (13%) conducted in 2001. The presence of this pathogen on cold-smoked salmon represents a serious public health concern, due to the increased consumption of this ready-to-eat food product. The subtyping of the different strains using MLVA seems to implicate a possible carryover of *L. monocytogenes* from the slaughter house to the processing plant. Therefore, suitable processing parameters and pre processing handling practice should be treated as important control measures to minimise the exposure to this pathogen. The present work confirmed the diversity of *L. monocytogenes* isolates based on its origin.It is suggested that MLVA is a suitable strategy for subtyping *L. monocytogenes*which could form part of a routine surveillance programme.

Chapter 5 Contamination pathway of *L. monocytogenes* in a processing plant for cold-smoked salmon in the Republic of Ireland (Part 1)

5.1 Summary

Having established the prevalence of *L. monocytogenes* in cold-smoked salmon, establishing the contamination route of *L. monocytogenes* is vital. In this chapter a smoked-salmon producing factory was monitored for the presence of *L. monocytogenes* and a possible route of contamination was established. Two independent studies were carried out to determine the contamination route. This chapter looks at the contamination pathway by examining the smoked-salmon factory in whole, the following Chapter 6 examines the processing of the cold-smoked salmon by tagging 60 individual fish (raw-salmon) and sampling after every important stage of processing to confirm the contamination pathway established in this chapter.

A cold-smoked salmon factory was surveyed for a period of one year (2008-2009) for the presence of *L. monocytogenes* in the processing line, processing environment, personnel, raw materials and product (cold-smoked salmon). The purpose of the study was to determine whether genetically similar strains colonise different environmental niches in the processing factory and thereby determining the possible contamination source or pathways. The processing factory was divided into four zones (1, 2, 3 and 4) based on the proximity to the cold-smoked salmon processing area.The overall prevalence of *L. monocytogenes* was 24.54 % (n = 444). The *L. monocytogenes* contamination pattern was identified by characterising 124 *L. monocytogenes* isolates (obtained from this survey) byMultiple Locus Variable number tandem repeats Analysis (MLVA). The isolates were divided into 8 MLVA types (Lm a, Lm b, Lm c, Lm, d, Lm e, Lm f, Lm g and Lm i). The final product (cold-smoked salmon) was contaminated with two major types of *L. monocytogenes*; one type originating from the raw material (Lm a) and the other type colonising the production line (Lm c) in zone 1. This suggests that, in addition to the fish processing line, *L. monocytogenes* contaminated

raw material can progress through the production chain and result in contamination of the final product. Each zone had one dominating strain type, thus leading to the hypothesis that specific *L. monocytogenes* strains may be better adapted to specific environmental niches in the processing factory. The results clearly indicated that the problematic sites were the raw material, cutting board, drains, floor, conveyer belt and slicer/skinner equipment. Although, these areas would be rigorously cleaned before the start of the production, there seems to be the existence of resistant *L. monocytogenes* strain types. In order to minimise the problem observed in this study, new cleaning and disinfection protocols should be considered.

Some results from this study has been accepted for publication in Food Research International (Page :212)

Key Words: *Listeria monocytogenes*, MLVA, cold smoked-salmon

5.2 Introduction

The contamination rate of seafood products with *L. monocytogenes* can vary from zero to 30 % (Embark, 1994; Jinneman *et al.*, 1999 andMiettinen and Wirtanen 2005).Vacuum packed cold-smoked salmon is a seafood product with wide ranging consumption patterns in Europe and thus is of considerable economic importance for the seafood market (Cardinal *et al.*, 2004). The prevalence of *L. monocytogenes*in freshly produced cold-smoked fish is relatively high and is typically between 10 to 40 % (Miettinen and Wirtanen 2006, Azevedo *et al.*, 2005, Vogel *et al.*, 2001, Rorvik *et al.*, 2000, Autio *et al.*,1999). The high prevalence could be due to the low smoking temperature (20 ºC) applied during the cold-salmon processing; as this condition would be ideal for the proliferation of *L. monocytogenes*if the raw salmon harboured the pathogen or acquired the pathogen from the processing environment.

Contamination of seafood with *L. monocytogenes* depends on many factors such as the cleaning and processing procedures, microbiological status of the raw fish and the existence of the surface persistent *L. monocytogenes* in the processing plant (Rorvik *et al.,* 1997, Autio *et al.,* 1999). Several authors have reported the colonisation of some subtypes of *L. monocytogenes* in fish processing plants, equipment, utensils and brine causing persistent contamination for months or even years (Rorvik *et al.,* 1995, Ahrens and Vogel, 2006 and Lopez, 2008). Numerous studies show that in-house *L. monocytogenes* flora contaminates seafood during processing (Autio *et al.,* 1999; Fonnesbech Vogel *et al.,* 2001 and Miettinen and Wirtanen 2006). However, there are indications that *L. monocytogenes* present in the raw material can proliferate and contaminate the final products, especially those that are not heat-treated before consumption (cold-smoked salmon; Eklund *et al.,* 1995; Fonnesbech Vogel *et al.,* 2001 and Miettinen and Wirtanen, 2006). Although the bacterium has been isolated from several different environments, it is insufficient to understand the overall ecology of the transmission dynamics of *L. monocytogenes*.

The ability to precisely track the strains or subtypes of *L. monocytogenes* present in the fish processing plant is critical in reducing the contamination of the raw and cold-smoked salmon. This study has focused on the ecology of *L. monocytogenes* in a cold-smoked salmon processing plant in Ireland by following the changes in its occurrence throughout the year. The aim of this study is to better understand the transmission of *L. monocytogenes* in the final product (cold-smoked salmon), by utilising the discriminative molecular typing method MLVA. Accordingly, MLVA typing was employed to fingerprint *L. monocytogenes* isolates to elucidate the diversity of the strains and to determine the existence of any persistent clones in the processing plant and thereby identify the sources of contamination routes within the processing plant

5.3 Materials and Methods

5.3.1 Sampling

A total of 12 surveys (a monthly survey) were conducted in a smoked salmon production plant located in Ireland. In order to investigate the persistence of contamination during different seasons the plant was surveyed from winter (Sep 2008 to April 2009) to summer (May 2009 to Aug 2009).

The processing plant was divided into four zones (Figure 5.1) based on the contact and proximity to the product (FASI, 2008). A total of 444 samples from four zones were sampled during the 12 month period. Thirty seven samples were examined each month and the samples were withdrawn from the same place to maintain consistency throughout the survey.

The survey samples were collected in three different ways depending on the nature of the sample. Samples from the equipment were swabbed (Conveyor belt, slicer, smoking tray, deboning pin, tables, vacuum packer, cold and frozen unit, filleting board and skinner), production environment (floor, drain, walls and switch), personal (gloves, aprons, boots, head protection and sanitizers) and the surrounding environment (waste collection point, service area, rubbish collection area, waste disposal site and traffic area). Each site was swabbed 3 times using sterile cotton swab moistened with 0.1 % peptone water and the swabs were pooled as one sample. The swabs were exposed for 60 seconds to the swabbing environment. The samples were transported under refrigerated conditions to the laboratory for further processing.

Samples of salmon were taken after each critical processing step (raw salmon, cured salmon, cold-smoked salmon and finally vacuum packed cold-smoked salmon). In all cases 50 g of the samples were taken and sealed in a sterile petri-plate and transported under refrigeration condition to the laboratory for further processing.

Liquid samples (drain, fish wash, brine and ice) were aliquots of 25 ml taken in sterile bottles and transported under refrigeration conditions to the laboratory for further processing.

All the samples were taken after 1 to 1 ½ h of complete running of the processing plant.

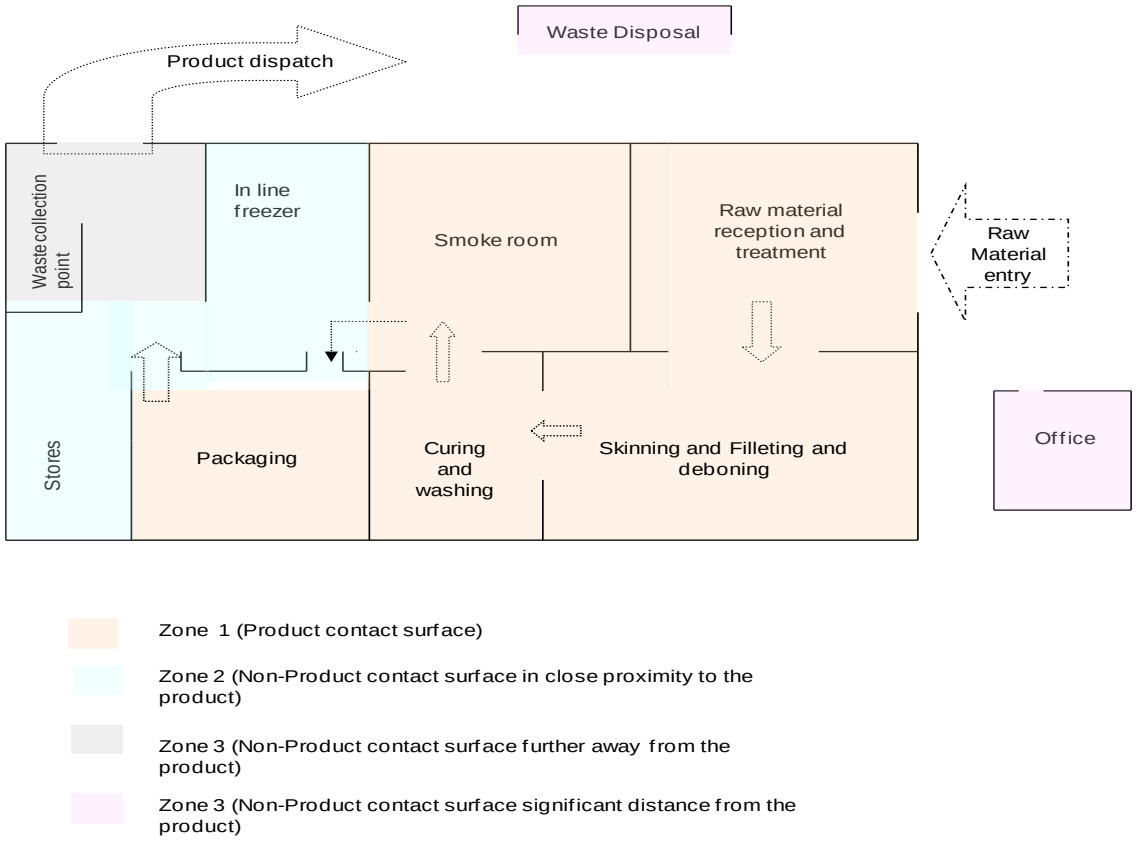


Figure 5-1: Schematic representation of the factory floor plan representing the four zones.

5.3.2 Detection

The detection of *L. monocytogenes* was carried out as described in the ISO 11290 -01 and 02. Upon arrival of the samples to the laboratory under refrigerated conditions, buffered *Listeria* enrichment broth (Scharlau-Chemie, Barcelona, Spain) was added to the swab samples as a

pre- enrichment step. The salmon samples (raw, cured, smoked and final product) were cut into 25 g each and were homogenised in medium speed for 1 min in a stomacher (Stomacher 400, Seward medical, England) in 225 ml buffered *Listeria* enrichment broth. Likewise, 25 ml of liquid samples were diluted with 225 ml of buffered *Listeria* enrichment broth. After the pre-enrichment step all the samples were incubated at 30 ℃ - 1 h in order to resuscitate stressed microorganism. The swabs were soaked in 20 ml of Listeria enrichment broth (Scharlau-Chemie, Barcelona, Spain) and incubated for 30 ºC for 1 h.

For the enumeration of *L. monocytogenes*, a volume of 0.1 ml from each homogenate was directly streaked onto each of 3 Palcam Agar plates (Oxoid, Hampshire, England) and was incubated at 37 ℃ for 24 - 48 h. From each plate, 5 colonies presumed to be *Listeria* spp. were streaked onto TSYEA plates (Scharlau – Chemie, Barcelona, Spain) and incubated for 24 h at 30 °C. Colonies were selected for typical appearance on TSYEA and submitted for Gram staining, catalase and oxidase test. Haemolytic activity and CAMP tests on sheep blood agar were performed for the *L. monocytogenes* confirmation.

5.3.3 MLVA typing

L. monocytogenes isolates (n = 124), representative of the positive samples were analysed for MLVA. Figure 5.2 represents the MLVA pattern in the 2 % agarose gel.

Refer section 2.3 (Chapter 2: Materials and Methods)

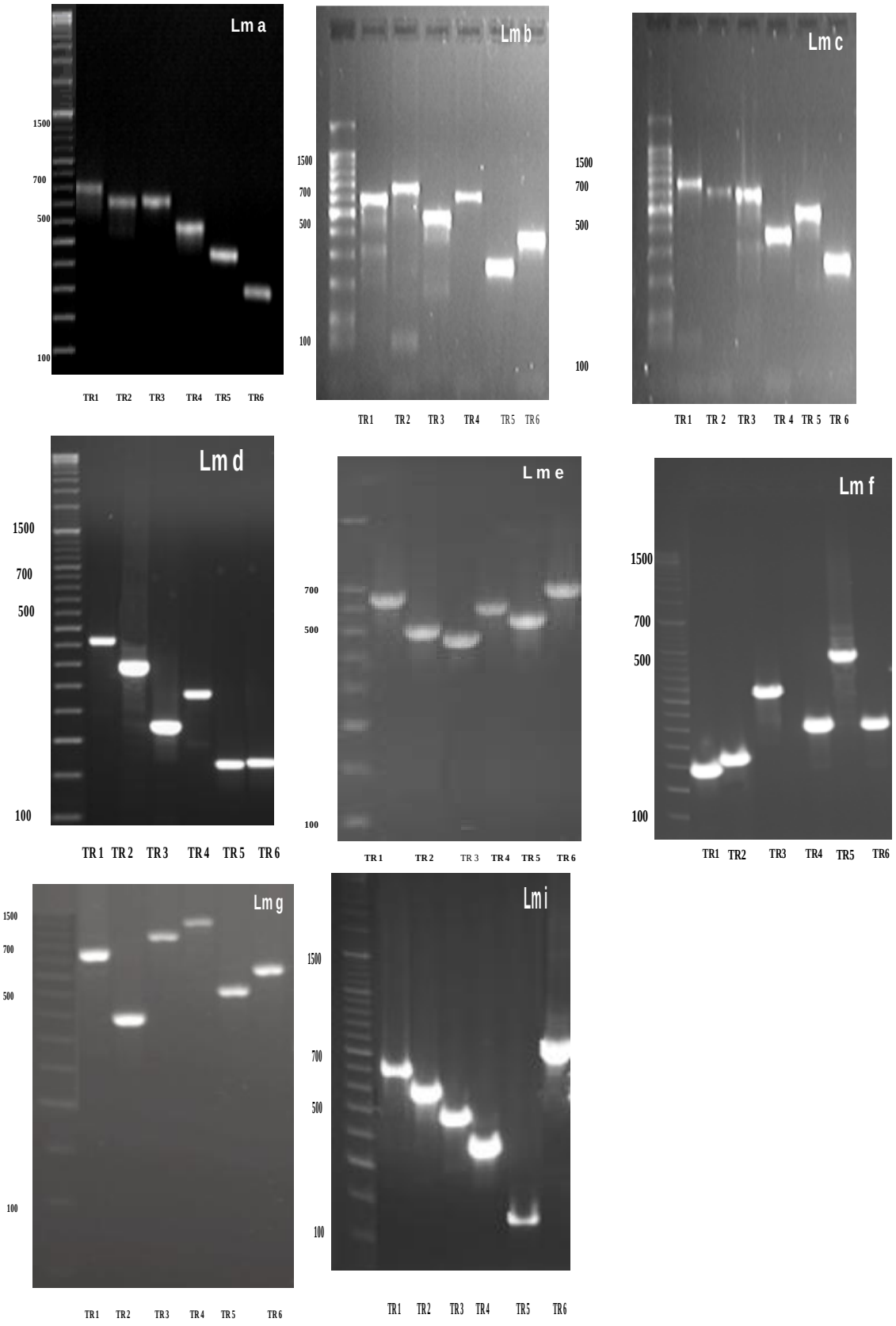


Figure 5-2: Eight MLVA pattern (Lma, Lmb, Lmc, Lmd, Lme, Lmf, Lmg and Lmi) on 2% Agarose gel, Lane 1- 100bp DNA ladder, Lane 2 to 7- TR 1 to TR6 aplicons

5.3.4 Data Analysis

The amplicons were sized and the estimated number of tandem repeats calculated using Quantity One software (Biorad, Hercules, CA). The allele strings were imported into a Bionumerics software package (version 4.5; Applied Maths, Sint-Martems-Latem, Belgium) and cluster analysis used the categorical coefficient and the Neighbour Joining algorithm. A dendrogram was constructed by the unweightedpair group method using average linkages (UPGMA), available in the PHYLIP package, to represent the genetic relationships of the MLVA profiles.

The use of categorical parameter implies that character states are considered unordered. The same weight is given to a large or small number of differences in the number of repeats at each locus. The genetic diversity at each TR was calculated using Bionumerics software package using Simpson's index.The TR was calculated from the below equation

Estimated number of tandem repeats = Band-Flanking region/ repeat unit.

5.4 Results and Discussion

5.4.1 Prevalence of *L. monocytogenes*

A total of 444 samples were tested during the period from Sep 2008 to Aug 2009. Of these, 24.54 % tested positive for *L. monocytogenes*. During the 12 month routine survey (Sep 2008 to Aug 2009), 37 samples were taken each month, the overall *L. monocytogenes*positive samples varied each month with the highest being in June 2009 with 45.9 % positive samples followed by July with 16 positive samples and Aug 2009 with 43.24 % positive samples (Figure 5.3). This could be related to seasonal variations with the highest temperature

recorded during June 2009 to Aug 2009 (Figure 5.3), thus potentially facilitating propagation of the pathogen. The lowest number of positive samples (2 to 3) was observed between Jan 2009 to Mar 2009 (Figure 5.3), which could be related to the low temperature recorded during that time period.

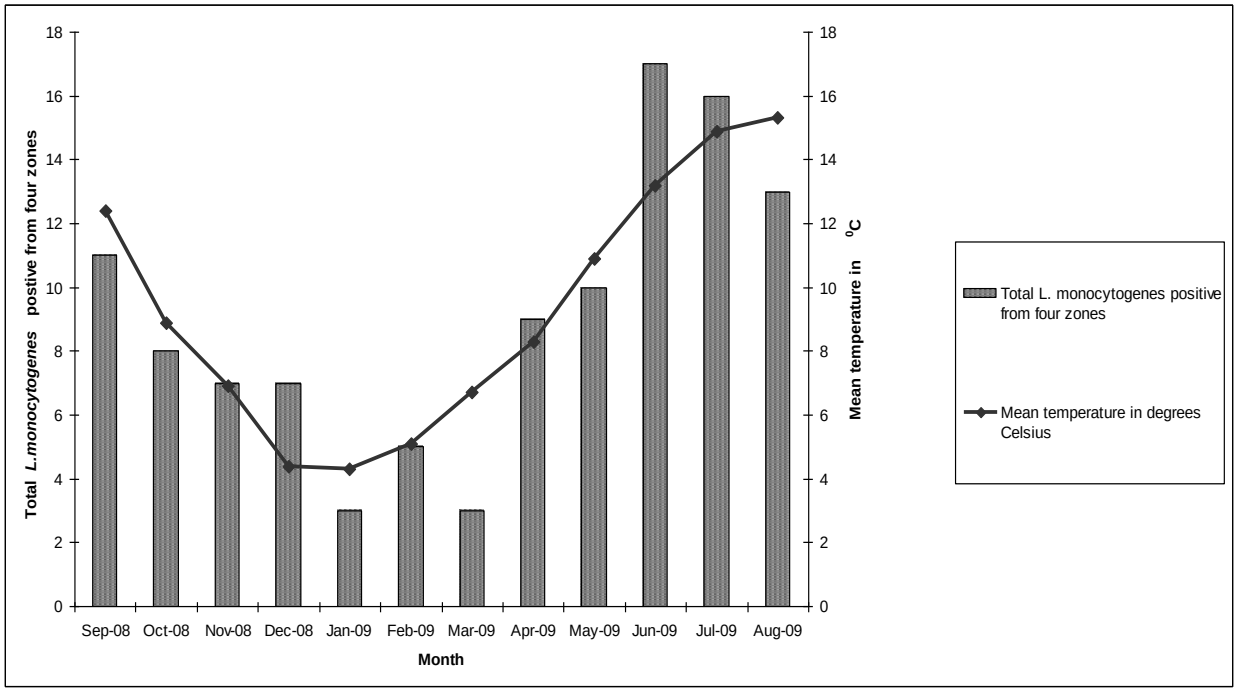


Figure 5-3: Total *L. monocytogenes* positive samples with the mean temperature and mean rainfall data recorded for the Dublin city (temperature data obtained form Irish metrological department)

Zone 1

Of the 216 samples tested in zone 1 (comprises of the raw materials or equipment) 36 samples were positive for *L. monocytogenes* (16.6 %). The overall contamination level varied each month, the highest level of *L. monocytogenes* positive samples were in the final product,

followed by the raw fish, cured fish, smoked fish and slicer/skinner. The knife, conveyor belt, deboning filleting board, food handlers, smoking tray, curing fish wash and raw fish wash were sporadically contaminated with *L. monocytogenes* with just 1 or 2 samples positive throughout the one year survey. The ice obtained during the defrosting process of the fish and the salts obtained during the curing process were negative for the presence of *L. monocytogenes* during the 12 month period.

Zone 2

This is the non-product contact surface in close proximity to the product. Out of the 132 samples tested in this zone 22 samples were positive for *L. monocytogenes* (26.6 %). The highest number of positives was found in the high risk drain with 4 samples tested positive. The rest of the positive samples were found in the drain, filleting drain, curing drain, goods reception floor, processing floor and cold and frozen units were sporadically contaminated with *L. monocytogenes*. Tables, doors and switches were negative for the presence of *L. monocytogenes*.

Zone 3

This zone comprises of the non-product contact surface which are further away from the sample. Out of the 36 samples tested in this zone, 16 samples were positive for *L. monocytogenes* (44 %). The waste collection point and low risk drain had the highest number of positive samples, while the personal safety item showed only one positive sample.

Zone 4

Out of the 60 samples tested at this zone (non-product contact, significant distance from the product), 35 samples (58.3 %) were positive for *L. monocytogenes*. The rubbish collection

area and waste disposal site had the highest number of positive samples. Access way, traffic area and service area were sporadically contaminated with *L. monocytogenes*.

In this study the ecology of *L. monocytogenes* in a cold-smoked salmon processing factory in the Republic of Ireland was investigated by following the changes in its occurrence in different types of *L. monocytogenes* strains for a period of one year. With an increase in listeriosis incidence across Europe and with the high consumption of cold smoked salmon, there is a need for vigilance in monitoring this pathogen (FSAI, 2008).

The prevalence estimated in this study was 24.54 %, and these results are in agreement with increasing trends seen elsewhere in Europe (Gudbjornsdottir *et al.*, 2004, Mclaughlin *et al.*, 1993). The increase in the prevalence found in this study when compared to the 2001 study conducted in Ireland (FSAI, 2001) could be due to the wide distribution of *L. monocytogenes* in the environment (Miettinen and Wirtanen, 2006, Farber and Peterkin, 1991) as this can cause contamination in raw fish and also during processing, pre- processing and handling (Miettinen and Wirtanen, 2006, Johansson *et al.*, 1999).

The plant was surveyed throughout the year to investigate if there were any change in the occurrence of *L. monocytogenes*. The *Listeria*contamination pattern gave some indications on the influence of weatherconditions and seasonal variation. The isolation of *L. monocytogenes* was highest during the summer months (June 09 – Aug 09) when the overall air temperature in Dublin city averaged between (12 - 15 ºC), this may explain the variation in the frequency of *L. monocytogenes*in the smoked salmon factory (Figure 5.3). The least isolations were found during the peak winter months where the temperature averaged between 2 – 5 ºC (Figure 5.3). This may highlight some seasonal influence on the shedding of the bacteria.

5.4.2 MLVA profiling of *L. monocytogenes* isolated from the four zones

A total of 124 isolates (Table 5.1) of *L. monocytogenes* were analysed for MLVA with 6 sets of primers and eight MLVA types were identified (Lm a, Lm b, Lm c, Lm d, Lm e, Lm f, Lm g and Lm i). Of the eight types, 4 MLVA types were dominant (Lm a, Lm c, Lm e and Lm d) and repeated isolation of the dominant strain each month was observed (Figure 5.4). The percentage isolation of *L. monocytogenes* strain types from each zone is illustrated in Figure 5.4.

The final product (cold-smoked salmon) was contaminated with two dominant strains, Lm a which originated in the raw fish and Lm c which predominantly dominated zone 1. Lm c was isolated from equipment which was in direct contact with the final product. Lm b was isolated sporadically from the final product. Two types of *L. monocytogenes* strains were isolated Lm c and Lm d from one food handler. The *L. monocytogenes* dominant subtype (Lm g) isolated from zone 3 or 4 did not contaminate final product.

Table 5-1: Represetation of the 124 *L. monocytogenes* isolates from zone 1 to zone 4

Type	Product/area	Total no of isolates
Lm a	raw fish	5
	cured fish	3
	smoked fish	2
	cold-smoked salmon	3
	curing fish wash	1
	raw fish wash	1
Lm c	Cured fish	1
	Smoked fish	2
	Cold-smoked salmon	6
	Knife	2
	Conveyor belt	2
	Deboning pin	2
	Slice/skinner	4
	Filleting board	1
	Smoking tray	1
	Food handlers	1
	Processing floor	1
	Filleting floor	1
	Cold-frozen unit	1
	Personal safety item	1
Lm b	Smoked fish	1
	Cold-smoked salmon	2
	Slicer/skinner	2
Lm d	Food handlers	1

Lmd	High risk drain	2
	Processing floor	2
	Filleting floor	1
Lm e	High risk drain	3
	Good inwards drain	5
	Filleting drain	2
	Curing drain	3
	Goods reception floor	1
	Processing floor	3
	Filleting floor	2
	Low-risk drain	2
Lm f	Cold and frozen unit	1
Lm g	Low risk drain	7
	Waste collection point	9
	Service area	1
	Rubbish collection area	11
	Waste disposal site	12
	Access way	2
	Traffic area	6
Lm i	Access way	1
	Traffic area	1

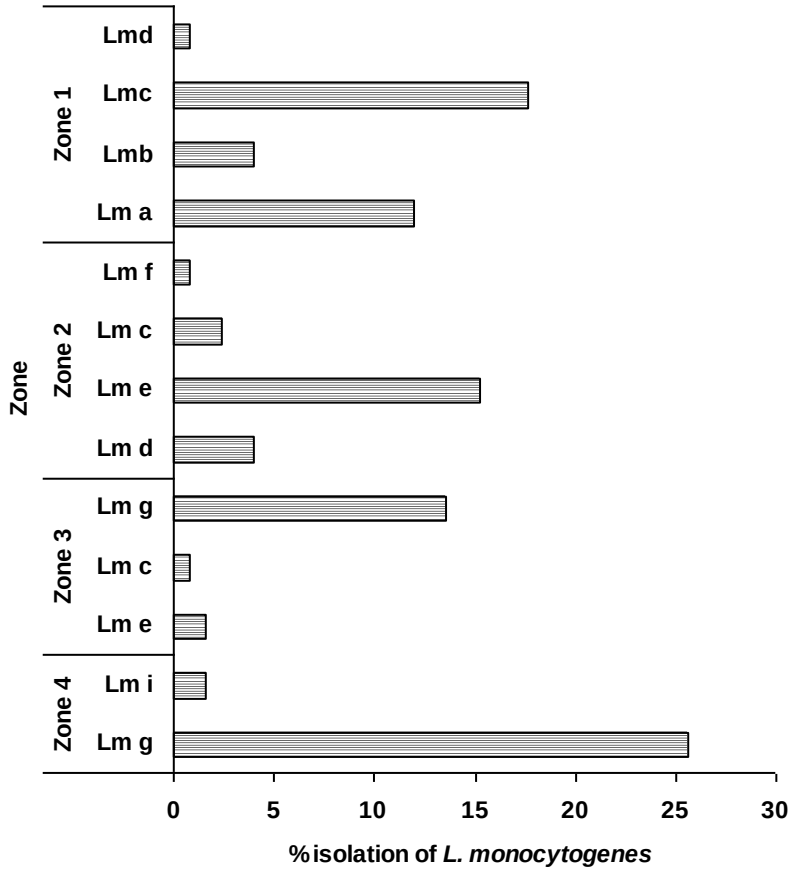


Figure 5-4: Percentage isolation of *L. monocytogenes* strains in each zone.

In zone 2, Lm e was predominantly isolated from drains and the processing floor. Other strains Lm d, Lm c and Lm f were sporadically isolated from zone 2 (Figure 5.4).

In zone 3 and zone 4, which were non-product contact surface zones and the surrounding environmental sites, one dominant strain type Lm g was isolated, which seems to have adapted to the environment outside the production line. Sporadically, Lm i was isolated from zone 4.

The clustering was carried out on the MLVA pattern of 124 *L. monocytogenes* strains from four different zones using Neighbour joining (NJ) algorithm. The Neighbour Joining method is a method for re-constructing phylogenetic trees, computing the lengths of the branches of

this tree. In each stage, the two nearest nodes of the tree are chosen and defined as neighbours in the tree. This is done recursively until all of the nodes are paired together.The cladogram thus constructed showed 8 distinct clusters (Figure 5.5). Different clusters are denoted with different colours in the final cladogram to show the genetic distinction among the 8 strain types.The subtypes which were isolated from the final product (Lm a, Lm b and Lm c) formed clusters close to each other, while the environmental strain type Lm g and the strain type isolated from drain and processing floor Lm e were clustered close to each other. This genetic distinction proves the genetic similarity between the strains originating in the product and adaptation of the specific strain type to each zone. Location of different strain types isolated from the four zones is illustrated in Figure 5.6.

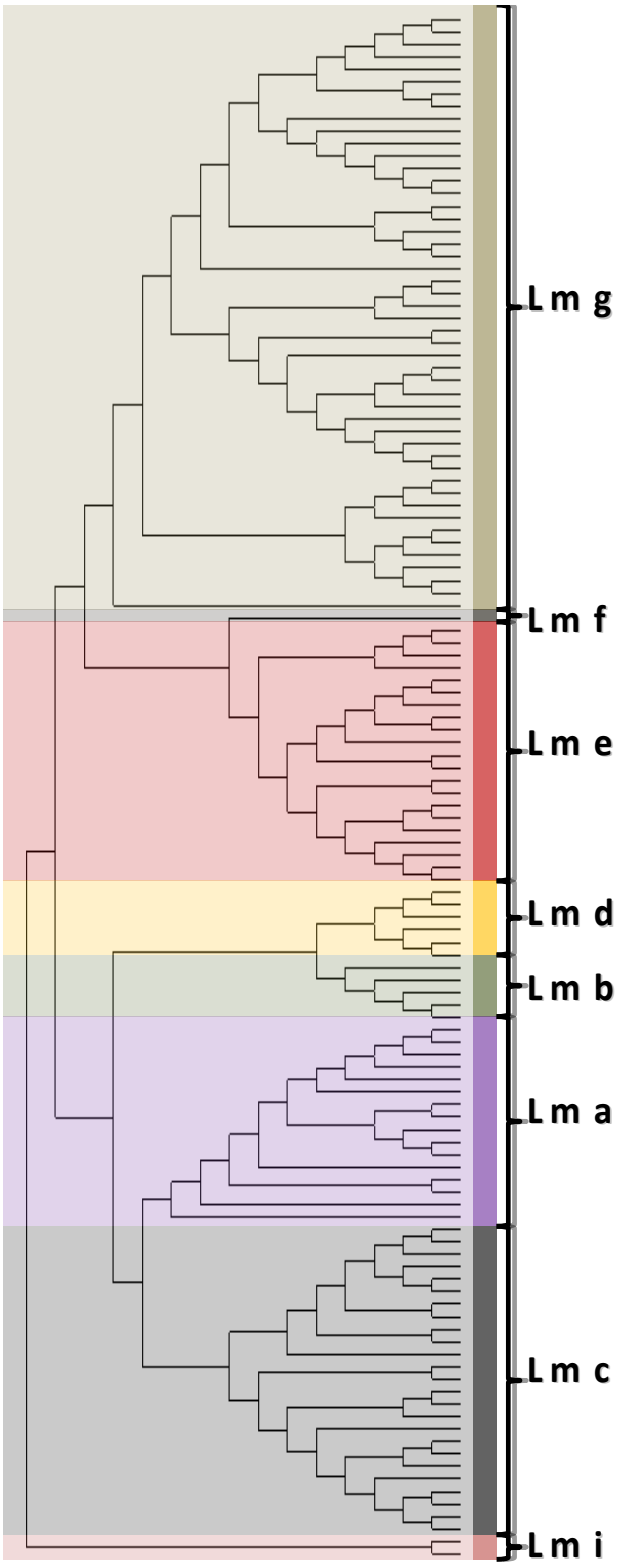


Figure 5-5: The Dendrogram (constructed by Neighbor-Joining method) show that the isolates can be classed as eight distinct MLVA types (Lm a, Lm b, Lm c, Lm d, Lm e, Lm f, Lm g and Lm I). Each clade in the dendogram represents particular genotypes, which within the context are specific MLVA strain types encountered in this study

The 124 *L. monocytogenes* isolates obtained from the four zones were genetically heterogeneous, and the isolates were differentiated into 8 MLVA types. The division of isolates into various types is consistent with the results from several studies of *L. monocytogenes* isolates in a factory (Miettinen and wirtanen, 2005 and Rorvik *et al.*, 2000). Four type strains, Lm a, Lm c, Lm e and Lm g, represented 77 % of the total isolates from the processing plant and its environment. The other types Lm b, Lm d, Lm f and Lm i were sporadically isolated.

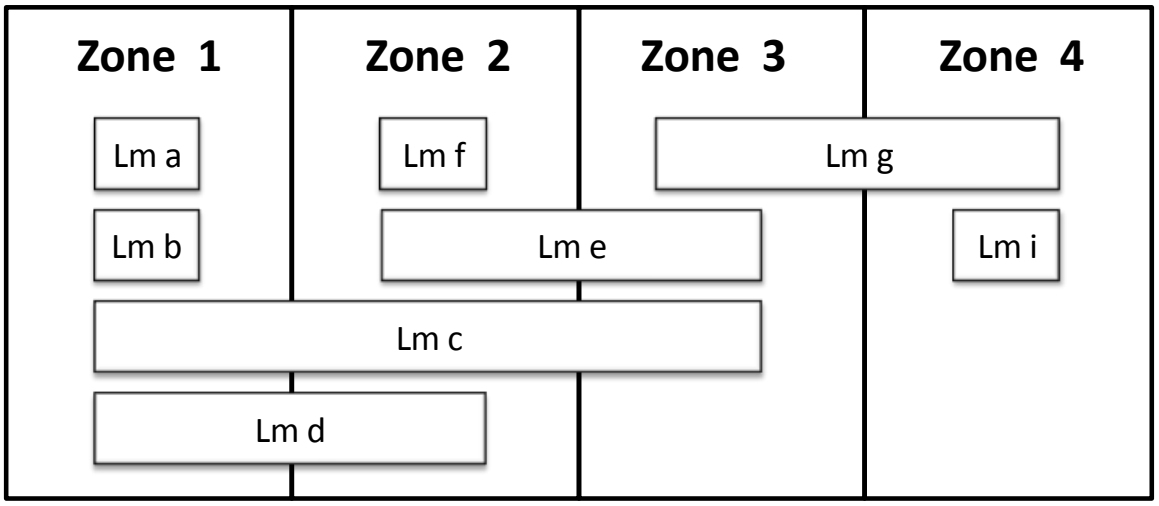


Figure 5-6 : Schematic representation of the location of different isolated in the four zones.

Food processing plants may be contaminated with *L. monocytogenes* from various sources including raw materials, equipment, processing environment and personnel. In this study, the final product cold-smoked salmon was contaminated with the same strain as that found in the raw material at the start of the process line (strain type Lm a) while further contamination appeared to occur further down the processing line (with Lm c and Lm b). The dendogram (Figure 5.5), shows that strain type isolated from the final product cluster together showing

genetic relatedness among Lm a, Lm b and Lm c.In certain cases the final product harboured two genetically related strain types (Lm a and Lm c) co-existing. Lm c could have been introduced into the processing line initially through raw material, this is evident from the genetic relatedness with the Lm a (isolated from raw material). These results thus support two hypothesis (i) the products do not constitute a particular microenvironment in which only one strain survive, (ii) some strains may have been introduced with the raw material, and found a niche in the processing factory, from where they are constantly shed during the processing thus contaminating products(Martinez *et al.*, 2003).

Despite rigorous cleaning procedure carried out by the processing factory surveyed before the start of the production, there seems to be a persistent strain (Lm c) existing on the equipment. This indicates the deficiencies in the cleaning and disinfection procedures and the need for new cleaning and disinfection strategies. Further research would be required to study the resistance to cleaning reagents.

In this study, only one *L. monocytogenes* positive samples were detected from theprotective clothing and footwear samples originatingfrom the smoked salmon processing plants. Two strain types (Lm c and Lm d) were isolated from personnel and personnel safety items. The presence of Lm c on personnel gloves could be due to handling of equipment contaminated with Lm c strain type. This could have been carried over to the personal safety item. Lm d isolated from personnel were also found in drain samples sporadically. Overall, there were few incidences of *L. monocytogenes* being isolated from personnel or safety items, thus indicating good hygiene practiced before and after production by the food handlers.

Zone 2, which comprises of drain and processing floor were contaminated with one dominate strain type Lm e. The strain type Lm e shared genetic similarity to type Lm g isolated from zone 3 and zone 4. The drain and processing floor are subjected to heavy chemical treatment

and despite these harsh treatments, Lm e has found a survival mechanism to overcome the harshness and has been repeatedly isolated from drains and processing floor. Despite being in close proximity to the processing line, there were no cross-contamination observed between the drain and floor sample with the final product, this could be due to the physical barrier across each zone and the change in the personal protection equipments by the food handlers while crossing each zone.

Zone 3 and Zone 4 were dominated by Lm g, and had the highest isolation rate when compared to other predominant strains in other zones (Figure 5.4).Survival of Lm g throughout the seasonal changes could be due to the survival mechanism with changes in temperature (4 – 16 ºC) and these two zones (3 and 4) are least subjected to chemical treatment in comparison to other two zones (1 and 2). It is well known that *L. monocytogenes* can survive through a wide range of temperatures (Ericsson *et al.*, 1997), this could be the reason for the repeated isolation of Lm g in most of the months surveyed. It can be summarised that there are clear difference in *L. monocytogenes* strains as some colonise fish and others environmental surfaces.Thus suggesting that *L. monocytogenes* strains might show site-specific endemism to particular niches in the production plant. These niches might impart strain-specific favouritism on viability and attachment properties of the bacteria. Further studies would be required to validate the hypothesis.

It is evident from this study that raw fish carry L. monocytogenes through to processing, therefore it is proposed that non-thermal technology such as ozonisation, pulsed electric field, irradiation or electron beam, could be employed to eliminate any naturally contaminated L. monocytogenes at the raw fish stage. It is therefore essential to minimize the occurrence of L. monocytogenes in raw fish stage to reduce the possibility of contamination of process equipment and formation of in-house strains. Implementation of food safety programme at the processing environment along with new disinfection strategies to control persistent L.

monocytogenes strains. These combined food safety strategies, at different levels (raw fish and processing factory) will help in controlling the human exposure of this harmful pathogen. The ability to carry out epidemiological investigations to determine the primary source of bacterial contamination is important so that preventive measures can be implemented to reduce *L. monocytogenes* prevalence and protect human health. In this study MLVA allowed for rapid and sensitive subtype discrimination of *L. monocytogenes*, thus facilitating identification of possible contamination pathways. MLVA is easy to perform and relatively fast, inexpensive and can be conveniently incorporated into any molecular laboratory without the need to acquire additional expensive equipment. Other than MLVA, rapid molecular methods such as RT PCR should also be explored. Molecular methods such as PFGE take up to a week to complete, whereas MLVA results can be available within a day (Murphy *et al.*, 2007).

5.4 Conclusions

In conclusion this study demonstrates that certain *L. monocytogenes* MLVA types may carry specific traits (growth over a wide range of temperatures, including at refrigeration temperatures, in high concentrations of sodium chloride and low concentrations of oxygen) that enable them to persist in the food processing environment. Presence of this pathogen on cold-smoked salmon represents a serious public health concern, due to the increased consumption of this ready-to-eat food product. The subtyping of the different strains using MLVA seems to implicate a possible carryover of *L. monocytogenes* from the raw fish and in-house strain to the final product. Therefore, suitable processing parameters and pre processing handling practices should be treated as important control measures to minimise the exposure to this pathogen. The present work confirmed the diversity of *L.*

*monocytogenes*isolates based on its origin. MLVA is a suitable strategy for subtyping *L. monocytogenes* as part of a routine surveillance programme.

Chapter 6 Tracking of *Listeria monocytogenes* during different stages of cold-smoked salmon processing in an Irish factory (Part 2)

6.1 Summary

This study was undertaken in order to confirm the contamination pathways established in the Chapter 5.The aim of this study was to characterise strains of *L. monocytogenes* isolated from different stages of the cold-smoked salmon processing and thereby establishing the contamination pathway of *L. monocytogenes* in the final vacuum packed cold-smoked salmon. In order to evaluate the incidence and distribution of *L. monocytogenes*, 60 raw whole salmon were tagged and sampled during different stages of cold-smoked salmon processing. The results highlighted that 28.33 % of the raw salmon were contaminated when compared to the 21.60 % of the final product. The *L. monocytogenes* strains (n = 60) isolated from the tracking studies were typed using Multi Locus Variable Number Tandem Repeat Analysis (MLVA); this method confirmed three types of MLVA profiles (Lm 1, Lm 2 and Lm 3). The strain type Lm 1 was isolated from raw salmon while Lm 2 was isolated after curing and Lm 3 was isolated after filleting and slicing the salmon. The hypothesis that raw fish was an important source of contamination of the processing plant could not be rejected. Contamination of the product occurred in specific processing steps (filleting, curing and slicing). This indicates that the persistence of in-house flora is not eliminated by regular cleaning of the slicing and filleting equipment. Presence of *L. monocytogenes* found after curing proves the halotolerance nature of the pathogen. In some final vacuum packed samples multiple strains of *L. monocytogenes* were isolated, this supports the hypothesis that the product does not constitute a particular microenvironment in which only one strain survive. This study points to the need for more stringent cleaning and sanitizing procedures to reduce the contamination of cold-smoked salmon with *L. monocytogenes*.

Some parts of this chapter have been accepted for publication inInternational Journal of Hygiene and Environmental Health(212).

6.2 Introduction

In Europe, seafood is the largest or second largest food-type involved in recalls because of microbiological contamination (Cardinal *et al.*, 2004). Hence the presence of *L. monocytogenes* in smoked-salmon, together with an understanding of the source and routes of contamination, is of major concern to those involved with food production, retailing and regulation. *L. monocytogenes* occurs naturally in raw fish materials (Fonnesbench Vogel *et al.*, 2001). It has been frequently isolated from coastal waters probably due to its from-land-to-water transmission (Colburn *et al.*, 1990 and Gram, 2001).

Contamination of cold-smoked salmon with *L. monocytogenes* depends on various factors such as the raw material, working habits, cleaning, processing procedures and existence of surface persistent *L. monocytogenes* strain types in processing facilities (Rorvik *et al.*, 1997; Autio *et al.*, 1999; Azevedo *et al.*, 2005; Miettinen and Wirtanen 2006). *L. monocytogenes* contamination in raw material (salmon) is an important factor as it will affect the safety of the final product (cold-smoked salmon) as no heat treatment is applied during its processing or before consumption. The prevalence of *L. monocytogenes* in raw fish varies between 0 to 30 % (Ben Embark *et al.*, 1997; Jinneman *et al.*, 1999; Hoffman *et al.*, 2003,Miettinen and Wirtanen 2006, Chitlapilly Dass *et al.*, 2010a and Chitlapilly Dass *et al.*, 2010b). As *L. monocytogenes* is regularly isolated from cold-smoked salmon, a better understanding of its colonisation of the product is vital; in order to develop and improve *Listeria* control strategies to reduce the number of *L. monocytogenes* cells and consequently reduce consumer risk.

L. monocytogenes isolation and subtyping are very critical steps in characterising the contamination patterns. Multi locus variable number tandem repeat analysis (MLVA) is a PCR-based method that subtypes organisms by determining the number of variable number

of tandem repeats (VNTR) found in multiple regions in the bacterial genome (Lindstedt *et al.*, 2008). This method allows for very sensitive subtype discrimination.

The aim of this study was to characterise strains of *L. monocytogenes* isolated from different stages of cold-smoked salmon processingby tracking the raw salmon from start to the finished product and withdrawing samples from the tagged salmon after every intermediate step of processing (raw, filleting, curing, cold-smoking, slicing and final vacuum packed cold-smoked salmon) and analysing for the presence of *L. monocytogenes*. This was done to establish possible routes of contamination of *L. monocytogenes* in cold-smoked salmon.

6.3 Materials and methods

6.3.1 Survey and sampling

A total of 12 surveys were undertaken from Dec 2008 – Nov 2009 in a cold-smoked salmon processing factory. A total of 60 raw salmon were tracked through the production line. Each month 5 raw salmon were tracked through the different stages of the smoked-salmon processing (raw, filleted, cured, smoked, sliced and final vacuum packed cold-smoked salmon) Figure 6.1.

Each of the raw fish analysed were tagged with a wire string and passed through the various intermittent stages of processing, 25 gm of sample were withdrawn from each critical processing step (raw, filleted, cured, cold-smoked, sliced and final vacuum packed cold-smoked salmon). The samples were collected in a sterile petri-plate and were transported under refrigerated conditions to the lab for further analysis. The samples were collected after 1 ½ h of complete running of the factory and were transported within 30 min to the lab for further processing.

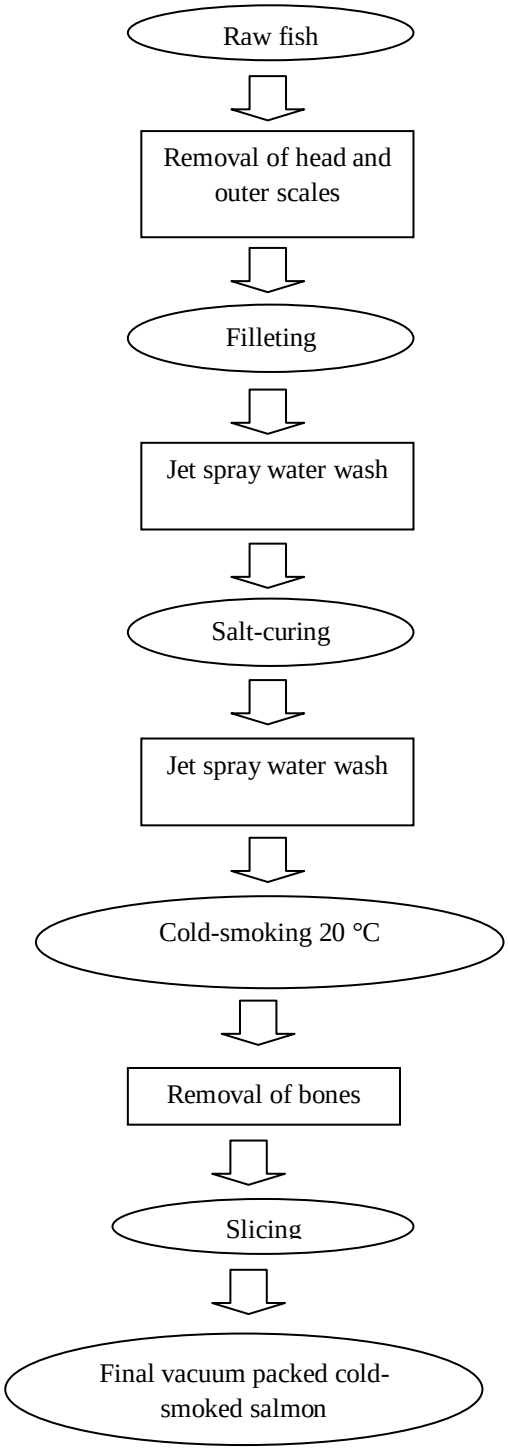


Figure 6-1: Schematic representation of different processing stages during the production of vacuum packed cold-smoked salmon. The stages represented in oval were taken for sampling.

6.3.2 Detection

Refer section 2.1 (Chapter 2: Materials and Methods)

6.3.4 MLVA typing

L. monocytogenes isolates (n = 60) were analysed for MLVA typing. Figure 6.2 represents the different banding pattern on 2% agarose gel.

Refer section 2.3(Chapter 2 Materials and Methods)

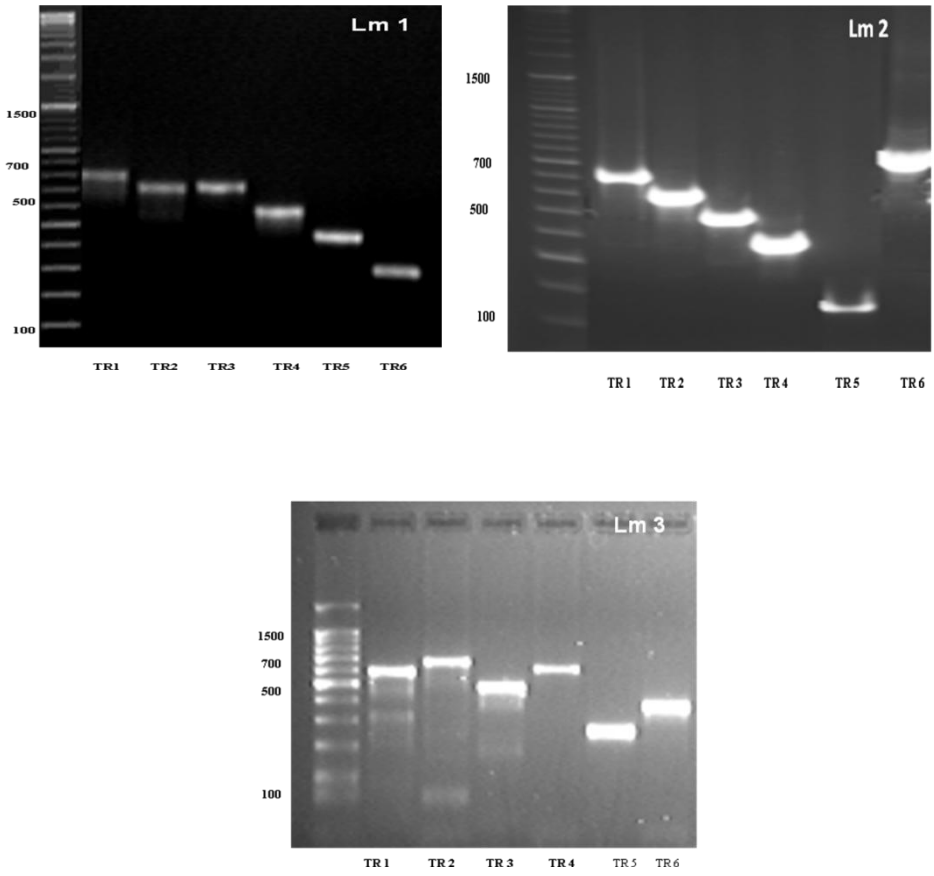


Figure 6-2: Three MLVA pattern (Lm 1, Lm 2 and Lm 3) on 2% Agarose gel, Lane 1- 100bp DNA ladder, Lane 2 to 7- TR 1 to TR6 aplicons.

6.3.5 Data Analysis

The amplicons were sized and the estimated number of tandem repeats calculated using Quantity One software (Biorad, Hercules, CA). The allele strings were imported into a Bionumerics software package (version 4.5; Applied Maths, Sint-Martems-Latem, Belgium). The genetic diversity at each TR was calculated using Bionumerics software package using Simpson's index.The distance between the isolates obtained from Bionumerics was exported into PHYLIP and the radial tree was constructed using Pearson and Ward correlation. Percentages of similarity are shown below the radial tree.

6.4 Results and Discussion

6.4.1 Incidence of *L. monocytogenes* in tested samples

A total of 60 raw salmon samples were traced through the different processing stages of cold-smoking for the presence of *L. monocytogenes* from Dec 2008 to Nov 2009. Of the 60 salmon samples tested, *L. monocytogenes* were isolated in varying degrees from the raw salmon (28.33 %), and the final vacuum packed cold smoked salmon (21.60 %; Figure 6.3). The contamination rates differed each month, with the highest recorded in March (100 %) with the all the samples tested positive and in October 80 % of the samples tested positive. Samples from December – February, April – August and November showed either a lower frequency of *L. monocytogenes* isolation or no contamination in the final product (Table 6.1).

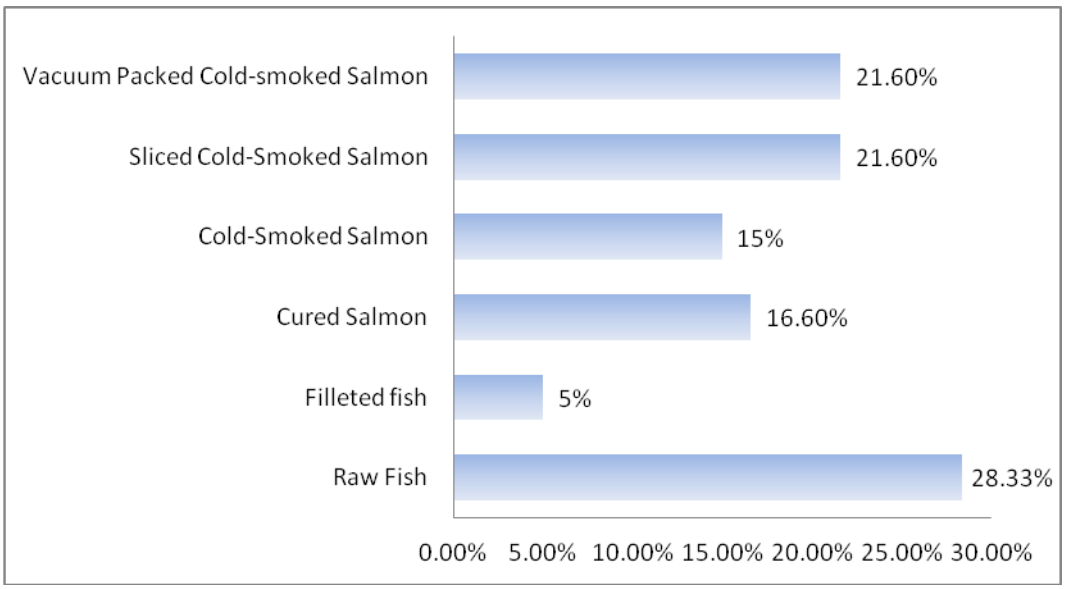


Figure 6-3: Percentage contamination of *L. monocytogenes* in raw fish, cured fish, cold-smoked fish, sliced cold-smoked fish and vacuum packed cold-smoked fish.

The recovery of *L. monocytogenes* from cold-smoked salmon highlights the continuing problematic nature of contamination of this food type. *L. monocytogenes* strains found in our studies were isolated from fish bred in coastal waters. This is in agreement with earlier studies by Huss et al. (1995) who suggested a higher incidence of *L. monocytogenes* in water bodies receiving heavy runoff from agricultural and urban areas. Previous studies also suggest that there are high incidences of *L. monocytogenes*presence in the raw fish (Embark *et al.*, 1994, Dauphing *et al.*, 2001, Nortan *et al.*, 2001, Timothe et al 2004, Gudmundsdottir *et al.*, 2005, Miettinen and Wirtanen, 2005 and Chitlapilly Dass 2010b).

The prevalence of *L. monocytogenes* in cold-smoked salmon has been reported to range from as low as0% (Guyer and Jemmi, 1990; Dillon and Patel, 1993) to much higher levels 30 % (Farber, 1991). Several studies conducted in various European countries on the prevalence of *L. monocytogenes* in cold-smoked salmon have stressed the importance of the problem of *Listeria* contamination which could lead to potential fatal listeriosis illness (Minttinen Wirtanen, 2006, Garrido *et al.*, 2009 and Chitlapilly *et al.*, 2010a). A survey conducted by

Gudmundsdottir *et al.*, (2005) in various smoked houses in Iceland found that 45% of the plant was contaminated with *L. monocytogenes*, mostly due to raw materials and processing environment. In their study it was found that well-maintained facilities had a lower incidence of *L. monocytogenes*.

Table 6-1: Frequency of *L. monocytogenes* isolation

Sample	Dec'08	Jan'09	Feb'09	Mar'09	Apr'09	May'09	Jun'09	Jul'09	Aug'09	Sep'09	Oct'09	Nov'09
Raw salmon		Lm 1		Lm 1			Lm 1	Lm 1			Lm 1	
Filleted smoked salmon									Lm3			
Cured salmon				Lm 1 Lm 2		Lm 2					Lm 1	
cold-smoked salmon				Lm 1 Lm 2							Lm 1	
Sliced smoked salmon				Lm 1 Lm 2				Lm 3	Lm 3		Lm 1	
Vacuum packed cold-smoked salmon				Lm 1 Lm 2				Lm 3	Lm 3		Lm 1	

6.4.2 MLVA subtyping

The application of epidemiological typing systems for *L. monocytogenes* is essential for understanding the source of contamination. Recent developmentsin molecular techniques have proven useful to the food industry to trace the source of contamination and assess whether the cleaning procedures are adequate (Murphy *et al.,* 2007, Miya *et al.,* 2008, Foley and Nayak *et al.,* 2009 and Chitlapilly Dass *et al.,* 2010b). MLVA has been used successfully to discriminate among the various *L. monocytogenes* strain type (Murphy *et al.,* 2007, Lindstedt *et al.,* 2008, Sperry *et al.,* 2008 and Smith, 2010, Chitlapilly *et al.,* 2010a and Chitlapilly et al 2010b)

A total of 60 *L. monocytogenes* isolates representing 3 isolates from each positive sample (Palcam selective media) were subtyped using 6 MLVA primer set. MLVA subtyping resulted in 3 different MLVA profiles (Figure4). All the isolates derived from the raw fish formed one MLVA type Lm 1. Strain type Lm 2 was isolated from cured salmon, smoked salmon and sliced cold-smoked salmon and the final vacuum packed cold-smoked salmon. Lm 3 was isolated from filleted cold-smoked salmon, sliced and final vacuum packed cold-smoked salmon. All the five samples analysed in March were contaminated with multiple strains of *L. monocytogenes* Lm 1 and Lm 2. The radial tree constructed with the 60 *L. monocytogenes* isolates showed 3 distinct clusters. Each cluster denoted one strain type thus showing the distinction among the isolates. The percentage of similarity among the three strain type isolated reflected the genetic relatedness among the three isolates (Figure6.4).

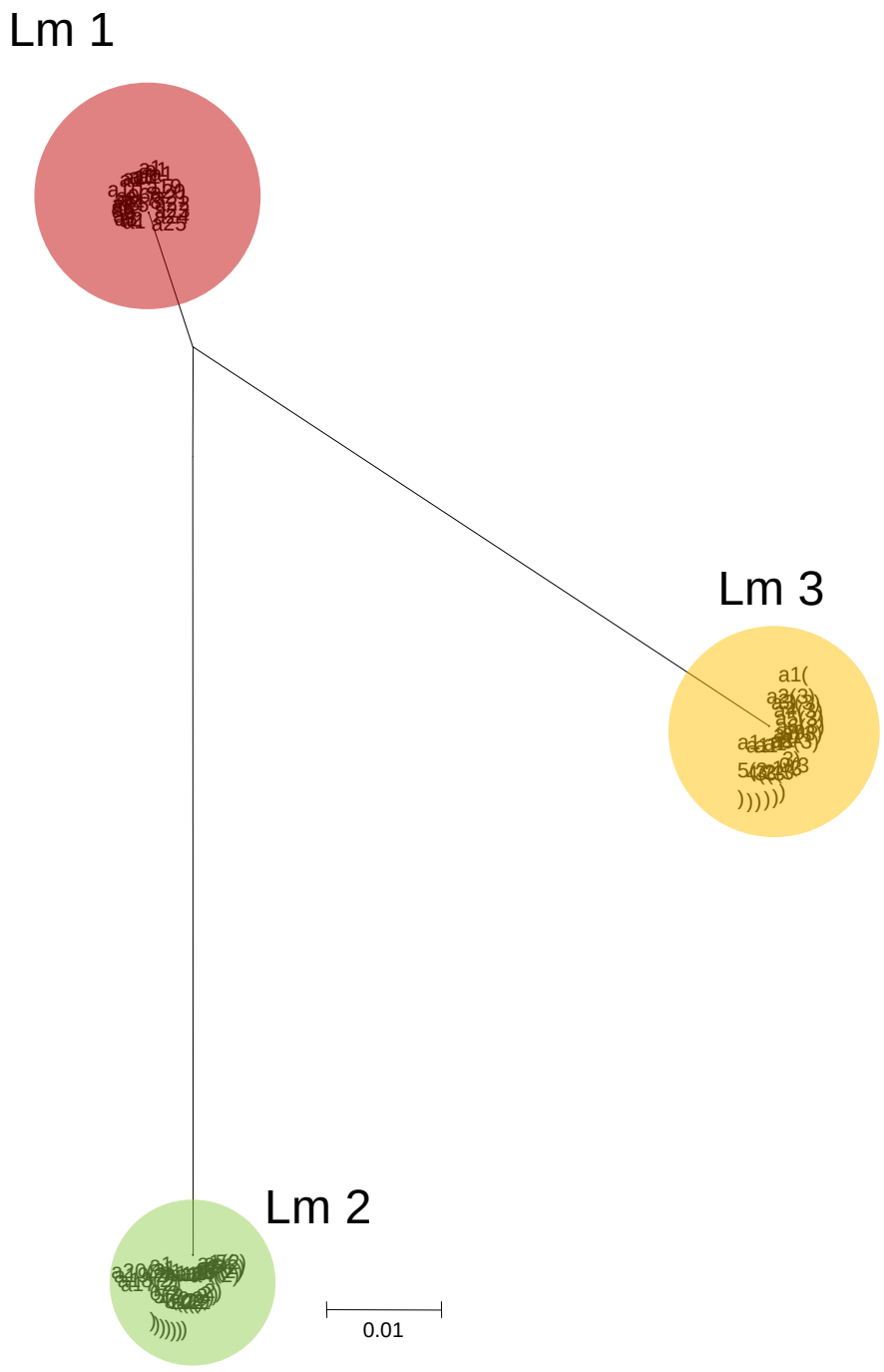


Figure 6-4: The radial tree was constructed with PHYLIP using Pearson and Ward correlation. The distance is shown below the radial tree. Each clade represents one MLVA type, Lm 1 Lm 2 and Lm 3.

Despite the presence of a higher percentage of *L. monocytogenes* in raw fish (28.33 %), there was only 21.60 % of *L. monocytogenes* isolated from the final product. The results obtained indicate that *L. monocytogenes* entering the plant *via* raw fish is not completely eliminated by the antibacterial step (curing with salt and cold-smoking at 20 °C). As *L. monocytogenes* can survive a wide rage of temperatures (1- 45 °C), high salt concentration (≤ 10 %) and pH 4.5 - 9.6, it will not be totally eliminated by salt curing and smoking at 20 °C. Initial contamination of the raw fish with *L. monocytogenes* (Lm1) and an inability of the antibacterial steps involved during processing plays a vital role for the persistent existence of Lm 1 in the final product. These results are in line with the work conducted by Medrala *et al.,* 2003 and Miettinen, 2006, where strains isolated from the raw fish were present in the final product and also the strains from the raw fish resulted in cross-contamination in the processing environment where similar strains were isolated from the floor and processing equipment.

In addition, to the Lm 1 strain isolated from raw fish there were two additional strains attained by the salmon during the intermediate processing stages (Lm 2 and Lm 3) which were not isolated from raw fish. Strain type Lm 2 was isolated from the salmon after curing and Lm 3 was isolated after filleting andslicing. Strain type Lm 2 and Lm 3 could have been an in-house flora which could have established themselves in the processing equipment and environment and were not eliminated by the cleaning protocols practiced by the processing factory. It has been confirmed that *L. monocytogenes* can establish itself and persist for long times, particularly in the processing plant environment (Unnerstad *et al.*,1996, Vogel *et al.*, 2001, Gudmundsdottir *et al.,* 2005 and Miettinen *et al.,* 2005). In the study conducted by Gudmundsdottir and co-workers (2005), they indicate that the filleting, slicing and brining processes in cold-smoked salmon processing may provide reservoirs for some *L. monocytogenes*. According to Vogel and co-workers (2001) the most critical steps of the production line were salting and slicing, mainly because of difficulties with cleaning the

equipment thoroughly. These findings are in line with the present study, as the strains Lm 2 was isolated after curing and Lm 3 isolated afterfilleting and slicing, indicating that potential problem sitescould be the curing vessel, slicing/ filleting board and knife, as these equipment would have undergone cleaning and sanitizing procedures before the start of the processing.

In some of the final product, in addition to Lm 1, there has been re-infection of the product with 'in-house' strains acquired along the intermediate processing stages (Lm 3), which was isolated from the filleting and slicing stages). Multiple strains of *L. monocytogenes* co-existed on the final vacuum packed cold-smoked salmon, these results are in line with the work done by Medrala *et al.*,(2003), Miettinen *et al.*, (2005), Gudmundsdottir *et al.*, (2005) and Garrido *et al.*, (2009).These results thus support two hypothesis (i) the products do not constitute a particular microenvironment in which only one strain survive, (ii) some strains may have been introduced with the raw material, and found a niche in the processing factory, from where they are constantly shed during the processing thus contaminating products (Martinez *et al.,* 2003).

To reduce the number of *L. monocytogenes* cells, the source of contamination must be identified. Fish tracking experimentsare essential to trace the route of contamination from raw to processed cold-smoked salmon. In the current study, MLVA typing of the isolates indicated that contamination with *L. monocytogenes*was mostly due to the raw fish contamination and direct contact with contaminated processing equipment, and it was also possible to identify specific areas (filleting, curing and slicing) at which contamination of the final product occurred.

6.5 Conclusions

The contamination pathways of *L. monocytogenes* are numerous, and raw fish could be a significant source of contamination for the processing environment and equipment. Preventing the introduction and spread of *L. monocytogenes* is important to avoid the risk of contamination in the final product. Because *L. monocytogenes*willcontinue to be introduced into the processing plant environment, controlmust be directed towards preventing the establishment and growthof this organism in these environments. The control optionsmust rely primarily on a proper cleaning and sanitation programs.However, production of products consistently free ofthe organism may be impossible due to the ubiquitous nature of the *L. monocytogenes*. The ability to carry out epidemiological investigations to determine the primary source of bacterial contamination is important so that preventive measures can be implemented to reduce *L. monocytogenes* prevalence and protect human health. In this study MLVA allowed for rapid and sensitive subtype discrimination of *L. monocytogenes*, thus facilitating identification of possible contamination pathways.

Chapter 7 : Dynamic modelling of *L. monocytogenes* growth in vacuum packed cold-smoked salmon under typical retail and consumer storage conditions.

7.1 Summary

A product specific model was developed and validated under dynamic temperature conditions to predict the growth of *L. monocytogenes* in cold-smoked salmon taking into account the retail and consumer phase of the food pathways. The variability in time and temperature during retail storage, consumer transport and consumer storage were included in the model. Vacuum packed cold-smoked salmon was inoculated with a 10CFU/g cocktail of 3 strains of *L. monocytogenes* and stored at 4, 8, 12 and 16 °C for 18 days. The growth kinetic parameters at each temperature were obtained by fitting the observed data to the primary model of Baranyi and Roberts. The maximum specific growth rate was further modelled as a function of temperature by the square root model. The model was validated under two scenarios of dynamic temperature conditions incorporating the fluctuations occurring during the various stages of the food pathways (retail and consumer phase) in the range from 4 to 16°C. Growth predictions for dynamic temperature scenarios were based on the square root model and the differential equations of the Baranyi and Roberts model, which were numerically integrated with respect to time. The model performance was based on the measures of bias factor (B_f), accuracy factor (A_f) and goodness of fit (GoF). Results showed that the model could adequately predict the growth of *L. monocytogenes* under the different temperature scenarios assayed. The values of $B_f$and $A_f$of the modelwere close to unity, indicating good agreement between observations and predictions. The model was also compared to two growth predictors; Combase and Seafood Spoilage and Safety Predictor (SSSP) and the predictions obtained gave anoverestimation of *L. monocytogenes* growth. This study illustrates the potential of dynamic modelling of *L. monocytogenes* growth for vacuum packed cold-smoked salmon from retailer to consumer as a means of evaluating the product safety at different stages of the food pathways.

Part of this chapter have been submitted for publication *Journal of Food Microbiology* and also presented at ICFHM conference (Page: 212).

7.2 Introduction

Predictive or quantitative microbiology employs quantitative terms (mathematical models) to express the effect of environmental conditions on microbial growth kinetics (Dalgaard and Jorgensen, 1998). Although it is generally accepted that this approach can be useful in predicting food quality and safety, high accuracy of the constructed models is a prerequisite for their application by the food industry. So far, the majority of published mathematical models dealing with either the growth or survival of pathogens, were developed using experiments conducted in liquid media or simulated foods under constant temperatures (Cheroutre-Vialette and Lebert, 2000).

Food distribution channels are often prone to temperature fluctuation during transit from production environment to consumer storage. Therefore, it is important to study the changes in microbial population that occur as a result of fluctuating temperatures. This knowledge would assist in predicting the actual impact of microbial contamination at the time of consumption (Dalgaard and Jørgensen 1998, Xanthiakos *et al.*, 2006, Panagou and Nychas 2008, Koutsoumanis *et al.*, 2010; Mejlholm *et al.*, 2010). It is important to be able to understand and predict the response of microorganism, more specifically *L. monocytogenes*, along its food pathways in actual food matrices. The term 'food-pathways' refers to the journey encountered by the food from its processing factory, distribution channel, retail storage, consumer travel (from retail storage to consumer storage) and finally consumer refrigeration storage.

To ensure proper sensory quality and food safety, developing a model under typical retail and consumer temperature practice using real food product would be ideal (Vermeulen *et al.*, 2011). This would provide an opportunity to develop product-specific models with more accurate food safety prediction. Thussuch models would assist in formulating safer food products and wouldavert much empirical microbial testing of food under constant environmental conditions.Hence in this study, the actual food matrix along with its background flora, time and temperature fluctuations encountered at the retail storage, consumer travel (from retail storage to consumer storage) and consumer refrigeration storage has been incorporated to provide more realistic predictions.

The aim of this thesis was to illustrate how food pathways may be modelled under dynamic conditions and to study the impact of post-processing storage conditions on the growth of *L. monocytogenes* in vacuum packed cold-smoked salmon. Thus, the objectives of the study were to (i) develop a product-specific model for the effect of temperature on the growth of *L. monocytogenes* in vacuum packed cold-smoked salmon, (ii) validate the model under 2 scenarios of fluctuating temperatures encountered under typical retail and consumer practice, (iii) compare the developed model with two growth predictors, Combase and Seafood Spoilage and Safety Predictor (SSSP).

7.3. Materials and Methods

7.3.1. Stock culture preparation

Three strains of *L. monocytogenes*, LMSS 05, LMSS 11 and LMSS 23 (these strains were from the collection of the strains isolated from cold-smoked salmon by Chitlapilly *et al.*, 2010) were used. Stock cultures were maintained in vials of 20 % glycerol at – 80 °C until

further use. The cultures were revived by adding 1 ml of 20 % glycerol stock to 9 ml of tryptic soy broth (TSB: Scharlau – Chemie, Barcelona, Spain) and incubated at 30 °C for 24 h. Before the start of the experiment, a loopful of each strain was transferred into 9 ml of TSB and subcultured twice at 30 °C for 24h.

7.3.2. Inoculation of smoked salmon and sampling

Fifty gramsquantities of vacuum packed cold-smoked salmon were obtained from the cold-smoked salmon processor in Dublin and inoculated with 3 strains cocktail. The cocktail was prepared by combining each of the 3 cultures (grown overnight in 10 ml TSB at 30 °C) in sterile tubes and centrifuged at 3000 x g at 4 °C for 30 min. The pellets were washed with sterile Ringer's solution (Oxoid LTD, UK) and centrifuged for 30 min at 3000 x g at 4 °C and the resulting pellets were resuspended in the Ringer's solution to a final volume of 5 ml. The cell level in the resulting composition was 6 log CFU/ml, as assessed by McFarland's standard (BioMérieux,Marcy-l'Etoile, France).

The 50 g pack of vacuum packed cold-smoked salmon was opened inside the laminar air-flow chamber using a sterile scissors and 200 µl of the appropriate diluted culture was added to the cold-smoked salmon sample to obtain 1 $\log_{10}$ CFU/ml. In order to obtain uniform distribution, the inoculum was spread on the surface of the smoked salmon using sterile spatula. The cold-smoked salmon sample was re-vacuum packed (Multivac, MSC, Ireland). The process of opening the vacuum pack, inoculation with cocktail strains of *L. monocytogenes* and re-vacuum packing took 15 min per sample and was done under sterile environment. Two hundred and sixteen packs were inoculated (54 packs for each temperature) and stored at 4, 8, 12 and 16°C in an incubatorat the respective temperatures (Friocell, MSC, Ireland). The experiment was replicated twice (n = 2).

7.3.3. Microbial Analysis

The samples were analysed everyday for 18 days (recommended shelf-life as provided by the manufacturer). Enumeration was done by transferring 25 g of smoked salmon aseptically into a stomacher bag and blending with 225 ml of 1/4 strength Ringer's solution in a stomacher (Lab blend 400, Seward Medical, London, UK) for 60 s at medium speed. One ml of the homogenate was taken and decimal dilution was prepared with Ringer's solution. 0.1 ml sample of three appropriate dilutions were spread in duplicates on Palcam (Scharlau – Chemie,Barcelona, Spain) for *Listeria* and incubated at 30 ℃ for 48 h and on plate count agar (Scharlau – Chemie,Barcelona, Spain) for total viable count at 24 ℃ for 48 h. Data from the plate count were log transformed.

7.3.4. Description of the applied mathematical model

L. monocytogenes growth data (log CFU/g) in vacuum packed cold-smoked salmon was fitted to the DMFit Excel add-in (Baranyi and Roberts, 1994). The Square root model was applied to model the maximum specific growth rate (μ_{max}) obtained at isothermal conditions as a function of the storage temperature studied (Ratkowsky *et al.*, 1983)

$$\sqrt{\mu_{max}} = b\,(T - T_{min}) \quad \text{.....Equation 7-1}$$

Where b is a constant, T (°C) is the storage temperature and $T_{min}$is the theoretical minimum temperature for growth of the organism, $T_{min}$was estimated by extrapolation of the regression line to $\mu_{max} = 0$.

The development of the 'salmon-model' was based on combining the prediction of *L. monocytogenes* growth under dynamic storage temperature (time – temperature) profiles of smoked salmon, *T (t)* and the square root model (Equation 7-1) for the estimation of the

'momentary' $\mu_{max}$and also with the differential equation of Baranyi and Roberts model Equation 7- 2 and 7-3 whicwere integrated with respect to time

$$\frac{d}{dt}y = \left(b(T(t) - T_{\min})\right)^2 \left(\frac{q}{q+1}\right)\left(1 - \frac{y}{y_{\max}}\right)x \text{ ..... Equation 7-2}$$

$$\frac{d}{dt}q = \left(b(T(t) - T_{\min})\right)^2 q \text{ ..... Equation 7-3}$$

Where t is time, y is the concentration of the microbial population at time t, $y_{max}$is the maximum population density and the parameter q denotes the concentration of a substance critical for microbial growth. Hence the model developed in this research work is referred to as the 'salmon-model'.

7.3.5 Validation under dynamic condition

The model developed (salmon-model) under isothermal conditions was validated against observed growth of the pathogen under two dynamic temperature scenarios which simulated temperature fluctuation from retail to consumer storage conditions. The vacuum packed cold-smoked salmon (50 g) were inoculated with 3 strain cocktail of *L. monocytogenes* (as described in section 2.2) and stored at specific temperatures. The storage temperature was monitored using data-logger- monitoring devices (Squirrel view 3400, MSC, Ireland). The packages were stored in high precision incubator (± 1 ˚C; Friocell, MSC, Ireland) at three consecutive stages: (1) retail storage, (2) consumer transport from retail to consumer storage and (3) consumer storage, for both the scenarios tested as indicated in Tables 7.1 and 7.2.

Table 7-1: Scenario 1: Representing cycle of the dynamic temperature profile for retail refrigeration, consumer shopping and consumer storage conditions.

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Retail Refrigeration	
Time (h)	**Temperature (°C)**
12	4
6	8
12	16

Consumer Shopping	
Time (min)	**Temperature (°C)**
30	Room temperature (±18 ˚C)

Consumer Storage	
Time (h)	**Temperature (°C)**
12	12
12	6

Final analysis for levels of *L. monocytogenes*

Table 7-2: Scenario 2: Representing cycle of the dynamic temperature profile for retail refrigeration, consumer shopping and consumer storage conditions.

Retail Refrigeration	
Time (h)	**Temperature (°C)**
12	4
6	8
12	16

Consumer Shopping	
Time (h)	**Temperature (°C)**
3	Room temperature (±18 ˚C)

Consumer Storage	
Time (h)	**Temperature (°C)**
12	12
12	6

Final analysis for levels of *L. monocytogenes*

The sampling for the levels of *L. monocytogenes* was done at the end the consumer refrigeration storage. The sampling was carried out for 18 days to cover the shelf-life (as recommended by the producers). The validation was done by numerically integrating equation 7-2 and 7-3 along with the time and temperature profile obtained for the two temperature scenarios in Excel 2007.

The temperature range chosen for the fluctuating temperatures were obtained from the Food Safety Authority of Ireland (2002) survey. In Ireland, 75 % of the retail refrigeration is between (4 - 16 °C), 62.5 % of households takefrom 30 mins up-to 3 h for travelling from the retail storage to consumer storage and 92 % of household stored food products under refrigerated condition are between 4 °C - 12 °C (FSAI, 2002).

7.3.6. Comparison of salmon-model with predictive software

The predictions obtained for *L. monocytogenes* from salmon-model were compared with those obtained from the growth predictor Combase (www.combase.cc) and the Seafood Spoilage and Safety predictor (SSSP) v. 3.1. Comparison of the predictions was based on the bias factor (B_f), the accuracy factor (A_f) and the goodness of fit (GoF; Ross, 1996; Mataragas *et al.*, 2006). Both programs can provide predictions for microbial growth under fluctuating temperature conditions. In SSSP, the temperature fluctuations recorded from this study can be uploaded and the predictions obtained from the developed model in this study are compared to those obtained using sliced vacuum packed cold-smoked salmon in contrast to Combase where the predictions are based on using synthetic media.

7.4. Results and Discussion

The ubiquitous and psycho to mesophlic (0 – 40 °C) nature of *L. monocytogenes* makes the control of this pathogen difficult as it can multiply at refrigeration temperatures which are the recommend storage conditions for lightly preserved food.

Several factors that would influence the growth of *L. monocytogenes* in vacuum packed cold-smoked salmon were considered before conducting this study such as, actual food matrix (vacuum packed cold-smoked salmon), background microorganisms, storage time and temperature, and consumer travel patterns from retail to consumer refrigeration storage conditions. To perform a more accurate challengetest, Garido *et al.*, (2009) recommends that ‘‘inocula concentration used experimentallyshould be similar to those detected naturally in the foodproduct but enough for precision counts”. In this study 1 log CFU/g was used for the challenge-test which is in line with what would be naturally found on vacuum packed cold-smoked salmon (Minnetan and Warten, 2005, Garrdo *et al.*, 2009, Chitlapilly Dass *et al.*, 2010a and Chitlapilly Dass *et al.*, 2010b).

The growth of *L. monocytogenes* in vacuum packed cold-smoked salmon was monitored at isothermal storage conditions from 4 – 16 °C. Fig 7.1 represents the growth of *L. monocytogenes* during 18 days of storage at 4, 8, 12 and 16 °C. The control sample tested negative for the presence of naturally contaminated *L. monocytogenes*. The variations in growth were a function of the storage temperature tested. According to Regulation (EC) 2073/2005, food safety criteria establish that ready to eat products should not exceed the limit of 100 CFU/g of *L. monocytogenes* throughout their shelf life. The regulation is applied to lightly preserved foods able or unable to support the growth of *L. monocytogenes* (Garrido *et al.*, 2009). The pathogens reached 2 log CFU/g ($\pm$ 2.5 log CFU/g) after 15, 3 and 2 days of storage at 4, 8 12 and 16 °C respectively.

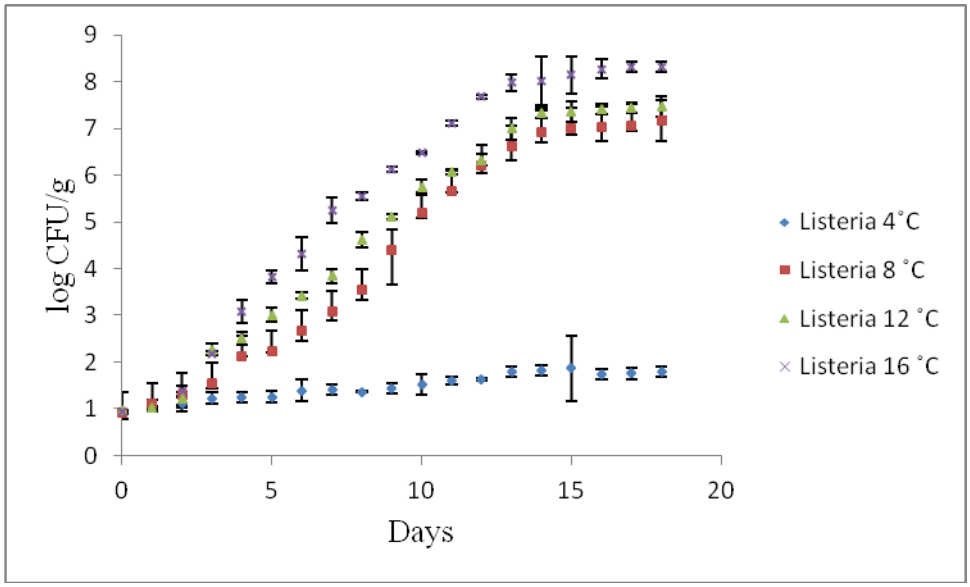

Figure 7-1: Growth curves of *L. monocytogenes* in vacuum packed cold-smoked salmon at different storage temperatures (4 °C (-▪-), 8 °C (-■-), 12 °C (- ▲-) and 16 °C (-x-)).

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The growth data under isothermal conditions (4, 8, 12 and 16 °C)were fitted to the DMFit Excel add-in, in order to calculate the maximum specific growth rate (μ_{max}), lag phase (λ) and maximum population density (MPD; Table 7.3).

Table 7-3 Parameters and statistics of the model of Baranyi and Roberts for the growth of *L. monocytogenes*in vacuum packed cold-smoked salmon (mean ±Standard error of two independent experiments with two replications each)

Temp (˚C)	Maximum specific growth rate μ_{max} (log CFU/h^{-1})	Lag phase λ (h)	Maximum population density y_{max} (log CFU/g^{-1})	Standard error of fit	R^2
4	0.031(± 0.003)	159(±26.5)	6.97(±0.18)	0.332	0.971
8	0.096(± 0.001)	93(±7.8)	7.82(±0.09)	0.411	0.983
12	0.153(± 0.007)	44(±10.1)	8.01(±0.17)	0.430	0.964
16	0.193(± 0.004)	23.4(±6.5)	8.53(±0.13)	0.451	0.982

The parameter h_o represents the 'work to be done' by the cell to adapt itself to the new environment and move from the lag phase to the exponential growth phase. The parameter h_o, related to the physiological state of the cells, was calculated as the product of $\mu_{max} \times \lambda$ as obtained under the isothermal condition investigated in this study. The effect of temperature on h_o is shown in Fig 7.2. At temperatures between 8 and 16 °C the average value of $h_o$was1.63 and 4.9 at 4 °C.

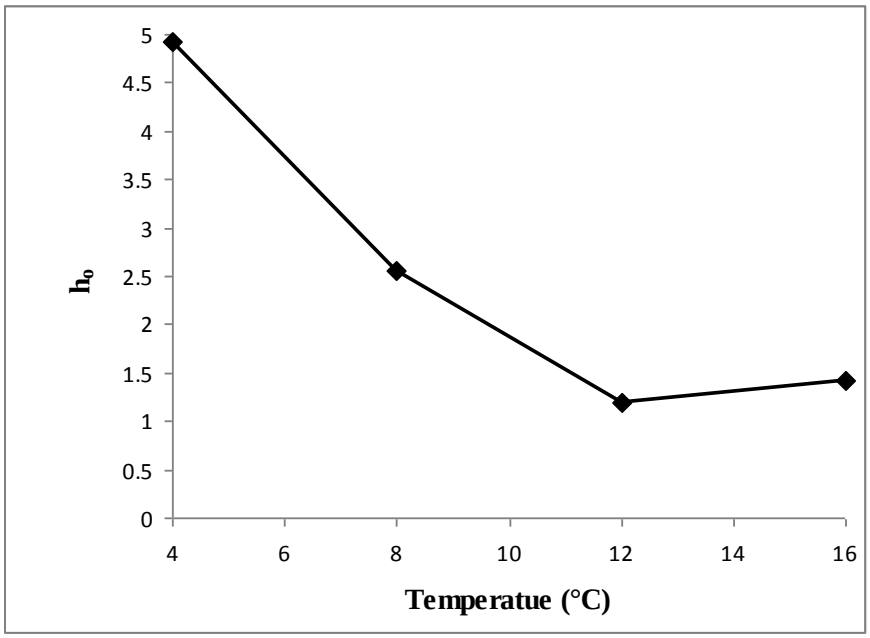


Figure 7-2: Effect of storage temperature on the adaptation work (h_0) of *L. monocytogenes* in vacuum packed cold-smoked salmon.Data points are mean (±standard error) of two independent experiments with two replications each.

The effect of temperature on the adaptation work parameter h_o required by the cell to adjust to new environment was relatively constant for storage temperatures 8 to 16 ºC (h_o= 1.63). At the storage temperature of 4 ºC, the value of h_o increased indicating that storage at low temperatures altered the physiological state of the bacteria. These findings are in line with other studies (Xanthiakos *et al.,* 2006; Panagou and Nychas, 2008) where the physiological

state of *L. monocytogenes* was relatively constant at temperatures between 10 to 16 ºC but increased substantially at lower temperatures.

The square root model was applied to model the maximum specific growth rate (μ_{max}) obtained at isothermal conditions as a function of the storage temperature studied (Fig 7.3). The T_{min} was calculated from the slope of the square root model and found to be - 4.23 ˚C which was in line with other studies on *L. monocytogenes* (Xanthiakos *et al.*, 2006). The square root model described the effect of storage temperature (r^2= 98.98 %) satisfactorily.

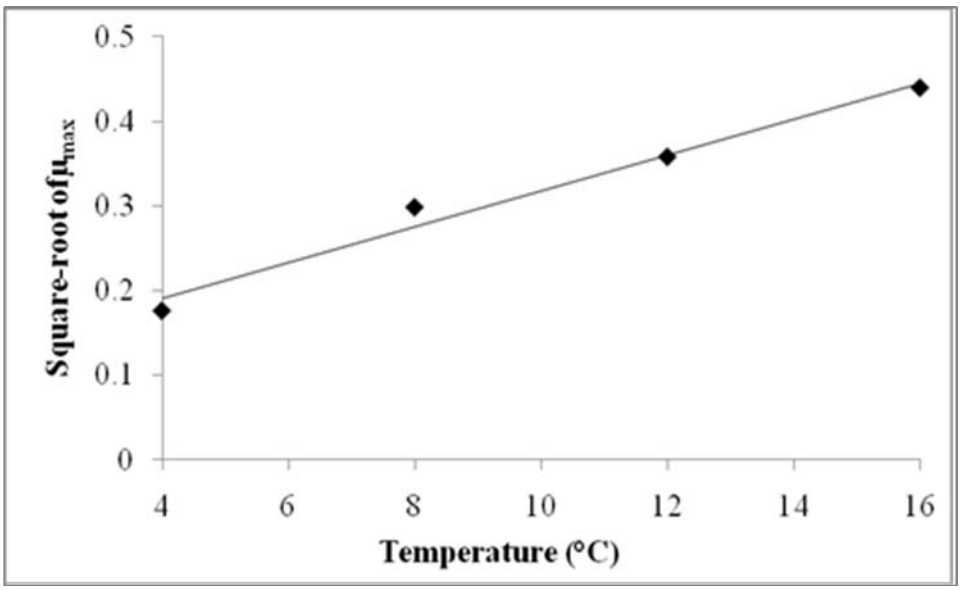


Figure 7-3: Square root type model for the effect of temperature on the maximum specific growth rate ($\sqrt{\mu_{max}}$) of *L. monocytogenes* in vacuum packed cold-smoked salmon. Data points are mean (±standard error) of two independent experiments with tworeplications each.

The salmon-model was validated against growth observed under dynamic conditions using 2 temperature scenarios mimicking retail storage conditions, consumer shopping habits and consumer refrigeration storage conditions (Table 7.1 and 7.2).

For the initial microbial population, the initial inoculum level (1 log CFU/g) determined by plate counting was used. The maximumpopulation (y_{max}) was taken as the mean value

estimatedfrom the individual curve fittings during storage under isothermal conditions (8.5logCFU/g). Predictions were based on two different values for h_o; the average value ofh_o observed at static storage temperatures from 8 to16°C was 1.63 andfor 4°C was 4.9. The comparison between predicted and observedgrowth of *L. monocytogenes* in cold-smoked salmon stored atdynamic temperature conditions for h_o= 1.63 is shown in Fig 7.4 and 7.5.

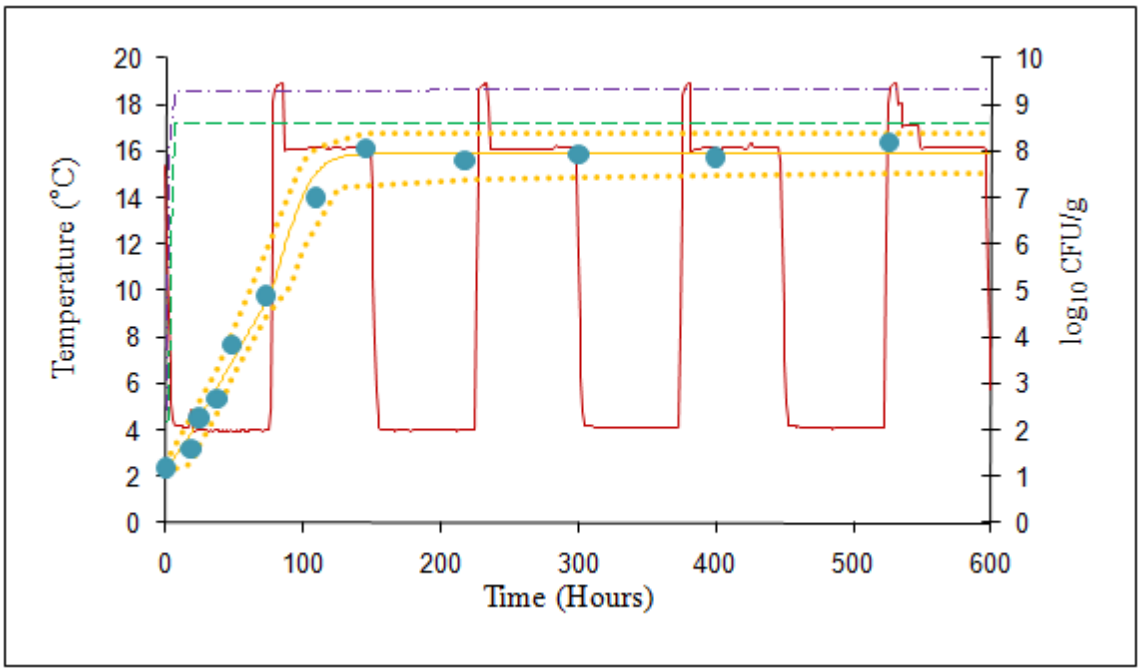


Figure 7-4: Comparison between the observed (-•-) and predicted (—) growth of *L. monocytogenes* in vacuum packed cold-smoked salmon under periodical changing temperature (Scenario 1). Comparison between the 'salmon-model' (—) and predictive program SSSP (— —) and Combase (— - — -) in scenario A.(…) indicate lower and upper 95% confidence intervals of the 'Salmon model'

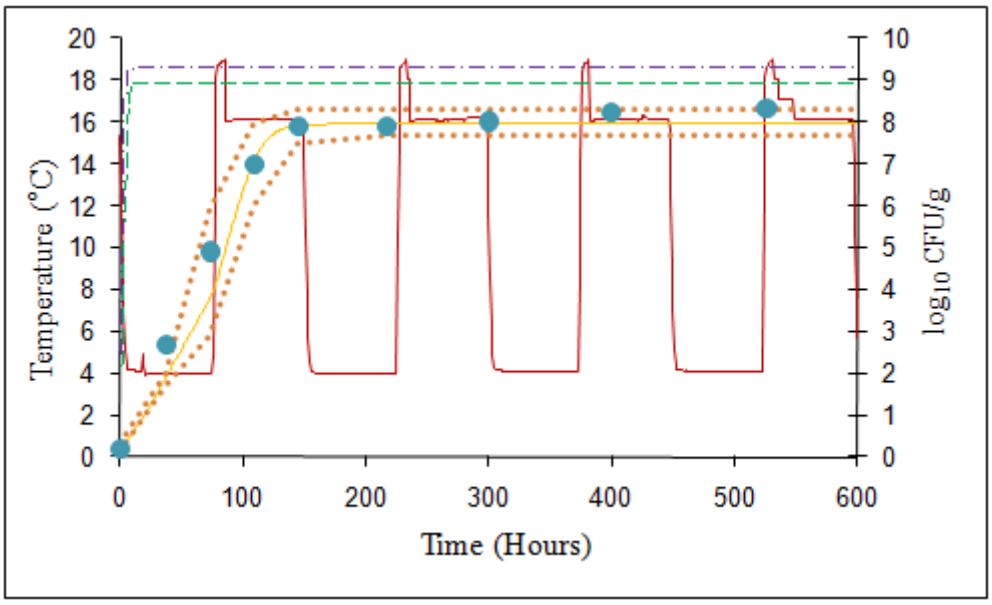


Figure 7-5: Comparison between the observed (-•-) and predicted (—) growth of *L. monocytogenes* in vacuum packed cold-smoked salmon under periodical changing temperature (Scenario 2). Comparison between the 'salmon-model' (—) and predictive program SSSP (— —) and Combase (— - — -) in scenario 2.(…) indicate lower and upper 95% confidence intervals of the 'Salmon model'

Predictions based on the value of h_o= 1.63 fitted accurately the growth of *L. monocytogenes* in both the scenarios tested. The prediction of the model was very poor when the average value of h_o observed at static temperatures 4 °C wasused, resulting in significant under-prediction of the model.These results are in agreement with other studies at low temperatures (Xanthiakos *et al.*, 2006, Panagou and Nychas, 2008, Koustsoumanias *et al.*, 2010 and Garrido *et al.*, 2010), who attributed this by the sudden cold shock that influenced the physiologicalstate of the organism.

In scenario 1, which follows the 30 min shopping regime, the pathogen reaches 2 log CFU/gat the end of 100 h (~ 4 days). In Scenario 2, the pathogen reaches 2 log CFU/g after 36 h of storage. In comparison to scenario 2, scenario 1 took longer time to reach the 2 log CFU/g levels. In both the scenarios even before reaching half of the recommended shelf-life

(as indicated on the products packaging) in this case 18 days, the products reached dangerous levels, pointing to the possible reduction in the shelf-life for safe usage of the product. In this study, it is evident that longer exposure of vacuum packed cold-smoked salmon during consumer transportation from retail to consumer refrigeration storage had an impact on the higher growth rate of *L. monocytogenes*. Thus, an improvement in the form of shorter transportation time and proper storage condition can be effective in reducing the proliferation of *L. monocytogenes*.

Comparison of salmon-model with the growth prediction software

The predictions of the salmon-model under dynamic temperature conditions were compared to the growth predictor software Combase and SSSP (Fig 7.4 and 7.5). The Combase model yielded significant over prediction of growth in comparison to the SSSP and salmon-model. SSSP growth model predicted values that were close to that of the salmon-model as indicated by the values of A_f, $B_f$and GoF in Table 7.4.

Table 7-4: Comparison of validation indices for the developed Salmon model and the other predictive programs for modelling the growth of *L. monocytogens* under periodical change in temperature**.**

Model	A_f	B_f	GoF
Salmon Model			
Scenario 1	1.061	1.043	0.419
Scenario 2	1.057	1.052	0.312
Combase growth predictor			
Scenario 1	1.198	1.182	1.731
Scenario 2	1.213	1.189	1.930
SSSP growth predictor			
Scenario 1	1.110	1.107	1.017
Scenario 2	1.127	1.093	1.009

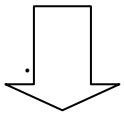
The bias and accuracy factor were assessed to understand the overall performance of the models. The values of B_f and A_f were close tounity, indicating good agreement between observations andpredictions.

The predictions of the salmon-model under fluctuating temperatures were compared with the simulated results obtained from SSSP and Combase. The predictions of SSSP were closer to the 'salmon-model' when compared to the Combase predictions. The predictions of SSSP were based on the product characteristics of pre-sliced vacuum packed cold-smoked salmon and the temperature input was imported from the data logger data used in recording the temperature fluctuation in this study. The over prediction of Combase predictor was due to the fact that this program was developed through experiments conducted in synthetic microbiological media or simulated foods and may thus overestimate the microbial growth

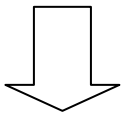
that actually occurs in real foods. Predictions based on such models are not necessarily valid in complex food environments because significant factors for microbiological growth such as food structure and microbial competition are not taken into account (Pin *et al.*, 1999; te Giffel *et al.*, 1999 and Koutsoumanis *et al.*, 2006).

Describing the growth of *L. monocytogenes* in vacuum packed cold-smoked along the food pathway is important, as this would aid in understanding the rate of contamination as the food moves along the retail to consumer refrigeration storage and also aid in the designing of the control measures. SSSP growth predictor was used in predicting the increase in the *L. monocytogenes* growth at each of the storage/shopping condition illustrated in Tables 7.1 and 7.2. The input used in simulating the retail, consumer shopping and consumer refrigeration storage in SSSP growth predictor is elaborated in Fig 7.6. SSSP growth predictor was used over the other software as it gave close predictions to the model developed in this study.

1 log CFU/g of initial level of *L. monocytogenes* contamination in vacuum packed cold-smoked salmon was used to predict the growth of *L. monocytogenes* at retail storage condition.



Growth levels of *L. monocytogenes* from the retail storage condition were used as initial feed for two consumer shopping scenarios 30 min and 3 h and levels of *L. monocytogenes* were obtained.



Growth levels of *L. monocytogenes* from the two consumer shopping scenarios were used as initial input for retail storage conditions to obtain the final levels of *L. monocytogenes* respectively for the 2 scenarios of shopping time.

Figure 7-6: Flow chart representing the application of the use of SSSP growth model in predicting the growth of *L. monocytogenes* at retail storage, consumer shopping and consumer storage conditions of vacuum packed cold-smoked salmon food pathways.

In Fig 7.7 and 7.8, the growth of *L. monocytogenes* at each storage/shopping condition is demonstrated. The temperature fluctuations encountered during retail storage on day 1 resulted in *L. monocytogenes* levels reaching 1.07 log CFU/g. Following a shopping period of 30 min, the *L. monocytogenes* counts were 1.12 log CFU/g, showing a 4.6 % increase from the retail storage. The population of *L. monocytogenes* after one day at consumer refrigeration storage conditions reached 1.21 log CFU/g which was a 13 % increase from the

retail storage. The limit of 2 log CFU/g was reached after 6 days of storage in the consumer refrigeration storage. The final level of *L. monocytogenes* after storage for 18 days was 8.6 log CFU/g.

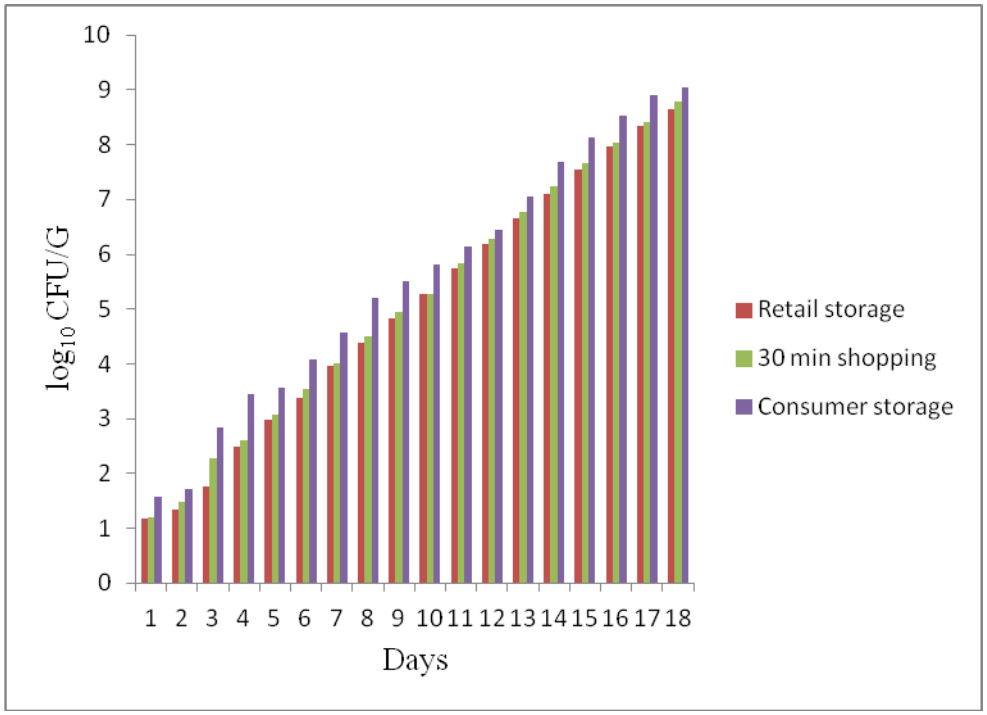


Figure 7-7: The growth of *L. monocytogenes* along the vacuum packed cold-smoked salmon food pathways of scenario 1 as predicted by SSSP growth predictor.

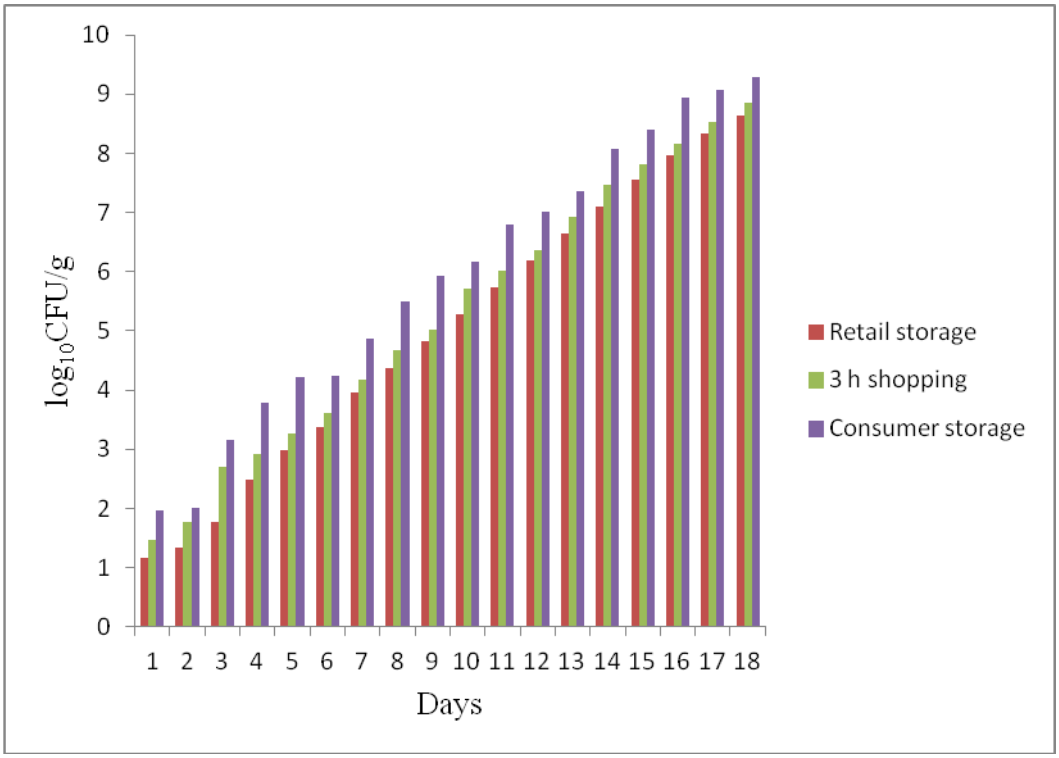


Figure 7-8: The growth of *L. monocytogenes* along the vacuum packed cold-smoked salmon food pathways of scenario 2 as predicted by SSSP growth predictor.

In the second scenario, after the retail fluctuation the level of *L. monocytogenes* on day 1 reached 1.04 log CFU/g, after 3 h of shopping the levels of *L. monocytogenes* were 1. 21 log CFU/g which was a 16 % increase from the retail storage conditions. The levels of L. monocytogenes after consumer storage of 1 day reached 1.39 log CFU/g showing a 33 % increase from the retail storage. The 2 log CFU/g limit was reached after 4 days of storage at the consumer refrigeration storage.

While assessing the individual parameters in the food-pathways (retail, consumer shopping and consumer refrigeration storage), the results showed that the consumer shopping time and temperature and the consumer refrigeration storage temperature are the most important factors affecting the concentration of *L. monocytogenes* at the time of consumption. This is in agreement with the conclusions of the available risk assessment studies of *L.monocytogenes* in ready-to-eat foods (Koutsoumanis, *et al.*, 2010), which stress theimportance of consumer

handling and storage temperature in consumerexposure to *L. monocytogenes* at the time of food consumption. The results from this study indicate that careful control of storage conditions can bring a significant reduction in levels of *L. monocytogenes* contamination; hence communication strategies should be focused on highlighting this to the consumers. This strategy should be directed to improve consumer awareness of the importance of correct storage conditions, where temperature abuse will be mostfrequent: unlike temperatures in a professional setting,it cannot be controlled by legislation (Cheroutre-Vialette and Lebert, 2000). Emphasis should be made on the health risk associated with consuming such products kept under abusive storage conditions, in particular preventive tips should be given to high risk populations.

7.5. Conclusions

The results obtained showed that the developed 'salmon-model' can effectively be used to study the *L. monocytogenes* growth behaviour at fluctuating temperature conditions. Such model could follow the microbial impact of different steps associated with retailing and consumer behaviour to food and this could be an important support to the food safety system. The modelprovides the sea food industry with a useful tool for effectivemanagement and optimization of product safety and maycontribute to more realistic estimations of safety risksrelated tovacuum packed cold-smoked salmon.Among predictive models used for comparison with the 'salmon-model', the SSSP growth predictor gave better prediction when compared to the Combase growth predictor, as SSSP growth predictions are based on work conducted on sliced vacuum packed cold-smoked salmon.

Chapter 8 : Growth modelling of 3-cocktail strains of *L. monocytogenes* and native microflora in vacuum packed cold-smoked salmon marketed in the Republic of Ireland

8.1 Summary

This study examined the growth characteristics of *Listeria monocytogenes* as affected by native microflora at refrigeration and abuse temperatures in vacuum packed cold-smoked salmon as retailed in the Republic of Ireland. A three strain cocktail of *L. monocytogenes* (isolated from a previously related study) was inoculated on vacuum packed cold-smoked salmon and stored at 4, 8, 12 and 16 °C for 18 days. The growth characteristics: lag phase duration (LPD, h), growth rate (GR, $\log_{10}$ CFU/h), and maximum population density (MPD, $\log_{10}$ CFU/g), of *L. monocytogenes* and the native microflora in vacuum packed cold-smoked salmon stored were determined by DM-Fit Excel add-in. At 4 °C, *L. monocytogenes* did not reach the stipulated 2 $\log_{10}$ CFU/g after incubation for 18 days, while *L. monocytogenes* reached 2 $\log_{10}$ CFU/g at abuse temperatures (8, 12, 16 °C) after 3 to 4 days ($\pm$ 2) of incubation. The pH decreased from 6.8 to 5.8 ($\pm$ 0.3) at 8, 12 and 16 °C after 18 days of storage, while a_w showed only a slight variation at all the temperatures tested. Square root model and inverse of square root model were applied to model thegrowth rate and lag phase duration as a function of storage temperature for both *L. monocytogenes* and native flora and the results were satisfactory ($R^2 > 0.9$) respectively. The GR, LPD and MPD of *L. monocytogenes* were restricted by the presence of native flora at refrigeration temperature of 4 ˚C. At higher temperatures the effect of native microflora was marginally diminished. The MPD of the native microflora was > 8 $\log_{10}$ CFU/g, whilst the MPD of *L. monocytogenes* were < 7.8 $\log_{10}$ CFU/g. This study demonstrates the risk of *L. monocytogenes* reaching unacceptable levels on vacuum packed cold-smoked salmon upon storage under abuse temperature and the role of native microflora in controlling *L. monocytogenes* under the studied conditions.

Some parts of this paper have been submitted for publication in Journal of Food Safety and also presented at Food Microbiology conference and Food Simulation conference (212-213).

Key words: *L. monocytogenes*, smoked salmon, square root model, inverse of square root modelnative microflora

8.1 Introduction

The growth behaviour of *L. monocytogenes* in vacuum packed cold-smoked salmon has been researched extensively. Studies have identified that salt, a_w, smoke components, lactate, pH, storage temperatures had effects on the growth of *L. monocytogenes* in smoked salmon (Augustin and Carlier, 2000, Lappi *et al.*, 2004; Tome *et al.*, 2007). Among these, temperature is considered the most important factor for spoilage (Koutsoumanis *et al.*, 2010). There were also studies which reported the influence of native microflora on the growth of *L. monocytogenes* in vacuum packed cold-smoked salmon during refrigeration temperature (Tome *et al.*, 2007 and Hwang and Sheen, 2009). The native microflora present on smoked salmon reaches 10^6–$10^8 \log_{10}$CFU/g after 3 weeks storage under vacuum at refrigeration temperature (Leroi *et al.*,2000), 60 % of the microflora is represented by lactic acid bacteria (LAB) while the remaining 40 % are Gram-negative microorganisms such as *Enterobacteriaceae, Shewanella putrefaciens, Photobacterium phosphoreum* and *Aeromonas* spp. which are characterized by a high spoiling potential and are responsible for unpleasant smell and taste (Miettinen *et al.*, 2006). This native microflora has a profound effect on the growth of *L. monocytogenes* and slows the growth rate of *Listera* (Hwang and Sheen, 2009). Nevertheless, these studies mainly reported the increase or decrease of cell countsof *L. monocytogenes* in vacuum packed cold-smoked salmon during storage. Data on *L. monocytogenes* and the native microflora, and the growth relationship between these two microflora in vacuum paced cold-smoked salmon are still limited and requires further investigation.

The objectives of this study were to examine the growth characteristicsof *L. monocytogenes* and native microflora in vacuum packed cold-smoked salmon at refrigerated and abuse temperatures typically encountered during retail and consumer storage. In this study, the change in the pH and a_w was also monitored throughout the shelf life of vacuum packed cold-smoked salmon and its effect on the growth of *L. monocytogenes* was demonstrated.

8.3. Materials and Methods

8.3.1. Stock culture preparation

Three strains of *L. monocytogenes,* LMSS 11, LMSS 17 and LMSS 23 (from the collection of strains isolated from vacuum packed cold-smoked salmon by Chitlapilly *et al.*, 2010b) were used. Stock cultures were maintained in vials of 20 % glycerol at - 80 ℃ until further use. The cultures were revived by adding 1 ml of 20 % glycerol stock to 9 ml of tryptic soy broth (TSB: Scharlau – Chemie, Barcelona, Spain) and incubated at 30 ℃ for 24 h. Before the start of the experiment, a loop-full of each strain was transferred into 9 ml of TSB and subcultured twice at 30 ℃ for 24h.

8.3.2. Inoculation of smoked salmon and sampling

Fifty grams of vacuum packed cold-smoked salmon were obtained from the manufacturer followed by inoculation with 3 strains cocktail of *L. monocytogenes*. The cocktail was prepared by combiningequal volumes of each of the 3 cultures (grown overnight in TSB at 30 ℃) in sterile tubes and centrifuged at 3000 x g at 4 ℃ for 30 min. The pellets were washed with sterile ¼ Ringer's solution (Oxoid LTD, UK) and centrifuged for 30 min at 3000 x g at 4 ℃ and the resulting pellets were resuspended in the ¼ Ringer's solution to a final volume of

5 ml. The cell level in the resulting composition was 6 $\log_{10}$ CFU/ml, as assessed by McFarland's standard (BioMérieux, Marcy-l'Etoile, France).

The 50 g pack of vacuum packed cold-smoked salmon was inside the laminar air-flow chamber using a sterile scissors and 200 µl of the appropriate diluted culture was added to the cold-smoked salmon sample to obtain 1 $\log_{10}$ CFU/ml. To attain uniform distribution, the inoculum was spread on the surface of the smoked salmon using sterile spatula. The cold-smoked salmon sample was re-vacuumed packed (Multivac, MSc, Ireland). The entire process of inoculation and re-vacuum packing took approximately 10 min. Two hundred and sixteen packs were inoculated (54 packs for each temperature) and stored at 4, 8, 12 and 16°C ($\pm$ 2 ˚C) in incubation chamber (Friocell, MSc, Ireland). The experiment was replicated twice (n = 2).

8.3.3. Microbial analysis

The samples were analysed everyday for 18 days (shelf-life as provided by the manufacturer). Enumeration was done by transferring 25 gm of smoked salmon aseptically into a stomacher bag and blending with 225 ml of ¼ Ringer's solution in a stomacher (Lab blend 400, Seward Medical, London, UK) for 60 s at medium speed. One ml of the homogenate was taken and decimal dilutions were prepared with ¼ Ringer's solution. 0.1 ml sample of three appropriate dilutions were spread platedin duplicates on Palcam (Scharlau-Chemie, Barcelona, Spain) for *Listeria* and incubated for 48 h at 30 °C and on plate count agar (PCA; Scharlau-Chemie, Barcelona, Spain) for the growth of native microorganism at 24 °C for 48 h. Data from the plate count were log transformed.

8.3.4 Determination of lag phase duration, growth rate, and maximum population density

L. monocytogenes growth data (log CFU/g) during storage at 4, 8, 12, and 16 ˚in vacuum packed cold-smoked salmon were fitted to the primary model of Baranyi and Roberts (1994) using the DMFit Excel add-in available at www.combase.com, to obtain the lag phase durations (LPD, h), growth rates (GR, $\log_{10}$ CFU/h), and maximum population density (MPD, $\log_{10}$ CFU/g).

The square root model was applied to model the maximum specific growth rate (GR) as a function of the storage temperature studied (Ratkowsky *et al.*, 1983)

$$\sqrt{GR} = b\,(T - T_{min}) \text{ ---------- Equation 8-1}$$

Where b is a constant, T (°C) is the storage temperature and T_{min} is the theoretical minimum temperature for growth of the organism, T_{min} was estimated by extrapolation of the regression line to $\sqrt{GR} = 0$.

The inverse of square root model was applied to model the LPD as a function of the storage temperature studied (Augustin *et al.*, 2000). The T_{min} of both lag phase and exponential growth phase is reported to be different; hence the inverse square root model was used to determine the T_{min} for lag phase, thereby determining the fast growing bacteria in the vacuum packed cold-smoking bacteria (Koutsoumanis *et al.*, 2010).

$$\sqrt{\frac{1}{LPD}} = b\,(T - T_{min}) \text{ ----------Equation 8-2}$$

Where b is a constant, T (°C) is the storage temperature and Tmin is the theoretical minimum temperature for growth of the organism, Tmin was estimated by extrapolation of the regression line to $\sqrt{\frac{1}{LPD}} = 0$.

8.3.4. Physicochemical estimation

The pH of cold-smoked salmon was measured using an Orion pH meter (Beverly, Mass., U.S.A.) for 18 days. The water activity (a_w) of vacuum packed cold-smoked was measured

using an AquaLab Model CX2 water activity meter (Decagon Devices, Pullman,Wash., U.S.A.) for 18 days. The pH and a_w were measured for each day for 18 days to cover the shelf life of the product. The samples were recorded as replicates of five and the mean was noted as final value.

8.4. Results and Discussion

8.4.1. Growth of *L. monocytogenes* and native microflora in vacuum packed cold-smoked salmon

The growth of *L. monocytogenes* and native microflora in vacuum packed cold-smoked salmon at 4, 8, 12 and 16 ℃ are shown in Fig 8.1. Several factors that would influence the growth of *L. monocytogenes* in vacuum packed cold-smoked salmon were considered before conducting the study such as storage temperature and the actual food (vacuum packed cold-smoked salmon) with its native microflora. To perform a more accurate challenge test, Garido, *et al.,* (2010) recommends that ‘‘innocula concentration used experimentally should be similar to those detected naturally in the food product but enough for precision counts”. In this study 1 $\log_{10}$ cfu/g was used as the initial innoculum level was similar to what would be naturally found on the product (Minnetan and Warten, 2005, Garrdo *et al.,* 2009, Chitlapilly Dass *et al.,* 2010a). With respect to the temperature range studied, this study took into consideration a survey conducted by the Food Safety Authority of Ireland (FSAI, 2002), which showed that in over 70% of domestic refrigerators were temperatures were reported to be above 6 ˚C. Research shows that native microflora have a profound effect of restricting the growth of *L. monocytogenes* (Hwang and Sheen, 2009), therefore in this study the native flora in its natural environment (vacuum packed cold-smoked salmon) were studied to estimate their role in the actual risk of *L. monocytogenes* growth under various storage temperature scenarios.

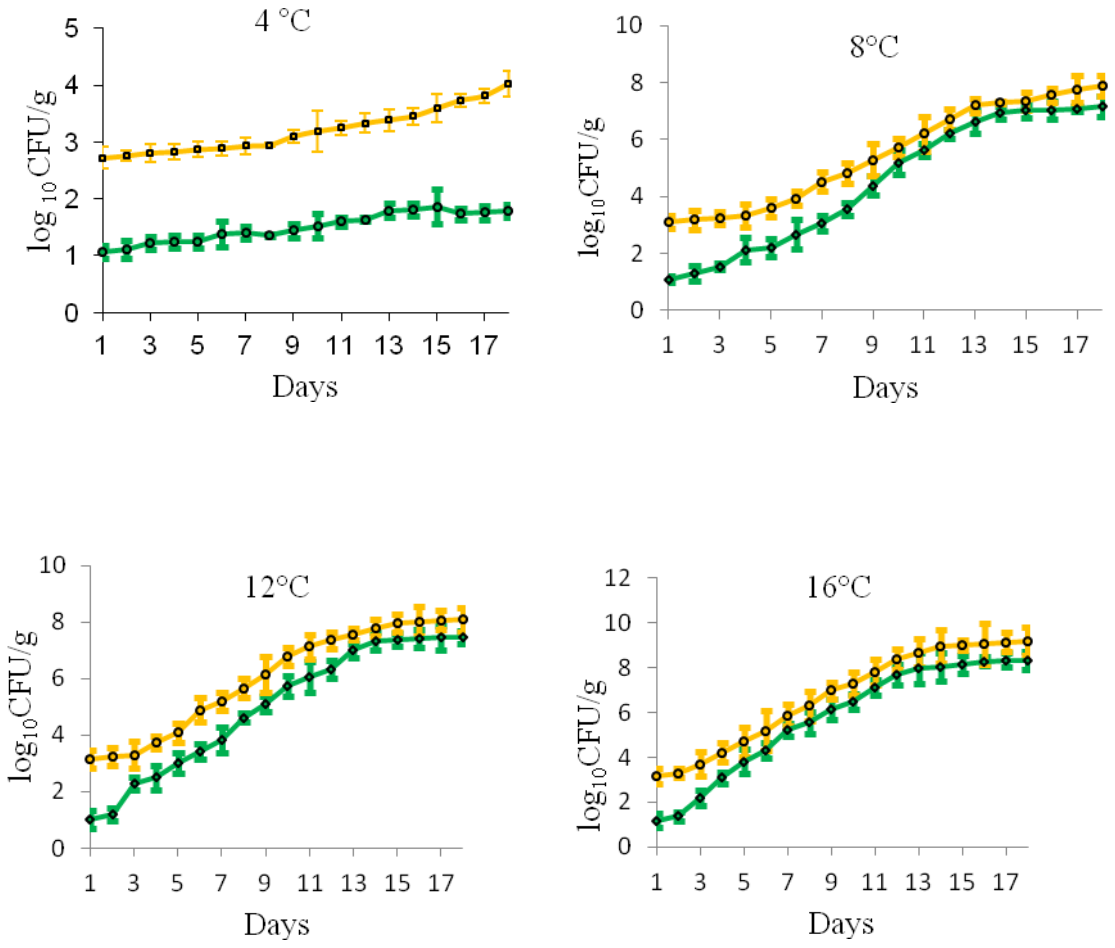


Figure 8-1: Growth of *L. monocytogenes* (——) and native microflora (——) at 4, 8, 12 and 16 °C in vacuum packed cold-smoked salmon.

The current legal contamination limit of lightly preserved food with *L. monocytogenes* is 2 $\log_{10}$ CFU/g in 25 g of the product (FSAI, 2002). This study demonstrates that at refrigeration temperature of 4 °C, *L. monocytogenes* did not reach the 2 $\log_{10}$ CFU/g after 18 days of incubation, whilst, at 8 °C, the 2 $\log_{10}$ CFU/g was reached after 5 days of incubation and at 12, 16 °C the 2 $\log_{10}$ CFU/g was achieved after 3 - 2 days of storage respectively.

The growth of native flora reached 5 $\log_{10}$ CFU/g at 4 ˚C after 18 days of storage, while at 8 ˚C it reached 7.5 $\log_{10}$ CFU/g and 8.5 and 9.1 $\log_{10}$ CFU/g after 18 days of storage at 12 and 16 ˚C respectively. Whereas, *L. monocytogenes* reached, 1.89 $\log_{10}$ CFU/g at 4 ˚C, 4.3 $\log_{10}$ CFU/g at 8 ˚C, 6.7 $\log_{10}$ CFU/g at 12 ˚C, and 7.8 $\log_{10}$ CFU/g at 16 ˚C after 18 days of

storages. The native microflora in vacuum packed cold-smoked salmon observed in this study were those cultivable on PCA. Researchers have demonstrated that 60 % of the native microorganism inhabiting vacuum packed cold-smoked salmon was mainly lactic acid bacteria (LAB), while the remaining 40 % were Gram-negative microorganisms such as *Enterobacteriaceae*, *Shewanella putrefaciens*, *Photobacterium phosphoreum* and *Aeromonas* spp. (Leroi *et al.,* 2000, Stohr *et al.,* 2001, Minetten *et al.,* 2005 and Chitlapilly Dass, *et al.,* 2011). The growth levels of *L. monocytogenes* appeared to be affected by the levels of native microflora, in particular when the native microflora reached its maximum growth at the end of the 18 days of storage. Hwang and Sheen (2009) reported that the growth of *L. monocytogenes* was restricted by the growth of native microflora in vacuum packed cold-smoked salmon, according to their research the native microflora was dominated by the presence of LAB. Similarly, Gimenez and Dalgaard (2004) reported that monoculture of *L. monocytogenes* grew to 8 $\log_{10}$ CFU/g in vacuum-packed cold-smoked salmon stored at 5 to 25 $^{\circ}$C. Gimenez and Dalgaard (2004) study showed that in the presence of native microorganism, *L. monocytogenes* reached only 2 to 4 $\log_{10}$ CFU/g.

Leroi, (2001) demonstrated that native flora was dominated by LAB, hence the presence of this bacteria could have slowed the growth of *L. monocytogenes* at all the temperatures tested. In this study the inhibitory characteristic of LAB on *L. monocytogenes* could be attributed to the listericidal activity an inherent property of LAB (Jorgensen *et al.,* 2000). These effects have been reported on vacuum packed cold-smoked salmon by various researchers (Nilsson *et al.,* 1999; Jorgensen *et al.,* 2000 and Leroi *et al.,* 2001).

The growth data obtained in this study were fitted to the DM-Fit Excel add-in, in order to calculate the maximum specific growth rate (GR, $\log_{10}$CFU/g), lag phase (LPD, h) and maximum population density (MPD, $\log_{10}$ CFU/g) (Table 8.1). The GR of *L. monocytogenes* ranged from 0.018 $\log_{10}$ CFU/g to 0.136 $\log_{10}$ CFU/g and the GR of native microflora ranged

from 0.021 to 0.211 at storage temperatures of 4 to 16 ˚C in vacuum packed cold-smoked salmon. There was a 2 fold increase in the GR of native flora from that of *L. monocytogenes* at all the storage temperature tested. These results showed that the native microflora grew at a faster rate when compared to *L. monocytogenes* and possibly could have lowered their growth rate. The LPD of the native microflora ranged from 193 to 23 h over the storage temperature range of 4 – 16 ˚C, these values were one fold less when compared to those of *L. monocytogenes* which ranged from 219 to 31 h. These results indicate that the fast growing native microflora having shorter lag phase may have inhibited the growth of *L. monocytogenes* which have longer lag phase. One of the reasons for the inhibition could be the depletion of the available nutrients on the vacuum packed cold-smoked salmon by the native microflora before the start of the exponential growth phase of *L. monocytogenes* (Garido *et al.*, 2010 and Koutsoumanis *et al.*, 2010). The MPD of *L. monocytogenes* were 2.9 to 7.6 $\log_{10}$ CFU/g at 4 to 16 ˚C, whereas the MPD of native microflora was approximately 4.6 to 9.13 $\log_{10}$ CFU/g. Combination of native microflora and refrigeration temperature could have restricted the growth of *L. monocytogenes* at 4 ˚C. The MPD of *L. monocytogenes* was significantly higher at 16 ˚C than those observed at 4, 8, and 12 ˚C. The growth of *L. monocytogenes* appeared to be more active at higher storage temperaturesand more competitive against the native microflora (Augustin and Carlier *et al.*, 2000 and Hwang and Sheen 2009).

Table 8-1: Parameters and statistics of the model of Baranyi and Roberts for the growth of Listeria monocytogenes in vacuum packed cold-smoked salmon (mean ±standard error of two independent experiments with two replications each)

	Temperature (°C)	GR (log_{10}CFU/h)	LPD (hours)	MPD (log_{10}CFU/g)	Standard error of fit	R^2
L. monocytogenes	4	0.018 (±0.002)	219 (±18)	2.907 (±0.004)	0.262	0.965
	8	0.089 (±0.008)	102 (±8.1)	4.352 (±0.009)	0.218	0.975
	12	0.156 (±0.005)	67 (±5.1)	6.374 (±0.002)	0.365	0.934
	16	0.187 (±0.003)	31 (±6.3)	7.668 (±0.0050	0.423	0.951
Native Microflora	4	0.021 (±0.004)	193(±21)	4.694 (±0.008)	0.421	0.981
	8	0.106 (±0.001)	94 (±13)	7.391 (±0.005)	0.381	0.923
	12	0.149 (±0.008)	51 (±9.3)	8.016 (±0.009)	0.265	0.945
	16	0.201 (±0.002)	23 (±3.2)	9.135 (±0.006)	0.324	0.931

8.4.2. Temperature dependence of GR and LPD on the growth of *L. monocytogenes* and native microflora

The GR of *L. monocytogenes* and native microflora was modeled as a function of storage temperature using the square root model (Fig 5.2 and 5.3). The LPD for *L. monocytogenes* and native microflora were modeled as a function of storage temperature using the inverse of the square root model (Fig 5.2 and 5.3). The 'reciprocal square root model' used is based on the assumption that the lag phase is inversely related to the specific growth rate (Baranyi and Roberts, 1994). It has been applied, for example, by Zwietering *et al.*, (1994) and Koutsoumanis and Nychas (2010), and has been tested positively for *Lactobacillus curvatus* and *L. monocytogenes* respectively. The equations obtained for GR and LPD for both *L. monocytogenes* and native microflora are as follows:

$$\sqrt{GRLm} = 0.026\ (T - 0.047) \quad \text{---------- Equation 8-3}$$

$$\sqrt{\frac{1}{LPD\ Lm}} = 0.009\ (T - 0.027) \quad \text{---------- Equation 8-4}$$

$$\sqrt{GR\ nm} = 0.024\ (T - 0.083) \quad \text{---------- Equation 8-5}$$

$$\sqrt{\frac{1}{\text{LPD nm}}} = 0.011\ (T - 0.019)$$ ---------- Equation 8-6

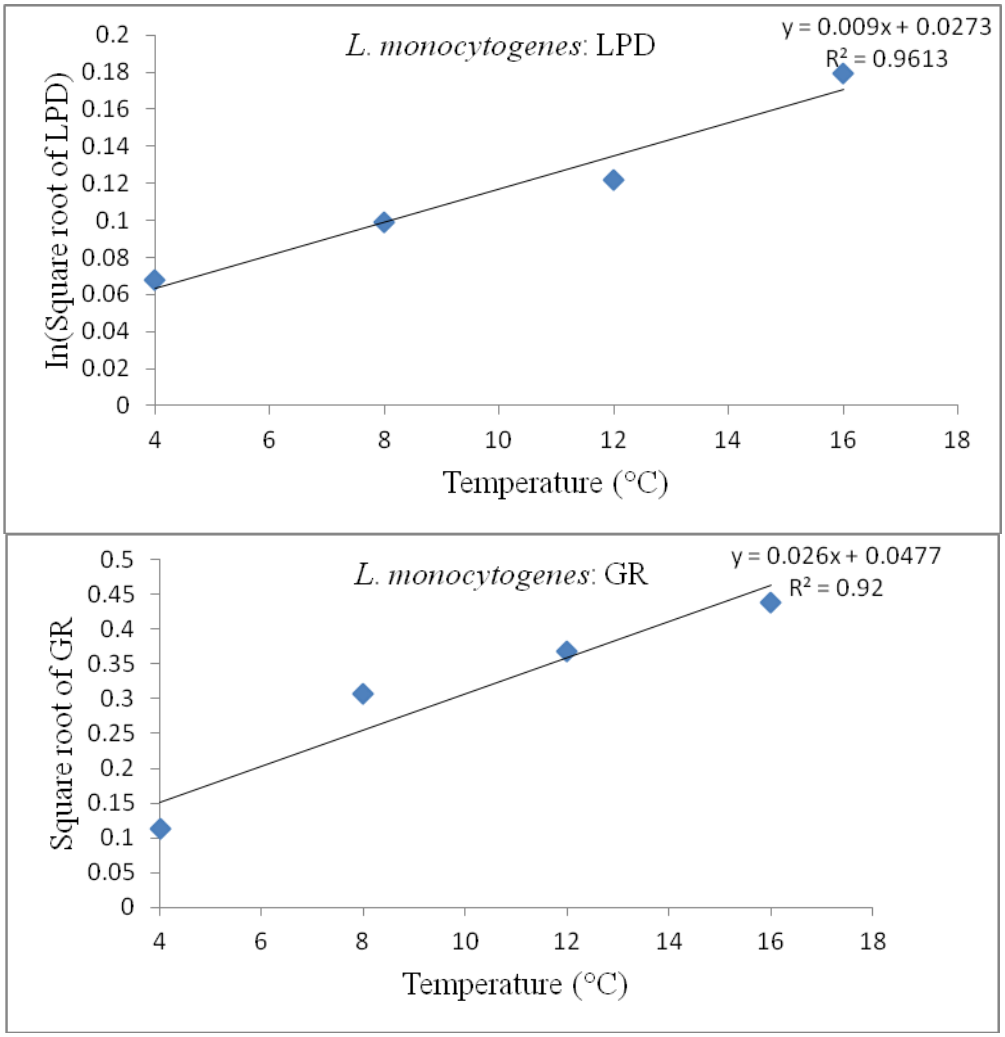


Figure 8-2: Square root type model and inverse square root type model for the effect of temperature on the GR, LPD of *L. monocytogenes* respectively. Data points are mean (±standard error) of two independent experiments with two replications each.

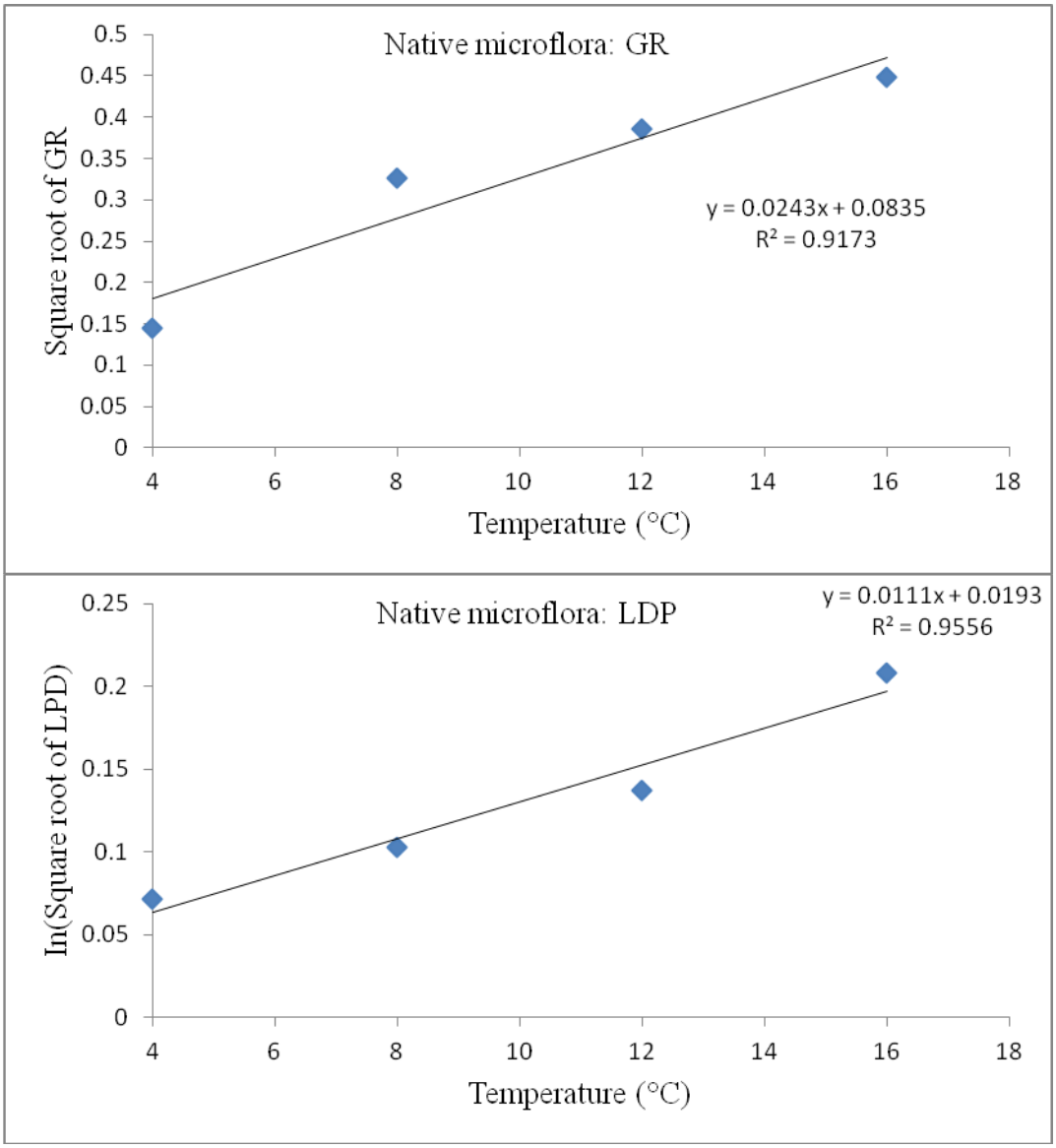


Figure 8-3: Square root type model and inverse square root type model for the effect of temperature on the GR, LPD of Native microflora respectively. Data points are mean (±standard error) of two independent experiments with two replications each.

The parameters coefficients of GR and LPD of both *L. monocytogenes* and native microflora are given in Table 8.2. The square root model and the inverse of square root model described the effect of storage temperature on GR and LPD respectively and showed satisfactory results ($R^2 > 9.0$). LPD prediction is complex as lag time duration may strongly depend on the initial physiological state of the cells and on the population size of the bacteria (Baranyi and Roberts, 1995; Baranyi, 1998). During lag phase, the bacteria undergo acclimatisation to its

new environment and germinate (Koutsoumanis, 2010). The LPD for native microflora is less when compared to LPD of *L. monocytogenes*. Thus the native microflora enters the exponential growth phase faster rate than *L. monocytogenes* and begin to utilize the available nutrients in the vacuum packed cold-smoked salmon. When the *L. monocytogenes* enters into the exponential growth phase, they would have to compete with actively growing native microflora and to utilize the nutrients in the vacuum packed cold-smoked salmon (Jacobson, 2006). This could be the reason for the difference in growth rate between *L. monocytogenes* and native microflora.

Table 8-2: Parameters coefficients of GR and LPD of both *L. monocytogenes* and native microflora. (mean ± standard error of two independent experiments with two replications each)

	GR (T_{min} °C)	**LPD (T_{min} °C)**
L. monocytogenes	- 2.04 (0.02)	- 3.26 (0.07)
Native microflora	- 3.15 (0.08)	- 3.9 (0.05)

8.4.3. Changes in the pH and a_w of cold-smoked salmon during the 18 days storage period

The a_w, at all the temperatures tested showed only 0.46 (± 0.03) units change during the 18 days (Fig 8.4) shelf-life of vacuum packed cold-smoked salmon, there was no significant change during shelf-life in all the storage temperatures studied.

pH of vacuum packed cold-smoked salmon at 8, 12 and 16 ˚C reduced from 6.8 to 5.8 (± 0.2) showing a one unit reduction, while at 4 ºC it showed 0.79 (± 0.01) unit reduction. The one unit reduction in the pH at 8 – 16 ˚C could be attributed to the presence of LAB which is part of native microflora and also higher growth rate of the native microflora at these higher temperatures owing to the higher utilization of nutrients. These bacteria have been associated

with the production of organic acids and thus turning the environment acidic (Leroi *et al.*, 2001).

Lactic acid bacteria can survive at low temperature (4 °C) and this could have slowed the growth of *L. monocytogenes* at 4 °C. The antilisterial activity of LAB has been attributed primarily to the production of antimicrobial compounds such as organic acids, hydrogen peroxide, and bacteriocins (Amezquita and Brashears, 2002). Several researchers have demonstrated the use of LAB as a protective culture against *L. monocytogenes* in various lightly preserved products and have proved to be beneficial (Djenane *et al.*, 2005 and Concha-Meye *et al.*, 2010). In a recent study LAB incorporated as an alginate film demonstrated that these films inhibit *L. monocytogenes* growth on vacuum packed cold-smoked salmon during refrigerated storage and did not alter the sensory quality (Concha-Meye *et al.*, 2010). Thus future studies focusing on the incorporation of LAB as a protective culture for controlling *L. monocytogenes* are warranted.

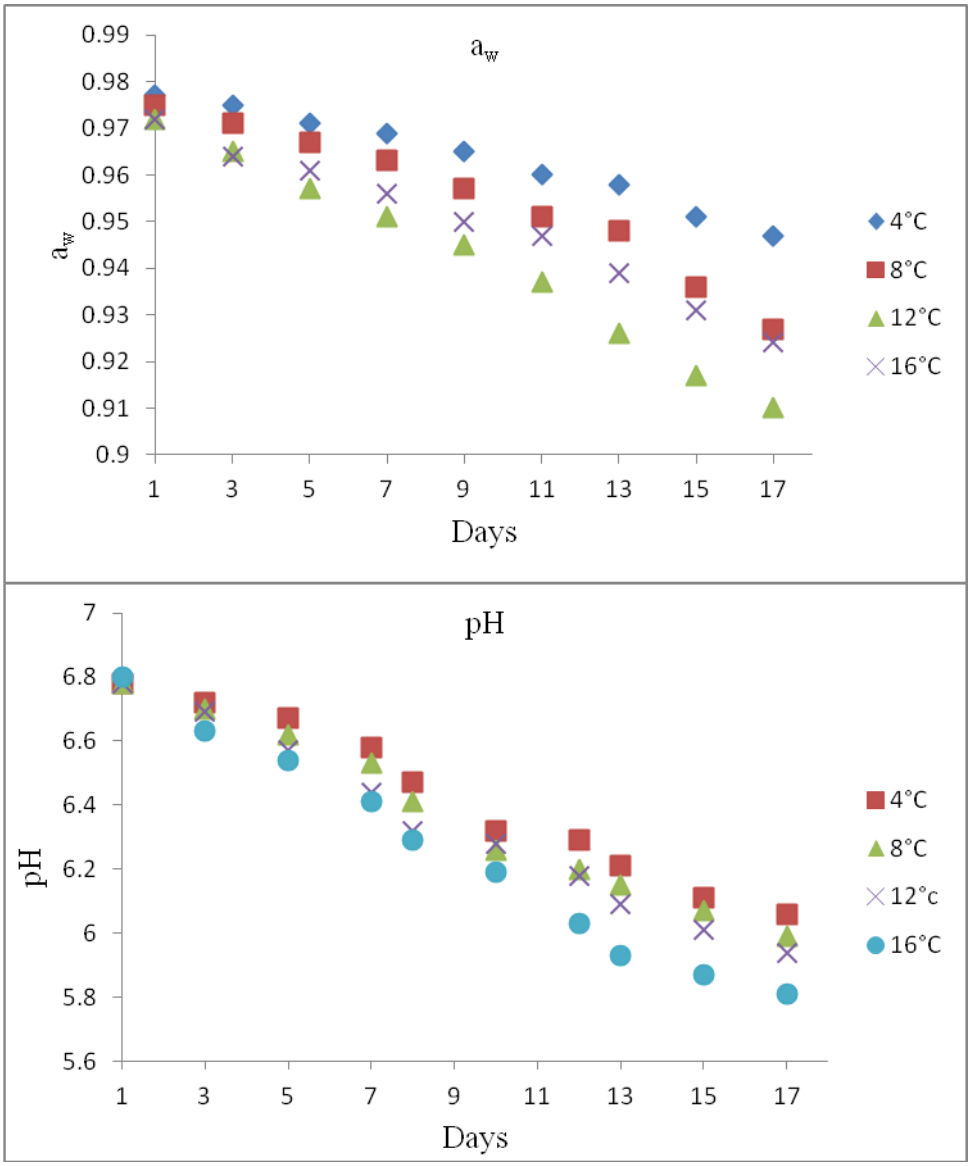


Figure 8-4: Change in pH and a_w of vacuum packed cold-smoked salmon recoded throughout the shelf-life of 18 days. The samples were recorded as replicates of five and the mean was noted as final value.

8.5. Conclusions

In this study, the growth of *L. monocytogenes* in vacuum packed cold-smoked salmon was dependenton the native microflora and the storage temperatures. The results showed that the GR, LPD and MPD of *L. monocytogenes* were affected by the growth of native flora during refrigeration storage (4 ˚C); the growth of *L. monocytogenes* did not reach 2 $\log_{10}$CFU/g at the end of the storage period of 18 days. This was deemed to be within the safety limits of the

presence of *L. monocytogenes* in ready to eat food. As storage temperatures to control the growth of *L. monocytogenes* in vacuum packed cold-smoked salmon can be difficult to maintain from retail until consumer consumption, investigations of novel techniques to control *L. monocytogenes* growth such as the incorporation of LAB merit further research with the aim of protecting public health.

Chapter 9 : Quantitative risk assessment of *Listeria monocytogenes* in cold-smoked salmon in the Republic of Ireland.

9.1 Summary

In this study, a quantitative Monte Carlo risk assessment model was developed to assess likely human exposure and the probability of human illness by *L. monocytogenes* on cold-smoked salmon in Ireland. A surveillance study conducted at the retail level served as the starting point for the model with a mean prevalence of *L. monocytogenes* in cold-smoked salmon of 22.1 % and a mean count on contaminated cold-smoked salmon of 2.60 $\log_{10}$ CFU/g (95 % confidence interval 0.00 – 4.53 $\log_{10}$ CFU/g). The model simulated likely growth conditions considering the uncertainty in transport and consumer storage conditions, while also assessing final risk of illness by taking into account the likely exposure frequency and levels. The model predicted the annual log probability of illness by consuming contaminated cold-smoked salmon in a low risk andhigh risk population, with mean values – 5.76 and – 1.63, respectively (assuming weekly consumption). The model sensitivity analysis highlights the importance of reducing the initial contamination levels of *L. monocytogenes*on raw fish and the maintenance of proper storage conditions. Various 'what-if' scenarios were studied to assess the likely impact on the log probability of illness per serving. Careful control of consumer storage temperature and time were identified as the best strategies to decrease the probability of illness. The quantitative risk assessment developed in this study may help risk managers to make informed decisions with regard to possible control measures for *L. monocytogenes*in cold smoked salmon and therefore improve food safety.

Some parts of this chapter have been submitted to the *International Journal of Food Microbiology*and is currently under review and also presented at the Food simulation conference (Page: 212-213).

9.2 Introduction

Risk assessment is the scientific process of determining the relationship between exposure to a given pathogen under a defined set of conditions and the likelihood of an adverse health effect or disease (Pouillot *et al.,* 2007). Quantitative Microbiological Risk assessment (QMRA) can help to obtain the necessary information regarding the severity of a health disturbance because it is based on knowledge concerning exposure to the pathogen and an individual's response.Some risk assessments have been conducted around Europe with regard to *L. monocytogenes* in cold-smoked salmon (Pouillot *et al.*, 2007, Lenhart *et al.*, 2008 and Garrido *et al.,* 2010). This chapter details the development of a risk assessment model for *L. monocytogenes* on cold-smoked salmon in Ireland and represents the first published study of its kind for the Republic of Ireland.

9.3. Material and Methods

9.3.1. Model development

The model developed in this study describes the probability of developing listeriosis following the consumption of cold-smoked salmon potentially contaminated with *L. monocytogenes*, taking into account primary data obtained in the exposure assessment step, including: occurrence and levels of *L. monocytogenes*at the point of sale, growth of *L. monocytogenes*from the point of sale to consumption, time and temperature fluctuations(at retail, consumer transport and consumer storage), serving size and consumption frequency of smoked salmon. This model describes human exposure as a distribution of ingested *L. monocytogenes*, taking into account the possible growth from market to table. The model was created in Microsoft Excel 2003 with the @Risk add-on package (Version 4.05, Palisade Corporation, New York, USA). Probability density distributions were used to take account

ofinherent uncertainty and variability in the input parametersin the model. The model was broken down into 3 main modules, as illustrated in Fig 9.1. The framework of the three modules is described below:

1. Exposure assessment: (a) Prevalence and initial contamination (retail storage): At this initial stage if the product is contaminated, it will have a certain level of pathogen at that point in time. (b) Consumer transport: The pathogen concentration could increase during consumer transport due to the time and temperature fluctuations during transit between purchasing and consumer storage. (c) Consumer storage: The pathogen could increase during storage depending on the consumer storage conditions, potentially leading to an increase in the final level of *L. monocytogenes*in the product. (d) Consumer consumption: The consumers will invariably eat different quantities (population variability) of the product, resulting in varying degrees of exposure. Various serving sizes have been observed among different groups of population.
2. Dose response: The dose response relates the amount consumed to a clinical outcome, in this study a listeriosis illness, and is used to translate exposure into a log probability of illness.
3. Risk characterisation: This stage combines the first two steps to characterise the risk of illness for the given simulated exposure level. The model simulated the annual risk of illness for a high risk and low risk population considering different consumption frequencies (i.e. monthly and weekly consumption).Each module was modelled with each proceeding module acting as an input into the next. The model provides a baseline description of listeriosis threat in Ireland by consuming cold-smoked smoked salmon contaminated with *L. monocytogenes*. Table 9.1 summarises all the main model inputs.

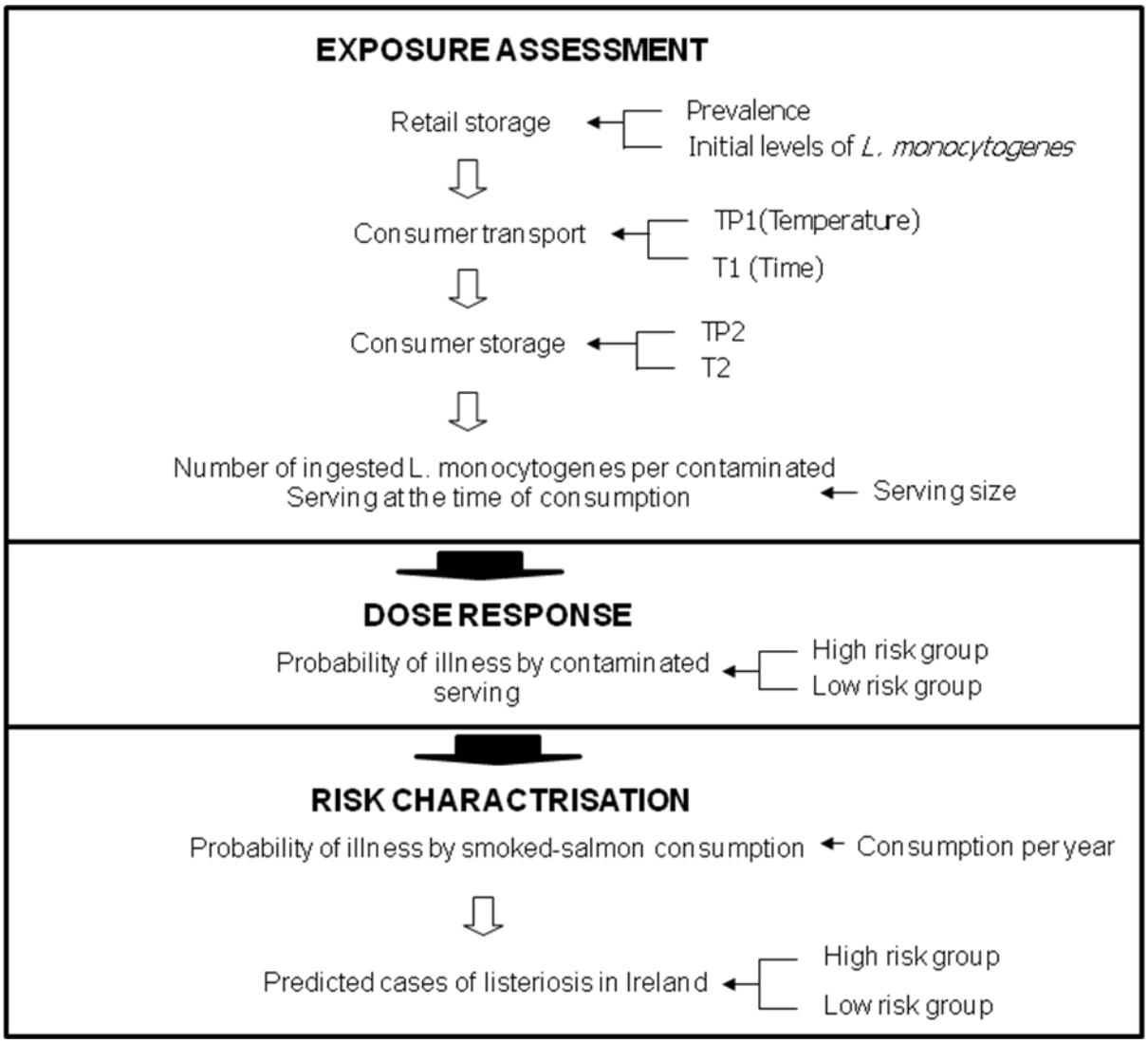


Figure 9-1 Schematic representation of the three modules used in the development of the baseline model of *L. monocytogenes* in smoked-salmon.

Table 9-1: Model Inputs used in building the risk assessment model

Description	*Units*	*Variable*	*Formula/model/values*
Prevalence	Percentage	Prev	Beta(26+1,120-26+1)
Initial contamination level	log CFU/g		Cumulative (x:s) Table 2
Time from retail fridge to consumer fridge	Hours	T1	Uniform (30,180)/24
Temperature consumer transport	ºC	Tp1	Uniform (4,8)
Temperature consumer storage	ºC	Tp2	FSAI, 2001 Table 3
Maximum storage time in consumer fridge	Days	T_{max}	30
Minimum storage time in consumer fridge	Days	T_{min}	21
Consumer storage time	Days	T2	Uniform (T_{min}:T_{max})
mCurv		m	1
nCurv		*v*	10
Bacterial adaptation		h_o	$\mu_{max} \times \lambda$
Growth constant		q_o	$1/\exp(h_0)-1$
Concentration of L. *monocytogenes* after consumer travel from retail to consumer storage	logCFU/g	y(T1)	Baranyi and Roberts model 1994 Equation (1)
Concentration of L. monocytogenes after consumer storage	logCFU/g	Y(T2)	Baranyi and Roberts model 1994. Same as Equation (1), with change in parameters $Y_{01,}$ $Y_{max1,}$ t1and μ_{max1} replaced by new parameter $Y_{02,}$ $Y_{max2,}$ t2and μ_{max2}
Serving size	g	S	Normal(35,26,Truncate(0,))

9.3.2. Exposure assessment

Prevalence and Initial concentration

The prevalence and levels of *L. monocytogenes* in vacuum packed cold-smoked salmon was estimated from a survey carried out in a retail outlet in Ireland between 2007 and 2008 (Chitlapilly Dass*et al.*, 2010a). The prevalence was described by a Beta distribution assuming

α equal to (21.6 + 1) and β equal to (120 - 21.6 + 1). The levels of *L. monocytogenes*in the unprocessed fishwere modelled by fitting a continuous cumulative distribution to observed data (Chitlapilly Dass*et al.,* 2010a; Table 9.2).

Table 9-2 Initial levels of *L. monocytogenes* in smoked-salmon at retail level (Chitlapilly Dass et al., 2010a).

L. monocytogenes count at the retail level (log_{10} CFU)	Probability
5	0.037
5.5	0.074
6.5	0.111
8	0.148
15	0.185
21	0.222
22	0.259
23	0.296
23	0.333
33.5	0.370
34	0.407
42.5	0.444
56	0.481
58	0.519
58.5	0.556
62	0.593
63	0.630
66	0.667
75	0.704
79.5	0.741
87	0.778
89	0.815
89.5	0.852
99.5	0.889
240	0.926
498	0.963

Consumer transport

The duration for consumer transport from retail to home refrigeration was described by a uniform distribution assumed a minimum and maximum time of 30 min and 3 hrs, respectively(FSAI, 2002). A uniform distribution is a rough modelling tool used to describe uncertainty when only the minimum and maximum value is known. The consumer transport temperature was also described by a uniform distribution, with a minimum and maximum temperature of 8 and 12 ºC (FSAI, 2002), respectively. The growth of *L. monocytogenes*during transportation from retail to consumer fridge was calculated using the growth model suggested by Baranyi and Roberts, 1994 (Equation 9- 1).

$$A(T1) = yo1 - \frac{In\left(1 + e^{-m(y_{max1} - y_{01})}\right)\left[-1 + e^{m\mu_{max1}\left[t1 + \frac{In\left[\frac{e^{-t1v}+q}{1+q}\right]}{v}\right]}\right]}{m} + \mu_{max1}\left[t1 + \frac{In\left[\frac{e^{-t1v}+q}{1+q}\right]}{v}\right]$$

----------Equation 9-1

Where: A(T1) is the concentration of *L. monocytogenes* following consumer transport (for length of time T1) from retail to consumer storage (log CFU/g), T1 is the time taken by the consumer to transport the smoked salmon from the retail outlet to the consumer fridge, y_{01} is the initial level of *L. monocytogenes* in smoked salmon at retail level, μ_{max1} is the maximumgrowth rate. The maximum population density (y_{max1}) for specific time and temperature combinations (8 and 12 ºC), where obtained from fitting the observed data (Chitlapilly Dass *et al.,* 2010a) in DMFIT Excel add-in.

The sigmoid curve parameters m and *v* determines the shape of the curve. The parameter m determines the curve's shape during the transition from the exponential to stationary phase. Therefore an empirical value, m = 1 is suggested for this curvature parameter (logistic potential growth). The rate *v* determines the rate of the transition fromthe lag to the exponential phase, therefore an empirical value of *v* = 10 is suggested for this curvature (Barayni and Roberts, 1994).

The values of m and *v* were assigned by Turner *et al.*,(1976) to obtain a unified generic growth model, (Baryany and Roberts, 1994) and q represents the physiological state of the cells and given a value 0.002 for *L. monocytogenes* (Panagou, 2008).

Consumer storage

Auniform distribution was used to define the consumer refrigeration temperature (Tp2); the data for consumer refrigeration was obtained from a survey conducted in Ireland (FSAI, 2002) as detailed in Table 9.3. The average refrigerator temperature recorded during the study ranged from 1.7 to 11.8 °C. Refrigerators in around half of the households surveyed had an average temperature above the recommended temperature range of 1-5 °C. The growth of *L. monocytogenes*during consumer storage was calculated using the growth model suggested by Baranyi and Roberts, (1994). The storage time (T2) was based on the shelf life printed on the smoked salmon packand was modelled using a uniform distribution, with the minimum storage time of 21 days (shelf life printed on the smoked salmon pack) and maximum time of 30 days (5 days added to the shelf life). Growth during consumer storage was also modelled using equation 1, with parameters $Y_{01,}$ $Y_{max1,}$ t1and μ_{max1} replaced by new parameters $Y_{02,}$ $Y_{max2,}$ t2and μ_{max2}, respectively. Where, Y_{02} is the initial level of *L. monocytogenes* in smoked salmon after retail storage, μ_{max2} maximumgrowth rate and

maximum population density (y_{max2}) which where obtained from fitting observed data (Chitlapilly Dass*et al.,* 2010a) in DMFIT Excel add-in. The parameter t2 represents the temperature during consumer storage.After modelling the growth resulting from the consumer storage the model yielded a final concentration of *L. monocytogenes*before consumption.

The overall equation 9- 2 was obtained by combining the two modules (consumer transport and consumer storage) to obtain the final concentration of *L. monocytogenes* before consumption. Where, Y(t) is the final concentration of *L. monocytogenes* (log CFU/g) after total storage time (i.e. retail storage, consumer transport and consumer storage time).

$$Y(t) = \frac{1}{mv}\left[-v\,In\left[1+e^{m(-y_{max1}+y_{01})}\left[-1+e^{m\mu_{max1}}\left[t1+\frac{In\left[\frac{e^{-t1v}+q}{1+q}\right]}{v}\right]\right]\right]+v\,In\left[1+e^{m(-y_{max2}+y_{02})}\left[-1+e^{m\mu_{max2}}\left[t2+\frac{In\left[\frac{e^{-t2v}+q}{1+q}\right]}{v}\right]\right]\right]\right.$$
$$\left.\mathrm{m}\left[\mathrm{t1v}-\mathrm{t2v}+\mathrm{In}\left[\frac{e^{-t1v}+q}{1+q}\right]-In\left[\frac{e^{-t2v}+q}{1+q}\right]\right]\right]$$

---------- Equation 9-2

Table 9-3 Temperature distribution in domestic refrigerators on the island of Ireland (FSAI, 2002)

Temperature (ºC)	Percentage
≤5	51
6-7	28
8-12	21

Consumption data

The level of *L. monocytogenes* in a smoked salmon serving was assumed to be evenly spread on the sample and the ingested dose was dependent on the number of *L. monocytogenes* present in 250 g of smoked salmon and the corresponding amount ingested. Accurate data about individual consumption patterns were not available. However, a consumption survey carried out in Ireland (IUNA, 2004) on ready to eat fishwas used (Table 9.4). The likely number of cells ingested per contaminated serving was calculated using equation 9-3

$$E = \text{Log}(10^{Y(t)} \times S) \quad \text{---------- Equation 9-3}$$

Where, Eis the level of exposure to *L. monocytogenes*per contaminated serving ($\log_{10}$ CFU/g), Y(t) is the final concentration of *L. monocytogenes* at the point of consumption and S is the serving size.

Table 9-4 Consumer consumption survey of ready-to-eat fish meals (IUNA, 2007)

	Age (Years)	Mean (g/d)	Standard deviation (g/d)	Median (g/d)	95 percentile (g/d)
Male	18-64	27	30	20	86
Female	18-64	20	23	14	69

9.3.3. Dose response model

The dose response curve was used to translate the simulated exposure into an estimate of risk of illness. A flexible Weibull-Gamma model,as suggested by Farber *et al* (1996), was used.The model incorporated two scenarios by dividing the population of Ireland into two sub-groups, low-risk individuals (healthy population) and high-risk individuals (infants, pregnant women, HIV and individuals above > 65 years of age). Data from the central statistic office (CSO, 2006) indicated that 7.41 % of the Irish population are children less than 4 years old, pregnant women account for 1.68 % of the population, 11.03 % of the population is over 65 years of age, while the prevalence of HIV patients is approximately 0.1 % (HPSC, 2008). The high risk category could be approximately 20.21 % (pregnant women, children <1 year, elderly > 65 years of age and HIV/AIDS patients) of the total population (as used by Cummins *et al.*, 2010). The flexible Weibull-Gamma model equation is described by the following equation 9-4:

$$P = 1-[1 + (10\text{^}E^{b})/ß]^{-\alpha}$$ ---------- Equation 9-4

Where, P is the probability of illness for an individual exposed to a dose E. The parameters α and β are host/pathogen relationship. For both sub-populations, α was selected as 0.25 and b as 2.14, whereas β is $10^{10.98}$ for the high-risk population and $10^{15.26}$ for the low-risk population (Farber *et al.,* 1996 and Bemrah *et al.,* 1998).The dose response model is illustrated in Fig 9.2.

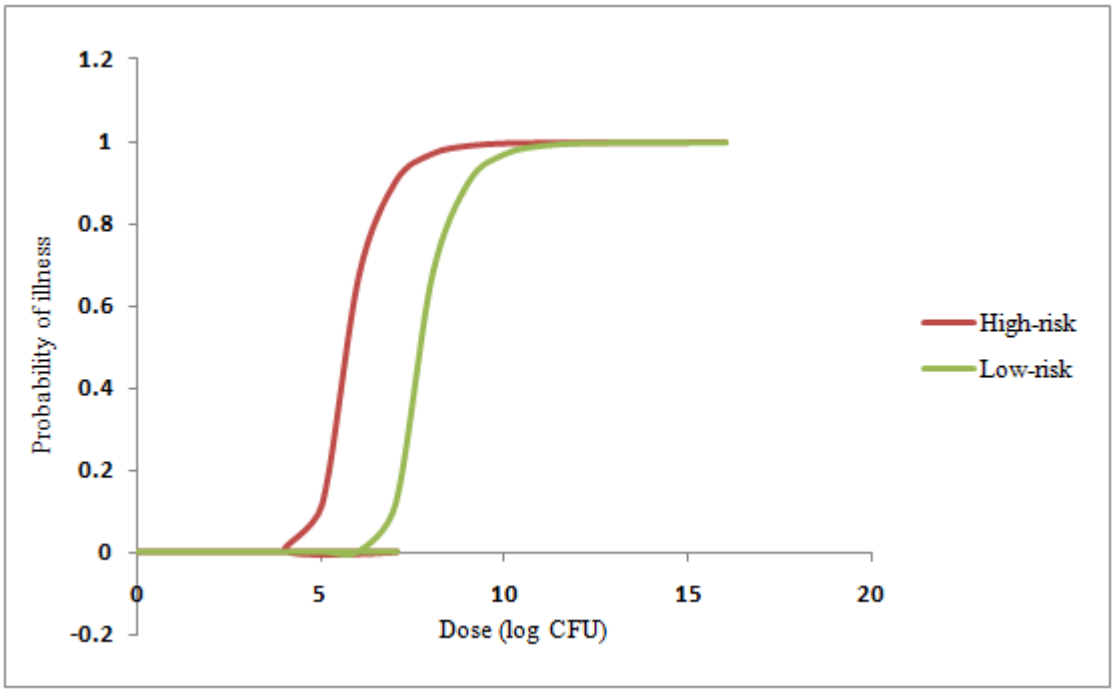


Figure 9-2 Dose response model for 'High risk' and 'Low risk' populations

9.3.4. Risk Characterisation

The annual probability of illness was estimated fromknowing the probability of illness per serving and assuming a frequency of consumption (i.e. weekly or monthly consumption frequency) as detailed in equation 9-5. The simulated annual log probability of illness was derived as an output in both the low and high risk population.

$$P_{ill} = 1 - (1 - P)^{n}$$ ---------- Equation 9-5

Where P_{ill} is the annual probability of listeriosis, P is the probability of illness per serving in the high risk (P_{HR}) or low risk population (from equation 9-4), and n is the consumption frequency (n = 52 for weekly consumption, and n = 12 for monthly frequency).

9.3.5. Model run

The input parameters were combined onto a spreadsheet (Microsoft Excel, 2003) running the @Risk add-on package (Palisade Software, Newfield, NY, USA) and the Monte Carlosimulation were performed using Latin hypercube random sampling. The simulation was performed using the parameters and calculations presented and the model was run for 10,000 iterations. A table summarising all the model inputs for the baseline model is provided in Table 9.1. The model resulted in a number of output distributions following the consumption of cold-smoked salmon contaminated with *L. monocytogenes*as detailed in Table 9.5.

Table 9-5 Simulated outputs from the risk analysis

Symbol	Description	Model/Distribution	Units
Y(t)	Final concentration of *L. monocytogenes*	Baryani and Roberts (1994), Equation (2)	log_{10}CFU
E	Number of bacetria ingested per contaminated seving	Log(10^Y(t) × S)	log_{10}CFU/g/serving/person
$P_{(HR\ and\ LR)}$	Probability of illness inhigh risk population and low risk population	$P_{(HR\ and\ LR)}=1-(1+(10\hat{}E^b)/ß)^{-\alpha}$ where ($ß_{LR} = 10^{15.26}$ for low risk population $ß_{HR}= 10^{10.98}$ for high risk population $\alpha = 0.25$ and $b = 2.14$ for both the population (Lindqvist & Westoo, 2000)	Probability
$P_{ill(HR\ and\ LR)}$	Probability of illness per year	$P_{ill(HR\ and\ LR)} = 1- (1 - P)^n$ Where, n = 12 for monthly consumption n = 52 for weekly consumption	Probability

9.3.6. Scenarios

In order to test possible risk management measures, various scenarios were proposed focusing on the parameters identified by the sensitivity analysis. The scenarios represented the individual effect of each control measure or a combination of multiple parameters. The changes were assessed based on the log probability of illness per serving in both the high risk and low risk populations and were compared with the baseline model. Scenario A looks at setting the maximum concentration of the pathogen at the time of purchase from the retail store to be truncated at 100 CFU/g, this scenario would represent strict adherence to the

safety criteria according to the current EU regulation with regard to *L. monocytogenes*. In Scenario B, the travel time from the retail cabinet to the consumer storage was set to a maximum of 30 min. Scenario C represents the situation where the consumer storage time was set to be a maximum of 15 days and minimum of 7 days. Scenario D was where the consumer storage temperature is set to be constant at 4 °C throughout the storage period. Scenario E is a combination of scenario C and D, combining the consumer storage time and temperature. Scenario F looks at the combined effects of the control measures in scenarios A, B, C and D. A table of scenarios used is given in Table 6. Values are the same as the baseline model except where stated.

Table 9-6 Changes in inputs from the baseline model used in the scenario analysis

Scenario	Initial contamination level (log_{10}CFU/g)	Consumer shopping time (min)	Consumer storage (Days)	Consumer storage temperature (°C)
Baseline model	Cumulative (1, 10^3)	Uniform (30 min – 3 h)	Uniform (21 – 30)	Uniform (3 – 10)
A	Same as baseline truncated at 10^2			
B		30		
C			7 – 15	
D				4
E			7 -15	4
F	Same as baseline truncated at 10^2	30	7-15	4

9.3.4 Model assumptions

The results of the risk assessment can be influenced by assumptions and hence results must be viewed in the context of theassumptions made (Cummins *et al.*, 2010).The following modelling assumptions have been made in the development of this risk assessment model:

- *L. monocytogenes*are assumed to be uniformly distributed on the surface of the cold-salmon.
- Consumption of cold-smoked salmon is the same for both high risk and low risk population categories.
- Consumers do not store cold-smoked salmon for more than 30 days.
- Consumers' behaviour (serving size and frequency) is the same throughout the year.

- The high risk category did not include people in the hospitals, treatment centres or crèches.
- As smoked-salmon are vacuum sealed at the end of the production, cross contamination was not modelled, cross contamination of opened packs were also not studied (at consumer level).
- A Weibull–Gamma model is assumed for the dose response model and may be a pessimistic model to use.
- The dose response model assumes all strains are virulent; this may be a pessimistic assumption giving rise to high risk estimates.

9.4. Results

9.4.1. Baseline model

This chapter details the development of a risk assessment model for *L. monocytogenes* in vacuum packed cold-smoked salmon in Ireland and represents the first published risk assessment study on *L. monocytogenes* in Ireland. Estimation of exposure to *L. monocytogenes* in cold-smoked salmon involves a number of complex and interrelated processes, describing the transmission of *L. monocytogenes* through the food pathway up-to the point of consumption.

The baseline model resulted in a number of output distributions which were used to predict the following: A) Final concentration (Y(t)) of *L. monocytogenes* after retail storage, consumer shopping behaviour and consumer storage. B) Number of *L. monocytogenes* (N) ingested per contaminated serving. C) Log probability of illness (P_{ill}) for a low risk and high risk individual in a year.

The predicated mean dose of *L. monocytogenes* ingested by people eating a contaminated fish was simulated as 3.66 log CFU/g (Fig 9.3). The frequency distribution, of the log probability of illness per serving in Ireland in both 'high risk' and 'low risk' groupsare given in Fig 9.4. The mean log probability of illness in the high risk group is – 3.4 log which is approximately 1 in 2,500 (people) risk of getting ill. The mean log probability of illness in low risk group is - 8.02 log, which is approximately 1 in >100 million (people) risk of getting ill (Table 9.7). The results highlight the significant risk to high risk population. Given some of the pessimistic assumptions used in the model (as above), the model may overestimate the risk, this is highlighted by the fact that the average number of reported listeriosiscases in Ireland is 37 per year (FSAI, 2007). There may also be some underreporting of cases which would result in some deviation.Not withstanding this, the model can be used to evaluate risk reduction measures

For this study the end of the production line was used as the first point in the model thus restricting the production processevaluation. Studies carried out by Pouillot and co-workers (2007) and Yang and co-workers (2006) on cross-contamination in kitchen from smoked salmon, concluded that cross-contamination in the home is a less important factor contributing to listeriosis risk when compared to the initial contamination levels and *L. monocytogenes* growth during home storage. Hence for this study, the baseline model integrated growth of bacteria at retail storage, consumer shopping behaviour and consumer storage at varying temperatures.

The model resulted in a number of output distributions which can be used to predict the log probability of illness for both low risk and high risk individuals following the consumption of contaminated smoked salmon. The distribution for prevalence and concentration of *L. monocytogenes*were based on the analysis of samples stored at dynamic temperatures, representative of the variable storage temperatures that these products can be expected to

experience in the retail and consumer home. This also took into account the consumer shopping time and temperature the product is exposed to.

The development of the quantitative risk assessment model based on various surveys, assumptions and the inclusion of input uncertainty has resulted in a helpful tool to evaluate the relationship between risk factors which may be used to mitigate *L. monocytogenes* risk. The model was simulated from retail level to the consumers' home; the interventions have been set at these steps for the food chain

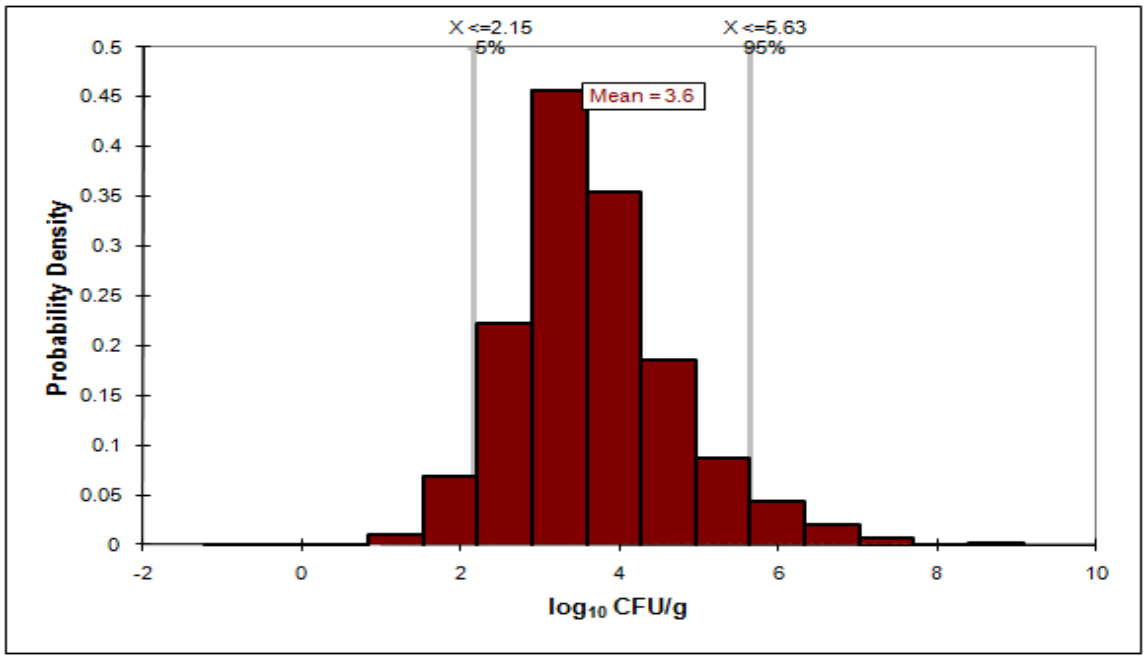


Figure 9-3 Simulated *L. monocytogenes* ingestion by consuming contaminated smoked-salmon

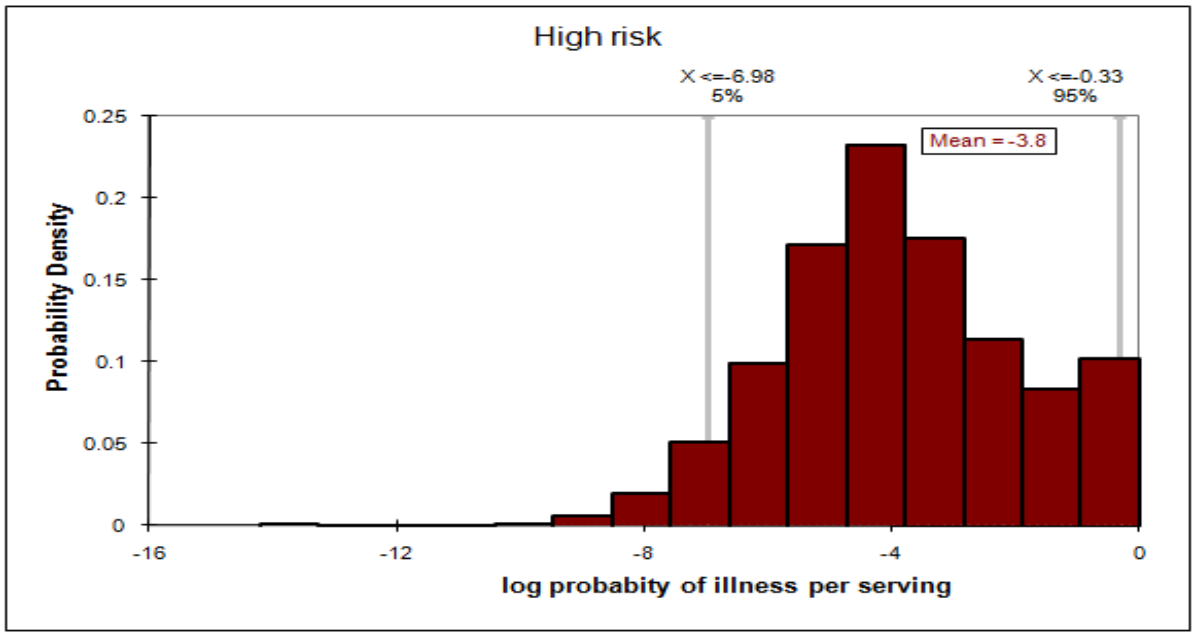


Fig 9.4a

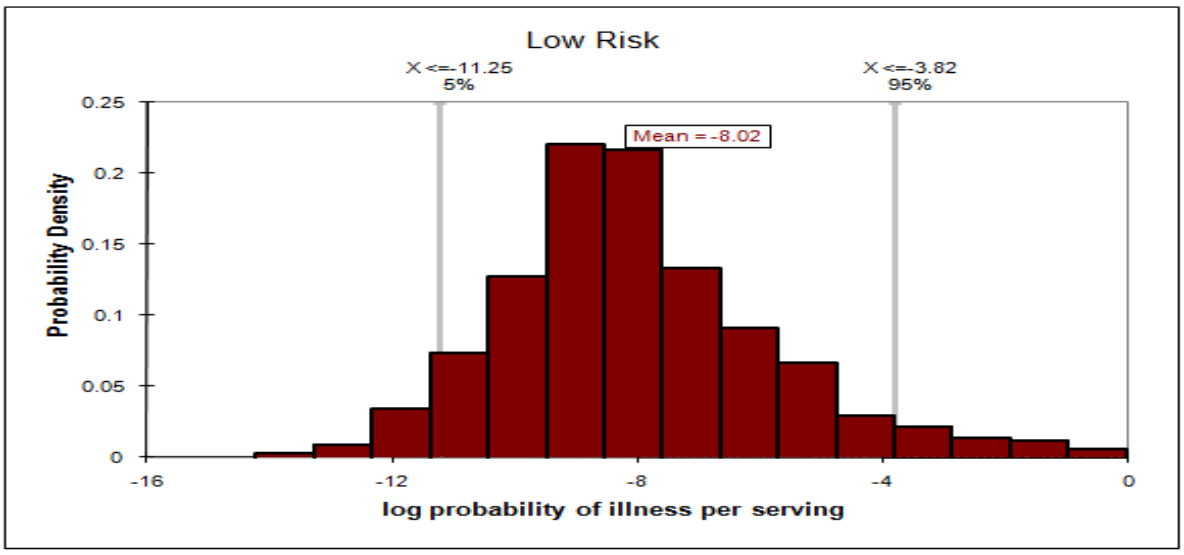


Fig 9.4 b

Figure 9-4a and b Frequency distribution of the log probability of illness in Ireland in both 'High risk and 'Low risk'.

Table 9-7 Simulated outputs for the baseline model for *L. monocytogenes* in smoked-salmon.

Output (units)	Mean	SD	Percentiles (log)				
			5th	10th	50th	75th	95th
Final concentration (log_{10}CFU)	2.60	0.83	1.1	1.28	2.21	2.80	3.14
Ingested dose (log_{10}CFU/g/serving/person)	4.7	0.80	3.01	3.85	4.15	4.61	5.31
Log Probability of illness per serving in the high risk (Probability)	- 3.8	0.94	-11.01	-10.93	-10.21	-9.72	-9.31
Log probability of illness per serving in the low risk (Probability)	-8.02	0.92	-12.01	-12	-11.98	-11.02	-10.94

9.4.2. Sensitivity to parameters

A sensitivity analysis indicated that probability of illness per serving was most sensitive to the input distribution initial contamination level, time and temperature during consumer storage.

The sensitivity analysis is a systematic evaluation of model inputs, parameters and assumptions (Cummins *et al.,* 2006). The sensitivity of the model inputs was measured by the spearman rank order correlation (Fig 9.5). The parameters that influenced the model predictions were the initial contamination level Y(t) correlation coefficient 0.97, time during consumer storage T1correlation coefficient 0.13 and the temperature encountered during consumer transport Tp2 correlation coefficient 0.06. The initial contamination level was found to be the most important factor responsible for the increase in the number of *L. monocytogenes* in cold-smoked salmon at the time of consumption. This could be controlled by eliminating or reducing the levels of *L. monocytogenes* contamination during the

production stage and before the final vacuum packaging of the cold-smoked salmon product.The sensitivity analysis also highlights that the risk management strategies should focus on controlling the consumer storage conditions, which can result in a significant reduction in risk. The significant parameters influencing final exposure to *L. monocytogenes* could be used to develop possible risk management strategies.

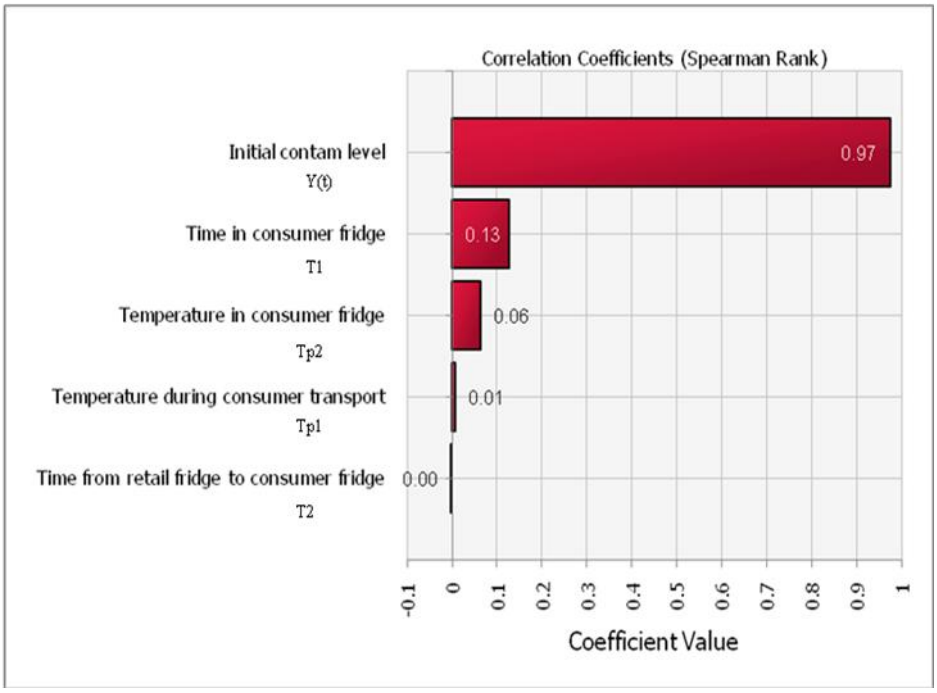


Figure 9-5 Sensitivity analysis for the annual risk of illness for high risk population.

9.4.3. Scenario analysis

Table 9.8 shows the simulated log probability of illness per serving in both the low and high risk population. The impact on the log probability of illness varied according to the scenarios tested. Reduction of consumer storage time and temperature had a significant effect in decreasing the log probability of illness in both the low and high risk population (scenarios B,

C, and E). The most significant reduction was scenario F (34.7 % and 17. 07% reduction, in both high and low risk population, respectively) which was a combination of low (< 3 log CFU/g) initial contamination, shortened consumer travel time and controlled consumer storage temperature and time. A reduction in initial count and consumer travel resulted in a moderate decrease (Table 8) in the log probability of illness in both high and low risk population (scenario A and E).

Table 9-8 Simulated log probability of illness for the tested scenarios

Scenario	Log probability of illness per serving High risk population (% change from baseline)	Log probability of illness per serving Low risk population (% change from baseline)
Baseline model	-3.8	-8.02
A	-4.1 (7.8 %)	-8.9 (10.9 %)
B	-4.6 (21.0 %)	-8.8 (9.7 %)
C	-4.3 (13.1 %)	-8.6 (7.2 %)
D	-4.8 (26.3 %)	-9.1 (13.4 %)
E	-3.8 (0 %)	-8.04 (2.4 %)
F	-5.12 (34.7 %)	-9.39 (17.07)

The “what-if” scenario analysis results indicated that, the temperature and the storage period inhousehold refrigerators (scenarios B, C and E) significantly influenced theexposure to *L. monocytogenes*following the consumption ofcontaminated cold-smoked salmon in Ireland. Secnario A had lower impact on the log probability of illness. Table 8 shows the percentage change of scenarios compared to the baseline model

The infectious dose of *L. monocytogenes* is 10^3 CFU and hence requires consumption of about 10,000 cells to cause infection (Chikindas and Gandhi, 2005). However, this estimate could vary depending on the health state of the population (Cummins *et al.*, 2010). Therefore it is important to avoid or minimise the growth and eventual increase of microbial load. This is attainable when the recommended time and temperature is maintained until consumption. Therefore, reduction in the temperatureof household refrigerators to 4°C without exceeding 15 days of storage period is the best strategy to reducethe log probability of illness in both low and high risk populations.

The results from this study indicate that a careful control of storage conditions can bring a significant reduction in risk. Unlike the conditions employed in professional settings, storage conditions implemented by the consumer cannot be controlled by legislation (Cheroutre-Vialette., 2000). This strategy should be directed to improve consumer awareness on the importance of correct storage conditions, where temperature abuse will be most frequent.Emphasis should be made on the health risk associated with consuming such products kept under abusive storage conditions, in particular preventive tips should be given to high risk populations.

Considering the results of the present study, the model is of practical value. Nevertheless, continuous studies on the levels of *L.monocytogenes* in cold-smoked salmon at the point of sales and the consumer behaviour of food handing and storage are necessary in order to improve this first risk assessment carried out in the Republic of Ireland.

9.5. Conclusions

Risk assessment is a scientific approach which is able to provide an estimate of the impact of contaminated food on public health. Risk assessment can be a valuable tool in assessing risk to human heath from consuming cold-smoked salmon. The model developed in this study will be useful for the regulatory authorities to evaluate the likely risk for *L. monocytogenes* related illnesses. The model provides a better understanding of the potential threat from *L. monocytogenes* in cold-smoked salmon. Further study on the levels of *L. monocytogenes* in smoked salmon at the point of sale and consumer behaviour regarding food storage, handling and consumption are necessary in order to improve the first approximation to listeriosis risk assessment in the Republic of Ireland.

Chapter 10 : General Conclusions and Recommendation for Future Work

10.1 General Conclusions

The overall aim of this project as detailed in chapter 1 was to investigate the prevalence and source of *L. monocytogenes* in different stages of vacuum packed cold-smoked salmon production chain /retail market and to develop a risk assessment model. This will enable a closer analysis of the source and contamination pathways of *L. monocytogenes* in Irish vacuum packed cold-smoked salmon and facilitate the calculation of risk associated with the consumption of smoked salmon contaminated with *L. monocytogenes*. This was achieved by addressing specific project objectives as detailed in chapters 3, 4, 5, 6, 7 and 8. These objectives were met and the following conclusions are reported below:

10.1.1 Identification and isolation of *L. monocytogenes* in vacuum packed cold-smoked salmon

Historically, it has been challenging to isolate *L. monocytogenes*from food or other samples and this explains why it remainedunnoticed as a major food pathogen until recently (Gasanov *et al.,* 2005).Once *L. monocytogenes* contaminates a foodprocessingplant it can survive there for a long time if the temperatureis low and the organism is protected by the food components (Fonnesbech Vogel *et al.,* 2001). For these reasons standardisation of methods for typing *L. monocytogenes* isolates is necessary in epidemiological surveys investigating food-born outbreaks. This investigation will aid in identifying the source of contamination and routes by which *L. monocytogenes* is distributed and how they contaminate vacuum packed cold-smoked salmon.

For the detection and identification of *L. monocytogenes;* phenotypic, biochemical and immunological assays and genotypic methodology are used. However, the time for preparation, skills and objectivity when evaluating resultsconsistently differ among these

methods (Zunabovic, 2010). In the study presented in this thesis, the official method for isolation (EN/ISO 11290 -01 and - 02) of *L. monocytogenes* from ready-to-eat food was assessed for its efficacy for isolating and detecting *L. monocytogenes*. The advantage of this method is that*Listeria* spp. are sensitive to the enrichment media which resuscitate the injured *Listeria* spp. and the selective plating technique uses chromogenic media for the identification of *L. monocytogenes* (Gasanov *et al.,* 2005).

The frequently recommended (ISO) *Listeria* agarsPalcam and Oxford do not distinguish between pathogenicand non-pathogenic *Listeria* spp. (Stam, 2007). Although,ALOA distinguish between the *L. monocytogenes* and *L. ivanovii*, Palcam had better rates of recovery when compared to ALOA and Oxford (Chitlapilly Dass, *et al.,* 2011).

The present study utilised culture-dependent molecular tracking techniques for identification and sub-typing of *L. monocytogenes*.The major advantage that moleculartechniques offer over conventional methods is thatthese are based on differences within the genome anddo not rely on the expression of specific antigenic factorsor enzymes to facilitate identification. They areextremely accurate, reliable and some can be performedin the same time frame as immunoassay methods. In this study, *L. monocytogenes* was grown on Palcam (selective culture method) and identified by 16S rRNA gene sequence analysis and sub-typed by MLVA.

The method of identification by 16S rRNA gene sequence analysis is rapid and accurate when compared to cultural methods, but it is cost intensive when compared to the ISO 11290-01 and -02 methods for detection. A drawback of this method is the requirement of specialised equipment, such as an automated DNA sequencer, to carry out the analysis.

In the recent years MLVA has become one of the most popularmethods for bacterial typing. In the prevalence study (Chitlapilly Dass *et al.,* 2010a), subtyping techniques aimed at

finding differences between unrelated strains. Thisunderlines the differences in regional prevalence of single genotypesand second genotype which is representative of the imported smoked –salmon. Most of the studies are representative of regional prevalence, the need for global studies to integrate information derived fromdifferent areas of the world is vital. Interestingly, there were some similarities between the allelic profiles between this study and that of Murphy *et al.,* (2007) study on Irish *L. monocytogenes* isolates.

MLVA can be used for global epidemiological surveillance, while providing better discriminatorypower and ensuring inter-laboratory data-sharing.MLVA is more desirable over typing techniques as their data are stored in a web server that can be queried by any useranywhere in the world (Murphy, *et al.,* 2007). Even if MLVA schemes are based on the tandem DNA repeats they can be identified by many allelic profiles (Keim, 2007).Ithas the potential to indicate relatedness of isolates and henceidentify outbreaks, based on MST comparisons with an activedatabase. MLVA subtyping of *Listeria* would support controlmeasures aimed at protecting public health.

In the ecology study, MLVA could easily distinguish between the *Listeria* isolates originating from different zones in a cold-smoked salmon processing factory, and thereby a contamination pathway could be established (Chitlapilly Dass, *et al.,* 2010b). The MLVA technique reported here is easy to perform, relatively fast, inexpensive and can be conveniently incorporatedinto any molecular biology laboratory without the need to acquireadditional expensive equipment. Whereas, PFGE may take up to aweek to complete (non-standardised), MLVA results can beavailable in less than eight hours following DNA extraction.Furthermore, this approach requires only a conventional PCRfollowed by standard agarose gel electrophoresis. It would berelatively easy to standardize the method, thereby facilitatinginter-laboratory data exchange, in a manner similar to PulseNet (Swaminathan *et al.,* 2001). However, as MLVA isbased on an estimation of the number of tandem repeats in

anisolate as indicated by amplicon size, the accuracy of the results may be lower than can be achieved by direct sequencing of eachamplicon (Lindstedt *et al.,* 2003, 2004). Access to 'Bionumerics' softwarepackage (Applied Maths, Sint–Martems–Latem, Belgium) orequivalent bioinformatics software is essential when using this approach (Murphy *et al.,* 2007).

10.1.2*L. monocytogenes'* prevalence in vacuum packed cold-smoked salmon

Having established the detection protocol, culture-depended molecular typing technique was utilised in determining the prevalence in vacuum packed cold-smoked salmon in Dublin, Ireland (Chitlapilly Dass *et al.,* 2010 a). This was the second study after nearly 9 years of the first recorded prevalence study in the Republic of Ireland (FSAI, 2001). The prevalence of *L. monocytogenes* (21.6 %) in smoked-salmon from various outlets recorded in this study showed 8.6 % increase when compared to the prevalence (13 %) conducted in 2001 by FSAI. The prevalence reported in this study is in line with other studies carried out in Europe where the prevalence range from 10- 40 % (Lindquist *et al.,* 2000, Delignette-Muller *et al.,* 2006, Pouillot *et al.,* 2007 and Garrido *et al.,* 2009).

One of the objectives of this prevalence study was to quantify levels of *L. monocytogenes* (in the various smoked-salmon brands tested), in order to estimateactual consumer exposure to the organism.The highestnumber of *L. monocytogenes* was found in a brand A and D ($>10^5$ CFU/g). Since several lots of the same producers showed elevated levelsof the pathogen, this could be related to an in-house contamination as the strains isolated from the lots showed genotypic similarities.

Despite the fact that the majority of vacuum-packed productsexhibited a label reading ‘‘use by date” and ‘‘store at 0–5 °C”, in reality many of the retail and domestic refrigerators are toowarm for the safe storage of food (reaching temperatures 9 °C; Johnson *et al.,* 1998), allowing the growth of the pathogen andother spoilage organisms in a shorter time (Francois *et al.,* 2006).Since storage time and temperature are important in controllingthe growth of *L. monocytogenes*, improved consumer educationconcerning refrigerator temperature control is needed (Kosa, *et al.,* 2007), together with more informative labelling of all RTE (manufacturers and in-storepackaged),preferably a safety-based use by date type (Cates, Kosa and Canavan, 2004).

As an extension of the prevalence study, a cold-smoked salmon factory in Dublin, Ireland was divided into various zones depending on its proximity to the product and the presence of *L. monocytogenes* was analysed for a period of one year (Chitlapilly Dass *et al.,* 2010 b). Overall prevalence of the zones were 24.54 %, while the highest was recorded in zone 4, which was 58.3 %, this zone was the outside the smoked-salmon production area. Zone 1 which was the product handing area recorded 16.6 % contamination with *L. monocytogenes*. This study shows ubiquitous nature of *L. monocytogenes* and its persistence in nature and on inanimate objects (wooden surface, stainless steel, salt).

As a part of the factory evaluation study, 60 individual raw salmon were tagged and traced for the presence of *L. monocytogenes* after every step of the processing of cold-smoked salmon. Of the 60 salmon samples tested, *L. monocytogenes* were isolated in varying degrees from the raw salmon (28.33 %), and the final vacuum packed cold smoked salmon (21.60 %).

Process contamination in particular has proved to be animportant source of *Listeria* contamination in food production andnumerous studies show that in-house *L. monocytogenes* floracontaminates seafood during processing (Autio *et al.,* 1999; Fonnesbech Vogel *et*

al.,2001; Medrala *et al.,* 2003). Nevertheless, there are indications that *L. monocytogenes* contaminates final products from seafood raw materials (Eklund *et al.*,1995; Fonnesbech Vogel *et al.,* 2001 and Chitlapilly Dass *et al.,* 2010b).

The greatest challenge in controlling *L. monocytogenes*in the food industry is therefore to prevent persistence of the bacterium in specific niches; whereroutine cleaning and disinfection are ineffective. The factory in which the study was conducted had implemented internalcontrol and sanitation procedures. However, the cleaningprocedures do not completely eliminate *L. monocytogenes* from the processing environment. Thiswas revealed by the observation that 16.6 % of samplestaken from surfaces in direct contact with food were stillcontaminated with *L. monocytogenes* after cleaning. Ifthe cleaning procedures are ineffective and theconditionsare favourable, biofilms incorporating *L. monocytogenes* may be formed on different surfaces (Beresford *et al.,* 2001). These attached bacteria may bevery pernicious, contaminating products in the processeither continuously or only occasionally, when a piece ofbiofilm is dislodged to the process (Pouillot *et al.,* 2007). The most problematicsites examinedwere conveyer belts and other transport systems, floorsand drains, cooking equipment andcutting boards. The results clearlydemonstrated the types of equipment requiring specialattention both with respect to hygienic design and whenplanning routinesfor cleaning and disinfection. In orderto solve the problems observed in this study, there is aneed for close co-operation between the suppliers of equipment, cleaning agents' manufacturer, the staff of cleaningcompanies and hygiene specialists from the foodindustry.

There istherefore evidence in the literature that *L. monocytogenes* may be avoided by vigorous cleaningand sanitation but that, due to the ubiquitous nature ofthe organism, sporadic contamination may still occur (Nesbakken, 1995; Fonnesbech Vogel *et al.,* 2001).

10.1.3*L. monocytogenes'* route of contamination in vacuum packed cold-smoked salmon

In this study, transmission dynamics of *L. monocytogenes*was established by tracing the contamination pathways of *L. monocytogenes* in final vacuum packed cold-smoked salmon.

In the first approach to establish the contamination pathways, the *L. monocytogenes* isolates obtained from the various brands of vacuum packed cold-smoked salmon as a part of the prevalence study was typed by MLVA, to study the relatedness among the isolates. Surprisingly, the *L. monocytogenes* isolates from Irish and the UK smoked salmon producer were genetically related whilst the *L. monocytogenes* isolate from the eastern European were genetically unrelated and formed a separate branch in the MST analysis. The Irish and UK smoked salmon producer used the same slaughter house in the UK to procure the raw-salmon; this could be related to the similarities in the isolates. This study brings about the 2 hypothesis for the possible route of contamination (1) raw salmon could have carried the *L. monocytogenes* isolates as a part of its natural flora which was supplied to the two processers (Irish and U.K.); (2) the slaughter house could have harboured an in-house flora which was responsible for the contamination, which could have been transferred to the two processors. The un-relatedness of the eastern European isolates to the other isolates could be due to the geographical distance of the two processors or the strain could be specific to the smoked-salmon processing factory or the slaughter house from where the salmon was procured. As this study looked at the relatedness among the different isolates obtained from various brands manufactured in Ireland and abroad a clear picture of the route of contamination could not be established. Nevertheless the study was able to establish relatedness among geographically distant processors (Irish and U.K.) who procured raw salmon from the same slaughter house, pointing to the fact that raw salmon entering the factory could be the cause of contamination in the final product.

In the second approach a smoked salmon processing factory in Ireland was investigated for a period of one year to establish a possible contamination pathway. The factory's zone one which was the product contact surface contaminated the final product; certain batches of the final product had more than one type of *L. monocytogenes*. The *L. monocytogenes* types that were isolated from the final product originated from raw fish, skinner and filleting board. There was no cross-contamination from the drain or garbage collection area into the food-handling area. This points that the strains present in product region could have originally come from the raw material and was not eliminated through cleaning. This study further confirms the prevalence study results, that raw salmon is the main cause of contamination of *L. monocytogenes* in smoked salmon followed by the in-house flora present in the equipment.

To further these results, 60 raw salmon were tagged along the production line. In addition to the strains originating from raw salmon, there where also strains of *L. monocytogenes* originating on the cold-smoked salmon after curing and filleting. When compared to the levels of *L. monocytogenes* present in the raw salmon there was a reduction of 6.74 % of *L. monocytogenes* after cold-smoking, this could be attributed to the bactericidal activity of brining and the smoke components used in the process of cold-smoking (Miettinen and Wirtanen 2006). These results are similar to that obtained in various studies where there was a reduction in the final number of *L. monocytogenes* after the process of cold-smoking (Fonnesbech Vogel *et al.,* 2001, Medrala *et al.,* 2003 and Gudbjornsclottir *et al.,* 2004).

Thus results from the three surveys carried out in this study to establish a possible contamination pathway, pointed out that *L. monocytogenes* from raw salmon could be the starting point of contamination and further along the production line in-house strains are shed on the smoked-salmon from the processing equipment, which would have formed a bio-film, as these strains were consistently isolated during the one year period of the survey.

This study could not be extended to trace the origin on *L. monocytogenes* in the raw fish from the fish farm and slaughter house due to time and resource restriction.

10.1.4 Development of product specific dynamic model for *L. monocytogenes* in vacuum packed cold-smoked salmon

The food supply chain is increasingly global in natureand relies on a complex system involving fish catchment, slaughter house, ingredients suppliers, smoked-salmon processors, distributors, importers, and food retailers in many different regionsof the world. To achieve safety of food from catchment to fork, and to reduce the frequency,impact and severity of food-borne illness outbreaks, there have to be effective workingpartnerships and cooperation throughout the entire food pathway's (Benson, 2010). However, the delivery ofsafe food depends on the pathway's weakest link, since this is where problems are mostlikely to occur.Breaks in the food supply pathway can occur at any time as food moves from fish farm to table.

The psychrotrophic nature of *L. monocytogenes*makes this pathogen in ready to eat products difficult in controllingsince refrigerationtemperature is used to increase the shelf life of this type of products.According to Regulation (EC) 2073/2005, food safety criteria establishthat ready to eat products should not exceed the limit of 100 CFU/gthroughout their shelf life. In this study a low inoculum size of *L. monocytogenes* along with actual product (smoked salmon) and its natural flora was used to study the growth of *L. monocytogenes* at abuse temperatures encountered along the food pathways which is described by the retail storage, consumer travel from retail to consumer storage and consumer storage. The results obtained confirm the growth dependenceon temperature, coinciding with some other researchers(Francois *et al.*, 2006; Glass & Doyle, 1989; Jacobsen & Koch,2006).

The temperature range used in this study was obtained from various survey results in Ireland on domestic and retail refrigeration (FSAI, 2002). It was evident in this study that when temperature was maintained at 4 °C throughout the shelf life, the *L. monocytogenes* (1 log CFU/g) inoculated on smoked-salmon does not reach 2 log CFU/g up-to 20 day. The shelf-life recommended on the package is 18 days, so under constant low temperature with low initial contamination the 2 log CFU/g is not reached within the recommended shelf-life. This suggests that, low temperature storage is the key for *L. monocytogenes* growth reduction withinthe self-life of product, with special emphasis once the productis purchased and remains under the responsibility of consumers (Pouillot *et al.,* 2007 and Garrido,*et al.*,2010).Therefore, for a better control of ready-to-eat products it would be necessary to lay down strict criteria of temperature(0–4 °C) throughout the food chain (including home storage).

Gay, (1996) emphasized that storage conditions, pre-incubation temperatureand inoculum concentration appear to influence the subsequentgrowth curve (specially lag phase) when a low inoculumwas used (1 log CFU/g). Another factor of interest when studying real contaminatedsamples is the effect of the background competitive flora on thegrowth rate of pathogen.The effect of competition between natural microbiota and *L. monocytogenes*observed in the present study is in accordance with thatreported by other authors (Cole, 2003 and Ross, *et al.,* 2000), reaching a maximum concentrationlower than predicted.

In this study, a link could be established between the food pathways starting from the retail storage up-to the consumer storage and a final count of *L. monocytogenes* could be established using fluctuating temperatures obtained from the Irish survey (FSAI, 2002). These results reflect the real life scenario with the temperature abuse. The model developed in this study predicted growth more accurately than the predictive models Combase and

SSSP. The predictive model Combase does not use the real food providing overestimation, while SSSP fared better in comparison to Combase as the experiments on SSSP were based on the real food (in this case, vacuum packed sliced cold-smoked salmon).

Prevention of *L. monocytogenes* contamination in smoked-salmon demands a combined effort of the wholefood chain from smoked-salmon processing company to retailer and consumer. This study can be (at least partially) attributed to the setting of the new European hygiene legislation andthe implementation of food safety management systems in the foodproducing companies.

10.1.5 Quantitative risk assessment of *L. monocytogenes* in vacuum packed cold-smoked salmon.

Microbiological risk assessment is an important tool for evaluating and communicating the impact of raw material quality, processing and changes on food safety. Quantitative microbial risk assessment (QMRA) is a unique scientific approach that links data from food (in the farm-to-fork approach) and the various data on human disease to produce a clear estimation of the impact of contaminated food on human public health. It is also the most powerful tool available today to clearly assess the efficacy of each possible mitigation strategy.In this study, *L. monocytogenes* in vacuum packed cold-smoked salmon was modelled from the end of the production line to consumption. The model predicted the log probability of illness by consuming contaminated vacuum packed cold-smoked salmon in a low risk andhigh risk population.

Minimising data gaps and assumptions are important steps towards producing a QMRA that provides better predictions. The baseline model integrated growth of bacteria at retail storage, consumer shopping behaviour and consumer storage at varying temperatures. Dose-response

assessment is the most difficult module for the evaluation of the risks related to food. In the present risk assessment we preferred to use the model published by Farber, (1996). The log probability of illness was carried out for high and low risk populations. The risk to the high risk category was 3 times greater than that for low risk category; this highlights the need for caution for the high risk category when eating vacuum packed cold-smoked salmon.

A sensitivity analysis indicated that probability of illness was most sensitive to the input distribution describing consumer storage time and temperature, growth occurring during the consumer storage, initial contamination level, growth during consumer transportation and prevalence. The sensitivity analysis provided the focus of future studies, and pointed out that the research should focus on collecting data on these variables to reduce the uncertainty in the estimation of the variability of the levels of *L. monocytogenes* (Lindqvist, 2000). The "what-if" scenario analysis results indicated that, the temperature and the storage period inhousehold refrigerators (Scenario B, C and E) seems to determine theexposure to *L. monocytogenes*from consumption ofcontaminatedvacuum packed cold-smoked salmon in Ireland.

The present risk assessment model makes it possible to define precise objectives and priorities for future studies.Considering the results of the present work, the model is of a practical value and the predictions by the model seemed to confirm surveillance and monitoring systems data of that smoked-salmon are high risk foods as far as listeriosis is concerned if they are stored under adverse storage conditions.

In conclusion, risk models can be beneficial to food industry because they can describe the conditions that can be applied to control a process or specify a formulation in order to minimize the risk of pathogen growth.

10.2 Recommendation for future work

Incorporation of Real-time PCR for the detection of *L. monocytogenes* in surveillance study would be advantageous as this technique allows the direct quantification of target DNA. The reaction mix of the Real-time PCR contains a fluorescent marker (SYBR Green) that binds specifically to double strand DNA of the *L. monocytogenes*. The increase of fluorescence after each successive cycle allows the direct quantification of target DNA. This method has been used to identify and quantify *L. monocytogenes* in foods and clinical samples in several studies. Establishing the actual number of contaminating *L. monocytogenes* cells in food and environmental samples is an important factor when investigating outbreaks of listeriosis. This technique was not incorporated in this study due to the requirement of specialized equipment and materials which substantially increase the cost of testing. For those reasons MLVA was used as next method of choice in this study, but for future studies Real-time PCR will prove advantageous, as they directly quantify the levels of the bacteria and will be useful for food testing and epidemiological investigations.

L. monocytogenes originating from the raw salmon was one of the main sources of contamination in vacuum packed cold-smoked salmon in this study, to precisely identify the route of contamination, sampling the salmon immediately after catchment would provide information to clarify if *L. monocytogenes* is originally part of the natural flora of the salmon. It would be interesting to sample the salmon entering the slaughter house to see if there was *L. monocytogenes* specific to the slaughter house. This experiment would provide answers for the treatment required to eliminate *L. monocytogenes* at the start of the cold-smoking process so that the pathogen is not carried on to the final product and also avoid cross contamination of the processing environment.

Presence of persistent *L. monocytogenes* in the processing equipment and environment were also responsible for contamination in the final product. It would be useful to study the physiological condition of the persistent bacteria to understand the mode of their survival and anchorage to in-animate objects. These items of equipment would have been put through rigorous cleaning regime; in-spite of these regimes the bacteria has found a mode to survive. For these reasons, this study would be useful to design cleaning protocols to target these specific physiological conditions responsible for its survival.

Investigation of non-thermal technology for the treatment of raw salmon and finished product would be useful to eliminate the pathogen before processing or after processing. Some of the non-thermal technology that could be compared for the efficacy in the reduction of *L. monocytogenes* and also maintain the quality parameters of salmon are ozone, E-beam, Gamma irradiation and plasma.

The quantitative microbial risk assessment carried out in this study considered the retail storage, consumer transport and consumer storage conditions. Temperature fluctuation occurring during the transportation from the end of production line to the retail distributors and its implication on the growth of *L. monocytogenes* was not considered. It was assumed that the temperature remained constant and levels of *L. monocytogenes* did not increase during the transport from production site to the distributors and then from the distributors to the retail storage. Future work on collecting data reflecting the above assumption would add knowledge to the QMRA established in this study.

Outline of the thesis

Chapter	Title	Summary	Outcome
1	Introduction and review of literature	- Survival characteristics of *Listeria monocytogenes* - Prevalence of *L. monocytogenes* in sea-food products in the EU and other countries - Aspects of microbial quantitative risk assessment - Aspects of microbial growth modelling	
2	General materials and methods	- Methods used in this study to detect, isolate and characterise *L. monocytogenes* from vacuum packed cold-smoked salmon - Inoculation of smoked salmon with *L. monocytogenes* - Statistical methods used in the evaluation of models applied in this study	
3	Development of rapid and reliable method for detection of *L. monocytogenes*	- Various selective plating methods tested for the isolation of *L. monocytogenes.* - Evaluation of the plating methods using 16S rRNA method developed in this study.	1. Paper accepted in the Journal of Food control 2. Poster presented at the 21st International ICFMH Symposium-Food Microbiology 2008, September 1-4, Aberdeen, United Kingdom.
4.	Prevalence of *L. monocytogenes* in vacuum packed cold-smoked salmon marketed in retail outlets in Dublin	- Prevalence of *L. monocytogenes* in cold-smoked salmon marketed in retail outlets in Dublin for a period of one year	1. Paper accepted in the Journal of Food Safety.
5.	Contamination pathways of *L. monocytogenes* in an Irish cold-smoked salmon processing factory	- Factory was divided into four zones and samples from each zone were analysed for the presence of *L. monocytogenes* - The *L. monocytogenes* isolates were characterised using MLVA to study the similarities of the isolates from various zones - Contamination pathways of *L. monocytogenes* in vacuum cold-smoked salmon was established	1. Paper accepted in the Journal of Food Research International.
6.	Tracking *L. monocytogenes* during different processing stages of vacuum packed cold-smoked salmon in a cold-smoked salmon processing	- 60 raw salmon were tagged and sampled after every critical step in the processing of vacuum packed cold-smoked salmon, to confirm the contamination pathways established in the previous Chapter (5)	1. Paper accepted in the International Journal of Food Hygiene and Safety.

	factory		
7.	Dynamic modelling of *L. monocytogenes* in vacuum packed cold-smoked salmon	- Development of a product specific growth model for vacuum packed cold-smoked salmon contaminated with *L. monocytogenes* was constructed that covering retail and consumer storage temperature range.	1. Paper in review in the journal of Food Protection 2. Paper presented at the 6th International Conference on Predictive Modelling in Foods 2009, September 8-12, The Renaissance, Washington, 999 Ninth Street NW, Washington DC, USA
8.	Growth modelling of *L. monocytogenes* in vacuum packed cold-smoked salmon at 4, 8, 12 and 16 °C.	- Determine of the kinetic parameters of both *L. monocytogenes* (isolates of *L. monocytogenes* obtained from vacuum packed cold-smoked salmon Chapter 4) and native microflora at 4 °C and at abuse temperatures 8, 12 and 16 °C. - The nature of growth relationship between native flora and *L. monocytogenes* was also studied.	1. Paper accepted in the journal of Food Safety. 2. Paper presented at the European Simulation – Food Simulation 2008, June 26-28, University College Dublin, Dublin, Ireland 3.Poster presented at the 8th Joint Meeting of Seafood Science and Technology Society of the Americas Atlantic Fisheries Technology Conference and 1st North Carolina Marine Biotechnology Symposium 2008, October 19-22, Wrightsville Beach, North Carolina, USA 4. Poster presented at the 21st International ICFMH Symposium-Food Microbiology 2008, September 1-4, Aberdeen, United Kingdom.
9.	Risk assessment of *L. monocytogenes* in vacuum packed cold-smoked salmon	- Development of quantitative risk assessment model of human listeriosis linked to the consumption of vacuum packed cold-smoked salmon.	1. Paper in International Journal of Food Microbiology 2. Paper presented at the 6th International Conference on Simulation and Modelling in the Food and Bio-Industry, FoodSimulation 2010 June 24- 16, CIMO Research Centre, Braganca, Portugal
10	General conclusions and recommendations for future work	- The chapter presents the conclusions, implications, limitation and recommendation for future work.	

List of Publication

Peer- reviewed papers

Chitlapilly-Dass, S., Abu-Ghannam, N., and Antony-Babu, S, Cummins, E. J.(2010) Ecology and molecular typing of *Listeria monocytogenes* in a processing plant for cold-smoked salmon in the Republic of Ireland.*Food Research International*. 43, 1529–1536

Chitlapilly-Dass, S., Abu-Ghannam, N., Cummins, E. J. (2010) Prevalence and typing of *Listeria monocytogenes* strains in retail vacuum packed cold-smoked salmon in the Republic of Ireland. Journal of Food Safety 31**,** 21–27

Chitlapilly-Dass, S., Abu-Ghannam, N., Cummins, E. J. (2011) Alternative identification by 16S rRNA gene sequence analysis for EN ISO 11290 -01 and -02 for detection of Listeria monocytogenes in cold smoked salmon. Food Control.42, 38-45

Chitlapilly-Dass, S., Abu-Ghannam, N., and Antony-Babu, S, Cummins, E. J.(2011) Tracking *L. monocytogenes* during different processing stages of vacuum packed cold-smoked salmon in a cold-smoked salmon processing factory. International Journal of Food Hygiene and Safety. 20, 181-186

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Chitlapilly-Dass, S., Abu-Ghannam, N., Panagou, E, Z. and **Cummins, EJ.** Dynamic modelling of *L. monocytogenes* in vacuum packed cold-smoked salmon. Journal of Food Protection. (in review)

Conference Presentation

Papers

Chitlapilly-Dass, S,N. Abu-Ghannam, E.J. Cummins (2008). Predictive modelling of *Listeria monocytogenes* in Irish smoked salmon. In *EUROSIS - FOODSIM 2008*, June 26-28, University College Dublin, Dublin, Ireland

Chitlapilly-Dass, S., Abu-Ghannam, N. and Cummins, E. (2009) Dynamic modelling of listeria monocytogenes growth in vacuum packed cold smoked salmon *6th International Conference on Predictive Modelling in Foods* The Renaissance, Washington, 999 Ninth Street NW, Washington DC, USA, , 08-SEP-09 - 12-SEP-09

Cummins, E., Chitlapilly Dass, S., Abu-Ghannam, N. (2010) Exposure assessment of Listeria monocytogenes in Irish cold-smoked salmon . In: Cadavez, V. and Thiel, D eds. *6th International Conference on Simulation and Modelling in the Food and Bio-Industry (FOODSIM'2010)* CIMO Research Centre, Braganca, Portugal, 24-JUN-10 - 26-JUN-10 , pp.144-146

Posters

Chitlapilly-Dass, S.,N. Abu-Ghannam, E.J. Cummins (2008). Modeling the lag phase of *Listeria monocytogenes* in vacuum packed cold-smoked salmon.In *8th* Joint Meeting Seafood Science and Technology Society of the Americas Atlantic Fisheries Technology Conference and 1st North Carolina Marine Biotechnology Symposium, October 19-22, 2008, Wrightsville Beach, North Carolina, USA

Chitlapilly-Dass, S., N. Abu-Ghannam, E.J. Cummins (2008).16S rRNA gene sequence analysis for identification of *Listeria monocytogenes.*In*21st International ICFMH Symposium-FOODMICRO 2008,* September 1- 4, Aberdeen, United Kingdom

Chitlapilly-Dass, S., N. Abu-Ghannam, E.J. Cummins. (2008) Growth modelling of *Listeria monocytogenes* in smoked salmon. In *21st International ICFMH Symposium-FOODMICRO 2008,* September 1-4, Aberdeen, United Kingdom.

Appendix 1

Colony morphology of *L. monocytogenes* on Palcam agar

Greyish-green or black colonies with black corona, opaque with smooth slimy surface

Palcam agar showing growth of *L. monocytogenes*

Colony morphology of *L. monocytogenes* on TSA

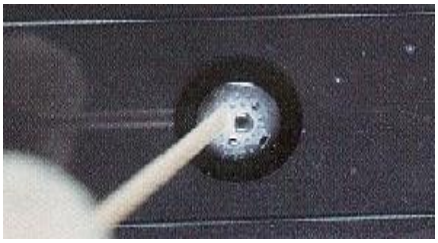
White to off-white raised smooth colonies, with opaque and slimy surface.

TSA showing growth of *L. monocytogenes*

Appendix 2

Catalase test

- Approximately 0.2 mL of hydrogen peroxide solution was placed on aclean glass slide
- Colony was carefully picked with disposable sterile loop
- The colony was spread on the surface of the hydrogen peroxide solution.
- The hydrogen peroxide solution covered the colony
- Vigorous bubbling occurred within 10 seconds



Positive catalase test showing vigorous bubbling in the presence of *L. monocytogenes* and hydrogen peroxide solution

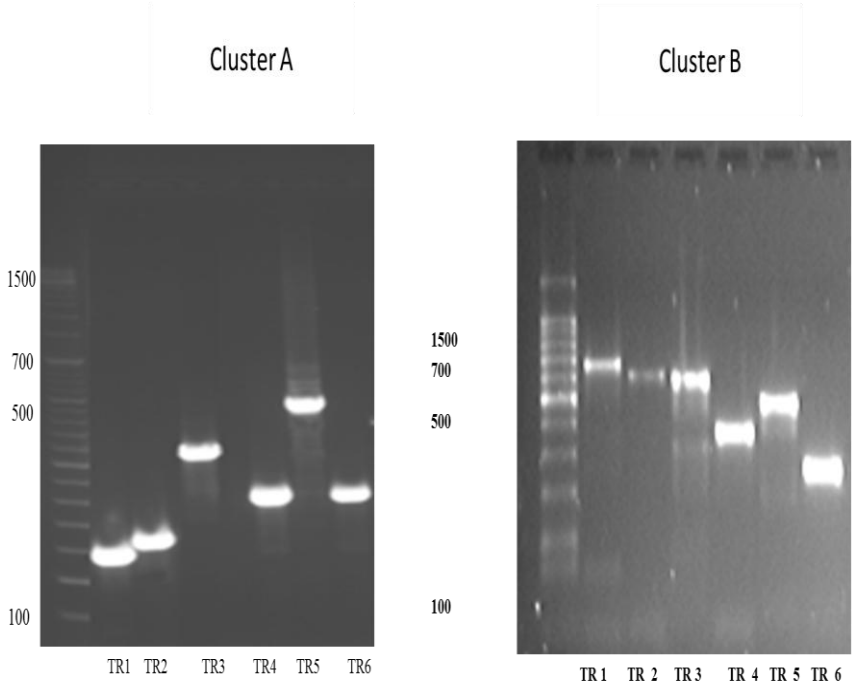
Impregnated oxidase test strip method

- Freshly grown colonies were picked with the help of disposable loop
- The swab containing the oxidase reagent was rubbed on top on the loop containing the colony. Blue colour appeared within 10 seconds.

Positive oxidase test showing blue colour in the presence of

L. monocytogenes and oxidase reagent

Appendix 3


Cluster A
Cluster B
1500
700
500
100
TR1 TR2 TR3 TR4 TR5 TR6
1500
700
500
100
TR 1 TR 2 TR 3 TR 4 TR 5 TR 6

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