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University College Cork, Ireland



Monitoring, understanding and controlling *Listeria* in food processing

Thesis presented by

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The thesis is submitted to the National University of Ireland, Cork for the degree of Doctor
of Philosophy

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Signed:

Dara Leong

Abstract

The objective of this study was to monitor, understand and help control *Listeria monocytogenes* and *L. ivanovii* in the food processing environment. As foodborne transmission has been identified as the primary route of human infection of *L. monocytogenes*, the contamination of foods during production poses a serious threat to public health. Although the incidence of listeriosis is relatively low, the mortality rate can be extremely high, especially in the immunocompromised. This work aimed to examine the prevalence of *L. monocytogenes* and *ivanovii* at the primary production stage and, through the typing of isolates, identify and subsequently examine persistent strains. A three year monitoring programme was conducted in 54 processing facilities which identified 86 distinguishable PFGE pulsotypes, 17 of which were seen to be persistent. The effect of management practices on the occurrence of *L. monocytogenes* was also examined. Separation of personal protective equipment in high and low risk areas, training being performed by management and the use of a power hose were seen to have an effect on *L. monocytogenes* occurrence and persistence. Challenge tests were also performed using varying methodologies on several types of food in order to establish whether the growth of *L. monocytogenes* was supported by these foods. Different methodologies were seen to affect the results in challenge tests performed in mushrooms while smoked salmon was seen to support *L. monocytogenes* growth regardless of methodology used. Finally, two closely related 1/2a *L. monocytogenes* strains isolated from smoked salmon were subjected to whole genome sequencing and their genomes were compared. The monitoring and examination of *L. monocytogenes* and *L. ivanovii* has been seen to reduce the occurrence in the food processing environment and therefore reduce the risk to public health.

Outputs related to this work

Publications

- Determination of *Listeria monocytogenes* growth during mushroom production and distribution. Leong, D., Álvarez-Ordóñez, A., Guillas, F. and Jordan, K., *Foods*, 2, 544-553. 2013
- Monitoring occurrence and persistence of *Listeria monocytogenes* in foods and food processing environments in the Republic of Ireland. Leong, D., Alvarez-Ordóñez, A. and Jordan, K., *Frontiers in Microbiology*, 5, 436. 2014
- Occurrence, persistence and virulence potential of *Listeria ivanovii* in foods and food processing environments in the Republic of Ireland. Álvarez-Ordóñez, A., Leong, D., Morgan, C.A., Hill, C., Gahan, C.G.M. and Jordan, K., *Biomed Research International*, Article ID 350526. 2015
- The challenge of challenge testing to monitor *Listeria monocytogenes* growth on ready-to-eat foods in Europe by following the European Commission (2014) Technical Guidance document. Álvarez-Ordóñez, A., Leong, D., Hickey, B., Beaufort, A., and Jordan, K., *Food Research International*, 75, 233-243. 2015
- Examination of *Listeria monocytogenes* in Seafood Processing Facilities and Smoked Salmon in the Republic of Ireland. Leong, D., Álvarez-Ordóñez, A., Zaouali, S., and Jordan, K., *Journal of Food Protection*, 78 (12), 2184-2190. 2015
- A note on challenge trials to determine the growth of *Listeria monocytogenes* on mushrooms (*Agaricus bisporus*). Leong, D., Alvarez-Ordóñez, A., Jordan, K., *Irish Journal of Agricultural and Food Research* 54 (2) 121-125. 2015
- *Listeria monocytogenes* in the Food Processing Environment. Jordan, K., Leong, D., Álvarez-Ordóñez, A., Springer Brief, Springer International Publishing. 2015
- *Listeria monocytogenes* in food: Control by monitoring the food processing environment. Jooste, P., Jordan, K., Leong, D., Álvarez-Ordóñez, A., *African Journal of Microbiology Research* 10 (1) 1-14. 2016
- A 3-year multi-food study of the presence and persistence of *Listeria monocytogenes* in 54 small food businesses in Ireland. Leong, D., NicAogáin, K., Luque-Sastre, L., McManamon, O., Hunt, K., Alvarez-Ordóñez, A., Scollard, J., Schmalenberger, A., Fanning, S., O'Byrne, C., Jordan, K., *International Journal of Food Microbiology*, 249, 18-26. 2017

Oral Presentations

- “Monitoring *Listeria monocytogenes* occurrence, persistence and patterns of contamination in Irish food dairy processing environments” The Ninth Cheese Symposium 2014
- “Monitoring occurrence and persistence of *Listeria monocytogenes* in foods and food processing environments in the Republic of Ireland” **safefood** *Listeria* Knowledge Network Annual Conference 2014
- “*Listeria* monitoring in Ireland” **safefood** *Listeria* Knowledge Network Annual Conference 2015

Poster Presentations

- Detection and examination of *Listeria monocytogenes* contamination in food processing facilities throughout Ireland in 2013. Food Integrity and Traceability conference 2014
- *Listeria monocytogenes* research support for the farmhouse cheese making industry in Ireland. The Science of Artisan Cheese 2014
- Monitoring *Listeria monocytogenes* occurrence, persistence and patterns of contamination in Irish ready-to-eat food processing environments over a three-year period. ISOPOL XIX 2016
- Monitoring *Listeria monocytogenes* occurrence, persistence and patterns of contamination in Irish ready-to-eat food processing environments over a three-year period. FoodMicro2016

Brief Overview

The initial focus of this work was based around a three-year sampling programme to examine *Listeria* in the food processing industry in the Republic of Ireland. The presence and diversity of *Listeria* strains was seen through PFGE subtyping.

Due to the common incidence of *Listeria* in both the food processing environment and in food products, the behaviour of the bacteria within food was evaluated through challenge testing using varying methodologies. Whether or not a food can be shown to support the growth of *L. monocytogenes* effects the regulations to which the food is subjected and so is of high importance to the food processing industry. Strains of *L. monocytogenes* which had been isolated during the sampling programme from appropriate foods were used for inoculation in the challenge tests in order to replicate real world contamination events.

The effects of management practices has previously been seen to have an effect on the prevalence of *L. monocytogenes* and so a detailed survey was conducted with food business operators involved in the sampling programme. This survey was largely based around cleaning and management practices and aligned these answers with the occurrence and persistence of *L. monocytogenes* found in 32 food businesses over two years.

Finally the whole genome sequences of two *L. monocytogenes* strains with similar PFGE profiles, both isolated from smoked salmon from separate facilities, were examined. Similar PFGE profiles, including the persistent pulsotype P59, had previously been seen in several food processing facilities during the sampling programme. This work aimed to evaluate the two strain's relatedness and therefore examine the accuracy of the use of PFGE as a typing technique for use in sampling programmes.

Chapter 1

Literature review

***Listeria monocytogenes* in the food processing environment**

**Invited Book Publication: *Listeria monocytogenes* in the food processing environment
Published as a Springer Briefs in Food, Health, and Nutrition 2015**

***Listeria monocytogenes* in food: Control by monitoring the food processing environment
Published in African Journal of Microbiology Research 2016**

1.1 Abstract

L. monocytogenes is a foodborne pathogen that is widely dispersed in the environment, being found in soil, water, and plant material, and can grow at refrigeration temperature and at unfavourable conditions of pH (up to pH 4.7) and salt (up to 10%). It can persist in the harsh conditions of the food processing environment from which it can contaminate food. Listeriosis, infection with *L. monocytogenes*, can be mild but the ability of the pathogen to cross the epithelial barrier of the intestinal tract, the blood brain barrier and the fetoplacental barrier can also result in more severe illness including bacteraemia and meningitis or spontaneous miscarriage. Although relatively rare, infection with *L. monocytogenes* can have a mortality rate of up to 30%, resulting in a serious hazard, particularly for the high risk groups of the elderly and immunocompromised individuals.

Healthy adults are generally unaffected by *L. monocytogenes*. However, in the susceptible populations (elderly, pregnant women and their unborn children, infants, and the immunocompromised) listeriosis is a serious disease that can occur in different forms: neuromeningeal (meningitis, encephalitis), maternal-neonatal (intrauterine infection, spontaneous abortion) and febrile gastroenteritis, and in serious cases it can lead to brain infection, sepsis and even death. As consumer demand for less processed, less preserved, longer shelf-life ready-to-eat food increases, the threat of *L. monocytogenes* to public health and the food industry continues to rise. In addition to being a public health threat, *L. monocytogenes* is a major economic burden on industry in terms of costs of analysis and potential product recalls.

Current knowledge suggests that cases of listeriosis are almost exclusively through foodborne infection. However, this critical transmission vector only became clear during the 1980s, principally as a result of a series of high-profile disease outbreaks, particularly the Canadian outbreak of 1981, linked to contaminated coleslaw. Awareness of its ubiquitous nature and understanding of its physiology and survival are important aspects of its control in the food processing environment with the aim of reducing the public health concern.

Appropriate methodologies are required for its detection and isolation. Characterisation of strains by pulsed field gel electrophoresis (PFGE) and other genotypic methods can facilitate identification of putative contamination routes. Whole genome sequencing (WGS) of outbreak strains is becoming a part of outbreak investigation. Such WGS will lead to a greater understanding of the physiology of the organism as well as contributing to understanding epidemiology and pathogenicity. However, despite the advances, the best mechanism of public health protection is still prevention. Awareness of its presence, and control by conventional hygiene methods or by novel biocontrol methods such as bacteriocins and bacteriophage will help prevent cross-contamination of food from the environment and therefore reduce the public health burden.

1.2. What is the Issue?

Listeria monocytogenes is a foodborne pathogen that causes the disease listeriosis. Although rare, the mortality rate of listeriosis is 25% worldwide (de Noordhout et al., 2014) and with a hospitalisation rate of >95% (Scallan et al., 2011) it ranks as the third most serious foodborne disease. The clinical manifestations of listeriosis have been reviewed and there have been many recent high-profile outbreaks of listeriosis worldwide that have resulted in numerous fatalities (Table 1.1) (Schlech, 2000) .

Among the many species of the genus *Listeria*, *L. monocytogenes* is the only one that causes disease in humans, apart from a few reported cases of disease caused by *L. ivanovii* (Guillet et al., 2010), although *L. ivanovii* can be pathogenic for animals. None of the other species of the genus have been reported to cause disease.

In the European Union, according to the latest EU summary report on zoonoses, zoonotic agents and food-borne outbreaks (EFSA, 2014), 1,642 confirmed human cases of listeriosis were reported in 2012, representing a 10.5% increase compared with 2011. The EU notification rate was 0.41 cases per 100,000 population, with the highest member state specific notification rates observed in Finland, Spain and Denmark. On average, 91.6% of the cases were hospitalised. This is the highest proportion of hospitalised cases of all zoonoses under EU surveillance. A total of 198 deaths due to listeriosis were reported by 18 member states in 2012, which was the highest number of fatal cases reported since 2006.

In addition to being a public health risk, *L. monocytogenes* is an economic burden on the ready-to-eat (RTE) food industry. Ready-to-eat foods are the most vulnerable to *L. monocytogenes* as they do not have a heating or other antibacterial step between production and consumption. The economic burden includes the cost of analysis of samples,

the costs, both financial and reputational, of recall of a contaminated product (Table 1.2) and the possible litigation costs, if the food is shown to have caused disease.

L. monocytogenes is ubiquitous in the environment and can be found in soil, water, faeces etc. Thus, it has been isolated from foods such as raw and unpasteurized milk, cheese, ice cream, raw vegetables, fermented meats and cooked sausages, raw and cooked poultry, raw meats, and raw and smoked seafood. It also has the ability to form biofilms which can contribute to its ability to colonise food processing facilities. It is also resistant to many of the stresses imposed in food processing such as salt (up to 10% salt), temperature (refrigeration temperatures), and detergents (many detergents). Therefore, it can survive in food processing environments and become persistent. Such persistence of *L. monocytogenes* has been shown, often for many years, at larger scale and smaller artisan facilities of different production sectors (Fonnesbech Vogel et al., 2001b; Fox et al., 2011; Giovannacci et al., 1999; Lawrence and Gilmour, 1995; Ojeniyi et al., 2000; Wulff et al., 2006).

Year	Place	No. of cases (deaths)	Cheese type	Serovar	Reference
2009/10	Austria/Germany Czech Republic	34 (8)	Quargel	1/2a (2 strains)	(Fretz et al., 2010)
2011-12	28 US states	146 (31)	Cantaloupe	Multiple strains of 1/2a and 1/2b	(CDC, 2011)
2012	14 US states	20 (4)	Ricotta salata cheese		(CDC, 2012)
2012	Spain	2	Fresh cheese	1/2a	(de Castro et al., 2012)
2013	5 US states	6 (1)	Farmstead cheeses		(CDC, 2013)
2014	2 US states	8 (1)	Dairy products		(CDC, 2014)
2013-2014	Denmark	41 (17)	Spiced lamb roll, pork, sausages, liver pâté and other meat products		Anonymous 2015a
2014-January 15	12 US states	35 (7)	Caramel apple	4b	CDC 2015a http://www.cdc.gov/Listeria/outbreaks/caramel-apples-12-14/
2015	4 US states	10 (3)	Ice cream		CDC 2015b http://www.cdc.gov/Listeria/outbreaks/ice-cream-03-15/

Table 1.1: Major outbreaks of foodborne listeriosis 2010-2015.

1.3 Regulations Relating to *L. monocytogenes*

In Europe, Regulation (EC) No 2073/2005 (EC 2005) sets the microbiological criteria for *L. monocytogenes* in foods that must be complied with. This regulation primarily covers RTE food products, and requires that *L. monocytogenes* must be absent from foods (10 x 25 g) intended for infants and for special medical purposes, and allows different criteria depending on the ability of the food product to support growth of *L. monocytogenes*. For RTE foods unable to support the growth of *L. monocytogenes*, the levels should be <100 CFU/g throughout the shelf-life of the product (5 x 25 g). On the other hand, for RTE foods that are able to support the growth of the bacterium, *L. monocytogenes* must not be present in 5 x 25 g samples at the time of leaving the production plant; however, if the producer can demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 CFU/g throughout its shelf-life, the level should be <100 CFU/g throughout the shelf life of the product (5 x 25 g).

In Canada (http://www.hc-sc.gc.ca/fn-an/legislation/pol/policy_Listeria_monocytogenes2011-eng.php) and Australia/New-Zealand (<http://www.foodstandards.gov.au/code/microbiolimits/Pages/Criteria-for-Listeria-monocytogenes-in-ready-to-eat-foods.aspx>), the regulations are in line with European regulations, allowing a differentiation between foods that can and cannot support growth.

However, in the USA there is 'zero tolerance' of *L. monocytogenes* (absence in 5 x 25 g of food is required at all times, and in the processing environment), where any occurrence is considered an offence (<http://www.fsis.usda.gov/wps/portal/fsis/topics/regulatory-compliance/Listeria>).

Further discussion on regulations in different jurisdictions are reviewed in a special issue of Food Control published in 2011 (Anonymous, 2011).

Year	Country	Number of recalls	Associated products
1991-2008	Canada	6	Frankfurters, pork, salami and others
1998-2008	United States of America	216	Frankfurters, sandwiches, ham, chicken, cheese, hot dogs, beef jerky and others
2009	United States of America	7	Meat, RTE meal
2010	Australia/New Zealand	31	Meat, cheese
2010	Canada	12	Meat, fish, eggs, cheese
2008	England	6	Meat, cheese
Sept. 2010-Dec. 2011	Ireland	6	Meat, fish, cheese
2014	United States of America	3	Dairy products
2014	United States of America	1	Soy products
2015	Ireland	1	Smoked Salmon
2015	England	1	Cheese
2015	United States of America	1	Green Beans
2015	United States of America	3	Apples

Table 1.2: Some food recalls associated with *L. monocytogenes*.

1.4 Occurrence and Persistence of *L. monocytogenes*

1.4.1 Occurrence of *L. monocytogenes* in Foods and Food Processing Environments

Because *L. monocytogenes* is ubiquitous in the environment and frequently present in the processing environment, it can contaminate food. *L. monocytogenes* is frequently present in raw foods of both plant and animal origin (including fish), and it can be found in cooked foods due to post-processing contamination. Thus, it has been isolated from foods such as raw and unpasteurized milk, cheese, ice cream, raw vegetables, fermented meats and cooked sausages, raw and cooked poultry, raw meats, and raw and smoked seafood. In addition, its ubiquitous presence also leads to the potential for contamination of the food processing environment, where occurrence and persistence of *L. monocytogenes* is frequent (Fox et al., 2011; Nakari et al., 2014; Vongkamjan et al., 2013).

A number of surveys of *L. monocytogenes* in foods (especially RTE foods) and processing environments within food processing facilities have been performed in recent years. Such surveys give valuable information for particular cases, but tend to be focused on a single analysis time at a few facilities. Surveys conducted over time at several processing facilities provide greater information on the ecology and persistence of *L. monocytogenes*. For instance, a European-wide survey on occurrence in different dairy and meat processing facilities over a 12-month period has also been reported (Muhterem-Uyar et al., 2015). Additionally, varying occurrence of *L. monocytogenes* has been reported in smoked fish products and processing facilities (Wulff et al., 2006), dairy processing facilities (Pritchard et al., 1995) and ready-to-eat food producing facilities (Kovacevic et al., 2013).

1.4.2 Occurrence of *L. monocytogenes* at Retail Level

Contamination of RTE foods by *L. monocytogenes* can occur at various stages of the processing and distribution chain, including at retail level, although studies of occurrence at retail level do not necessarily imply that contamination occurred in the retail environment. Cross-contamination with *L. monocytogenes* at retail has been identified as the main source of *L. monocytogenes* in RTE deli products (Sauders et al., 2009; Tompkin, 2002b; Vorst et al., 2006). Data from some surveys have indicated that RTE deli products handled at retail level have a significantly higher *L. monocytogenes* prevalence than products pre-packed by the manufacturer and not handled at retail (Gombas et al., 2003). For instance, Gombas et al. (2003) analysed 31,705 samples from retail markets in the USA and found an overall *L. monocytogenes* prevalence of 1.82%, with the prevalence ranging from 0.17 to 4.7% among the product categories tested. Interestingly, these authors observed significantly ($p < 0.001$) higher prevalence for in-store packaged samples than for manufacturer-packaged samples of luncheon meats, deli salads, and seafood salads.

It is important to note that recently conducted risk assessments for *L. monocytogenes* in deli meats indicated that the majority of listeriosis cases and deaths associated with deli meats are probably due to contamination of products at retail (Endrikat et al., 2010; Pradhan et al., 2010). Endrikat et al. (2010) estimated that 83% of human listeriosis cases and deaths attributable to deli meats are due to retail-sliced products, and Pradhan et al. (2010) performed a risk assessment using product-specific growth kinetic parameters that indicated that 63 to 84% of human listeriosis deaths linked to deli ham and turkey can be attributed to contamination at retail. Occurrence and cross-contamination at

retail level do not attract much research, but are obviously an important source of listeriosis.

1.4.3 Persistence of *L. monocytogenes* in Processing Environments

The persistence of *L. monocytogenes* in the food-processing environment is well-documented but poorly understood (Carpentier and Cerf, 2011b; Lomonaco et al., 2009). This is partly due to the loosely defined term “persistence”. Generally, strains of *L. monocytogenes* that have been repeatedly isolated from the same environment over a long period of time for example, over 6 months, are thought of as being persistent. Persistence of *L. monocytogenes* isolates has been shown, often for many years, at larger scale cheese production facilities (Lomonaco et al. 2009), smaller artisan facilities (Fox et al., 2011), in the salmon industry (Tocmo et al., 2014), in meat processing plants (Gómez et al., 2015) and in poultry production plants (Lawrence and Gilmour, 1995; Ojeniyi et al., 2000). Nevertheless, although it is probable that these strains are surviving and persisting in the food-processing environment, it is also possible that consistent contamination from outside sources, for example, from raw materials, act as a continuous source of particular *L. monocytogenes* strains (Carpentier and Cerf, 2011b).

The survival of *L. monocytogenes* in food processing conditions which would be inhospitable to most bacteria can be due to several factors including: (1) ability to grow at a wide range of temperatures, especially refrigeration temperatures (Schmid et al., 2009), (2) resistance to acid stress, (3) resistance to desiccation (Takahashi et al., 2011), (4) resistance to sanitation agents and (5) biofilm formation (Galvão et al., 2012; Gandhi and Chikindas,

2007). This ability to survive where other bacteria cannot allows *L. monocytogenes* to grow with little competition from other bacteria. Persistent strains do not appear to have any particular resistance genes to help them survive and persist in the environment, but *L. monocytogenes* strains in general are hardy and resistance to various stresses is commonly seen (Carpentier and Cerf, 2011b). These characteristics allow *L. monocytogenes* to survive and possibly even thrive in environments which would be considered unfavourable for general bacterial growth.

A major step to discourage bacterial growth in food processing is storage at refrigeration temperatures of 4 °C. Although the majority of food pathogens cannot grow at this temperature, *L. monocytogenes* can. Therefore, refrigerated storage essentially selects for *L. monocytogenes* growth. Cold shock proteins have been shown to be essential for *L. monocytogenes* ability to survive at low temperature as well as its ability to survive osmotic stress (Schmid et al., 2009). An alternative sigma factor σ^B , encoded by *sigB*, plays a vital role in *L. monocytogenes* stress response. The *sigB* gene has been shown to be vital in the survival of *L. monocytogenes* in prolonged cold storage (Moorhead and Dykes, 2004).

Harbourage sites are also a very important factor in the persistence of *L. monocytogenes*. When used correctly, cleaning and sanitising procedures should be adequate to remove *L. monocytogenes* from the environment (Cruz and Fletcher, 2012). However, a harbourage site could be an area where sanitation agents do not properly reach so *L. monocytogenes* is not properly removed. When used correctly and in a high enough dosage, *L. monocytogenes* does not seem to have increased resistance to disinfectants when compared to other bacteria (Kastbjerg and Gram, 2012; Lourenço et al., 2009). However, a harbourage site may be an area where the disinfection product reaches but at a lower concentration and it may not be properly dried so that a sub lethal amount of the

product remains in the site. This may allow *L. monocytogenes* strains sufficient time to develop a resistance to the product so that a community of *L. monocytogenes* which is resistant to the cleaning product develops. This strain could then be spread out from the harbourage site to contaminate other areas of the facility (Carpentier and Cerf, 2011b).

Biofilm formation is an important factor in the survival of *L. monocytogenes* strains in the environment (Figure 1.1) (Harvey et al., 2007). Strong adherence to surfaces, and especially biofilm formation, may contribute to the ability of *L. monocytogenes* to survive cleaning procedures. Bacteria in a biofilm display altered behaviour in comparison to the behaviour of planktonic cells. This can include increased adherence, increased resistance to stresses and increased tolerance to disinfectants (Bremer et al., 2006). Bacteria in a biofilm may display altered gene expression, cell morphology, growth rate and can produce extracellular polysaccharide (EPS) which has a protective effect and has been seen to be important in biofilm formation (Chae et al., 2006). The biofilm structure itself helps to protect *L. monocytogenes* from both physical and chemical stresses (Cruz and Fletcher, 2011). Although the adhesion ability of *L. monocytogenes* is affected by conditions of low temperature, varying pH and low nutrient availability commonly found in a food processing facility (Galvão et al., 2012), biofilms have been routinely identified in multiple food processing facilities worldwide (Cruz and Fletcher, 2011; Latorre et al., 2010a). The wear of equipment over time may facilitate the formation of biofilms as the bacteria can attach to scratches or imperfections which develop in the equipment (Latorre et al., 2010a). Although disinfectants and sanitisers may be effective against planktonic cells, their effect on biofilms can be variable (Bremer et al., 2006). Norwood and Gilmour found statistically greater mean adherence ability among persistent strains compared to presumed non-persistent strains (Norwood and Gilmour, 1999). However, the results were not entirely consistent as some

individual non-persistent strains showed high adherence. Using a microtitre plate assay method, Djordjevic et al. (2002) did not find higher adherence among persistent strains. In a study by Lunden et al. (2000), it was shown that persistent strains showed enhanced attachment over short periods of time, although some presumed non-persistent strains matched, or in some cases surpassed, the levels of attachment of persistent strains after 72 h. A recent study found better adherence of persistent strains than sporadic strains from the dairy environment (Latorre et al., 2011). Higher biofilm formation among persistent compared to presumed non-persistent strains from bulk milk samples was also described by Borucki et al. (2003). Latorre et al. (2010b) conducted a study monitoring the epidemiology of *L. monocytogenes* strains on a dairy farm, in which they postulated that biofilm formation was responsible for repeated contamination events during the study period. The work, including typing of *L. monocytogenes* strains isolated from bulk milk and milking equipment, and examination of biofilms on the milking equipment, supported the view that the ability of *L. monocytogenes* to form biofilms is important in persistence of strains.

In addition, it has been shown that strongly adherent *L. monocytogenes* strains have an increased invasive ability in both cell cultures (Kushwaha and Muriana, 2010b) and *in vivo* in mouse assays (Kushwaha and Muriana, 2010a). Therefore, the *L. monocytogenes* strains in a biofilm have may have increased virulence compared to planktonic *L. monocytogenes* cells. This further increases the need to eliminate persistent *L. monocytogenes* biofilms from the food processing environment.

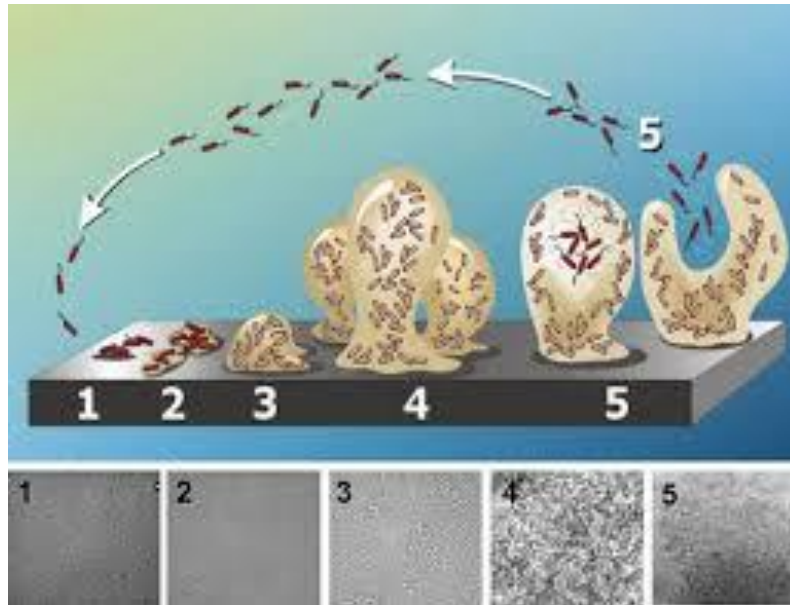


Figure 1.1: Schematic diagram of formation and development of biofilm.

1.4.4 Stress Response and Sigma B

L. monocytogenes has the ability to survive and even grow under stress conditions e.g. at refrigeration conditions, or in the host. Survival and adaptation in the host has recently been reviewed by (Gahan and Hill, 2014).

Sigma factors contribute to stress survival in bacteria. A sigma factor (σ) is a specialised protein subunit that is required for initiation of RNA synthesis. Along with the RNA polymerase, it binds to a specific promoter sequence and in that way determines which genes are transcribed. Different bacteria have a different number of sigma factors, but all cells have primary sigma factors which direct transcription of essential genes, and alternative sigma factors, the activity of which depends on the environmental conditions in which the cells exist. The larger the number of sigma factors a cell has, the greater the ability it has to adapt to stressful environmental conditions. Some of the common sigma factors include σ^{70} , σ^{38} , σ^{28} and σ^{32} . σ^{32} (RpoH) for example (the heat shock sigma factor), is turned on when the bacteria are exposed to heat. Due to the higher expression, the factor will bind with a high probability and in doing so other heat shock proteins are expressed. This enables the cell to survive higher temperatures. Some of the enzymes that are expressed on activation of σ^{32} include chaperones, proteases and DNA-repair enzymes. The system is quite complex as there are anti-sigma factor proteins and anti-anti-sigma factor proteins.

In *L. monocytogenes*, *sigB* encodes σ^B which contributes to stress survival of *L. monocytogenes* under acid and osmotic stress and also has a role in stationary phase stress response (O'Byrne and Karatzas, 2008). It also directly upregulates virulence genes, and is responsible for regulation of >100 genes (Mujahid et al., 2013). Gene deletion has been

used to study the function of σ^B in regulating stress and virulence genes (Wiedmann et al., 1998). For a review of alternative sigma factors and their role in virulence, see Kazmierczak et al. (2005).

1.4.5 Virulence and Virulence Factors

In order to cause an infection, *L. monocytogenes* has many obstacles to overcome. It must first resist the passage throughout the intestinal tract, recognize and target human cells, adhere to and enter into them, delay phagosome maturation, escape into the cytoplasm, control the production of different factors such as toxins, and identify pathways to infect other cells (Camejo et al. 2011). The expression of several virulence factors makes all this possible. Having developed a large arsenal of virulence determinants, *L. monocytogenes* is capable of infecting a large variety of cells, tissues and organs. Table 1.3 outlines some of the major virulence factors important in listeriosis and their functions in infection. Additional virulence factors absent from Table 1.3 include over 20 additional internalins and products of the genes *plcA*, *mpl* and *plcB* which are located on the *Listeria* pathogenicity island-1 (LIPI-1).

Virulence factor	Gene	Function	Note	Gene family
InlA	<i>inlA</i>	Binding to E-cadherin	Truncated versions of InlA have been seen to have reduced virulence	Internalin family
InlB	<i>inlB</i>	Binding to hepatocyte growth factor receptor	Additionally acts as a co-factor in bacterial invasion	
PfrA	<i>prfA</i>	Major regulatory factor	Functions in regulating virulence genes in the mammalian host environment	<i>Listeria</i> pathogenicity island 1(LIPI-1)
LLO	<i>hly</i>	Haemolysin used in lysing from vacuole		
ActA	<i>actA</i>	Re-arranges host actin by polymerisation for movement at temperatures over 30°C	Additional function in adhesion, bacterial aggregation	
LLS	<i>lls</i>	Haemolysin which confers increased virulence	Only associated with lineage 1 strains	<i>Listeria</i> pathogenicity island 1(LIPI-3)

Table 1.3: Some of the major virulence factors important in listeriosis infection.

1.5 Methods for Analysis of *L. monocytogenes*

1.5.1 Methods of Detection

L. monocytogenes contamination usually occurs in very low numbers both in foods and in the processing environment so it is vital that any analysis performed includes one or more enrichment steps which inhibit other microflora, and allow both the increase of *L. monocytogenes* to sufficient numbers to allow detection and the recovery of injured/stressed cells. Three methods of analysis are most commonly used: the International Standard (ISO-11290) method (Figure 1.2) which uses a two-step enrichment in Fraser broth, the United States Department of Agriculture (USDA) method (Figure 1.3) which uses a two-step enrichment in University of Vermont media (UVM) and the One-broth *Listeria* method (Figure 1.4) which has been approved for use by the Association Française de Normalisation (AFNOR) and takes considerably less incubation time and yields results in 2 days as opposed to the 4-5 days needed for the other two methods (Gómez et al., 2013; Zhang et al., 2007). All these methods involve plating on *Listeria* selective agar (traditional or chromogenic agars) and require confirmation of isolates as *L. monocytogenes* by biochemical or molecular tests.

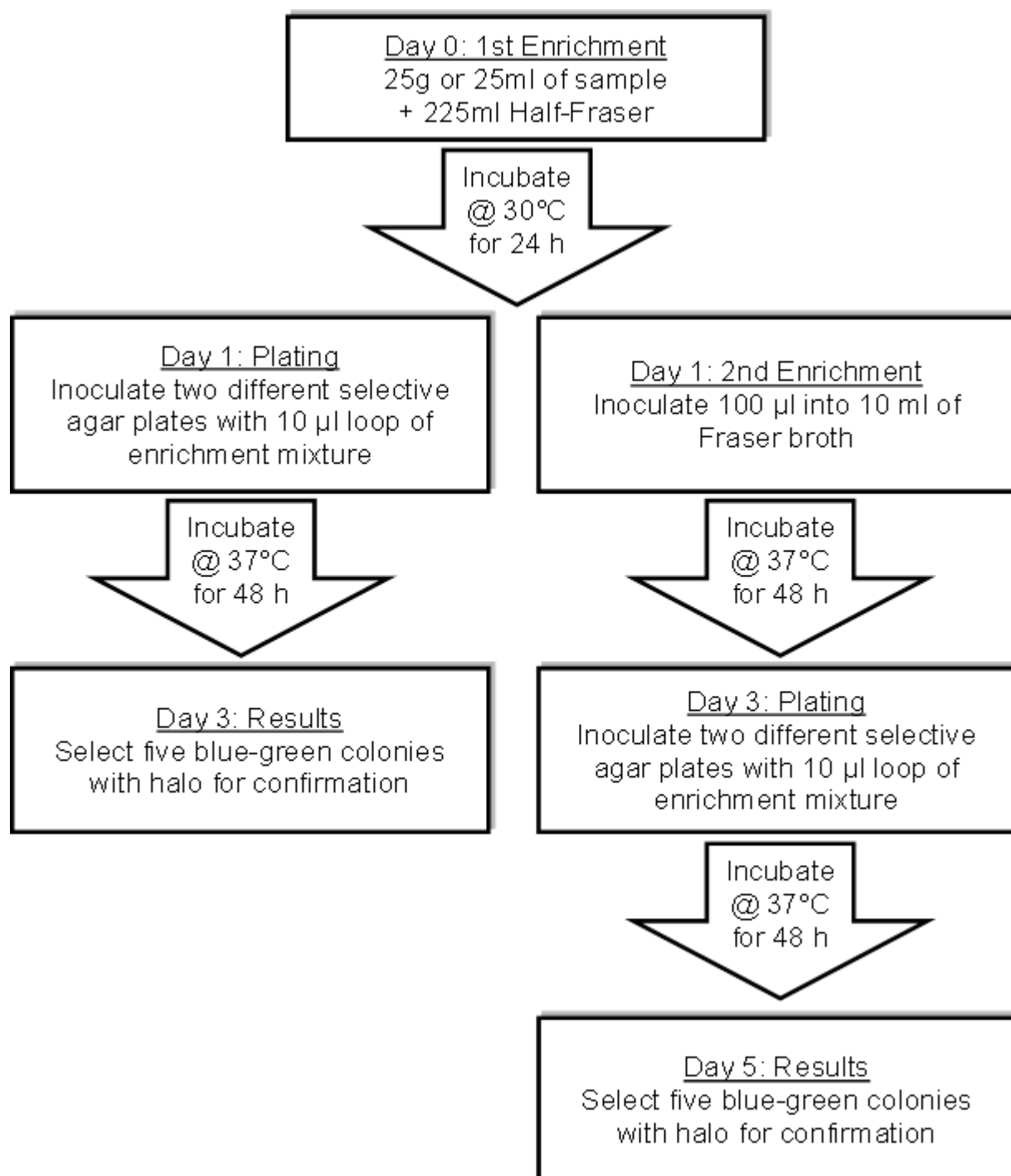


Figure 1.2: Flowchart for the ISO analysis method.

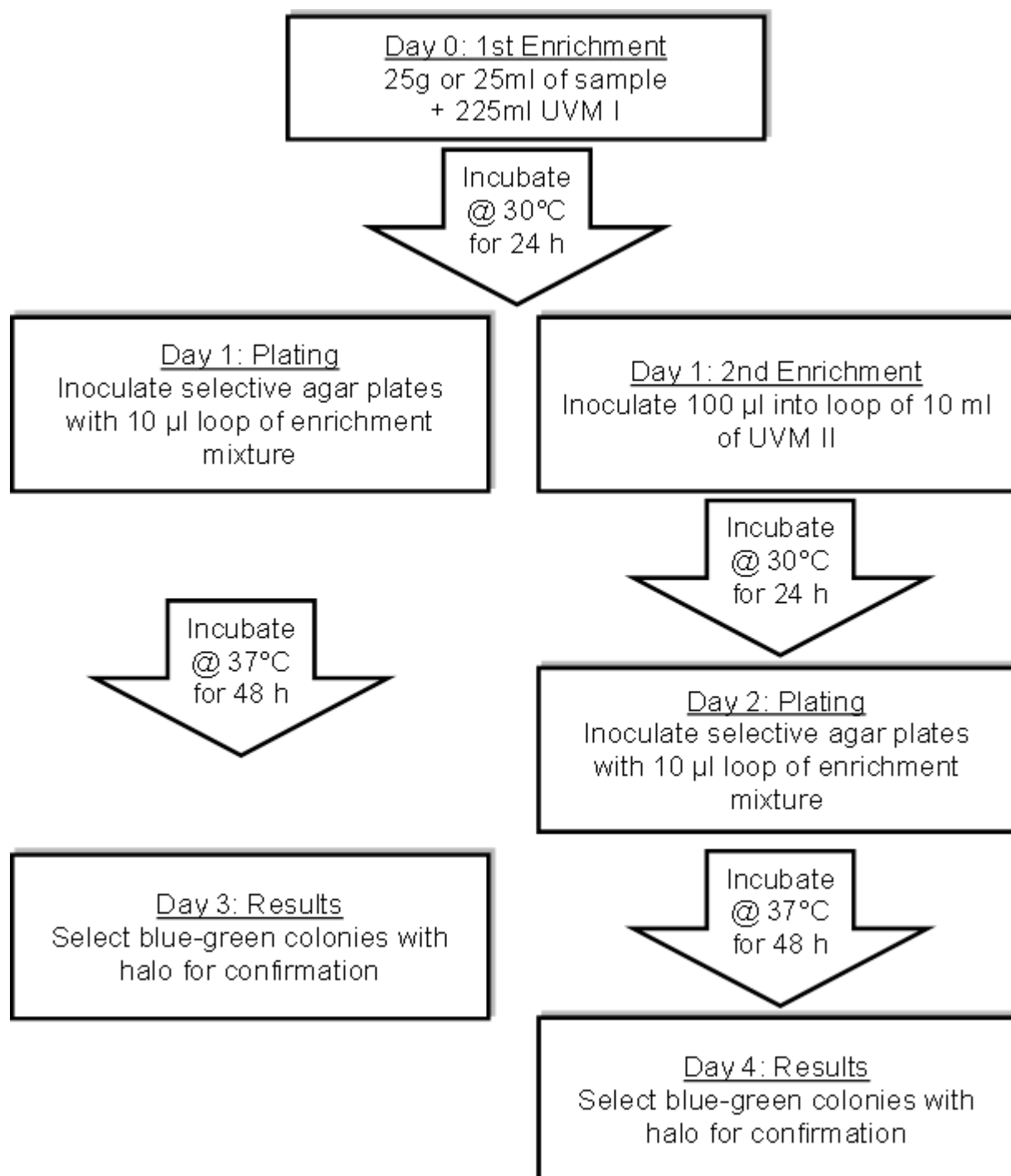


Figure 1.3: Flowchart for the USDA analysis method.

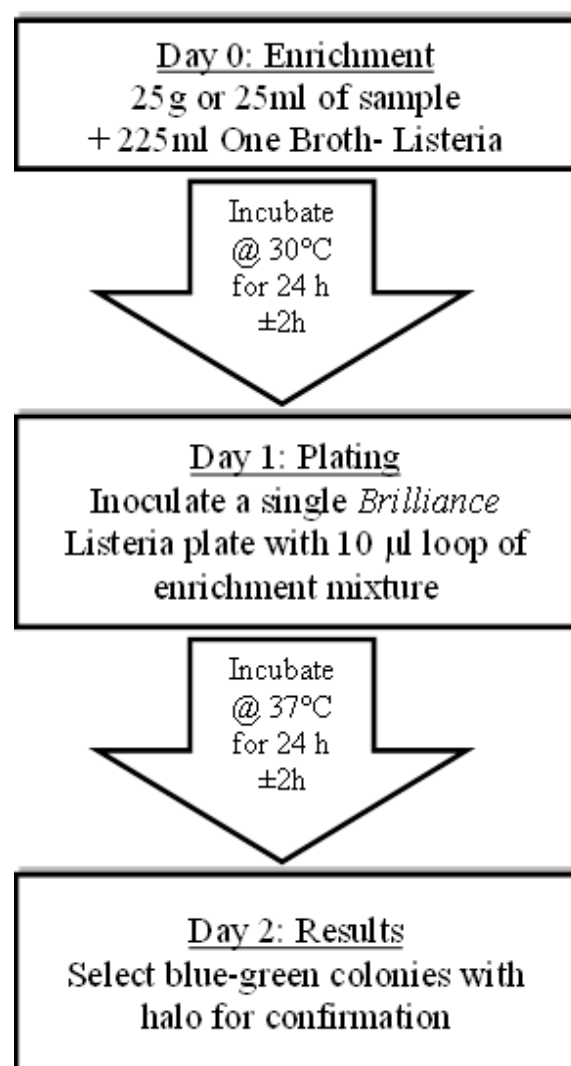


Figure 1.4: Flowchart for the ONE-Broth analysis method.

The use of selective agar in *L. monocytogenes* isolation gives an initial result that is presumptive positive, but confirmation of the *L. monocytogenes* isolates is needed as false positives can and do occur. Polymixin Acriflavine Lithium Chloride Ceftazidime Aesculin Mannitol (PALCAM) and *Listeria* selective agar (Oxford formulation) are both recommended for use in the ISO method although other *L. monocytogenes* selective agars give similar and sometimes even better results. *Listeria* selective agar (Oxford formulation) utilises several inhibitory components as well as the hydrolyzation of aesculin and ferrous iron to differentiate *L. monocytogenes*. However, some strains of *Enterococcus* can also grow on this medium and may exhibit a weak aesculin reaction. PALCAM agar often gives many false negatives as *L. innocua*, which can have higher growth rates than *L. monocytogenes* during enrichment, appears similar to *L. monocytogenes* on this agar. Other *L. monocytogenes* selective agars, including the chromogenic agars *Listeria* Ottavani & Agosti (ALOA) and Brilliance *Listeria* Agar (BLA) are based on the phospholipase C activity and β -glucosidase activity of *L. monocytogenes*. Some *L. ivanovii* strains can also display similar activity and appear analogous to *L. monocytogenes* (Becker et al., 2006). Other *Listeria* species can display similar growth to *L. monocytogenes* on ALOA and BLA, e.g. round, smooth blue/green colonies (Figure 1.5). Often, the only visual difference between *L. monocytogenes* and other *Listeria* species on ALOA or BLA is whether or not a halo is produced. This can be misinterpreted if a nearby *L. monocytogenes* colony produces a halo close to another non-halo producing *Listeria* species on the plate. Rapid' *L. mono* uses the phospholipase activity combined with the fermentation of xylose to differentiate *L. monocytogenes* from other *Listeria* species and results can be obtained for *L. monocytogenes* within 24 h as opposed to BLA and ALOA where 48 h of incubation is usually needed. Overall, although the use of selective agar to identify *L. monocytogenes* is generally

quite accurate, confirmation by other means should always be performed before any conclusions are drawn. Common methods of confirmation of *L. monocytogenes* include confirmation by PCR, API kits and sequencing, which is becoming increasingly popular as costs of sequencing are reduced.

PCR is a relatively simple assay which involves the amplification of a DNA fragment. PCR methods for *L. monocytogenes* confirmation generally focus on virulence genes of *L. monocytogenes* as *L. innocua* and *L. monocytogenes* share a similar genome with the exception of certain key, virulence clusters. With conventional PCR, the DNA fragments need to be amplified, dyes added and the fragments are then run at the end-point on an agarose gel and separated by gel electrophoresis. From start to finish, conventional PCR may take several hours.

Alternatively, real-time PCR adds a fluorescent probe to the DNA fragments during replication which allows the results to be viewed during the amplification of the DNA fragments which reduces considerably the time taken to view the results. The *hly* gene is commonly used in both conventional (Gawade et al., 2010) and real-time PCR (Rodriguez-Lazaro et al., 2004). Although real-time PCR is more expensive and requires more expertise than conventional PCR, it offers a distinct advantage as conventional PCR can only give a positive/negative result whereas with real-time PCR, *L. monocytogenes* can be measured quantitatively and not just qualitatively. The use of real-time PCR (RTi-PCR), in combination with traditional culture, to detect the presence or absence of *Listeria* has also been explored in recent years (Dalmasso et al., 2014; Rossmanith et al., 2010). By amplifying *Listeria*-specific genes through PCR and quantifying them by the detection of a fluorescent probe attached to the DNA fragments, even low numbers of the bacteria can be detected within a few hours (after enrichment) as opposed to the several days it takes to complete traditional

plating techniques. For best use, RTi-PCR should be combined with the traditional methods so that isolates can be obtained from the traditional method for strain typing. PCR is not suitable for direct detection of *L. monocytogenes* in food as it lacks the required sensitivity, may be subject to inhibition by food ingredients and can detect the presence of DNA from live as well as dead cells.

There is a wide range of different test methods for *Listeria* spp. and *L. monocytogenes* that have been reviewed previously (Välimaa et al., 2015). These include antibody-based tests, enzyme linked immunosorbent assay (ELISA), immune-capture methods, molecular methods targeting different genes and biosensor methods. Commercial kits are available for many of these methods, but it is not within the scope of this review to detail all of these methods.

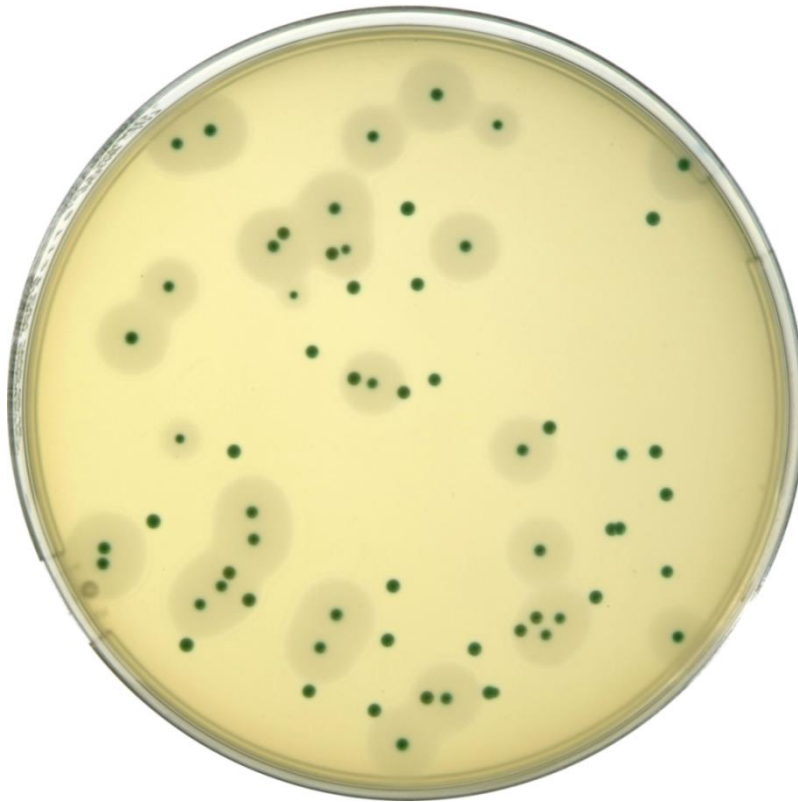
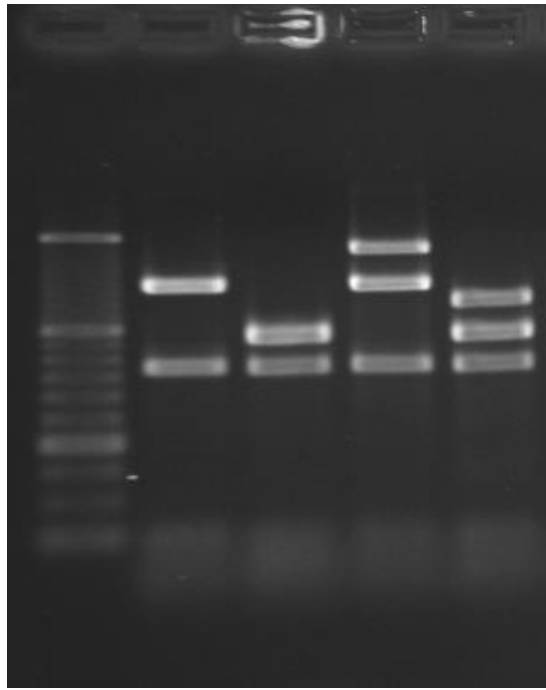


Figure 1.5: ALOA agar plate showing blue-green colonies of *L. monocytogenes* with a halo and other *Listeria* species appear as blue/green colonies with no halo.

1.5.2 Characterisation of Isolates

In order to identify the source or route of contamination, it is necessary to identify the strain type of *L. monocytogenes* contaminating the food or the processing environment rather than just give a positive/negative result. Differentiation of *L. monocytogenes* strains by serotyping is one of the oldest methods of typing and is based on the somatic (O) and flagellar (H) antigen differences between strains. As more exacting typing techniques have since been developed, serotyping of strains now offers little in terms of strain identification but can be helpful in the characterisation of strains (Morobe et al., 2012). Thirteen serotypes are currently recognised which can be broadly split in 4 different serogroups. Doumith et al. (2004) have developed a widely used multiplex PCR which can be used to divide *L. monocytogenes* strains into their serogroup (Figure 1.6) (Doumith et al., 2004). However, to further differentiate strains into their serotype, testing with antisera needs to be performed, which can be prohibitively expensive. Some reactions in antisera testing can be variable, for instance, currently serotypes 4b and 4e cannot be separated by this method. The vast majority of listeriosis outbreaks, approximately 90%, are caused by 1/2b and 4b serotypes, both of which are commonly found in food and food processing facilities. In general, serotype 1/2a has been isolated most frequently from food and the food processing environment (Leong et al., 2014; Shen et al., 2013). Although it is thought that some serotypes may be generally more virulent than others, currently all *L. monocytogenes* strains must be treated as virulent. Therefore, the identification of certain serotypes in a food or a processing facility does not mean that they will or will not cause disease.

Based on sequencing, *L. monocytogenes* is sub-divided into 4 evolutionary lineages (I, II, III, and IV) which have different but overlapping sources of origin, for review see (Orsi et al., 2011). Most *L. monocytogenes* isolates belong to lineages I and II, which generally harbour the serotypes more commonly associated with human clinical cases. Lineage II isolates (which includes most serotype 1/2a strains) are common in foods, widespread in natural and farm environments, and are commonly isolated from animal listeriosis cases and sporadic human clinical cases. Lineage I isolates (which includes most serotype 1/2b and 4b strains) are associated with the majority of human listeriosis outbreaks. Lineage III and IV strains are generally rare, although some serotype 4b strains can be from lineage IV, and are predominantly isolated from animal sources. Attempts to identify phenotypic traits specific to lineages have been for the most part unsuccessful. However, some generalisations on phenotypic traits of lineages can be made. Lineage II isolates generally have more plasmids and seem to be more resistant to bacteriocins than lineage I isolates. They also frequently have a premature stop codon in *InlA* leading to a truncated protein (Chen et al. 2011), and mutations in *prfA*. Lineage I isolates, on the other hand, can carry listeriolysin S (Cotter et al. 2008).



(A)

Serovar	O-antigens	H-antigens
1/2a	I, II	A, B
1/2b	I,II	A, B, C
1/2c	I, II	B, D
3a	II, IV	A, B
3b	II, IV, (XII), (XIII)	A, B, C
3c	II, IV, (XII), (XIII)	B, D
4a	(V), VII, IX	A, B, C
4ab	V, VI, VII, IX, X	A, B, C
4b	V, VI	A, B, C
4c	V, VII	A, B, C
4d	(V), VI, VIII	A, B, C
4e	V, VI, (VIII), (IX)	A, B, C
7	XII, XIII	A, B, C

(B)

Figure 1.6: (A) Agarose gel electrophoresis of DNA fragments generated by multiplex PCR to determine *L. monocytogenes* serogroup as in Doumith et al. (2004). Lane 1: O' RangeRuler 50bp (Biolabs, England); Lane 2: 1/2a, 3a serogroup; Lane 3: 1/2b, 3b, 7 serogroup; Lane 4: 1/2c, 3c serogroup; Lane 5: 4b, 4d, 4e serogroup.

(B) 13 Serovars of *L. monocytogenes* and their antigen reactions. Antigens in parentheses result in variable reactions. O-antigen III has been omitted from this table as the reaction to O-antigen III is variable for every serovar.

The gold standard for *L. monocytogenes* sub-typing remains pulsed field gel electrophoresis (PFGE), although other methods do offer advantages. PFGE is quite expensive, takes several days and requires trained staff to perform. However, it offers better discriminatory power than most other methods and can be compared between labs if performed according to international standard practices (PulseNetUSA, 2009). On the other hand, sequencing techniques have much better inter-laboratory comparison as they are not subject to interpretation as PFGE profiles can be.

Briefly, PFGE involves the lysis of cells to release the genomic DNA, the immobilisation of the DNA by trapping it in an agarose plug, the restriction digest of the DNA by specific enzymes and the migration of the DNA by gel electrophoresis over a long period of time, generally 21 hours. The restriction by a specific restriction digest enzyme gives a distinct pattern of bands, a PFGE pulsotype, which can be used to identify a strain. Generally, two separate restriction digests are performed in two separate PFGE runs which gives a much better differentiation than the use of a single enzyme (Borucki et al., 2004). The resulting PFGE pulsotypes can then be analysed by specialised software in order to accurately compare PFGE pulsotypes and the percentage similarity between strain patterns observed can be calculated (Figure 1.7). In this way, the same strain found in more than one area of a processing facility or over a period of time can be identified and the likely route/source of contamination may be identified (Strydom et al., 2013).

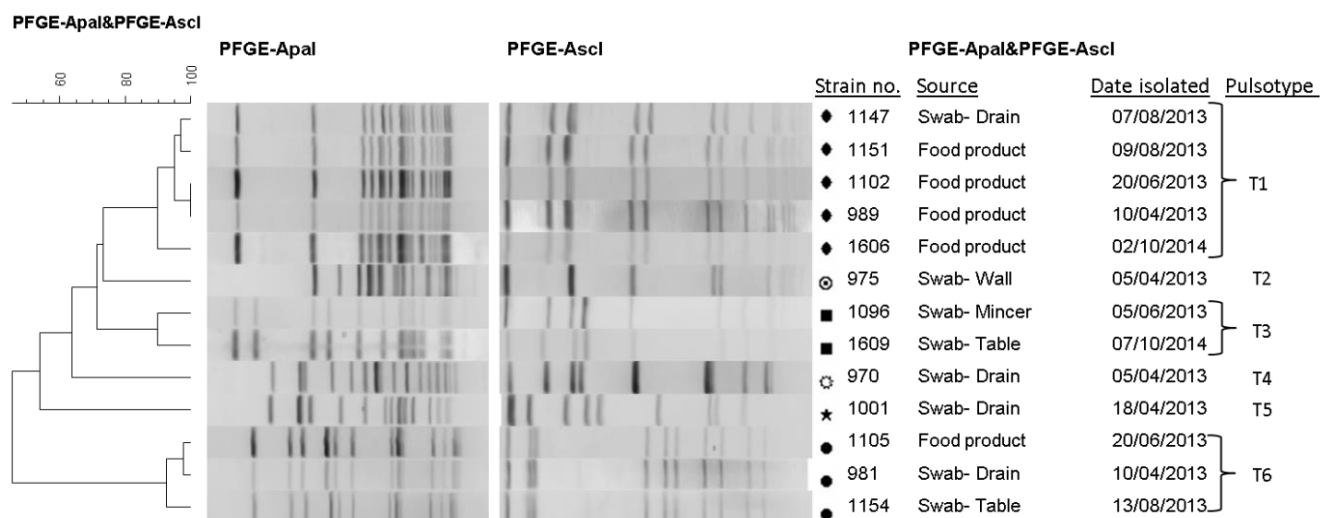


Figure 1.7: PFGE profiles (digested with two restriction enzymes: *Apal* and *Ascl*) of *L. monocytogenes* isolated from a single food business facility. Isolate similarity dendrogram was generated using BioNumerics version 5.10 software (Applied Maths), using the unweighted pair group method with arithmetic mean (UPGMA) and the Dice coefficient with tolerance and optimization settings of 1%. Pulsotype T1: Persistent pulsotype seen in both food and environmental swabs; Pulsotype T3: Persistent pulsotype isolated from environmental swabs 16 months apart; Pulsotype T6: Evidence of transfer between food and environment; Pulsotypes T2, T4 and T5: Sporadic contamination of the facility with various pulsotypes.

Sub-typing of isolates, using methods such as Pulsed Field Gel Electrophoresis (PFGE), allows analysis of the molecular diversity of *L. monocytogenes* strains present in processing facilities. Strains recurring in the processing environment over time (persistent strains) can be identified (Stessl et al., 2014). Persistent strains in the environment represent an increased risk of contamination of food products. Control of these persistent strains in particular, is an important part of a food processing facility food safety programme. After characterising the molecular diversity of isolates in the environment in question, putative routes of transmission and/or sources of entry into the environment can be identified. Muhterem-Uyar et al. (2015) identified three potential contamination scenarios that can increase the risk of food contamination: hot-spot contamination (where a specific area is contaminated), widespread contamination (where contamination is spread throughout the facility) and sporadic contamination (where non-persistent contamination occurs on an irregular basis). Visualisation of the contamination on a facility map can help identify the putative contamination routes (Dalmasso and Jordan, 2013). Thus, control strategies can be adjusted/targeted to remove the source of contamination and interrupt the route of transfer to the food. Analysis of such results can not only identify persistent strains, but can also identify an area which may be colonised by a particular strain, leading to possible recontamination events. It can also be used to prevent the spread of strains throughout the facility.

Multilocus sequence typing (MLST) is also commonly used in strain typing, by sequencing a specific set of alleles of housekeeping genes and analysing the variations in the sequences, it allows identification of strain differences. Although less discriminatory than PFGE, the evolutionary distance between strains can be measured, by inspecting the

number of alterations in the sequences, which cannot be performed by PFGE (Haase et al., 2014).

PCR to detect different genes present in *L. monocytogenes* strains is also commonly used for strain characterisation. The presence/absence of different genes can be a good indication of whether or not a strain is virulent or whether it possesses genes which may help it to persist in a food processing facility. Several genes, such as the stress survival islet SSI-1 and the Tn6188 transposon, which confers resistance to certain quaternary ammonium compounds, have been identified which appear to confer advantages to strains which may help them to survive in the seemingly inhospitable environment of a processing facility (Müller et al., 2013b; Ryan et al., 2010). Similarly several genes which contribute to virulence have been identified, for example listeriolysin S (LLS) and *actA*, and the use of PCR to detect these genes can help to evaluate a strains ability to cause disease (Cotter et al., 2008b; Jacquet et al., 2002).

Other options for characterisation of *L. monocytogenes* isolates include Multiple-Locus Variable Tandem Repeat Analysis (MLVA), ribotyping, phenotypic or biochemical arrays and Fourier Transform infrared spectroscopy (Stessl et al., 2014).

In recent years, the price of whole genome sequencing (WGS) has lowered significantly, allowing the use of WGS in more routine applications. As opposed to PFGE or MLST, WGS examines the entire sequence of a genome, rather than just parts of it, and so gives a much higher strain differentiation (Gilmour et al., 2010). Individual genes can also be examined through the use of WGS. For example, in the Quargel cheese outbreak in Austria in 2009/2010, WGS was used to identify 2 distinct 1/2a *L. monocytogenes* strains (QOC1 and QOC2) which overlapped to form the outbreak (Rychli et al., 2014). Through WGS, specific genes which contribute to invasion and survival were also identified including the presence

of a *vip* homologue in QOC2 which encodes a surface protein, likely responsible for the higher invasion efficiency of QOC2 in comparison to QOC1. Another advantage of whole genome sequencing is that, as the entire genome sequence is obtained, previous MLST and MLVA data can be compared to sequences obtained through whole genome sequencing by which the relatedness of the strains can be analysed.

There are still many problems associated with the use of WGS including the challenges involved in storing the large amounts of data generated and a lack of sufficient internet connection/speed which may particularly be a problem in developing countries and so may restrict the use of WGS as a tool to examine global prevalence and transfer of strains. Similarly, basic epidemiology, surveillance and food monitoring infrastructure is needed in some developing countries before strain examinations can move towards genome sequencing. Currently, there is no generally agreed upon pipeline for data analysis and well-trained bioinformaticians are required for data handling and interpretation, particularly due to the rapid development of new software and programmes in recent years (FAO, 2016). Despite these problems, as costs continue to fall, WGS is increasingly being used in outbreak investigations as it offers a much more comprehensive overview of a strain and gives a significantly higher confidence in strain identification.

1.6 Challenge Studies to Determine the Ability of Food to Support Growth of *L.*

monocytogenes

Certain foods are categorized in a higher risk category for contamination with *L. monocytogenes*. These are ready-to-eat (RTE) foods (including soft cheese, RTE meats and smoked fish), since the heat step of cooking, which would kill any *L. monocytogenes* present, is missing in these foods. Thus, if the food product is able to support the growth of *L. monocytogenes*, bacterial numbers can reach high levels, even at refrigeration temperatures, posing a health risk for consumers.

Determining the ability of RTE foods to support the growth of *L. monocytogenes* is important, especially in those jurisdictions where there is not a “zero tolerance” policy for *L. monocytogenes* (e.g. Europe, Canada, Australia). The ability of *L. monocytogenes* to grow in food products may be estimated based on specifications of the physico-chemical characteristics of the product, consultation of the available scientific literature, or predictive mathematical modelling. There are many tools that support predictive modelling of *L. monocytogenes* in food. These include for example, general pathogen models such as Combase (www.combase.eu) and Pathogen Modelling Programme (PMP; <http://pmp.errc.ars.usda.gov/PMPOnline.aspx>), and more specific *L. monocytogenes* models such as those at <http://safesmokedfish.food.gov.uk/>, or <http://fssp.food.dtu.dk/>. Such predictive models are useful, but for many reasons, including the possibility of overestimation/underestimation of growth in food products, in most cases growth assessment will involve laboratory-based studies, so-called challenge tests. From a public health perspective, overestimation of growth is a ‘fail-safe’ scenario, although such

overestimation can be inaccurate from a food producer's perspective. For example, in 40% of cases, Combase predicted growth in cheese when no growth was seen in growth experiments (Schvartzman et al., 2011). It was further shown that the growth characteristics of *L. monocytogenes* were different in liquid and solid matrices (Schvartzman et al., 2010).

A challenge test can be defined as a laboratory-based study that measures the growth of *L. monocytogenes* in artificially contaminated food stored under foreseeable abuse conditions of transportation, storage at retail and at consumer level. Performing challenge tests to assess growth of *L. monocytogenes* on foods is not simple, since different RTE foods may require different laboratory approaches. However, in order to harmonise the laboratory methodology, some agencies have published guidelines in the last decade for the execution of challenge tests. The Food Standards Agency of New Zealand has recently published guidelines for undertaking challenge studies (FSANZ, 2014), although this document is not specifically related to *L. monocytogenes*. On the other hand, Canada also has guidelines which specifically relate to *L. monocytogenes* (Health-Canada, 2012). In Europe, in order to facilitate the task of performing challenge studies, the European Union Community Reference Laboratory for *L. monocytogenes* (EURL Lm) prepared a Technical Guidance document in 2008 (EC, 2008). This guidance document, which was aimed at describing the microbiological procedures for determining growth of *L. monocytogenes* using challenge tests in the frame of the application of Regulation (EC) No. 2073/2005, has been recently updated (EC, 2014). The European Guidance document of 2014, recently reviewed by Alvarez-Ordóñez et al. (2015), helps the Food Business Operator to decide whether a challenge test would be required for their food product, and describes the laboratory methodology that must be followed when carrying out a challenge test. This guidance document differentiates two types of challenge tests: the ones that determine growth

potential of an inoculated strain or strains and those that calculate the growth rate of the strain(s). Growth potential is defined as the difference between the \log_{10} CFU/g at the end of the shelf-life and the \log_{10} CFU/g at the beginning of the test. When this difference is greater than 0.5 \log_{10} CFU/g the food is classified into RTE foods able to support the growth of *L. monocytogenes*. Alternatively, when the difference is less than 0.5 \log_{10} CFU/g, the food is classified into RTE foods unable to support the growth of *L. monocytogenes*. The growth rate is on the other hand calculated from the growth curve as the slope of the straight line resulting from plotting the \log_{10} of cell numbers against time in the exponential phase of growth. The growth rate is an important parameter of the growth curve which depends on the inoculated strain(s), the intrinsic properties of the food (e.g. pH, NaCl content, a_w , associated microflora, antimicrobial constituents), and extrinsic properties (e.g. temperature, gas atmosphere, moisture). Once the growth rate is known for a given food at a given temperature it is possible to estimate the concentration of *L. monocytogenes* at a given day of the shelf-life if the initial concentration is known. It is also possible to extrapolate the growth rate at a given temperature to predict growth rates at other temperatures in the same food.

1.7 Control of *L. monocytogenes*

Control is a more practical approach. Such control can be achieved by attention to detail in hygiene strategies, monitoring occurrence of the organism or using novel control methods such as bacteriocins and bacteriophage.

1.7.1 Novel Methods of Control

In recent years, in addition to novel technologies such as high pressure processing and pulsed electric field, novel methods for control of pathogens (and spoilage organisms) has focused on the use of natural anti-microbial agents such as bacteriocins and bacteriophage.

Bacteriocins: Bacteriocins are ribosomally-synthesised peptides that are pore-forming agents, which act by disrupting the integrity of the target cell membrane. They have the potential to inhibit other bacteria, including pathogens, in many cases resulting in cell death. Therefore, they have potential as a mechanism to control *L. monocytogenes*. The spectrum of activity can be broad, where a wide variety of unrelated species are inactivated, or narrow, where only closely related species are inactivated. To date, insufficient data has been generated to obtain a complete picture of the potential use for many bacteriocins. The current regulatory situation dictates against the use of bacteriocins as biocontrol agents as in many cases there is currently insufficient supporting data to assure the regulatory authorities of their efficacy and safety, for more information, see review (Cotter et al., 2013).

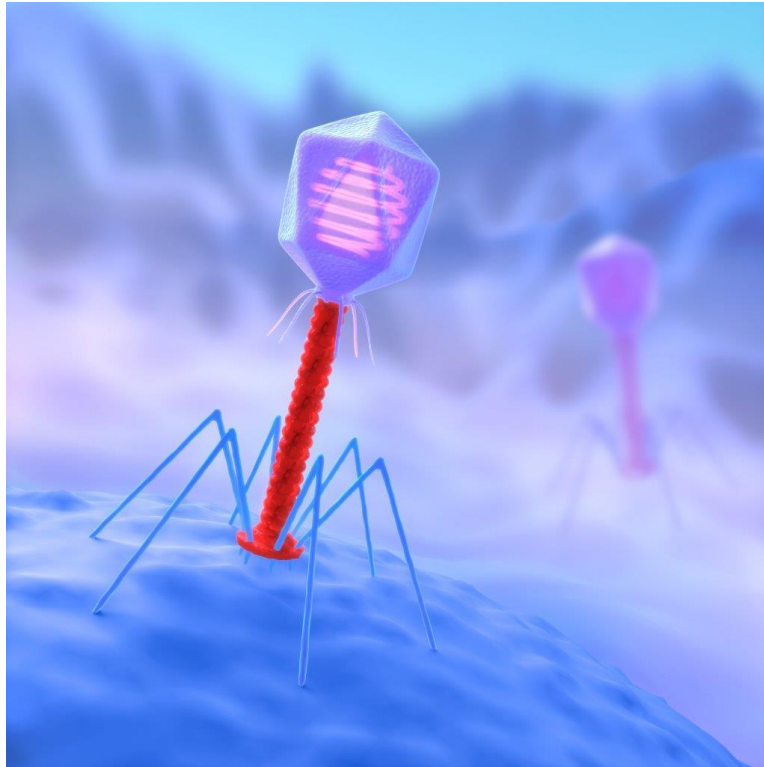
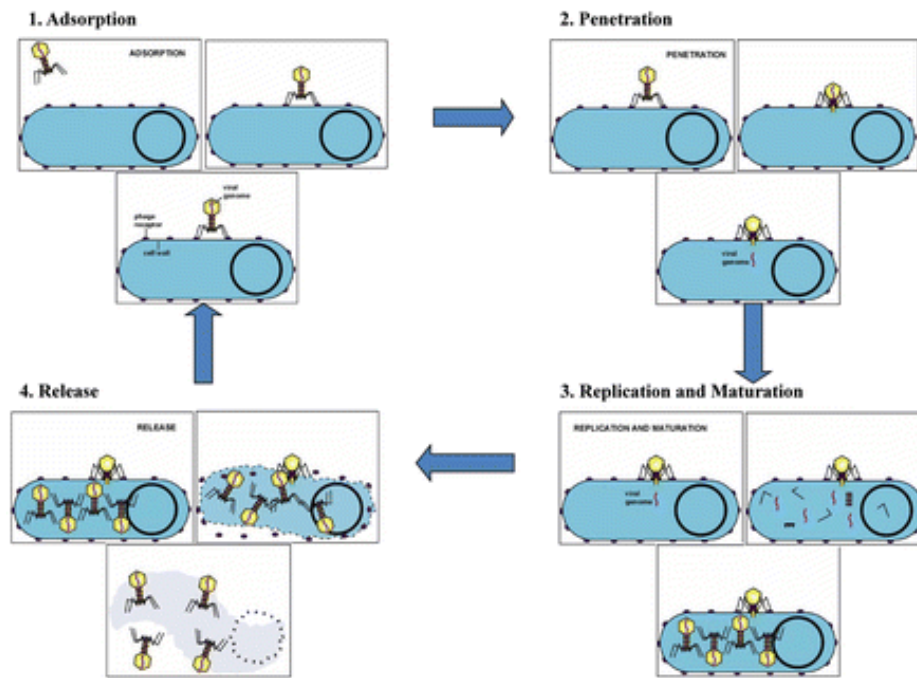


Figure 1.8: Image of a bacteriophage attacking a bacterium.



Step 1: Bacteriophage initially adheres to the surface of the bacterial cell through attachment sites on the phage, adsorbing to receptor sites on the host bacterium

Step 2: Phage injects its genome into the bacterial cytoplasm

Step 3: Phage then replicates its genome and uses the bacterium's metabolic machinery to synthesize phage enzymes and phage structural components.

Step 4: The phage particles are then constructed and undergo maturation and release of the intact bacteriophages

Figure 1.9: Life cycle of a lytic bacteriophage.

Bacteriophages are viruses that infect and can kill bacteria and are logical candidates for biocontrol of *L. monocytogenes* in food (Figure 1.8). They exhibit a high degree of specificity towards their target host bacterium, and as a result, are safe for use in food processing, considering they will have no detrimental effect on the microflora of the eventual consumer, nor will they have an effect on any other desired bacteria in the food. They also have other desirable attributes, including a relative stability during storage, and the ability to self-perpetuate. Of particular importance in terms of suitability for biocontrol of *L. monocytogenes* is finding a virulent bacteriophage phage that is strictly lytic, rather than a lysogenic phage which can be genetically unstable. Lytic phages are genetically stable, will always kill infected cells, and cannot therefore integrate its genome into that of the bacterial chromosome (Figure 1.9). It is also of critical importance that the full genome sequence of such phage is known, and that any phage applied to food does not encode any virulence factors or toxins which may be harmful (Hagens and Loessner, 2010).

The consensus among microbiologists is that bacteriophages do not have any known adverse effects on humans, animals, or the environment. For this reason, many scientists and food safety experts predict that bacteriophages could become a useful tool in the reduction of pathogens in the food chain. However, there are concerns that limited safety data testing has been undertaken, although bacteriophages have been widely used for treatment of human diseases in the former Soviet Union (Chanishvili, 2012).

The renewed interest into use of bacteriophage as biocontrol agents has resulted in the development of several commercial products designed for this purpose, such as LMP-102 phage preparation (now more commonly known as ListShieldTM) and ListexTM. Although products have been approved for use in some countries, their use is not permitted in others.

For a review on biocontrol for the control of *L. monocytogenes* with bacteriophage, see (Strydom and Witthuhn, 2015).

1.7.2 National Monitoring Programmes

Monitoring the food processing environment for the presence of *L. monocytogenes* can be an effective mechanism in its control (Dalmaso and Jordan 2013). Indeed, EU regulations require that food processing environments are sampled, although they don't state the number of samples to be taken, or the frequency of sampling (EC, 2005a).

In an attempt to control *L. monocytogenes*, the Austrian cheese industry has instigated a voluntary sampling programme aimed at early detection of *L. monocytogenes* followed by targeted intervention strategies. The Austrian *Listeria* monitoring programme comprises four levels of investigation; Level 1 deals with the routine monitoring of samples, Level 2 is an intervention phase if positive results are detected, Level 3 is an intensive sanitation phase and requires confirmation of successful control.

Level 1: Routine monitoring. Samples associated with cheese processing (such as smear, brine or wash water) are analysed at least every month. Smear liquid can be used to spread on the surface of cheese and is a good matrix to monitor cross-contamination. Where smear is not used, brine or wash water (used to clean trolleys or trays) can be used. Alternatively, drain water can be a good sample matrix for detection of processing facility contamination. Negative results are certified and used by the company management to document the status of safety. If *L. innocua* or other non-pathogenic *Listeria* are detected, an inappropriate status of hygiene is recorded as it is possible that pathogenic *L.*

monocytogenes are introduced by the same route as the non-pathogenic species. Reconsideration of hygiene measures are recommended to the company management.

Level 2: Intervention. If *L. monocytogenes* is detected, an intervention phase is initiated. An increased number of samples are collected by the factory personnel from sources which have shown contamination and from additional sources (tanks, racks, conveyor belts, etc.). The intervention examination is intended to clarify the extent of the contamination scenario. It should also help the manufacturer to decide whether a risk for cross-contamination to processed food has arisen. Isolates from food contact materials are treated as if those would have been isolated from the food commodity itself. In parallel, investigations of cheese samples according to the legal requirements determine whether a FPE contamination has already reached the food batch. If yes, and a test indicates that a food batch does not comply with the legal requirements then the batch should not be delivered or should be recalled from the market (internal recall).

Level 3: If the intervention examination confirms the monitoring result, a scrupulous sanitation of the FPE in addition to routine procedures is strongly recommended (Level 3). The sanitation usually cannot be performed without advice from external experts. The sanitation should be systematic, include a crucial survey of all factors that might drive the contamination scenario. This in particular includes a critical review of hygiene barriers, internal traffic management, the maintenance of buildings and rooms, and the cleaning and disinfection procedures applied. Typing of in-house strains supports the sanitation specialist to trace the contamination to hotspots from where *L. monocytogenes* might re-contaminate. A heavily contaminated FPE is difficult to sanitise. In most cases the goal is to control the contamination to spots from where a food batch contamination can be

excluded. This status of a co-existence of FPE contamination with pending food processing is a fragile reality in many food processing enterprises and should be monitored carefully.

1.7.3 Control of *L. monocytogenes* in the Processing Environment

It is relatively difficult to maintain a completely *L. monocytogenes*-free processing environment as many varying factors can have an effect on the occurrence of *L. monocytogenes* in the processing facility. These can include for example, contaminated incoming raw materials, staff members acting as *L. monocytogenes* carriers, insufficient cleaning strategies and sampling programmes in place, the facility design to prevent contamination, the location of the facility near a farm etc. Another major factor in the occurrence of *L. monocytogenes* is the awareness of the processing facility management and staff. The operation of a processing facility requires constant vigilance against bacterial contamination through various methods, and lack of awareness in this area can lead to more significant problems in end products which can result in product recalls, damage to company reputation, lawsuits, illnesses or even death. Thus, sampling and analysis are key factors in successful control. If occurrence is detected it can be eliminated through targeted intervention measures that help to prevent product contamination.

A major factor in keeping a facility free of *L. monocytogenes* is the design of the facility itself. Non-purpose designed facilities are common especially in industries such as farmhouse cheese making where converted farm buildings may house the food processing facility. These facilities may not be correctly designed or equipped to prevent contamination and redesign of the building itself is often necessary in these cases. The separation of raw

materials and finished products as well as the presence of boot wash areas, hand washing areas etc. are vital in preventing the spread of *L. monocytogenes* in a facility. The design of a facility should also allow clean-in-place (CIP), where all areas of the facility and all equipment therein can be completely cleaned without having to remove/dismantle equipment and with little or no manual input from the operator (Bremer et al., 2006).

The existence of harbourage sites, areas where disinfectants/sanitiser cannot properly reach, are a frequent source of *L. monocytogenes* contamination. Harbourage sites may be due to ill design, unsuitable materials/equipment or even to damaged materials. Due to the inaccessible nature of these sites, disinfectants/sanitiser may not be able to reach properly or may only reach in lower concentrations than would be needed to inhibit the bacteria therein. One theory suggests that constant low level of disinfectant in harbourage sites such as this may allow bacterial strains to evolve tolerances against certain chemicals being used. If bacteria then proliferate out from this site, the strain may have increased tolerance against the chemical even used in its intended concentration (Lundén et al., 2003). However, this theory is not strongly supported; general correlations between persistent strains and sanitiser resistant strains are not often seen (Heir et al., 2004). Any facility designed specifically for food processing should attempt to be free of harbourage sites and food processors should make every effort to remove any harbourage sites which may exist in a non-purpose designed facility. Unfortunately this can be extremely difficult and generally, harbourage sites remain an inherent danger in terms of contamination in food processing facilities (Carpentier and Cerf, 2011b).

Although final product testing is important in *L. monocytogenes* control programmes, it does not give information on the source and routes of product contamination. On the other hand, environmental testing is a more effective way to monitor

hygiene and prevent contamination events (Tompkin, 2002). Tracing the source of *L. monocytogenes* is critical in the control of the organism in a localised environment, although *L. monocytogenes*' ubiquitous nature makes it difficult to positively identify the source of contamination in some occasions. The potentially long incubation time for *L. monocytogenes* to cause disease can also make it difficult to trace the disease to a specific food and source of contamination (Goulet et al., 2013). It is therefore important to remove as many sources of contamination as possible from the food processing environment to reduce the possibility of food contamination.

Of utmost importance when sampling a processing environment for *L. monocytogenes* is actively looking for it, as opposed to selecting for negative results in order to adhere to regulations. Sampling directly after disinfection or cleaning or sanitation, for example, should be discouraged, unless the sampling is being used to evaluate the efficacy of the cleaning procedures. Proper sampling of a processing environment should include several areas in which contamination is most likely to occur, including both food contact and non-food contact surfaces. One of the most common areas to be contaminated are floor drains as any contamination throughout the facility is likely to be washed through the drain where *L. monocytogenes* can persist in a harbourage site (Carpentier and Cerf, 2011b). Sampling should be done with a sponge-type swab, allowing sufficient surface area to be sampled. Adequate sampling will allow problems of contamination to be pre-empted and addressed in a timely manner. *L. monocytogenes* contamination of food products is a much more serious problem which requires significantly more intervention than contamination at the processing stage.

The following guidelines may help in tackling problems with *L. monocytogenes*.

- Understand the nature of *L. monocytogenes* contamination and take it seriously.

Most food processing environments are contaminated to some extent. Adequate sampling for *L. monocytogenes* will help identify an issue, which should be addressed immediately. Regulations should be taken seriously and a food processing environment monitoring plan developed as a core activity of Good Hygiene Practices (GHP).

- Choose the right sampling sites and methodology. Sample the processing environment with a view of finding the organism. The most informative sampling sites can vary depending on the food commodity produced. Consider the difference in information that will be achieved from sampling of food contact materials *versus* non-food contact materials. Sampling is the most critical procedural step and, if done inappropriately, is of little benefit. Use swabs that have enough contact surface to sample the 900 cm² mentioned in many guidelines (Figure 1.10). Choose sampling sites from manufacturing or handling steps that are applied on most of the products produced (e.g. conveyor belts before packaging, slicer blades).



Figure 1.10: The type of swab that can be used for sampling for *L. monocytogenes* – a good surface area can be sampled.

- Choose the right sampling frequency. Recommendations on sampling frequency can only be expressed in general terms. If a food processing environment (FPE) is being sampled for the first time, use a broad sampling approach. If the contamination status is already known, test a restricted number of sampling sites frequently rather than a lot of sampling sites only once. Sampling frequency can be reduced if negative results are shown, but should be increased again if positive results are detected or if there are changes to the processing environment or manufacturing process. Sampling frequency should be dynamic.
- Establish critical control areas. To facilitate prioritisation of counter-measures, clearly define critical control areas (CCA) where FPE contamination is not acceptable under any circumstances. It makes a difference whether a *L. monocytogenes*-positive drain is located in a general processing area or if it is located where food is handled prior to packing. Critical control areas should be clearly marked (e.g. by marks on floors, in construction maps) and hygiene barriers should prevent CCAs from being visited or trespassed by unqualified personnel. Hygiene barriers, such as footbaths and change of personal protective clothing should reduce the risk of cross-contamination with *L. monocytogenes*. The high hygiene standard that should exist in CCAs can only be monitored by taking an appropriate number of FPE samples.
- Trace the route of transmission of isolates most importantly in CCAs. To combat contamination it is vital to keep all isolates at a safe and appropriate place (e.g. a contract laboratory). Use molecular typing to identify the putative routes of transmission of a pathogen in the facility, if possible (Figure 1.11). To reduce the costs, start with combating contamination in a CCA where the risk for contamination of the food commodity is the highest.

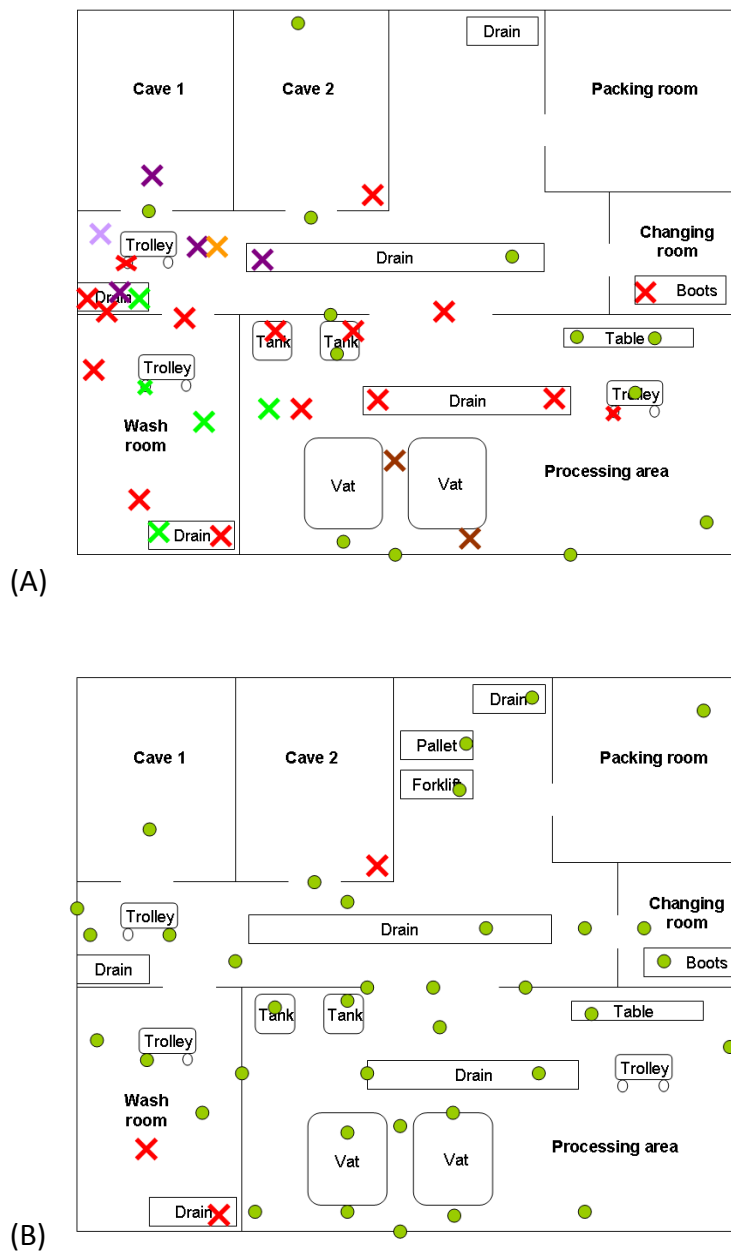


Figure 1.11: Sampling plan results mapped for a food processing facility before (A) and after (B) corrective action was implemented to control *L. monocytogenes* (Dalmasso and Jordan 2014). Green spot – *L. monocytogenes* negative sample; coloured X –different colours indicate strains with a different Pulsed Field Gel Electrophoresis profile. The sampling times were approximately one month apart.

- Be particularly aware at times of construction. During building work, hygiene measures are usually difficult to maintain at a food processing facility. On the one hand, craftsmen of various occupations with no training in hygiene need to have access to the FPE. Recommending the use of hygiene protection (overshoes, overcoats) to craftsmen is frequently in vain because it limits their maneuverability. Building material, often stored outdoors before use, needs to be carried around. Insects and rodents can get access to the FPE. On the other hand, the FBO frequently needs to produce food in processing rooms adjacent to the reconstruction area. Be aware of increased risk of cross-contamination during such construction periods, and construct physical barriers between food production and construction. Try to prevent access of craftsmen to production areas as much as possible. Observe careful and intensified sanitation programmes in the processing areas during the construction phase, and sanitise the entire FPE after completion of the construction phase. Verify the success of this process by subsequent sampling of the FPE.
- In cases of widespread contamination, critically review the floor sanitation procedures applied. If FPE monitoring demonstrates a widespread contamination of a genetically indistinguishable *L. monocytogenes* strains, re-consider your sanitation procedure (what sanitiser is used? Is it used appropriately? Are all areas covered? Are all the surfaces allowed to dry off before food production begins again?), and the workflow system. Use drain water sampling to control the efficiency of sanitation.
- Structure your data and use a processing facility map (roughly drawn) to document your progress and efforts. Safe food production is possible even if there is contamination of a FPE. However, the following criteria must be met:
 - The extent of contamination must be known (implies intensified sampling)

- Contamination must be never detected in the food commodity produced
- FPE contamination must be infrequent (reported only irregularly)
- Contamination must be detectable only in compartments where the risk for cross-contamination is low
- The food produced must not support growth of *L. monocytogenes* on its surface

Documentation is critical in any FBO communication process, either within an operation or with regulators or specialists from the outside. Documentation of ingredients and raw materials used as well as any contamination patterns is essential. A map of the facility (roughly drawn) can help with this.

To demonstrate that the FBO has met these requirements, is necessary to organise the data into a structured decision making process. Seek the advice of experts that help to facilitate the decision making process.

1.8 Conclusions

Despite extensive research, outbreaks related to *L. monocytogenes* continue and issues like host factors effecting pathogenicity and virulence factors are not fully resolved. As *L. monocytogenes* is ubiquitous in the general environment, elimination of the organism is an unreasonable objective. Therefore, control of *L. monocytogenes* is vital in addressing prevention of listeriosis. Awareness of the prevalence of *L. monocytogenes* in food processing facilities and use of appropriate control measures are important tools in the efforts for such control. Process control sampling and analysis are an important aspect of control measures. Reducing occurrence in the food processing environment reduces the risk of cross-contamination to food, and therefore has an impact on public health.

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Chapter 2

A 3-Year Multi-Food Study of the Presence and Persistence of *Listeria monocytogenes* in 54 Small Food Businesses in Ireland.

Dara Leong Chapter Contributions:

Experimental:

- Performed sample analysis on samples from 34 food businesses
- Performed PFGE analysis on isolates from 42 food businesses

Results Interpretation:

- Analysed all data and performed general comparative analysis on all isolates

Manuscript Preparation:

- Major contributor to the preparation of the manuscripts

Monitoring occurrence and persistence of *Listeria monocytogenes* in foods and food processing environments in the Republic of Ireland

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A 3-year multi-food study of the presence and persistence of *Listeria monocytogenes* in 54 small food businesses in Ireland

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2.1 Abstract

The problem of assessing the occurrence of the food-borne pathogen *Listeria monocytogenes* in the food chain, and therefore the risk of exposure of the human population, is often challenging because of the limited scope of some studies. In this study the occurrence of *L. monocytogenes* in food from four major food groups, dairy products, meats, seafood and vegetables, and associated food processing environments in Ireland was studied over a three-year period. Fifty-four small food businesses participated in the study and sent both food and environmental samples every 2 months between 2013 and 2015. *L. monocytogenes* was isolated using the ISO11290 standard method. Confirmation of *L. monocytogenes* and identification of serogroups were achieved using a multiplex PCR assay, and for some isolates serotype was determined using commercial antisera. Pulsed field gel electrophoresis (PFGE) analysis was performed on all isolates allowing the relatedness of isolates from different food businesses to be compared nationwide. In total, 86 distinct pulsotypes were identified. The overall occurrence of *L. monocytogenes* in food samples was 4.2%, while in environmental samples it was 3.8%. In general, the occurrence of *L. monocytogenes* in food businesses decreased over the course of the study, presumably reflecting increased awareness and vigilance. The majority of the pulsotypes detected were unique to a particular food group (63/86), while only three pulsotypes were found in all four food groups investigated. The highest occurrence in food was found in the meat category (7.5%) while seafood had the lowest rate of occurrence (1.8%). Seventeen of the pulsotypes detected in the study were persistent, where persistence was defined as repeated isolation from a single facility with a minimum time interval of 6 months. Using PFGE, 11 of the pulsotypes identified in this study were indistinguishable from those of 11 clinical isolates

obtained from patients in Ireland over the last 4 years, highlighting the fact that these pulsotypes are capable of causing disease. Overall, the study shows the diversity of *L. monocytogenes* strains in the Irish food chain and highlights the ability of many of these strains to persist in food processing environments. The finding that a significant proportion of these pulsotypes are also found in clinical settings highlights the need for continued vigilance by food producers, including frequent sampling and typing of isolates detected.

2.2 Introduction

Listeria monocytogenes is an opportunistic pathogen and it is the aetiological agent responsible for listeriosis cases in humans and a variety of animals. Human listeriosis is linked to the consumption of contaminated food and generally affects pregnant women and immunocompromised individuals, including new-borns and elderly people (Scallan et al., 2011). Listeriosis in adults is often manifested as a mild gastroenteritis and in some cases it can lead to more severe symptoms, which can lead to life-threatening illnesses, including endocarditis, encephalitis or meningitis, and severe sepsis (Roberts and Wiedmann, 2003; Vazquez-Boland et al., 2001). The incidence of human listeriosis is relatively low, however over the last few years (2008–2014) the number of recorded cases in Europe has increased significantly (EFSA, 2015). Furthermore, those infected by *L. monocytogenes* can suffer from a mortality rate of 20-30% (Silk et al., 2012), the third highest among all foodborne pathogens (Goulet et al., 2013).

As a foodborne pathogenic bacterium, in addition to being a public health problem, *L. monocytogenes* is of greatest concern to the ready-to-eat (RTE) food industry as there is no cooking or other microbial inactivation step between production and consumption. As *L. monocytogenes* is a psychotrophic facultative anaerobe, its occurrence in RTE refrigerated foods is of particular importance, particularly in the elderly population where a three-fold increase in listeriosis has been reported in the UK since the 1990s (Gillespie et al., 2006). It is ubiquitously found in a variety of environments, such as soil, water, animals and humans, and is therefore very difficult to eliminate from the food processing environment. Thus, preventing cross-contamination from the processing environment to food is essential in RTE

processing facilities. Regulatory compliance for the RTE food industry is challenging. Analysis for *L. monocytogenes* is expensive and results can cause product recalls and withdrawals (Gandhi and Chikindas, 2007), which are necessary from a public health perspective.

L. monocytogenes can be found in raw products and RTE foods, such as delicatessen meats, soft cheeses or smoked fish (Jensen, Björkman, et al., 2016). Due to its psychotrophic nature, RTE foods stored at low temperatures are particularly vulnerable to the possibility of growth, and its ability to survive and grow in the presence of many food preservation systems, such as low pH and high salt concentrations (Ryan et al., 2008) increase the risks. Any level of contamination could cause a problem if *L. monocytogenes* is able to survive and grow and therefore strict microbiological criteria are applied. In the European Union (EU), in food products intended for infants and for special medical purposes, the absence of *L. monocytogenes* in 10 x 25 g of product is required. For foods capable of supporting growth of *L. monocytogenes*, the food business operator (FBO) must demonstrate (by a challenge study) that the numbers will not exceed 100 CFU/g during the shelf-life of the food. If such data is not available, absence in 5 x 25 g is required. For RTE products not capable of supporting growth of *L. monocytogenes*, the numbers must not exceed 100 CFU/g during shelf-life (EU, 2005). In the United States of America, absence of *L. monocytogenes* is required in all cases, even in food processing environments. In Canada, Australia and New Zealand the regulations are similar to those in the EU (FSANZ, 2014; HealthCanada, 2011).

Under current regulations, samples positive for *L. monocytogenes* have been reported at retail in fish products, soft, semi-soft and hard cheeses, and RTE meat and fresh cut vegetable products (EFSA, 2015; Lubert et al., 2011). Indeed, in 2014 the European Food Safety Authority reported the non-compliance of RTE foods at processing and retail, and the

proportion of non-compliant units at processing level was considerably higher than at retail (EFSA, 2015).

RTE food processing environments are recognised as an important source of *L. monocytogenes* contamination (Tompkin, 2002a). Therefore, it is important for food businesses to have an *in situ* surveillance programme to monitor and control routes of contamination and cross-contamination in order to limit the risk of *L. monocytogenes* in the final product. Such environmental monitoring programmes are mandatory in the USA (USFDA, 2003) and recommended in the EU (EU, 2005). These approaches play a crucial role in monitoring, facilitating the identification and tracking of *L. monocytogenes* along the food chain and within food processing facilities, and can have an impact on avoiding cross-contamination to food (Lappi et al., 2004). Whole genome sequencing (WGS) of isolates from such monitoring programmes may facilitate studies on isolate characterisation (Stasiewicz et al., 2015).

Persistence of *L. monocytogenes* in food processing facilities, generally regarded as the repeated isolation of strains with indistinguishable PFGE profiles at intervals of 6 or more months apart (Leong et al., 2014), is of particular relevance. Pathogen monitoring programmes using molecular sub-typing techniques (e.g. PFGE or WGS) may be helpful in identifying persistent isolates within food processing facilities (Fox et al., 2015).

The aim of this study was to assess the occurrence and persistence of *L. monocytogenes* in 54 Irish food processing facilities over a three-year period (March 2013 to December 2015), through regular monitoring of ready-to-eat foods and processing environments, followed by the molecular characterization of the *L. monocytogenes* strains isolated, while making FBOs more aware of the issues relating to the organism. The isolates obtained were compared to other food processing and clinical isolates. The application of this approach is

discussed as a means of improving food safety in the processing environment and protecting public health.

2.3 Materials and Methods

2.3.1 *L. monocytogenes* Sampling Programme

Over three years, from 2013 to 2015, 54 food processing facilities submitted samples for detection of *L. monocytogenes*. These included 16 dairy, 18 meat, 15 seafood and five vegetable producers. The majority of these food processors (51) produce ready-to-eat foods. Every six months, sample kits were sent to the food producers; each consisting of a polystyrene box (DS Smith, UK), six pre-moistened 3M swabs (Technopath, Ireland), a sterile liquid container (VWR, Ireland), two sterile bags (VWR, Ireland), two cable ties and two ice packs.

Each food producer submitted samples every two months generally consisting of a sample set of six environmental swabs and two food samples. Food processors were given detailed instructions on how to sample and were requested to swab from a drain, a shelf and the floor (an area of approximately 1 m²). Processors were free to choose the location of the remaining swabs, depending on the layout and design of the particular facility. Food samples were requested to be at the point of leaving the facility. Liquid samples could also be sent if the producer wished to test brine, water, milk etc. Following sampling, the sample kit was sent by overnight courier to the appropriate laboratory for testing; Teagasc Food Research Centre Moorepark (TFRCM), National University of Ireland, Galway (NUIG), University College Dublin (UCD) or University of Limerick (UL). Several food processors missed one or more sampling points. However, all submitted sample sets in all three years of the programme.

2.3.2 Analysis of Samples

At all four sites (TFRCM, NUIG, UCD and UL), analysis of samples for the presence of *L. monocytogenes* was performed according to the ISO 11290-1 method, except that only one chromogenic agar was used for the initial isolation (Leong et al., 2014). Initial plating was performed on either Agar *Listeria* acc. to Ottavani & Agosti (ALOA) or Brilliance *Listeria* Agar (BLA) plates which were incubated for 48 h at 37 °C and then examined for typical *L. monocytogenes* colonies (blue/green colonies with a halo). From each positive plate, two presumptive positive colonies were restreaked to a second chromogenic agar plate (ALOA, BLA, or Oxford *Listeria* selective agar) and incubated for 48 h at 37 °C. Colonies which retained typical *L. monocytogenes* appearance were restreaked to a general agar; Brain Heart Infusion (BHI) or Tryptic Soy Agar (TSA) and incubated at 37 °C for 24 h. Bacterial mass from these plates was re-suspended in cyroinstant tubes and kept at -20 °C for bio-conservation and further analysis.

Up to four isolates were retained from each positive sample; two from each positive enrichment. Food samples were tested following their “best before date” to avoid causing recalls which would have prevented food processors from engaging with the project.

2.3.3 Isolate Confirmation

All isolates were confirmed as *L. monocytogenes* by multiplex PCR as described previously (Ryu et al., 2013). DNA used in multiple PCR analyses was extracted from presumptive *L. monocytogenes* isolates using the QIAgen Mini kit (Qiagen, Ireland).

2.3.4 Serogrouping and Serotyping

Serogrouping was performed by multiplex PCR as previously reported (Doumith et al., 2004). Serotyping was performed using antisera testing (Denka Seiken UK Ltd, Coventry, UK) as previously described (Fox et al., 2009).

2.3.5 PFGE

PFGE was performed according to the International Standard PulseNet protocol (PulseNetUSA, 2009) with the restriction enzymes *Sgs1* (formerly *Asc1*) and *Apa1*, in two separate experiments. Isolate similarity dendrograms were generated using Bionumerics version 7.5 software (Applied Maths, Belgium), by the unweighted pair group method with arithmetic mean (UPGMA) with tolerance and optimization settings of 1%. Comparisons with pulsotypes from other countries were made using BioNumerics 'bundles'.

2.3.6 Statistical Analysis

The Kruskal Wallis Test was used to analyse occurrence data between different food sectors.

2.4 Results

2.4.1 *L. monocytogenes* Occurrence

In total, 5869 samples from 54 food processing facilities were analysed for the presence of *L. monocytogenes* from 2013 to 2015. This included 4667 processing environment samples and 1202 food samples (Table 2.1). The average number of samples submitted by each food processing facility was 108.7 (Standard Deviation 29.6). Ten food processing facilities maintained a 0% *L. monocytogenes* prevalence over the three years of sampling; these included one dairy facility, three meat facilities and six seafood facilities. Thirty-two food processing facilities had an overall occurrence between 0-5%, seven between 5-10% and five between 10-20% (Table 2.2).

Figure 2.1 shows scatter plots of the annual occurrence of *L. monocytogenes* in processing facilities in the four different food sectors. In the dairy and meat sectors, the average value and the amount of variation decreased over time. In the seafood sector, the occurrence was relatively low and apart from one facility in 2015, there was little difference between 2013 and 2015, although there was a decrease in 2014. There was a low number of vegetable processing facilities involved, but the occurrence was relatively high. The number of facilities with zero occurrence increased over time. The mean general prevalence of *L. monocytogenes* decreased from 4.8% in 2013 to 3.7% in 2014 and 3.2% in 2015. The mean prevalence of *L. monocytogenes* in food samples decreased from 5.1% in 2013 to 4.7% in 2014 and 2.7% in 2015 (Table 2.1). Overall, 29 companies showed a decrease in occurrence between 2013 and 2015, 10 had 0% occurrence during all three sampling years and 13 showed an increase in occurrence from 2013 to 2015.

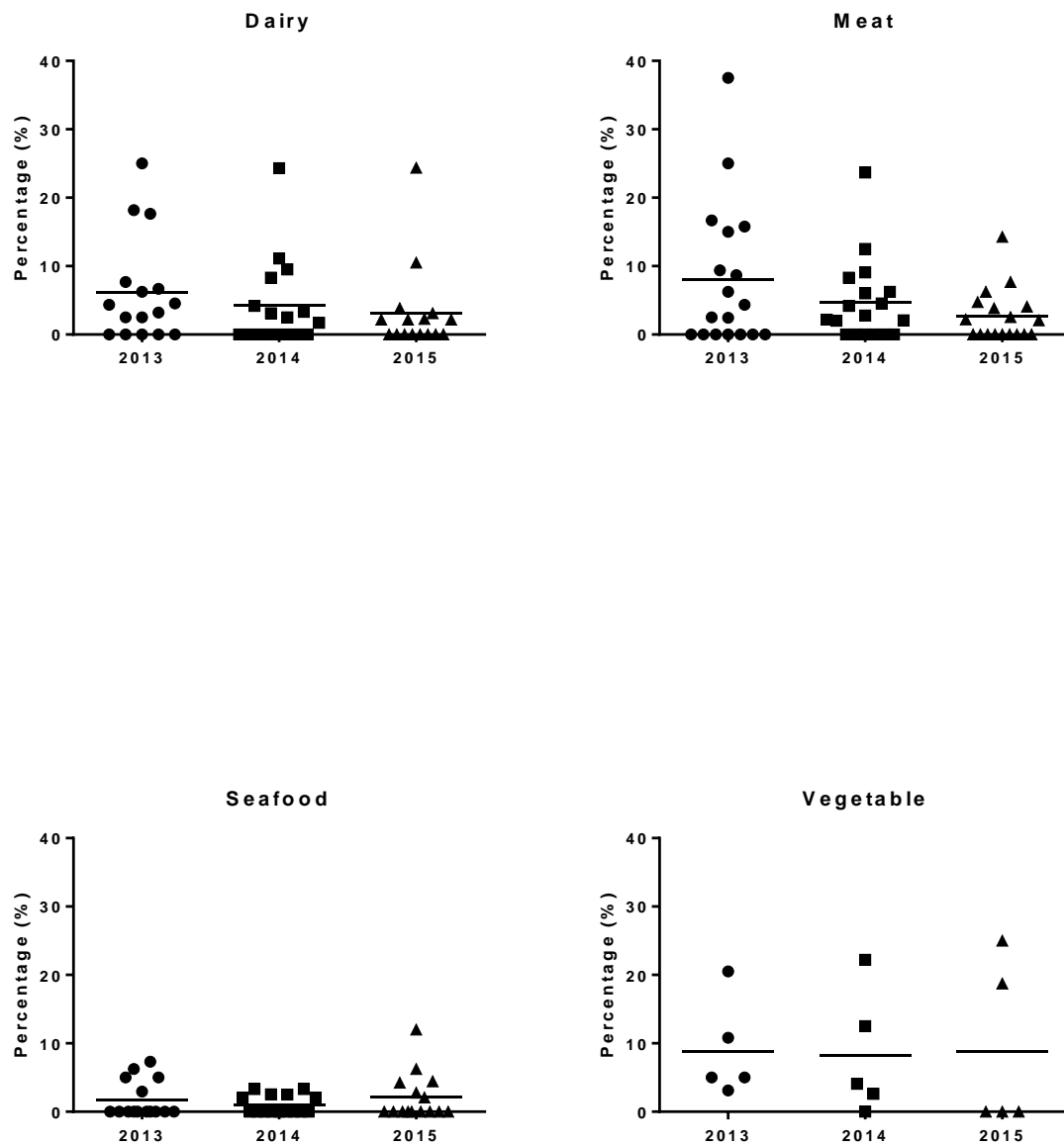


Figure 2.1: Scatter plots of the annual occurrence of *L. monocytogenes* in processing facilities in the four different food sectors. The line in each plot is the average. Each symbol represents a facility, with different symbols for each year.

	2013	2014	2015	Total
No. of samples taken	1696	2102	2071	5869
No. of positive samples	81	78	67	226
% of positive samples	4.78%	3.71%	3.24%	3.85%
No. of environmental samples taken	1345	1654	1668	4667
No. of positive environmental samples	63	57	56	176
% of positive environmental samples	4.68%	3.45%	3.36%	3.77%
No. of food samples taken	351	448	403	1202
No. of positive food samples	18	21	11	50
% of positive food samples	5.13%	4.73%	2.73%	4.17%

Table 2.1: Summary of results of the annual occurrence of *L. monocytogenes* in processing environments and food obtained from 54 food businesses in Ireland.

Overall		2013				2014				2015			
Facility no.	% positives	Environment	Number positives (%)	Food (%)	Environment (%)	Food (%)	Environment (%)	Food (%)	Environment (%)	Food (%)	Environment (%)	Food (%)	Number positives (%)
Dairy													
D1	0.74%	P46	1(3.33%)	0(0%)		0(0%)		0(0%)		0(0%)			0(0%)
D2	2.26%		0(0%)	P25	1(11.11%)	P31	2(5.56%)	0(0%)		0(0%)			0(0%)
D3	1.53%	P44	1(3.7%)	0(0%)		0(0%)		0(0%)	P24	1(2.78%)			0(0%)
D4	1.43%		0(0%)	0(0%)		0(0%)		0(0%)	P71	1(2.78%)	P82		1(6.25%)
D5	2.77%	P10	1(2.78%)	0(0%)	P8	1(4.16%)	0(0%)	0(0%)	P73	1(4.16%)			0(0%)
D6	0.00%		0(0%)	0(0%)		0(0%)		0(0%)		0(0%)			0(0%)
D7	0.61%		0(0%)	0(0%)	P51	1(2.27%)	0(0%)			0(0%)			0(0%)
D8	5.74%	P21	1(3.85%)	P21	1(10%)	P54, P81	4(13.33%)	0(0%)	P78	6(6.74%)	P6		1(3.03%)
D9	18.58%	P6, P18, P44	2(7.41%)	0(0%)	P46	9(36%)	0(0%)	0(0%)	P7, P10, P23, P28, P48, P70	21(24.14%)			0(0%)
D10	0.76%		0(0%)	0(0%)		0(0%)		0(0%)	P5, P31, P69	1(2.86%)			0(0%)
D11	6.70%	P9, P50	3(8.57%)	P20	1(20%)	P10, P44	3(7.1%)	P10	1(16.7%)	P47, P83	1(2.5%)		0(0%)
D12	4.31%		0(0%)	0(0%)		0(0%)	P46	1(10%)	P6, P85	3(3.33%)	P6		2(7.69%)
D13	1.54%	P42	1(8.33%)	0(0%)		0(0%)		0(0%)		0(0%)			0(0%)

D14	2.22%	P60	1(3.33%)	0(0%)	P59	1(3.33%)	0(0%)	0(0%)	0(0%)
D15	4.26%	P6, P44, P67	4(11.76%)	P2, P67	2(20%)	0(0%)	0(0%)	0(0%)	0(0%)
D16	7.29%	P32 , P41	3(9.38%)	0(0%)	P20, P32 , P37	4(13.33%)	0(0%)	0(0%)	0(0%)
Meat									
M1	2.99%	P16	1(4.17%)	0(0%)	P16 , P38	3(7.89%)	0(0%)	0(0%)	0(0%)
M2	2.75%		0(0%)	0(0%)		P13 , P32	2(20%)	0(0%)	P13 1(16.67%)
M3	2.68%	P44 , P54	3(12.5%)	0(0%)		0(0%)	0(0%)	0(0%)	0(0%)
M4	0%		0(0%)	0(0%)		0(0%)	0(0%)	0(0%)	0(0%)
M5	2.63%	P10, P12	2(9.52%)	0(0%)		0(0%)	0(0%)	0(0%)	0(0%)
M6	1.96%		0(0%)	0(0%)	P18	1(2.78%)	0(0%)	P79 1(8.33%)	0(0%)
M7	8.09%	P32 , T63	3(10%)	P32 , P37	3(30%)	P53	1(2.78%)	P32 , P58, P65	3(30%) 0(0%) P27, P77, P82 1(8.33%)
M8	10.81%	P33 , P66	3(50%)	0(0%)		0(0%)	P33	1(20%)	0(0%) 0(0%)
M9	2.19%		0(0%)	0(0%)	Untypeable	1(2.78%)	0(0%)	P26, P69	2(5.26%) 0(0%)
M10	2.94%	P56	1(3.33%)	0(0%)	P40, P44	1(2.78%)	P44	2(16.67%)	0(0%) 0(0%)
M11	9.68%	P32 , P37	3(15.79%)	0(0%)	P13, P46	2(9.09%)	0(0%)	0(0%)	P32 1(25%)
M12	5.48%	P66	1(5.88%)	0(0%)	P34	1(3.33%)	0(0%)	P35	2(16.66%) 0(0%)
M13	0%		0(0%)	0(0%)		0(0%)	0(0%)	0(0%)	0(0%)
M14	1.92%		0(0%)	0(0%)		0(0%)	P52	1(10%)	P14, P59 1(3.23%) 0(0%)

M15	7.50%	P46	1(20%)		0(0%)	P10	1(16.67%)		0(0%)	P10	1(4.17%)		0(0%)
		P2, P31 , P44 ,		P31 ,									
M16	20%	P59 , P61	4(15.38%)	P39	5(50%)	P44 , P59	4(14.29%)	P31	5(50%)	P59 , P75	2(9.52%)		0(0%)
M17	0%		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)
M18	1.38%		0(0%)	P36	1(25%)		0(0%)		0(0%)		0(0%)		0(0%)
Seafood													
S1	3.20%	P30	2(6.67%)		0(0%)		0(0%)		0(0%)	P59	1(2.78%)	P29	1(11.11%)
S2	0%		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)
S3	1.22%		0(0%)		0(0%)	P6	1(3.33%)		0(0%)		0(0%)		0(0%)
S4	2.42%	P45, P61	2(8.33%)		0(0%)		0(0%)	P61	1(8.33%)		0(0%)		0(0%)
S5	2.21%		0(0%)		0(0%)		0(0%)		0(0%)	P44, P46	3(7.14%)		0(0%)
S6	0.98%		0(0%)		0(0%)		0(0%)		0(0%)	P44	1(2.78%)		0(0%)
S7	0%		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)
S8	3.13%	P64	2(6.67%)		0(0%)	P64 , P74	1(3.33%)		0(0%)	P64	1(2.78%)		0(0%)
S9	0%		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)
S10	0%		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)
S11	3.75%		0(0%)		0(0%)		0(0%)		0(0%)	P17, P62	3(12%)		0(0%)
S12	0%		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)
S13	3.05%		0(0%)	P58	1(12.5%)	P45	1(2.63%)		0(0%)	P17	1(2.86%)	P45	1(8.33%)
S14	0%		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)
S15	3.17%	P45, P51, P80	3(8.57%)		0(0%)		0(0%)	P57	1(25%)		0(0%)		0(0%)

Vegetable													
V1	19.83%	P2, P18	4(13.33%)	0(0%)	P1, P2, P4, P15, P18, P20	8(25.81%)	0(0%)	P1, P2, P3, P10, P18, P31, P49	12(30%)	0(0%)			
V2	1.48%		0(0%)	P10, P15	2(20%)	0(0%)	0(0%)		0(0%)				0(0%)
V3	2.31%	P18, P46	1(3.85%)	0(0%)	P55	1(2.7%)	P68	1(8%)	0(0%)				0(0%)
V4	2.38%	P2, P6, P44	2(6.67%)	0(0%)	P31	1(3.57%)		0(0%)	0(0%)				0(0%)
V5	17.04%	P6, P17, P38	7(23.33%)	P17	1(11.11%)	P19, P20, P53, P63	4(11.11%)	P6, P59	2(16.67%)	P2, P20, P59, P72, P76	5(13.89%)	P48, P61, P84	4(33.33%)

Table 2.2: Complete results of the occurrence of *L. monocytogenes* in processing environments and food obtained from 54 food businesses in Ireland. Persistent pulsotypes are indicated in bold.

There was an uneven distribution of participants in the surveillance programme across the country (Figure 2.2). Considering this limitation, no geographical differences were observed in distribution of *L. monocytogenes*. Any differences observed could be due to the different number of samples received from the different locations. Additionally, no seasonal difference was observed in the occurrence of *L. monocytogenes* over the three years (data not shown).

Different industry sectors had differing rates of occurrence in samples ($p < 0.05$). Including food and processing environment samples, the industry with the lowest prevalence was the seafood industry, in which 1.7% of 1621 samples were positive for *L. monocytogenes*. The dairy industry had 3.7% *L. monocytogenes* positives from 1920 samples and the meat industry had 4.2% *L. monocytogenes* positives from 1681 samples. The highest processing environment prevalence occurred in the vegetable industry with 9.5% of 474 samples positive for *L. monocytogenes* as opposed to 4.1% in both the dairy and meat environmental samples and 1.6% in the seafood environmental samples. Positive food samples were obtained from all industry sectors (Table 2.2, Table 2.3).

Food Category	No. Process Environment Samples	% Positive	No. Food Samples	% Positive
Dairy	1512	4.2	408	2.2
Meat	1332	3.5	349	7.5
Seafood	1349	1.6	272	1.8
Vegetables	474	9.5	173	5.8
<i>TOTAL</i>	<i>4667</i>	<i>3.8</i>	<i>1202</i>	<i>4.2</i>

Table 2.3: Breakdown of the occurrence of *L. monocytogenes* in processing environments and food by food sector obtained from 54 food businesses in Ireland over three years.

2.4.2 Selection of Isolates for Characterisation

Ten percent of the positive samples yielded more than one PFGE pulsotype. When all four isolates from the same sample belonged to the same PFGE pulsotype, only one isolate was carried forward for further study. If differing pulsotypes were seen from the same positive sample, a representative isolate of each pulsotype was carried forward. Only isolates which were confirmed as *L. monocytogenes* by multiplex PCR were retained for further study. This approach yielded 255 isolates from 226 positive samples.

2.4.3 Serogrouping and Serotyping

Multiplex PCR was performed to serogroup all 255 isolates, resulting in 43.9% of isolates in the 1/2a-3a serogroup, 27.5% of the isolates in the 4b-4d-4e serogroup, 16.1% of the isolates in the 1/2b-3b-7 serogroup and 12.2% of the isolates in the 1/2c-3c serogroup (Table 2.4). Serotyping was also performed on 110 of these isolates; all isolates in each serogroup belonged to a single serotype (see Table 2.4). The serotypes 4b and 4e cannot currently be differentiated with the available antisera. All isolates, except one untypeable isolate, belonged to lineage I (111 isolates) or lineage II (143 isolates).

Serogroup testing ¹		Serotype testing ²	
Serogroup	No. of isolates	Serotype	No. of isolates
1/2a-3a	112	1/2a	50
1/2b-3b-7	41	1/2b	16
1/2c-3c	31	1/2c	16
4b-4d-4e	70	4b/4e	28
Untypeable	1		
Total number of 255 isolates tested		Total number of 110 isolates tested	

Table 2.4: Serogroup/serotype testing of the *L. monocytogenes* isolates obtained from a 3-year surveillance programme of processing environments and food obtained from 54 food businesses in Ireland.

¹ Serogroup testing by the method of Doumith et al., 2004

² Serotype testing using antisera from Denka Seiken UK Ltd, Coventry, UK

2.4.4 PFGE

Pulsotype numbers (P numbers) were assigned to PFGE pulsotypes based on their relatedness. The 255 isolates were assigned to eighty-six different pulsotypes. Several pulsotypes occurred in multiple industry sectors, but only three pulsotypes, P44, P46 and P59 occurred in all industry sectors (Figure 2.3).

Overall, there was great diversity in the isolates obtained, as seen in the minimum spanning tree (Figure 2.4). The majority of pulsotypes (69/86) were not seen to persist at a given facility and are likely to represent an incidence of sporadic contamination rather than persistent contamination. Except for 2 cases, all strains within a single pulsotype belonged to the same serogroup.

The distribution of the pulsotypes around the country can be seen in Figure 2.5. From these data it is clear that certain pulsotypes are prevalent on the island of Ireland and persistent isolates are found in each of the 4 food groups included in the study.

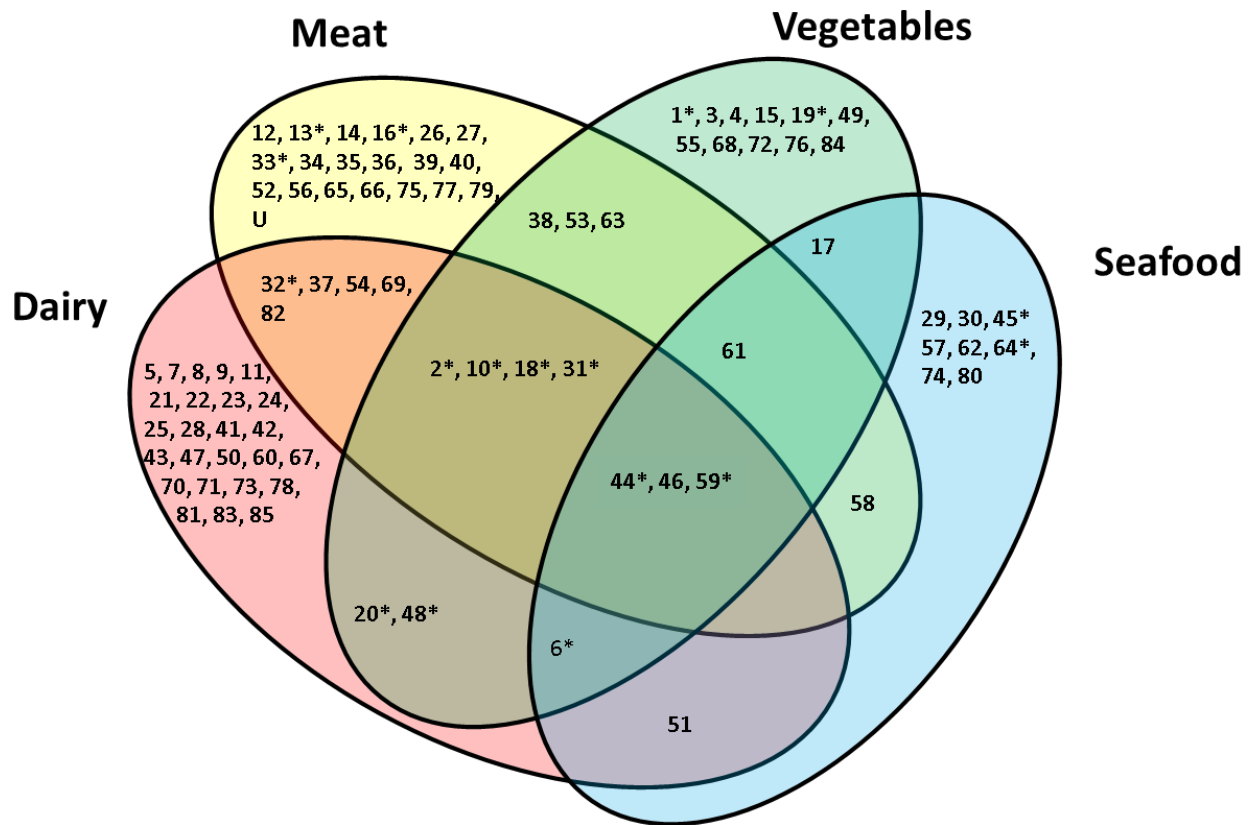


Figure 2.3: Distribution of the different *L. monocytogenes* pulsotypes obtained different food sectors in processing environments and food obtained from 54 food businesses in Ireland. * = These pulsotypes were persistent (isolated more than once at least 6 months apart in a single facility)

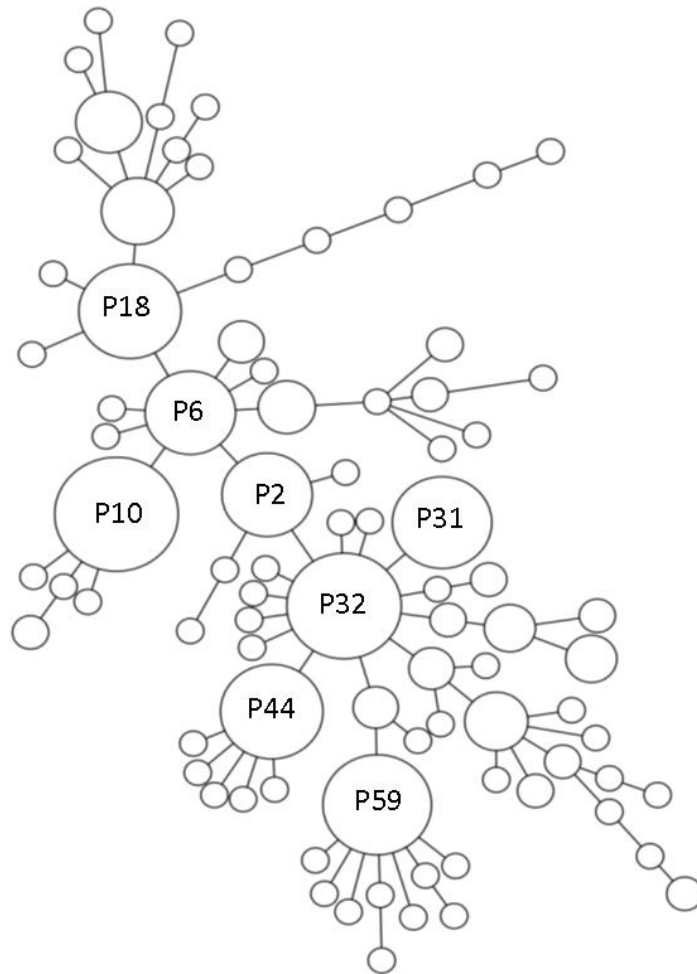


Figure 2.4: Minimum spanning tree of *L. monocytogenes* pulsotypes obtained from processing environments and food from multiple food sectors from 54 food businesses in Ireland. This was created in Bionumerics (version 7.5) using default settings except maximum distance between nodes in the same position of 12. Pulsotypes containing 10 or more strains are identified.

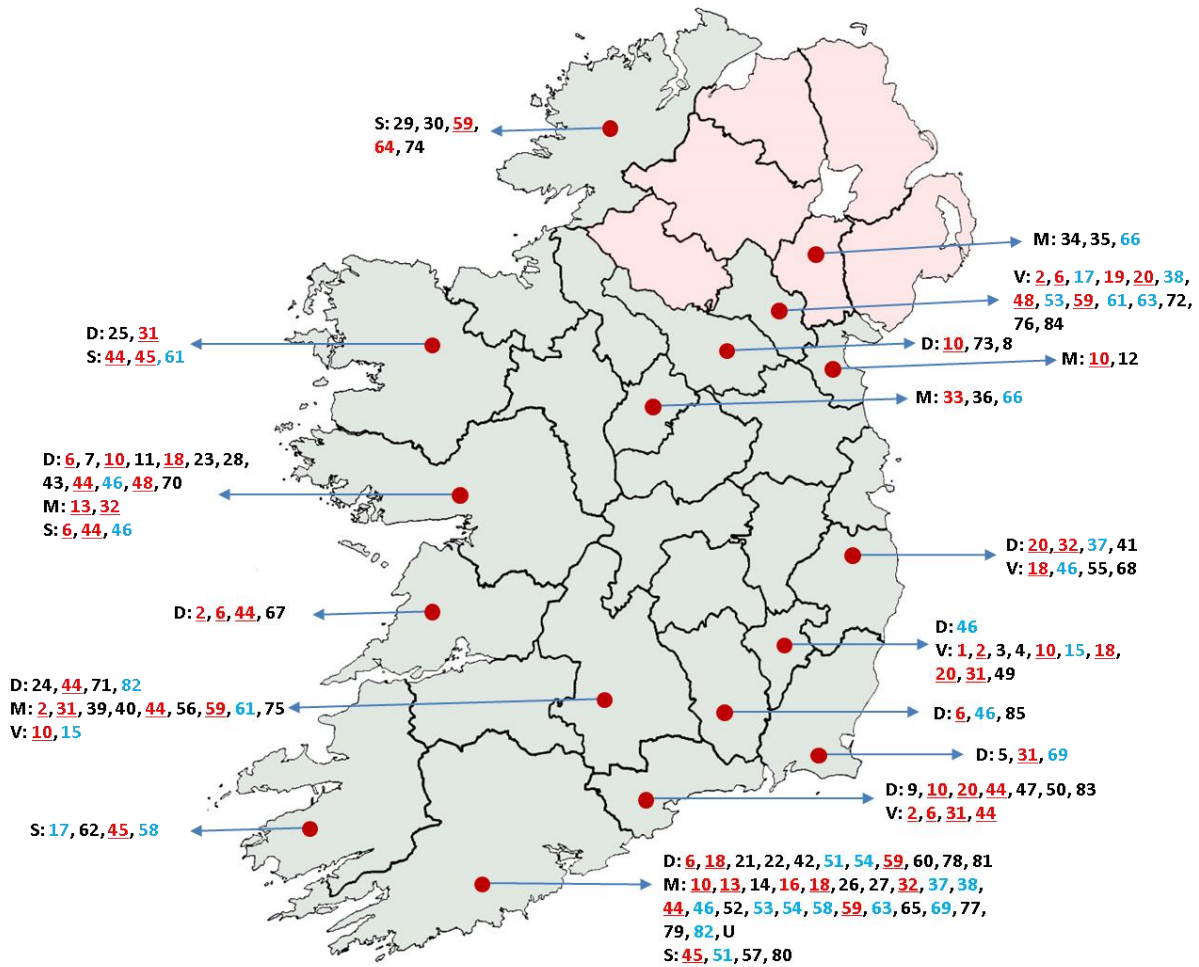


Figure 2.5: Summary of the *L. monocytogenes* pulsotypes detected in each county throughout Ireland. Unique pulsotypes are in black, persistent pulsotypes are in red, persistent pulsotypes found at multiple locations are in red and underlined, sporadic pulsotypes found in multiple sites are in blue and. D – dairy; M – meat; S – seafood; V – vegetable. U- untypeable strain.

2.4.5 Persistence

PFGE analysis also allowed for the identification of persistent strains, defined as indistinguishable strains (by PFGE analysis) isolated at least six months apart from the same processing facility (Figure 2.6). Sixteen processing facilities had at least one persistent *L. monocytogenes* strain over the three-year period. Seventeen different pulsotypes were observed as being persistent. Five pulsotypes were observed to persist in multiple facilities; P59 in two facilities, P6 in two facilities, P10 in three facilities, P32 in three facilities and P44 in three facilities. Cases of persistence decreased in several facilities over the three-year sampling period. In six facilities, (D16, M1, M3, M7, M8 and M10) persistence was observed in 2013 and/or 2014 but no persistence was observed in 2015 (Table 2.2).

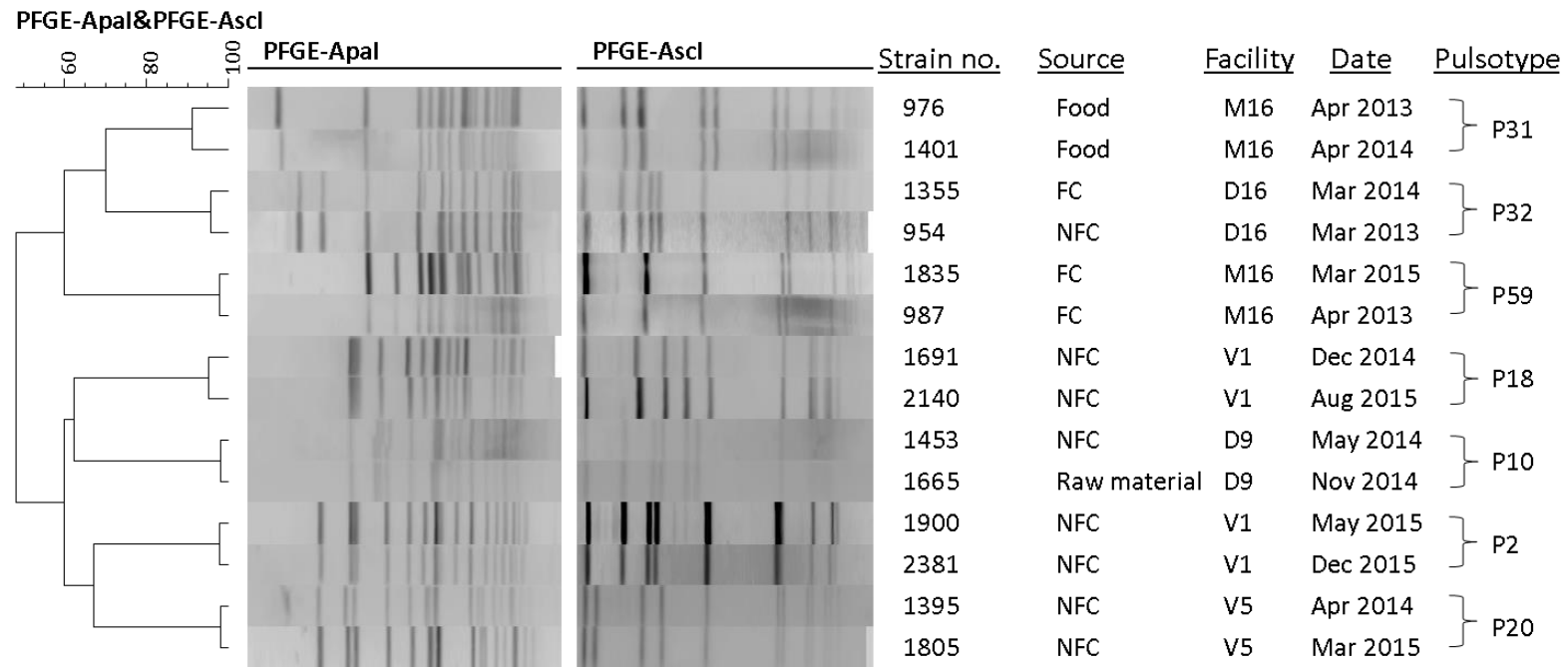


Figure 2.6: Examples of *L. monocytogenes* pulsotypes, persistent for at least 6 months within a single facility, isolated from processing environments and food obtained from 54 food businesses in Ireland. FC= Food contact area, NFC= Non-food contact area.

2.4.6 Comparison with Pulsotypes of Clinical Isolates

From 2012 to 2015, there were 25 *L. monocytogenes* clinical isolates obtained at the National Reference Laboratory for *Listeria*, *Salmonella* and *Shigella*. The PFGE profiles of the isolates from the current study were compared with these 25 clinical isolates using Bionumerics. Eleven of the industry/food pulsotypes showed close similarity with the clinical pulsotypes (Figure 2.7). These 11 pulsotypes were identified in 26 facilities and were found in both processing environment and food samples from all sectors. Seven of these 11 pulsotypes were identified as persistent in one (P2, P31, P45 and P48) or several (P6 and P32) facilities, and one of them (P44) occurred in all industry sectors.

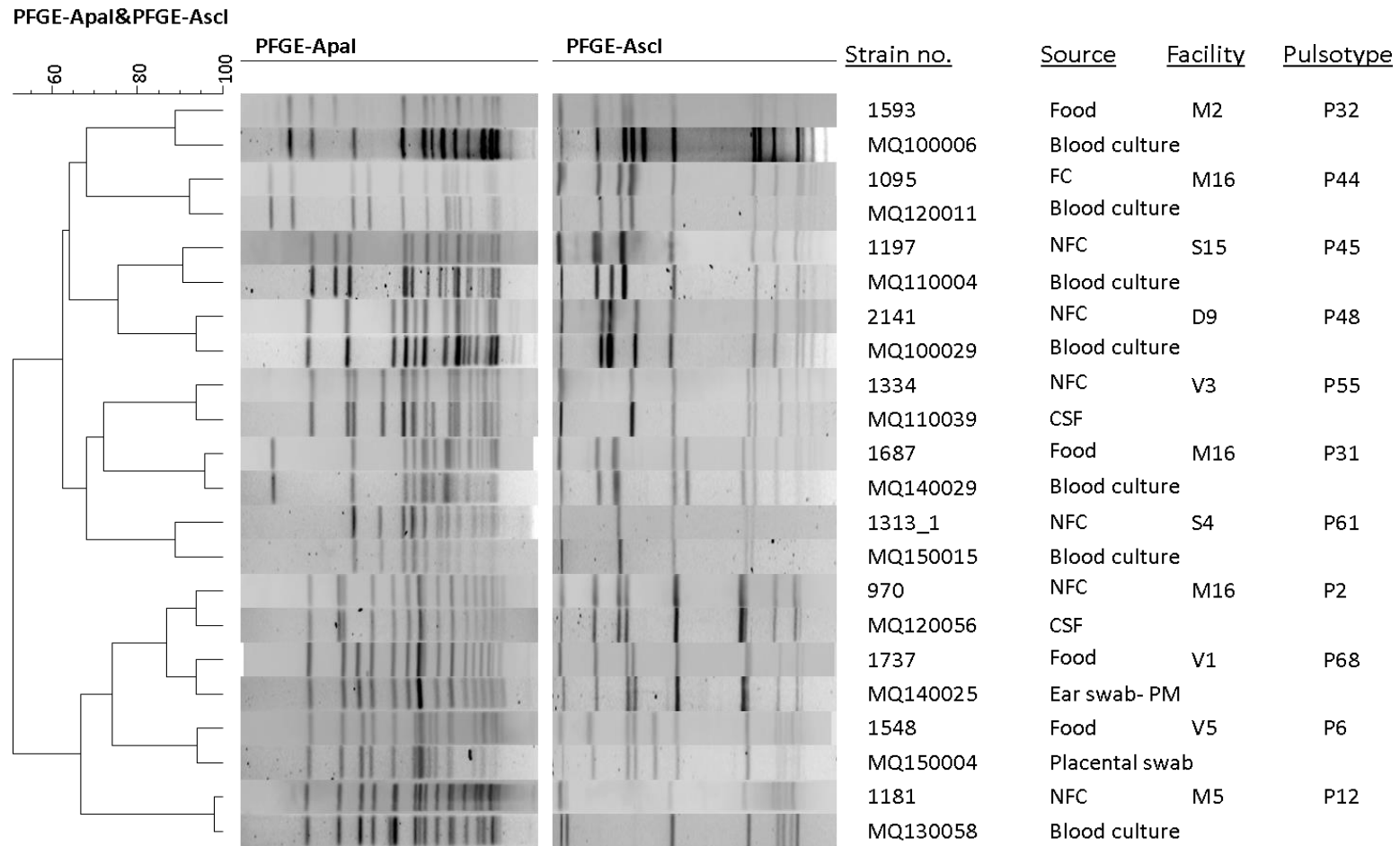


Figure 2.7: Dendrogram showing pulsotypes of clinical isolates in common with strains isolated from food and food processing facilities.

2.4.7 Comparison with Pulsotypes Identified Internationally

The 86 pulsotypes obtained were compared with pulsotypes obtained in 5 other countries, including United States of America, Australia, United Kingdom, France and Romania. Of the 86 pulsotypes obtained in Ireland, 32 were seen internationally, with 11 of the pulsotypes observed internationally being persistent in this study. P44, P46 and P59, which were obtained in all food sectors in this study, were also seen in at least one other country.

2.4.8 Evidence of Cross-Contamination from the Processing Environment to Food

There was evidence of cross-contamination between the processing environment and food (indistinguishable pulsotypes found in processing environment samples and food samples) at 12 facilities, representing all food sectors (Table 2.2). In two cases, transfer of more than one pulsotype occurred. The cross-contamination included seven different persistent pulsotypes (P6, P31, P32, P33, P44, P45 and P59) and five sporadic pulsotypes (P10, P17, P21, P61 and P67).

2.4.9 Reasons for a Large Increase/Decrease in Occurrence.

In some instances, there was a notable difference in the occurrence of *L. monocytogenes* at processing facilities from one year to the next. In two such cases, discussions with the business owners highlighted changes in management practices that

coincided with the change in occurrence. In one facility, D9, there was an increase from 5% occurrence in 2013 to 25% occurrence in 2014 (Table 2.2). This increase coincided with installation of new equipment. In a second facility, D15, there was a decrease from 15% occurrence in 2013 to 0% occurrence in 2014 (Table 2.2). This decrease coincided with inclusion of a peracetic acid final rinse in the cleaning and disinfection scheme.

2.5 Discussion

Many of the studies available in the literature on *L. monocytogenes* occurrence/surveillance were performed at a single facility over time or at a single time-point in many facilities. Such surveys provide an important perspective on the problem of *L. monocytogenes* occurrence. However, the lack of long term continuity and the use of sampling and analytical methods which vary from one study to the next limit their impact. Structured continuous surveillance with some degree of standardisation of methods, as undertaken in this study, is necessary to establish valid conclusions on occurrence and persistence over time.

During the course of this study, the overall occurrence of *L. monocytogenes* in the processing environment of the 54 facilities decreased from 4.7% in 2013 to 3.4% in 2015, while in the foods tested, the decrease was from 5.1% to 2.7%. All companies included in the study submitted samples in all three sampling years, and while there was variability in occurrence among individual facilities (29 showed a reduction in occurrence from 2013 to 2015, 12 showed no change between both years and 13 showed an increase between 2013 and 2015), a trend towards a reduction in occurrence over the sampling period was observed. Although the use of mean values across years may offer a somewhat shallow overview of the results, when combined with the reduction of variation in occurrence in individual facilities, as seen in Figure 2.1., the trend towards reduction is supported. Over the course of the study, the results of analyses were given to the food business owners every two months, explaining the significance of the results, and a series of workshops were undertaken with the aim of upskilling and further informing the food business operators about *L. monocytogenes*. Additionally, the food business owners or their quality staff were

responsible for taking the samples. It is considered that this also helped build awareness and understanding. A trend towards a decrease in *L. monocytogenes* persistence over the 3-year period observed in the majority of facilities (e.g. facilities D16, M1, M3, M7, M8 and M10) indicated that the approach of surveillance combined with awareness, which was created by sending the results to the companies after each sampling occasion, can have an impact on good management practices and can contribute to reducing *L. monocytogenes* occurrence (Hoffman et al., 2003; Lappi et al., 2004). The decrease in *L. monocytogenes* occurrence and persistence in food processing environments observed over the 3-year period in the current study contributed to a reduced risk of cross-contamination to food, which was reflected in the reduced occurrence in food observed in 2015. The fact that no attempt was made to “police” the sampling regime, may have biased the positive rate for some facilities making it difficult to establish with certainty if increasing the sampling regime produced a positive effect on the incidence of *L. monocytogenes* in the food industry.

Previous studies have shown the occurrence of *L. monocytogenes* in various food sectors. In smoked fish, a previous study showed that 25 out of 90 food samples were positive for *L. monocytogenes*, of which four exceeded the level of 100 CFU/g (Uyttendaele et al., 2009). In another study of raw and smoked fish and processing environments (over 1,000 samples tested), *L. monocytogenes* was isolated from 3.8% of the raw fish samples (0 to 10%, depending on the plant), and 1.3% of the finished product samples (Thimothe et al., 2004b). Different fish type/species may have contributed differently to the occurrence of *L. monocytogenes* in the processing environment, as well as the time of year and the turnover in the processing plant at a given time (Fonnesbech Vogel et al., 2001a). In this study 1.8% of 272 food samples from seafood processors were positive for *L. monocytogenes*. The

occurrence is considerably lower than that reported in the recent EU baseline survey, where the average EU occurrence was about 10% (EFSA, 2013b).

It has been documented previously that dairy farms can be a source of *L. monocytogenes*, either in animal faeces or the wider farm environment, at a prevalence of about 20% (Nightingale et al., 2004) or in bulk tank milk at a prevalence of 1-12% (Oliver et al., 2005), subsequently entering the milk processing environment, where contamination of milk and dairy products can occur. Post-pasteurization contamination of dairy products with *L. monocytogenes* occurs during the processing, packaging and storage of food. Studies have shown varying degrees of contamination of dairy products, from 0.47% to 7% (EFSA, 2013a; Fox et al., 2009). An occurrence of 3.7% of *L. monocytogenes* in dairy samples (1,920 samples, both food and processing environment samples) seen in this study is in line with other studies in the dairy sector. There was little difference in occurrence rates in the dairy sector over the course of the study which may be due to the fact that dairy processors are likely to be already aware of the risks of *L. monocytogenes* and have already taken steps to reduce contamination.

Extensive testing of RTE meat in the US over a 10 year period (1900-1999) revealed that contamination by *L. monocytogenes* varied according to meat product type (Levine et al., 2001), while the prevalence in production environments can vary from 0% to over 14% (Rivera-Betancourt et al., 2004). Thus, the reported prevalence of 3.5% in the Irish meat industry (1332 samples tested) is relatively low compared to other studies, although the average occurrence of 7.5% (349 samples tested) in food samples is surprising. The high occurrence is due, in part, to three meat processing facilities that did not produce RTE meat, but were included in the surveillance programme. The occurrence at these facilities was 8.1%, 10.8% and 20.0%.

The largest disease outbreak related to *L. monocytogenes* occurred in the U.S.A. in 2011 and was associated with consumption of contaminated cantaloupe (Laksanalamai et al., 2012), highlighting the risks associated with fruit and vegetables. Several publications on *L. monocytogenes* on fruit and vegetables report contamination of around 2% or lower, although some reports indicate higher contamination, of up to 85% in the case for Malaysian beansprouts (Arumugaswamy et al., 1994). Low concentrations have also been reported in frozen vegetables and on plant machinery in a facility producing frozen vegetables (below 2%). The authors suggested that some level of cross-contamination may have occurred in the facility that contaminated the food products (Aguado et al., 2004). In the present study, the vegetable processing environment was the most highly contaminated, and had the greatest diversity of pulsotypes. This may reflect the ubiquitous nature of *L. monocytogenes* and its association with soil. Cross-contamination of *L. monocytogenes* from the vegetable processing environment to the produce was seen, and at 5.8% occurrence, vegetable contamination was the second highest identified.

A large variability existed in *L. monocytogenes* occurrence among the food businesses. Thus, while *L. monocytogenes* occurrence was observed at above 10% for 5 facilities, 10 facilities showed a 0% occurrence over the 3-year period. It is tempting to speculate that appropriate management and hygiene practices implemented in each of these latter facilities may have contributed positively to the *L. monocytogenes*-negative status. This finding warrants further study to explore the nature of the microbiome that may have existed and which potentially could have contributed to this observation (Fox et al., 2014; Hoelzer et al., 2012). It is well recognised that changes in the occurrence of *L. monocytogenes* in food production facilities are often associated with certain types of intervention. For example, in one facility where there was an increase in occurrence from 5

to 23% between the years 2013 to 2014, while in another a decrease from 14 to 0% was recorded. The former coincided with the installation of new equipment, while the latter was attributed to the introduction of a 0.5% peracetic acid rinse cleaning in the protocol, suggesting that management practices can influence occurrence of *L. monocytogenes* in processing facilities.

All the isolates obtained during this surveillance were characterised by molecular methods, which allowed for the identification of patterns of contamination, which were reported to the food producer when they were available. The fact that 80.2% of the 86 distinct pulsotypes isolated along the food chain were classified as non-persistent indicates the diversity of strains that exists in the food processing environment. Continuous sporadic contamination identified in a processing facility could be regarded as an indication of a breakdown in hygiene protocols and, therefore, addressing such an issue, from a food safety standpoint, may reduce the risk of the food products being contaminated.

To address persistent contamination requires a different approach than that required to address sporadic contamination. The identification of persistent strains may be a symptom of process control failures or resistance of the strains to the cleaning methods used, and therefore the strains continue to exist in the manufacturing facilities. Based on the PFGE patterns and the definition used in this study, 16 facilities showed persistent contamination, while 28 facilities showed sporadic contamination but no persistent strains. All facilities which showed persistent contamination were also seen to have sporadic contamination. This would indicate a general need for updating both the cleaning procedures, with the aim of eliminating persistent strains, and the hygiene barrier systems, with the aim of preventing initial and sporadic contamination events.

Seventeen out of the eighty-six distinct pulsotypes identified along the food chain in the current study were considered persistent, according to the definition of persistence previously given. The failure to find other persistent pulsotypes does not necessarily indicate their inability to persist in the environment but could also reflect their lower relative abundance in the environment or even the existence of limitations/inconsistencies in the sampling regimes used. More extensive sampling could have resulted in the repeated isolation of strains that were isolated infrequently following the current sampling approach.

The occurrence of apparently persistent strains could also be due to re-contamination of environments from the exterior of the processing facility. However, if that was the case, persistence would have occurred in the external source. Persistence in food facilities is thought to primarily arise because of the availability of suitable sites (so-called harbourage sites) within the facility that can sustain a population, in combination with the genetic properties of particular strains that allow them to colonise those sites, e.g. sanitiser resistance, ability to use different carbon sources, ability to form biofilms, etc. (Carpentier and Cerf, 2011a). In the current study, several pulsotypes were considered as persistent in more than one facility, which suggests that strains belonging to those pulsotypes may have some superior survival or colonisation abilities in comparison to strains from other pulsotypes. Whole genome sequencing analysis and further phenotypic characterisation of these strains may help confirm whether this is the case. On the other hand, the identification of persistent pulsotypes common to several facilities might be due to the higher relative abundance of those pulsotypes in the environment.

Cross-contamination from the processing environment to food has been previously reported and indeed has been shown to be the cause of disease outbreaks (McCollum et al., 2013; Pérez-Rodríguez et al., 2008). Evidence of cross-contamination was seen in 12 of the

54 facilities in this study where indistinguishable pulsotypes were seen in both food and processing environment samples. This could be cross-contamination from the processing environment to the food or vice versa. Further research would be required to distinguish between these two scenarios. Furthermore, as this was a general study on occurrence, rather than one focused on contamination events, the number of food samples (about 36 from each company over 3 years) may not have been high enough to draw conclusions on sources of contamination.

From the 255 isolates included in this study, 43.1% of isolates belonged to lineage I and 56.1% belonged to lineage II. This is in general agreement with other studies where lineage I and II isolates are found frequently and lineages III and IV isolates are rarely found (Chenal-Francisque et al., 2013; Orsi et al., 2011). Serotypes identified in this study are in line with the general prevalence of serotypes found in the processing environment. Namely, the highest prevalence of 1/2a strains, followed by 4b, 1/2b and 1/2c (Todd and Notermans, 2011). Because of the ease of analysis, serogrouping by PCR is more frequently undertaken than serotyping through the use of antisera, yet there is little information correlating serogroup with serotype. In this study, 100% of isolates in serogroups 1/2a-3a, 1/2b-3b-7, 1/2c-3c and 4b-4d-4e belonged to serotypes 1/2a, 1/2b, 1/2c and 4b-4e, respectively. Similar results were obtained by Marugesen et al., indicating validity in serogroup rather than serotype analysis as a single serotype is significantly overrepresented in each serogroup (Murugesan et al., 2015).

L. monocytogenes strains have the ability to cause foodborne disease and indeed some strains show a variable ability to cause disease. Indeed, several reports have described apparently avirulent *L. monocytogenes* strains with polymorphisms in the *InlA* gene leading to a truncated non-functional protein (Chen et al., 2011). The comparison of the 255 isolates

with the 25 clinical isolates from Ireland showed that 11 pulsotypes from the food/processing environment surveillance matched 11 of the pulsotypes of clinical isolates. Seven of these 11 pulsotypes were persistent, and eight of them were found in several facilities, among these was one pulsotype, P44, which was found in all four food categories and showed a PFGE pattern indistinguishable from that of a blood isolate obtained from a patient in 2012. This strain was repeatedly found in the food chain over the entire three-year period of the study (2013-2015). This indicates that some strains frequently present in food processing environments which are capable of persisting and contaminating food products are closely related to strains that are capable of causing disease. Further investigation of these pulsotypes through whole genome sequencing analyses and phenotypic characterisation may reveal further information on their virulence traits.

Global clones of *L. monocytogenes* are known to exist (Chenal-Francisque et al., 2013). The results of this study support the existence of global clones as 32 of the 86 pulsotypes seen were also identified in other countries, 11 of which were seen to persist in the food processing environment. The significance of such global clones in terms of clinical cases is not clear, although 10 of the pulsotypes identified internationally also appeared as clinical isolates. The movement of strains into and out of the island of Ireland is likely facilitated by an open economy where there is a continuous large scale movement of goods and people.

2.6 Conclusions

In conclusion, this 3-year study has shown the prevalence of *L. monocytogenes* in 54 food processing facilities in Ireland and highlighted the diversity of *L. monocytogenes* strains that exist in the food sector, particularly in the vegetable sector. The overall rates of occurrence in food and food processing environments are broadly in line with reports from other countries. The finding that many of these strains have similar PFGE profiles to clinical isolates highlights the public health risk that this pathogen presents. The awareness and vigilance created by an extensive 3-year surveillance programme can contribute to a reduction of *L. monocytogenes* in food and food processing environments, leading to a decreased risk to public health.

2.7 Acknowledgements

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Chapter 3

Monitoring the Occurrence and Persistence of *Listeria ivanovii* in 48 Food Processing Facilities and Foods Produced Over a 12-month Period

Dara Leong Chapter Contributions:

Experimental:

- Performed sample analysis and PFGE analysis on samples from 48 food businesses
- Performed PCR and sequencing of *sigB* genes

Results Interpretation:

- Analysed all data and performed general comparative analysis on all isolates

Manuscript Preparation:

- Major contributor to the manuscript preparation

Occurrence, persistence, and virulence potential of *Listeria ivanovii* in foods and food processing environments in the Republic of Ireland

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3.1 Abstract

The aim of this study was to assess the occurrence of *L. ivanovii* in foods and food processing environments in Ireland, to track persistence, and to characterise the disease causing potential of the isolated strains. Although *L. ivanovii* human infections are rare, they do occur and ruminant infections are common. *L. ivanovii* contamination can also be indicative of a breakdown in hygiene procedures and therefore indicative of possible *L. monocytogenes* contamination areas, hence this information is important in preventing the production of contaminated food. A total of 2,006 samples (432 food samples and 1,574 environmental swabs) were collected between March 2013 and March 2014 from 48 food business operators (FBOs) belonging to different production sectors (dairy, fish, meat, and fresh-cut vegetable). Six of the forty-eight FBOs had samples positive for *L. ivanovii* on at least one sampling occasion. *L. ivanovii* was present in fifteen samples (fourteen environmental samples and one food sample). All but one of those positive samples derived from the dairy sector, where *L. ivanovii* prevalence was 1.7%. Six distinguishable pulsotypes were obtained by PFGE analysis, with one pulsotype being persistent in the environment of a dairy food business. Sequence analysis of the *sigB* gene showed that fourteen isolates belonged to *L. ivanovii* subsp. *londoniensis*, while only one isolate was *L. ivanovii* subsp. *ivanovii*. Cell invasion assays demonstrated that the majority of *L. ivanovii* strains were comparable to *L. monocytogenes* EGDe in their ability to invade CACO-2 epithelial cells whilst four isolates had significantly higher invasion efficiencies.

3.2 Introduction

The genus *Listeria* is at present comprised of fifteen low G+C content Gram-positive species. These are the *Listeria* sensu stricto species *L. monocytogenes*, *L. marthii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. ivanovii*, the distantly related species *L. grayi*, and the very recently described species *L. rocourtiae*, *L. fleischmannii*, *L. weihenstephanensis*, *L. floridensis* sp. nov., *L. aquatica* sp. nov., *L. cornellensis* sp. nov., *L. riparia* sp. nov., and *L. grandensis* sp. nov. (den Bakker et al., 2010; den Bakker et al., 2014). Of these, only *L. monocytogenes* and *L. ivanovii* are recognised as pathogenic for warm-blooded hosts. While *L. monocytogenes* causes a severe foodborne disease in humans as well as invasive infections in a range of other mammals, *L. ivanovii* is almost exclusively linked to infections in sheep and cattle, although sporadic cases of *L. ivanovii* associated human infections have been reported (Guillet et al. 2010; Snapir et al., 2006).

Due to its foodborne transmission, research on *L. monocytogenes* has received special attention in the last decades. Indeed, studies on occurrence and distribution of *L. monocytogenes* in foods and food processing environments are numerous and report variable prevalence. As an example, recent surveys carried out in the United Kingdom (Meldrum et al., 2010), Greece (Sakaridis et al., 2011), Sweden (Lambertz et al., 2012), Ireland (Khen et al., 2015; Leong et al. 2015), and various countries in Europe (Austria, Romania, Spain, and the Slovak Republic) (Muhterem-Uyar et al., 2015) have reported *L. monocytogenes* prevalence ranging from 2.5 to 38%. There is less information available in the literature on the occurrence and distribution of other *Listeria* species along the food chain, although it appears that, apart from *L. monocytogenes*, *L. innocua* is the most

frequently isolated *Listeria* species (Chambel et al., 2007; Gebretsadik et al., 2011). Regarding *L. ivanovii*, a few reports exist which describe a low occurrence, generally of <2% (Antoniollo et al., 2003; Chambel et al., 2007; Gebretsadik et al., 2011), although little or no information is available on its occurrence in Irish food industries.

Bacterial persistence, defined as repeated isolation of an indistinguishable (by pulsed field gel electrophoresis [PFGE]) isolate at sampling times greater than 6 months, is a great concern for food industries since it can lead to the repeated contamination of food with spoilage or pathogenic microorganisms and has been demonstrated to recurrently happen for strains of *L. monocytogenes* (Fox et al., 2011). A similar phenomenon could also occur for other members of the genus *Listeria*, including *L. ivanovii*. In fact, a study by Vázquez-Villanueva et al. has provided evidence for the persistence of a *L. ivanovii* subsp. *ivanovii* isolate in a Spanish cheese factory (Vázquez-Villanueva et al., 2010). These authors found a common PFGE pulsotype in both ewe's and goat's raw milk batches tested over a 6-month period and on the inner surfaces of raw milk bulk tanks and the milk dump tank at the cheese factory.

Both *L. monocytogenes* and *L. ivanovii* are facultative intracellular bacteria capable of crossing the intestinal barrier and proliferating within macrophages and epithelial and endothelial cells and ultimately inducing cell-to-cell spread (Vázquez-Boland, Kuhn, et al., 2001). Interestingly, it is well known that *L. monocytogenes* isolates vary considerably with respect to virulence capacity and disease causing potential, with some isolates being incapable of invading gastrointestinal cells due to the expression of a truncated virulence factor, internalin A (Jacquet et al., 2004; Van Stelten et al., 2010). Whether similar heterogeneity in disease causing potential is also present in *L. ivanovii* remains unexplored.

The aim of this study was to assess the occurrence of *L. ivanovii* in foods and food processing environments in the Republic of Ireland, to track persistence of the isolates, and to characterise the disease causing potential of the isolated strains.

3.3 Materials and Methods

3.3.1 Detection of *L. ivanovii* in Food and Environmental Samples

From March 2013 to March 2014, a total of 48 food processing facilities from various food sectors, that is, dairy (18 facilities), meat (12 facilities), seafood (8 facilities), fresh-cut vegetable (6 facilities), and miscellaneous (4 facilities), were sampled bimonthly. The selection of food processing facilities allowed coverage of major geographic areas of the Republic of Ireland.

Sampling packs, which consisted of a polystyrene box (DS Smith, UK) containing six pre-moistened 3M sponge-stick swabs (Technopath, Ireland), a sterile liquid container (VWR, Ireland), two sterile bags (VWR, Ireland), two cable ties, and two ice packs, were sent to all participating food processing facilities. Food business operators (FBOs) received detailed instructions which included information on how to take swab samples, which areas to sample, the type of food samples required, and the packaging and shipment of the samples to the laboratory. For food samples, FBOs were instructed to send two food samples which were at the stage of being ready to be sent from the processing facility.

Every second month, FBOs took 6 environmental samples and sent them to the laboratory by overnight courier along with 2 food samples. Thirty-seven FBOs were initially enrolled in the monitoring programme and 11 further FBOs later showed their interest in joining the collaborative network at different stages during the sampling year. On the other hand, 3 FBOs no longer wished to take part in the analysis or went out of business and several other companies missed one or various sample submissions throughout the sampling period.

Samples were analysed by following the ISO 11290-1 method for detection of *L. monocytogenes*, except that only one chromogenic agar was used. After the environmental swabs arrived at the laboratory, 100 mL of half Fraser broth (VWR, Ireland) was added to bags containing 3M stick-sponge swabs, after which they were incubated at 30 °C for 24 h. Then, a 0.1 mL aliquot was transferred to 10 mL of full Fraser broth, which was further incubated at 37 °C for 48 h. In addition, a 0.02 mL aliquot of the 1st enrichment broth was plated onto Agar *Listeria* according to Ottaviani and Agosti (ALOA) agar plates (Biomérieux, UK), which were incubated at 37 °C for 48 h. After incubation of the full Fraser broth, 10 µL was streaked onto ALOA agar plates, which were again incubated at 37 °C for 48 h. For liquid or food samples, 225 mL of half Fraser broth was added to 25 mL or 25 g of randomly selected analytical units of the food samples. Samples were then homogenized in a stomacher (Colworth Stomacher 400) for 4 min and incubated at 30 °C for 24 h. Subsequently, analysis of samples was continued by following the same approach used for environmental samples. After incubation, ALOA agar plates were examined for typical *L. monocytogenes*/*L. ivanovii* colonies (blue-green colonies with opaque halo). After confirmation of *L. monocytogenes* /*L. ivanovii* isolates (performed as described below) sampling results were regularly communicated to collaborating FBOs.

3.3.2 Molecular Characterization of *L. ivanovii* Isolates

Two characteristic *L. monocytogenes*/*L. ivanovii* colonies for each positive enrichment were streaked first onto Brilliance *Listeria* Agar (BLA) plates (Fannin, Ireland), which were incubated at 37 °C for 48 h, and then onto Brain Heart Infusion (BHI) agar plates,

which were incubated at 37 °C for 24 h. Cryoinstant tubes (VWR, Ireland) were prepared by resuspending the bacterial mass from BHI agar plates and were kept at –20 °C for bioconservation.

Isolates were differentiated as *L. monocytogenes* or *L. ivanovii* by multiplex PCR as described previously (Ryu et al., 2013) and *L. ivanovii* were confirmed by *sigB* sequencing as described below. PFGE analyses with the restriction enzymes *Ascl* and *Apal* were carried out on all confirmed *L. ivanovii* isolates according to the International Standard PulseNet protocol (PulseNetUSA, 2009). Isolate similarity dendrograms were generated for PFGE analysis using the BioNumerics version 5.10 software (Applied Maths, Belgium), by the unweighted pair group method with arithmetic mean (UPGMA) with tolerance and optimisation settings of 1%, as previously described (Fox et al., 2012). In addition, representative isolates from each pulsotype were subjected to real-time PCR analyses for differentiation of *L. monocytogenes* and *L. ivanovii* through amplification of *hly* as described by Rodríguez-Lázaro et al. and of *actA* as described by Oravcová et al. (Oravcová et al., 2006; Rodríguez-Lázaro et al., 2004). The *sigB* gene of *L. ivanovii* isolates was amplified using Taq DNA polymerase (Thermo Scientific, Ireland) with primers sigB-F (AATATATTAATGAAAAGCAGGTGGAG) and sigB-R (ATAAATTATTTGATTCAACTGCCTT) at 95 °C for 2 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Ireland) and sequenced by Source Bioscience services. Phylogenetic relationships between sequences were analysed using the web service <http://www.phylogeny.fr/> as described previously (Dereeper et al., 2008).

3.3.3 Invasion of CACO-2 Cells by *L. ivanovii* Isolates

The epithelial cell invasion assay was based upon the protocol of Nightingale et al. (Nightingale et al., 2005a). CACO-2 human intestinal cells (originally derived from human colon adenocarcinoma) were routinely maintained and grown in Dulbecco Modified Eagle Medium (DMEM) (Sigma-Aldrich, Ireland), supplemented with 10% Foetal Bovine Serum (Gibco, Ireland), 1% Penicillin-Streptomycin (Sigma-Aldrich), and 1% nonessential amino acids (Sigma-Aldrich) in a 37 °C incubator supplemented with 5% CO₂. Cells were counted using a haemocytometer and trypan blue exclusion to a cell density of 2×10^5 cells/mL of medium and seeded into each well of a 24-well tissue culture plate (Sarstedt), in triplicate. Cells were allowed to grow to a confluency of 80% over 48 h. Twenty-four hours prior to the assay, cells were washed and incubated in antibiotic-free DMEM.

Cultures of *L. monocytogenes* EGDe, *L. monocytogenes* PMSC1, or *L. ivanovii* strains were grown overnight in BHI at 37 °C with shaking. One mL of the overnight culture was subsequently pelleted by centrifugation and then washed in PBS, diluted to a final concentration of 2×10^7 CFU/mL, and resuspended in antibiotic-free DMEM. Precise numbers of bacterial CFUs added to wells at T₀ were calculated subsequently following plate counts.

Growth medium was removed from the CACO-2 cells in each well and cells were washed once with sterile PBS and 1 mL of bacteria in antibiotic-free DMEM was added (giving a multiplicity of infection of 100). Cells were incubated for 1 h at 37 °C/5% CO₂ to allow for internalisation of the bacteria. Subsequently, the bacterial inoculum was removed and the monolayer was washed once with sterile PBS. Fifty µg/mL gentamicin (Sigma) was

resuspended in antibiotic-free DMEM, applied to the monolayer, and incubated for one further hour to kill extracellular bacteria. This was followed by lysis of the entire monolayer with ice cold sterile water containing 0.1% of TritonX-100. One hundred μ L of the lysate was serially diluted and plated onto BHI agar (in triplicate for each well) which was incubated at 37 °C overnight.

Data were expressed as mean \pm SEM of at least three biological replicate samples. Data were transformed to log base ten prior to one-way Analysis of Variance (ANOVA) which was used to test the significance of differences in three or more groups followed by a post hoc test (in this case, Dunnett). In all cases, $P < 0.05$ was considered to be statistically significant. Graphs and statistical calculations were prepared using GraphPad Prism 5 (San Diego, California).

3.4 Results

From March 2013 to March 2014 a total of 2,006 samples (1,574 environmental samples and 432 food samples) were analysed following the ISO 11290-1 standard methodology. *L. ivanovii* was present in fifteen of the 2,006 samples tested (prevalence of 0.75%), accounting for 14 environmental samples (environmental prevalence of 0.83%) and one food sample (prevalence in food samples of 0.23%). All but one positive environmental sample derived from processing facilities of the dairy sector, where *L. ivanovii* prevalence was 1.7%. These isolates were obtained from non-food contact surfaces such as drains, floors, and pooled water on floors. The non-dairy isolate was obtained from a seafood processing environment (floor), while the positive food sample was obtained from meat sausages. No positive samples were observed in processing facilities of the fresh-cut vegetable sector. It is important to note that only six of the forty-eight processing facilities analysed had samples positive for *L. ivanovii* on at least one sampling occasion, with prevalence rates at those six facilities ranging from 1.8% to 13.1% (Table 3.1).

Facility no.	% positives (No. of samples)	March 13		May 13		July 13		September 13		November 13		January 14		March 14	
		Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods
Dairy															
1	13.1 (61)			T3				T3		T3		T3		T3	
2	0 (56)														
3	0 (54)														
4	0 (55)														
5	0 (64)														
6	2.4 (41)							T5				-		-	
7	0 (37)	-	-							-	-				
8	0 (40)														
9	0 (53)														
10	4 (50)					T2								T4	
11	0 (24)	-	-				-	-	-			-	-		
12	4.5 (44)	T1				T1		-	-			-	-		
13	0 (55)														
14	0 (56)														
15	0 (31)									-	-	-	-	-	-
16	0 (55)														
17	0 (22)	-	-	-	-	-	-					-	-		
18	0 (24)	-	-	-	-	-	-							-	-
Meat															
19	0 (15)	-	-					-	-	-	-	-	-	-	-
20	0 (54)														
21	0 (56)														
22	1.8 (54)										T6				
23	0 (32)	-	-	-	-	-	-								
24	0 (35)	-	-	-	-	-	-								
25	0 (15)	-	-	-	-	-	-			-	-	-	-	-	-
26	0 (6)			-	-	-	-	-	-	-	-	-	-	-	-
27	0 (40)									-	-	-	-		
28	0 (46)									-	-				
29	0 (25)									-	-	-	-		
30	0 (47)									-	-				

Seafood														
31	0 (34)													
32	0 (51)													
33	0 (34)		-			-		-						
34	0 (53)													
35	0 (49)												-	-
36	0 (53)													
37	12.5 (8)	-		-	-		-	-		-	T4		-	-
38	0 (30)	-		-	-		-	-					-	-
Vegetable														
39	0 (55)													
40	0 (24)							-		-		-	-	-
41	0 (55)													
42	0 (48)			-			-							
43	0 (52)													
44	0 (45)												-	-
Miscellaneous														
45	0 (56)													
46	0 (34)							-		-		-	-	-
47	0 (32)	-		-	-		-	-						
48	0 (46)										-		-	

Table 3.1: Occurrence and pulsed field gel electrophoresis characterisation of isolates from *L. ivanovii* positive samples, listed according to processing facility, sampling month, sample type, and pulsotype, for example, T1. Empty white boxes indicate no *L. ivanovii* detected in submitted samples. “—” indicates non-submission of samples during that sampling month.

PFGE analysis was performed for all confirmed *L. ivanovii* isolates in order to track persistence events in the food processing environment (Figure 3.1). Six distinguishable pulsotypes were observed. In two dairy processing facilities (FBO 1 and FBO 12), *L. ivanovii* strains with indistinguishable PFGE profiles were isolated at various sampling times during the monitoring programme. For FBO 1, *L. ivanovii* isolates belonging to the same pulsotype were obtained from drains, floors, and pooled water on floors in May 2013, September 2013, November 2013, January 2014, and March 2014 (10-month persistence). For FBO 12, two *L. ivanovii* strains with indistinguishable PFGE profiles were isolated from drains in March 2013 and July 2013.

In order to characterise the *L. ivanovii* isolates at the subsp. level, the *sigB* gene was sequenced for representatives of the six distinguishable pulsotypes (Figure 3.2). Analysis of *sigB* sequences showed that five of the six pulsotypes (which correspond to 14 of the 15 positive samples) belonged to *L. ivanovii* subsp. *londoniensis*, while the remaining pulsotype (T6, with only one strain isolated from meat sausages) was *L. ivanovii* subsp. *ivanovii*.

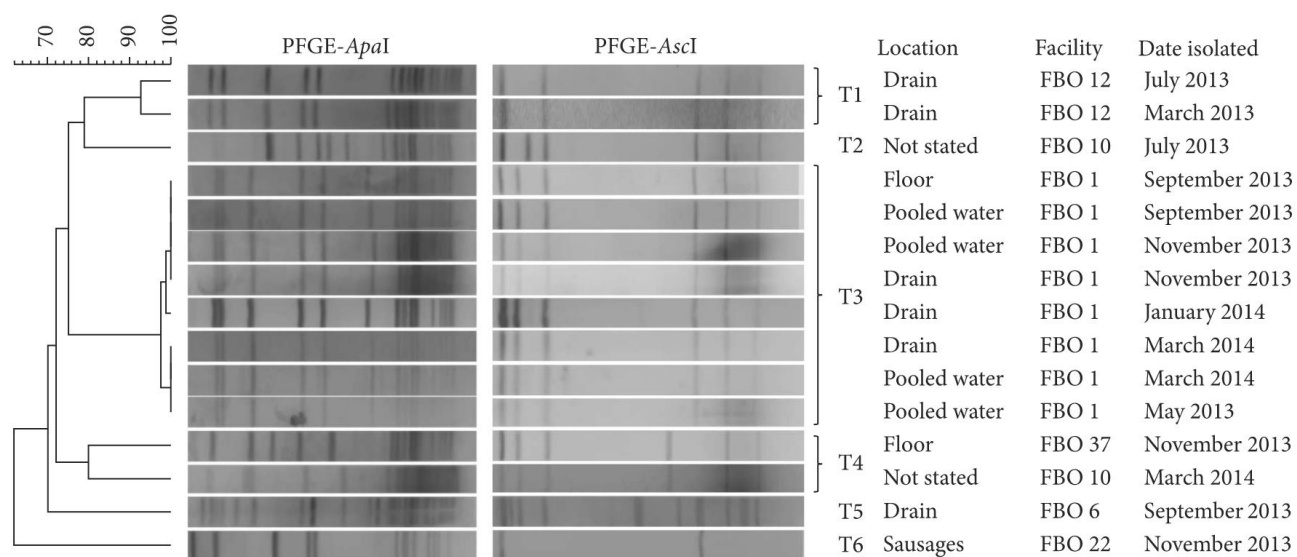


Figure 3.1: Dendrogram of PFGE pulsotypes of *Listeria ivanovii* isolates obtained from food and processing environment samples from the Republic of Ireland analysed from March 2013 to March 2014.

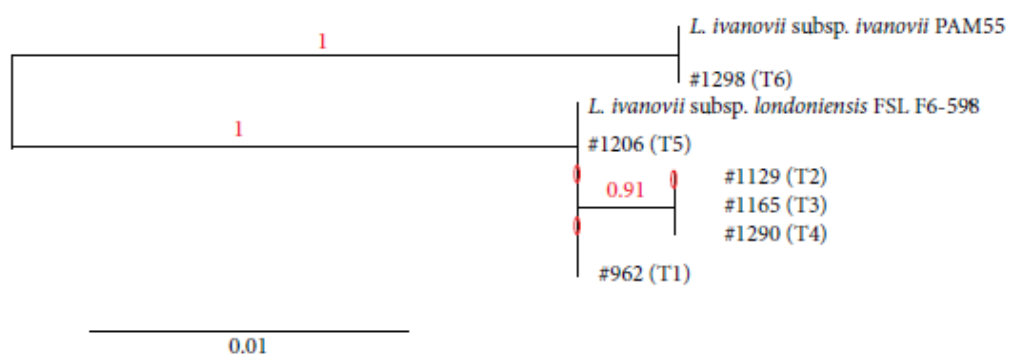


Figure 3.2: Phylogenetic tree (based on the sequence of the *sigB* gene) for the reference *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis* strains and representatives of the six *L. ivanovii* pulsotypes found in the current study.

When incorporating the PFGE profiles obtained in the current study to the *Listeria* spp. collection of profiles available at Teagasc Food Research Centre Moorepark, it became apparent that several isolates originally confirmed as *L. monocytogenes* by following the real-time PCR approach described by Rodríguez-Lázaro et al. presented PFGE profiles indistinguishable from the ones obtained in this study (Rodríguez-Lázaro et al., 2004). Some of these strains were analysed by multiplex PCR and actually confirmed as *L. ivanovii* (data not shown). Subsequently, the real-time PCR protocol described by Rodríguez-Lázaro and co-authors was applied to representative strains of the six pulsotypes observed in the present study (Figure 3.3(a)). Amplification of the target *hly* gene occurred for both *L. monocytogenes* positive control strains used, with Ct values of 17.9 and 18.2, while late amplification of the target gene was observed for the *L. ivanovii* isolates tested, with Ct values ranging from 26.1 to 32.7. In addition, the real-time PCR methodology described by Oravcová et al. for confirmation of *L. monocytogenes* based on the amplification of the *actA* gene was also tested with representative strains of the six *L. ivanovii* pulsotypes, and similarly late amplification events occurred, with Ct values ranging from 26.8 to 35.32, in contrast to Ct values of 18.4 and 20.0 observed for *L. monocytogenes* isolates tested (Figure 3.3(b)).

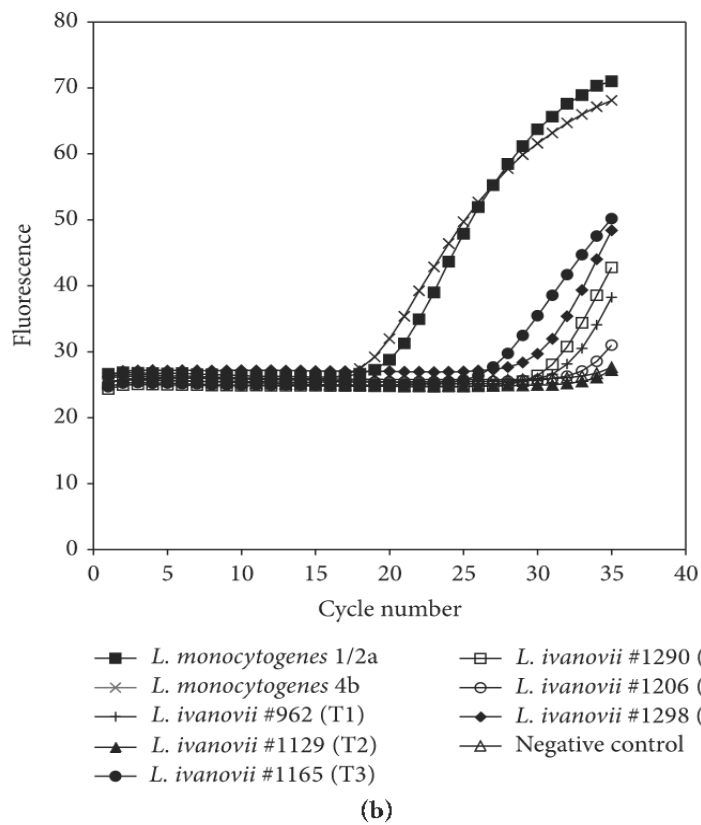
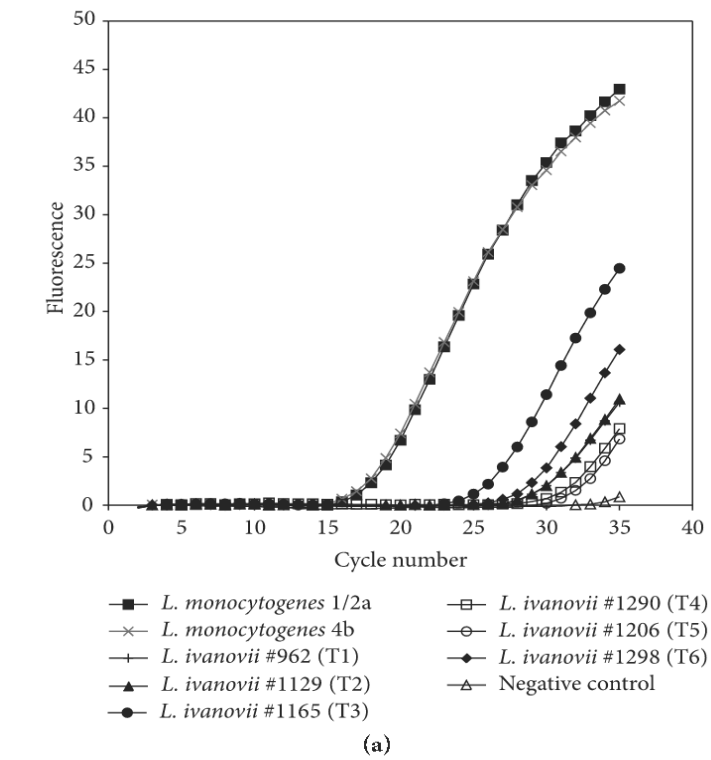


Figure 3.3: Amplification plot for *hly* (A) and *actA* (B) in *L. ivanovii* following the rt-PCR methodology described by Rodríguez-Lázaro et al. and Oravcová et al., respectively.

In order to determine the ability of various *L. ivanovii* strains to invade gastrointestinal epithelial cells, a standardised CACO-2 invasion assay (Nightingale et al., 2005a) was carried out. Representative strains from 4 of the 6 pulsotypes were compared to an invasive laboratory strain of *L. monocytogenes* (strain EGDe) as well as a non-invasive *L. monocytogenes* strain carrying a defined premature stop codon in the *InlA* gene (PMSC1) (Nightingale et al., 2005a). The assay clearly differentiates between invasive and non-invasive *L. monocytogenes* isolates (Figure 3.4) and invasion efficiency of wild-type *L. monocytogenes* and the PMSC1 strain were roughly equivalent to results in previous studies (Ciolacu et al., 2015; Nightingale et al., 2005a). *L. ivanovii* strains were generally highly invasive with 7 out of 9 strains demonstrating levels of invasion that were equal to or higher than those of *L. monocytogenes* EGDe. Two strains (1261 and 1167) were moderately less invasive than *L. monocytogenes* EGDe, but none of the isolates demonstrated an invasion phenotype that was comparable to the PMSC1 *L. monocytogenes* isolate. Interestingly, four *L. ivanovii* isolates (1017, 1165, 1262, and 1290) were significantly ($P < 0.05$) more invasive than *L. monocytogenes* EGDe.

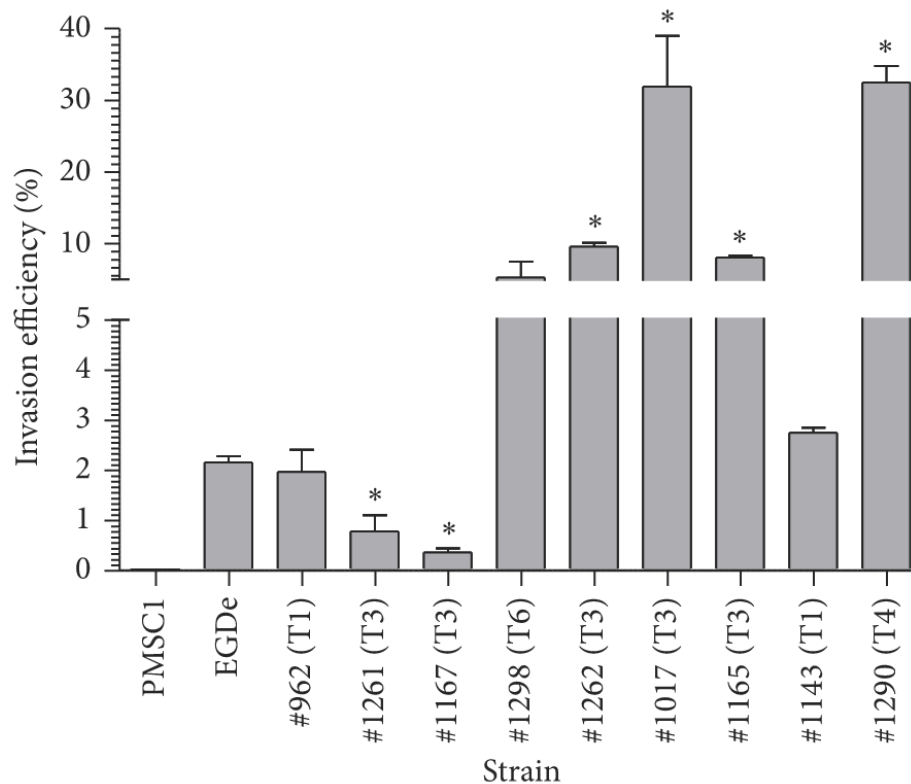


Figure 3.4: Invasive potential of wild-type *L. ivanovii* isolates in a CACO-2 epithelial cell assay. The strains were incubated with CACO-2 cells in vitro for one hour and levels of bacterial invasion were subsequently measured. For comparison, invasive (EGDe) and noninvasive (PMSC1) strains of *L. monocytogenes* were also examined. Data represents % invasion efficacy (relative to *Listeria* numbers initially added per well). Statistical significance was determined using one-way ANOVA and the Dunnett post hoc test with all strains compared to *L. monocytogenes* EGDe (* $P < 0.05$). All strains displayed statistically higher ($P < 0.05$) levels of invasion efficiency relative to the PMSC1 strain.

3.5 Discussion

The occurrence of *L. ivanovii* in foods and food processing environments was evaluated for the first time in the Republic of Ireland by bimonthly testing, over a one-year period, of samples from forty-eight processing facilities. The observed *L. ivanovii* prevalence was in general low (0.75%). The prevalence in food samples was especially low at 0.23% while environmental prevalence occurred at a higher rate of 0.83%. This difference between food and environmental occurrence rates may reflect successful management practices and hygiene barriers which are preventing contamination of food from the processing environment. The general prevalence agrees with the few reports available in the literature which also describe low *L. ivanovii* prevalence in the range 0–2% (Antoniollo et al., 2003; Chambel et al., 2007; Gebretsadik et al., 2011). However, the results showed that *L. ivanovii* occurrence depended on the food sector. Thus, while a higher prevalence of 1.7% was observed for the dairy sector, very low prevalences (0.2% and 0.3%, resp.) were found for the meat and seafood sectors and no positive samples at all were obtained for the fresh-cut vegetable industry sector (278 samples analysed). It is important to note that *L. ivanovii* predominantly infects small ruminants and cattle, which can act as reservoirs. Ruminants can carry *L. ivanovii* and contamination of milk can occur. Interestingly, three of the four dairy business operators that had positive samples (FBO 1, FBO 10, and FBO 12) produce cheese using milk from their own herds of cows or goats. Farming activity is carried out in those cases at facilities close to the cheese making facilities. This may potentially pose a further risk of processing environment contamination by *L. ivanovii*.

A survey regarding *L. monocytogenes* occurrence was conducted in parallel and showed that *L. monocytogenes* was present in 4.6% of samples analysed, with similar rates in food and environmental samples (Leong et al. 2015). In most sampling occasions when *L. ivanovii* was detected, no *L. monocytogenes* contamination was observed. However, there were three sampling occasions (Facility number 1: Environment, May 13; Facility number 1: Environment, November 13; Facility number 22: Foods, November 13) at which both *L. ivanovii* and *L. monocytogenes* isolates were identified, and in the particular case of Facility no. 1, both *L. ivanovii* and *L. monocytogenes* were isolated from the same samples (a drain and pooled water in the wash room) on November 13.

Molecular analysis of *L. ivanovii* isolates obtained throughout the monitoring programme showed that fourteen of the fifteen isolates (including all dairy isolates) belonged to *L. ivanovii* subsp. *londoniensis*, while only an isolate from meat sausages was *L. ivanovii* subsp. *ivanovii*. Interestingly, all environmental isolates were *L. ivanovii* subsp. *londoniensis*, while the only food isolate was *L. ivanovii* subsp. *ivanovii*. Whether *L. ivanovii* subsp. *londoniensis* is widely more prevalent in the environments than *L. ivanovii* subsp. *ivanovii* or this is a particular phenomenon observed in processing facilities in Ireland remains to be elucidated.

Persistence of *L. ivanovii*, considered for this study as the detection of isolates with indistinguishable PFGE profiles at times six months or more apart, was observed for a cheese processing facility (FBO 1), where a persistent *L. ivanovii* subsp. *londoniensis* pulsotype (T3) was detected repeatedly over a 10-month period (from May 2013 to March 2014) in several non-food contact environments (drains, floors, and pooled water on floors).

In addition, another pulsotype (T1), which cannot yet be considered as persistent, was found in drains of a cheese factory (FBO 12) at times four months apart (March to July 2013). These two cheese processing facilities were the ones with the highest *L. ivanovii* occurrence (13.1% and 4.5%, resp.). Long-term survival of strains in a food processing facility, such as these, confers a higher risk of bacterial transfer to food and therefore a higher risk of human exposure to the microorganism. Bacterial persistence in food processing environments can be due to the existence of harbourage sites that are colonised by bacteria and cannot be effectively cleaned or disinfected or can be due to the enhanced ability of some particular strains to grow or survive and therefore persist in industrial settings (Carpentier and Cerf, 2011a). Thus, strains with increased resistance to sanitisers, higher adaptability to stress, or better ability to form biofilms might be better suited to persist in inhospitable environments such as those prevailing in food industries. Persistence of *L. ivanovii* in food processing environments has been also previously reported by Vázquez-Villanueva et al. who identified a persistent *L. ivanovii* subsp. *ivanovii* pulsotype from ewe's and goat's raw milk samples from asymptomatic animals at farm level and from swabs obtained from the inner surfaces of raw milk truck tanks and the milk dump tank at the cheese factory level (Vázquez-Villanueva et al., 2010).

The current study also gives evidences that misidentification of *L. ivanovii* isolates as *L. monocytogenes* could occur when following the standard methodology for detection of *L. monocytogenes* in food and environmental samples. *L. ivanovii* strains are phosphatidylinositol-specific phospholipase C positive, and as such they grow in standard selective *L. monocytogenes* chromogenic agar plates forming colonies with the same characteristics as *L. monocytogenes* (blue-green colonies surrounded by an opaque halo on

ALOA plates). Genes within the *prfA* virulence gene cluster are habitually used as target genes for *L. monocytogenes* confirmation PCR methodologies (e.g., *hly* and *actA*). The *prfA* virulence gene cluster is present between the *prs* and *ldh* genes in the pathogenic *L. monocytogenes* and *L. ivanovii* but is absent from the non-pathogenic *Listeria* species (Cai and Wiedmann, 2001). Two widely used rt-PCR methodologies specifically designed for the detection and quantification of *L. monocytogenes* and based on the amplification of the *hly* and *actA* genes (Oravcová et al., 2006; Rodríguez-Lázaro et al., 2004) were applied to the set of *L. ivanovii* strains isolated in the present study. The results showed that a late amplification (but earlier than the negative control) of both target genes occurred for *L. ivanovii* isolates, which could lead to an erroneous interpretation of results. Indeed, the Teagasc Food Research Centre Moorepark culture collection contained various strains originally classified as *L. monocytogenes* by following the approach described by Rodríguez-Lázaro et al. that were subsequently identified as *L. ivanovii* during the course of this study. These results show the need for fine-tuning of the currently available molecular methodologies for confirmation of *L. monocytogenes*. Incorporation of such molecular tools able to rapidly and successfully discriminate *L. ivanovii* from *L. monocytogenes* is also advisable when implementing monitoring programmes focused on *L. monocytogenes*.

L. ivanovii is known to cause disease predominately in ruminants but has been associated on occasions with human disease (Guillet et al. 2010; Snapir et al., 2006) and is considered to be a potential opportunistic pathogen of humans. To date, studies examining the virulence characteristics of *L. ivanovii* have examined individual reference strains rather than collections of isolates. These studies indicate that *L. ivanovii* is capable of cellular

invasion, often at levels in excess of *L. monocytogenes* (Karunasagar et al., 1993; Longhi et al., 2014; Schlech et al., 1994). *L. ivanovii* is also capable of lysis of the host cell phagosome and actin polymerization but is perhaps less effective than *L. monocytogenes* in cell-to-cell spread and intracellular multiplication (Engelbrecht et al., 1998; Karunasagar et al., 1993; Schlech et al., 1994). The findings of this study support previous studies and demonstrated that some wild-type isolates of *L. ivanovii* are more invasive than a clinical *L. monocytogenes* reference isolate (EGDe). Indeed, the majority of isolates in this study were capable of highly effective cellular invasion, suggestive of some degree of disease causing potential. Further analysis is needed to ascertain the precise disease risk associated with these strains but the results suggest that such isolates may pose a health risk for immunocompromised individuals (Guillet et al. 2010).

3.6 Conclusion

In conclusion, *L. ivanovii* prevalence in foods and food processing environments in the Republic of Ireland is low but cannot be considered negligible in processing facilities from the dairy sector, where contamination of environments through contaminated raw milk and persistence of isolates with good abilities to grow/survive in industrial settings in particular environments can occur, leading to a higher risk of contamination of processed foods. Although *L. ivanovii* is mainly linked to infections in sheep and cattle, recent reports have highlighted its disease causing potential in humans (Guillet et al., 2010; Snapir et al., 2006) and the findings of this study demonstrated that the strains described are capable of invasion of human epithelial cells in vitro. These findings emphasise the need for dairy processors to be vigilant in order to avoid potential public health risks associated to *L. ivanovii* contamination.

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Chapter 4

Challenge Testing: A Comparative Study of Procedural Variation in Challenge Tests Evaluating the Growth of *Listeria monocytogenes*

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- Major contributor to the manuscript preparation

The challenge of challenge testing to monitor *Listeria monocytogenes* growth on ready-to-eat foods in Europe by following the European Commission (2014) Technical Guidance document

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4.1 Abstract

European Regulation (EC) No. 2073/2005 lays down the microbiological criteria for certain microorganisms in foods and the implementing rules to be complied with by food business operators (FBOs) in Europe when implementing general and specific hygiene measures. In relation to *Listeria monocytogenes*, this regulation covers primarily ready-to-eat (RTE) food products, and requires different microbiological criteria depending on the ability of the food product to support growth of *L. monocytogenes*. In addition, this regulation establishes that food safety is the responsibility of the FBO. The FBO can conduct studies to evaluate the growth of *L. monocytogenes* that may be present in the product during the shelf-life under reasonably foreseeable storage conditions of distribution, storage and use in order to investigate compliance with the criteria throughout the shelf-life of the product. The European Union Community Reference Laboratory for *L. monocytogenes* published a revised technical guidance document in June 2014 for conducting shelf-life studies on *L. monocytogenes* in RTE foods. This review article describes the recently published European guidance document, with special focus on the design of challenge studies to determine the growth potential of *L. monocytogenes* on foods. Information is given particularly on what a challenge test is and when one is advisable. The factors to be considered and the laboratory methodology to be applied when performing a challenge test to determine the growth potential of *L. monocytogenes* in a defined food matrix are also described. Results of recent research articles applying challenge tests to determine the growth of *L. monocytogenes* in a range of foodstuffs are summarized and discussed. Finally, recommendations for obtaining data that can contribute to any further revision of the

guidance document and for addressing the main challenges of challenge testing for FBOs are presented.

4.2 Introduction

European Regulation (EC) No. 2073/2005 (EC, 2005b) lays down the microbiological criteria for certain microorganisms in foods and the implementing rules to be complied with by food business operators (FBOs) when implementing general and specific hygiene measures. In relation to *L. monocytogenes*, this regulation covers primarily RTE food products, and requires the following: (i) in RTE products intended for infants and for special medical purposes *L. monocytogenes* must not be present in 10 × 25 g; and (ii) in RTE products other than those for infants and special medical purposes different microbiological criteria apply depending on the ability of the food product to support growth of *L. monocytogenes*. Thus, for RTE foods unable to support the growth of *L. monocytogenes*, the levels should be <100 CFU/ g throughout the shelf-life of the product (n = 5; c = 0). On the other hand, in RTE foods that are able to support the growth of the bacterium, *L. monocytogenes* must not be present in 5 × 25 g samples at the time of leaving the production plant; however, if the producer can demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 CFU/g throughout its shelf-life, a level of <100 CFU/g is allowable throughout the shelf life of the product (n=5, c=0).

In addition, this regulation establishes that the safety of the food is the responsibility of the FBO who can conduct studies to evaluate the growth of *L. monocytogenes* that may be present in the product during the shelf life under reasonably foreseeable storage conditions of distribution, storage and use in order to investigate compliance with the criteria throughout the shelf-life of the product. This triggers the question on how the FBO decides if the product is able or unable to support the growth of *L. monocytogenes*, and how

compliance with the 100 CFU/g limit throughout the shelf-life can be demonstrated. In this regard, the Directorate-General of Health and Consumers (DG SANCO) of the European Commission published a document directed at Food Business Operators who produce ready-to-eat (RTE) foods aimed to help them to demonstrate to the satisfaction of the competent authority that their products comply with the Community Regulation, to understand the range of different approaches available to help establish a safe product shelf-life in relation to *L. monocytogenes*, and to classify their products into RTE foods in which growth of *L. monocytogenes* can occur or in RTE foods in which growth of *L. monocytogenes* will not occur during their shelf-life (DGSANCO, 2008).

Determining the ability of foods to support the growth of *L. monocytogenes* is not simple since many RTE foods are traditionally produced in local regions using variable formulations which may have an impact on the fate of *L. monocytogenes*. The Food Standards Agency of New Zealand has recently published guidelines for undertaking challenge studies (FSANZ, 2014), although this document is not specifically related to *L. monocytogenes*. On the other hand, Canada also has guidelines which specifically relate to *L. monocytogenes* (Health-Canada, 2012). In Europe, in order to facilitate the task of performing challenge studies, the European Union Community Reference Laboratory for *L. monocytogenes* prepared a technical guidance document in 2008 in collaboration with seven laboratories, including six National Reference Laboratories for *L. monocytogenes* (EC, 2008). This guidance document was aimed at describing the microbiological procedures for determining growth of *L. monocytogenes* using challenge tests in the frame of the application of the Regulation (EC) No. 2073/2005. The content of this technical guidance document has been reviewed by previously (Beaufort, 2011).

However, feedback from food processors and independent laboratories indicated a need for the revision of the guidance document and to develop a more user-friendly set of guidelines to facilitate such analyses. In September 2012, the revision of the “EURL Lm Technical Guidance document for conducting shelf-life studies on *L. monocytogenes* in ready-to-eat foods” commenced. The European Union Community Reference Laboratory for *L. monocytogenes* established a working group of representatives of 10 national reference laboratories, 1 associate national reference laboratory and 1 laboratory on behalf of a national reference laboratory, and the updated version of the technical guidance document has been recently published (EC, 2014).

This review article describes the above mentioned, recently published European guidance document, with special focus on the design of challenge studies to determine the growth potential of *L. monocytogenes* on foods. Particularly, information is given on what a challenge test is, when one is advisable, the factors to be considered and the laboratory methodology to be applied when performing a challenge test to determine the growth ability of *L. monocytogenes* in a defined food matrix. Moreover, results of recent research articles applying challenge tests to determine the growth of *L. monocytogenes* in a range of foodstuffs are summarised and discussed. Finally, recommendations for obtaining data that can contribute to any further revision of the guidance document are presented.

4.3 Application of Challenge Tests on *L. monocytogenes* in the Food Industry Following the E.U. Technical Guidance Document

The growth ability of *L. monocytogenes* in food products may be estimated based on specifications of physico-chemical characteristics of the product, consultation of the available scientific literature, or predictive mathematical modelling. However, in most cases growth assessment will involve laboratory-based studies, so-called challenge tests. A challenge test can be defined as a laboratory-based study that measures the growth of *L. monocytogenes* in artificially contaminated food stored under foreseeable abuse conditions of transportation, storage at retail and at consumer level. As a primary objective, challenge tests aim to determine whether or not a particular food product has the ability to support growth of *L. monocytogenes*. An indication of the growth potential is obtained from the difference between the \log_{10} CFU/g at the end of the shelf-life and the \log_{10} CFU/g at the beginning of the test. When this difference is greater than 0.5 \log_{10} CFU/g the food is classified into RTE foods able to support the growth of *L. monocytogenes*. Alternatively, when the difference is less than 0.5 \log_{10} CFU/g, the food is classified into RTE foods unable to support the growth of *L. monocytogenes*. Performance of challenge tests is not needed for many food products. Consultation of available scientific literature and specifications of physico-chemical characteristics of the product will help decide whether a challenge test is required or not, based on the evidence that *L. monocytogenes* does not represent a risk or does not have the ability to grow in the product (Figure 4.1). Indeed, challenge tests for *L. monocytogenes* would not be needed for the following food categories:

- Foods which are intended to be cooked or subjected to any other bacterial inactivation step before human consumption.

- Foods which have received heat treatment or other processing effective to eliminate *L. monocytogenes*, when recontamination is not possible after this treatment (e.g. products treated in their final package).

- Fresh, uncut and unprocessed vegetables and fruits, excluding sprouted seeds (these are classified under primary production).

- Bread, biscuits and similar products.

- Bottled or packed waters, soft drinks, beer, cider, wine, spirits and similar products.

- Sugar, honey and confectionery, including chocolate and cocoa products.

- Bivalve molluscs.

- Food grade salt.

- Frozen products.

- Foods with $\text{pH} \leq 4.4$ or $a_w \leq 0.92$ or $\text{pH} \leq 5.0$ and $a_w \leq 0.94$, conditions which are already known as unable to support the growth of *L. monocytogenes*. Also, historical data on prevalence of *L. monocytogenes* in the specific RTE food at the end of shelf-life and particularly on results of durability studies (the number of samples exceeding 100 CFU/g) and outputs of predictive microbiology modules may be useful in deciding whether a challenge test is required or not for a particular foodstuff.

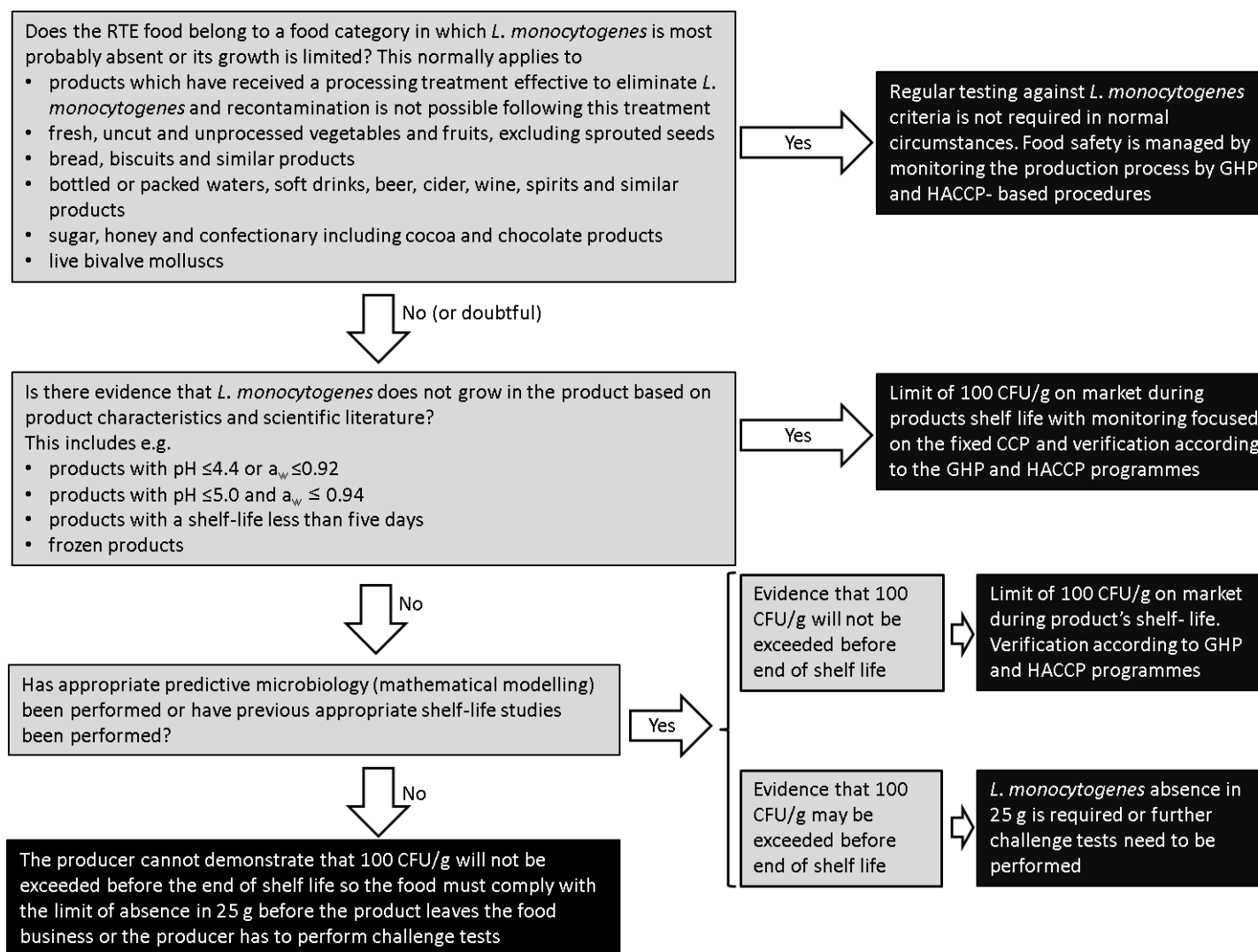


Figure 4.1 Decision tree showing the schematic steps to follow to determine on whether a challenge study is necessary (EC, 2005b).

The following factors must be considered when performing a laboratory challenge test to assess growth potential by following the updated version of the EURL Lm Technical Guidance document for conducting shelf-life studies on *L. monocytogenes* in RTE foods (Table 4.1):

(i) Number of batches: the number of batches to be included in the design of the challenge test will depend on the available information on probability of growth and inter-batch variability of pH and water activity (a_w). Predictive microbiology tools such as growth/no growth boundary modules or “inter-batch variability” calculators can be used for this purpose. If the growth probability is low or the inter-batch variability of pH and a_w regarding the growth of *L. monocytogenes* can be considered negligible it is possible to limit the study to one single batch. On the other hand, if the growth probability and inter-batch variability are high at least three batches need to be tested.

(ii) Bacterial strains: to account for variation in growth and survival among strains of *L. monocytogenes*, challenge tests must be performed with a mixture of at least two strains. One of them must be a strain with known growth characteristics, while the other strain/s can be freely chosen and will ideally be originally isolated from the food product being analysed. This second strain can also be isolated from environments, outbreaks or can be a collection strain. The European Union reference laboratory for *L. monocytogenes* has recently constituted a set of strains from various origins (meat, dairy products, fish) and various genoserotypes (II and IV). These strains were selected for their growth ability in harsh conditions of temperature, pH and a_w , according to the literature. The growth of these strains under harsh conditions (8 °C, pH = 5 or a_w = 0.95) has been characterised and their use is recommended when performing challenge tests (EURL-Lm, 2013).

(iii) Inoculum preparation: bacterial strains must be firstly inoculated in a non-selective medium (e.g. Brain Heart Infusion [BHI] broth) incubated at an optimal temperature (e.g. 30 or 37 °C) for the required time to reach the early stationary phase of growth (e.g. overnight), and then they must be subcultured in a non-selective medium and incubated at a temperature close to the actual storage temperature of the product to be tested (e.g. 7 °C, or 10 °C when considering refrigerated RTE foods) for the required time to reach early stationary phase. This allows for bacterial adaptation to the environmental temperature conditions prevailing during the challenge test in the food product. Extra stresses of relevance may be also added. Finally, individual cultures must be combined in equal quantities and serial dilutions must be prepared to obtain an inoculum at the expected concentration to be used for inoculation of the food.

(iv) Food inoculation: the method of inoculation of the food product with the cocktail of *L. monocytogenes* strains must be in such a way that it does not compromise the intrinsic properties (physico-chemical characteristics) of the food. For this reason, the inoculum volume must not exceed 1% of the mass (or volume) of the test unit. In addition, the inoculation must mimic realistic scenarios of food contamination by *L. monocytogenes*. In order to minimise the measurement uncertainty, the contamination level must be targeted at around 100 CFU/g. Several methods of inoculation can be considered. Inoculation can be performed at surface to mimic contamination of a specific part of the food product along the food chain. However, for foods considered to be homogeneous (e.g. ground food) or foods prepared by mixing several materials (e.g. mixed salad), inoculation “in depth” would be the best option. Other techniques (e.g. dipping) can be used if it can be demonstrated that the intrinsic properties of the food are not changed. Packaged foods can be removed from their packaging, inoculated and then repacked under similar gas conditions as an

unopened pack (consumer pack), or maintained in its packaging and contaminated through a septum.

(v) Storage conditions: conditions of storage (temperature, time and package) of inoculated foods must comply with the conditions to which the product is most likely to be subjected in the food chain, until its final consumption. Storage time must be equivalent to the shelf life of the food product. Regarding storage temperature, abuse temperature(s) must be considered in order to avoid underestimation of *L. monocytogenes* growth. When the FBO has its own data on the first two stages of the cold chain (from manufacturing to retail, and storage at retail) or national information is available, the use of this information is preferred to select the storage times/temperatures to be used. In that case, the 75th percentile of the observed data should be used. However, if no data are available and the shelf-life of the product is ≤ 21 days the following default conditions must be used: 8 °C for one third of the total shelf life of the product (representing from manufacturing to retail), 12 °C for the second third of the total shelf life of the product (representing storage at retail), and 12 °C for the last third of the total shelf life of the product (representing consumer storage). If the shelf life is > 21 days the following default storage conditions must be used: 8 °C for 7 days (manufacturing to retail), 12 °C for half of the remaining shelf life (storage at retail) and 12 °C for the other half of the remaining shelf life (storage at consumer).

(vi) Analysis of inoculated test units: numbers of *L. monocytogenes* must be determined at least at the beginning of the challenge test and at the end of the shelf life of the product by following the standard method EN ISO 11290-2 for enumeration of *L. monocytogenes*. Additionally, further test points can be included in the experimental design in order to detect potential peaks in growth/inactivation across the shelf life. The use of alternative analytical methods is acceptable when the methods are validated against the

reference method and if a proprietary method, certified by a third party in accordance with the protocol set out in EN/ISO Standard 16140 or other internationally accepted similar protocols, is used. Other methods shall be validated according to internationally accepted protocols and their use authorised by the Competent Authority. Associated microflora of the product must also be enumerated at the start and end of the challenge test following relevant standard methodology for the organisms and food type concerned. Physico-chemical characteristics of the food (at least pH and a_w [alternatively NaCl content or moisture]) must be also determined. In the case of foods packed under modified atmosphere or vacuum packed it is desirable to also monitor gas atmosphere at day “0” and day “end” of the challenge test.

(vii) Analysis of non-inoculated test units: non-inoculated test units must be checked for the presence of *L. monocytogenes* by following the standard method EN ISO 11290-1 for detection of *L. monocytogenes*. Only those batches showing absence of *L. monocytogenes* must be subjected to artificial contamination and challenge testing. Some uninoculated samples can be kept and in case of a positive detection of *L. monocytogenes*, durability studies on naturally contaminated food may be undertaken by determining bacterial numbers over time (under foreseeable storage conditions) by following the EN ISO11290 methodology. Associated microflora of the product and physico-chemical characteristics of the food must be also determined for non-inoculated samples.

(viii) Calculation of growth potential: for each batch, the growth potential (in \log_{10} CFU/g) is estimated as the difference between the median of *L. monocytogenes* numbers at the end of the challenge test and the median of *L. monocytogenes* numbers at the beginning of the challenge test. The highest value obtained among all tested batches is retained as the

growth potential. When the growth potential calculated is $>0.5 \log_{10} \text{CFU/g}$ it is considered that the food product supports growth of *L. monocytogenes*.

2008 European Guidance document		2014 European Guidance Document
Number of batches	At least 3	- If growth probability is low or inter-batch variability of pH and water activity is negligible: 1 batch - If growth probability and inter-batch variability are high: at least three batches
Choice of strains	A mixture of at least 3 strains. One must be a reference strain. The other strains must be isolated from the same or a similar food matrix	A mixture of at least two strains. One of them must be a strain with known growth characteristics. The other/s strain/s can be freely chosen
Inoculum preparation	First subculture in a non-selective medium at a temperature (37 °C) favourable to optimal growth of <i>L. monocytogenes</i> Second subculture at a temperature close to the temperature of the product, in order to adapt the strain to the storage conditions	First subculture in a non-selective medium at an optimal temperature (e.g. 30 or 37 °C) Second subculture at a temperature close to the actual storage temperature of the product
Food inoculation	The inoculum should not exceed 1% of the volume of the test unit The contamination level must be targeted at 50 CFU/g and should not exceed 100 CFU/g Several methods of inoculation can be considered depending on the product tested	Inoculum volume must not exceed 1% of the mass (or volume) of the test unit The contamination level must be targeted at around 100 CFU/g Several methods of inoculation can be considered depending on the product tested
Storage conditions	-When FBO has its own data on the first two stages of the cold chain (from manufacturing to retail, and storage at retail) or there exists national information available, the use of this information is preferred to select the storage time and storage temperature to be used -If no data are available: 8 °C (1/3 of shelf life), 12 °C (1/3 of shelf life), and 12 °C (1/3 of shelf life)	-When FBO has its own data on the first two stages of the cold chain (from manufacturing to retail, and storage at retail) or there exists national information available, the use of this information is preferred to select the storage temperature to be used -If no data are available: 8 °C (1/3 of shelf life), 12 °C (1/3 of shelf life), and 12 °C (1/3 of shelf life)
Analysis of inoculated test units	-Enumeration of <i>L. monocytogenes</i> : at least at the beginning of the challenge test and at the end of the shelf life of the product (3 test units at each time) by following the standard method EN ISO 11290-2	-Enumeration of <i>L. monocytogenes</i> : at least at the beginning of the challenge test and at the end of the shelf life of the product (3 test units at each time) by following the standard method EN ISO 11290-2

	<ul style="list-style-type: none"> -Associated microflora: at the start and end of the challenge test following relevant standard methodology -Physico-chemical characteristics of the food (at least pH and water activity): at least at the beginning and end of the challenge test 	<ul style="list-style-type: none"> -Associated microflora: at the start and end of the challenge test following relevant standard methodology - Physico-chemical characteristics of the food (at least pH and water activity): at least at the beginning and end of the challenge test
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Table 4.1 Major points in the European Technical Guidance documents of 2008 (EC, 2008) and 2014 (EC, 2014).

4.4 Available Literature on Application of Challenge Tests

Since the publication of the first EURL Lm Technical Guidance document for conducting shelf-life studies on *L. monocytogenes* in RTE foods in 2008, a number of studies have been conducted applying challenge tests to determine the ability of a wide range of foodstuffs to support *L. monocytogenes* growth (Table 4.2). However, strict adherence to the guidelines was not observed in most cases — one or more of the criteria were modified. Table 4.2 summarises the main findings of recent research articles on challenge studies.

Reference	Product tested	Number of batches	Choice of strains	Inoculum preparation	Food inoculation	Storage conditions	Microbiological analysis of inoculated test units	Result: Growth/ no growth
(Mejlholm et al., 2012; Mejlholm et al., 2008)	Shrimps in brine and brined and drained shrimps	Not stated	Four <i>L. monocytogenes</i> strains isolated from seafood	Two subcultures at 25 °C for 24 h and 10 °C for 2 to 3 days	Shrimps in brine: 0.1% (vol/wt) of the cocktail (10^5 CFU/ml) Brined and drained shrimps: 1% (vol/wt) of the cocktail (10^4 CFU/ml)	Shrimp in brine, and brined and drained MAP shrimp were stored at 7–8 °C or 15°C	Enumeration of <i>L. monocytogenes</i> according to ISO 11290-2	No growth
(Uyttendaele et al., 2009)	Mayonnaise-based deli-salads, cooked meat products and smoked fish	Not stated	Three <i>L. monocytogenes</i> strains	Subculture for 24 h at 30 °C	Inoculation (0.3-1.0 mL) on the surface (meat and fish product) or as in depth (deli-salad) inoculation in ca. 100 g of food sample to obtain a level of ca. 50–100 CFU/g	Packed samples (air, vacuum or modified atmosphere) kept for their shelf-life at 4 or 7 °C or a variable temperature schedule (1/3 of shelf-life at 4 °C and 2/3 of shelf-life at 7–8 °C as defined by FBO)	Enumeration of <i>L. monocytogenes</i> according to ISO 11290-2 using a reduced detection limit	Growth in 18 of 182 Mayonnaise-based deli-salads. Growth in 61 of 92 meat products. Growth in 12 of 25 smoked fish products
(Garrido et al., 2010)	Sliced ready-to-eat ham	One batch	One <i>L. monocytogenes</i> strain isolated from sliced-	Subculture at 30 °C for 18 h	Inoculation (1 mL) on 25 g of ham to give a concentration between 5 and	Packaged samples stored at 5 °C and 9 °C for 15 days	Enumeration of <i>L. monocytogenes</i> according to ISO 11290-2. Total	Growth

			cooked ham at retail		10 CFU/g		microorganisms, Lactic acid bacteria	
(Skalina and Nikolajeva, 2010)	Shrimp–tomato salad, smoked ham salad and garlic cheese salad	Not stated	Three <i>L. monocytogenes</i> strains (a type strains, one isolated from frozen Pollock loins, and one isolated from sausages)	Two subcultures at 37 °C for 12 h	Inoculation (0.1 mL) of 10 g of salad to obtain a level of 20 to 40 CFU/g	Storage at refrigerator temperatures (3 °C and 7 °C) for 48 h	Enumeration of <i>L. monocytogenes</i> according to ISO 11290-2. TVC, staphylococci and <i>Enterobacteriaceae</i> .	Growth
(Augustin et al., 2011)	Pork pie, smoked herring, sliced cooked ham, cooked chicken, and surimi salad	Pork pie: 1; herring: 4; ham: 7; chicken: 2; surimi salad: 3 batches	One <i>L. monocytogenes</i> strain	Exponential cells: 37 °C - 16h, 37 °C - 8h, 9 °C - 6d (BHI). Starved cells: 30 °C - 24h, 25 °C - 20h (TSBYE), 30 °C - 24h (0.85% NaCl)	Inoculation at the surfaces or homogeneous contamination depending on the type of product	Stored at 8 °C for the shelf life	Enumeration of <i>L. monocytogenes</i> according to ISO 11290-2 Aerobic microorganisms and mesophilic lactic acid bacteria	Growth
(Vermeulen	Smoked	Three	Three <i>L.</i>	Two	Inoculation (200	Storage for 8	Enumeration of <i>L.</i>	Growth

et al., 2011)	salmon	batches	<i>monocytogenes</i> strains (one from cheese, one from pate, one from tuna-deli-salad)	subcultures at 37 °C for 24 h and at 7 °C until the early stationary-phase	µl) of 200 g salmon samples with the cocktail to obtain a concentration of ca. 50 CFU/g	days at 2 °C, followed by 10 days at 4 °C and 13 days at 8 °C as agreed upon with the FBO	<i>monocytogenes</i> according to ISO 11290-2 Total psychotropic count, LAB and <i>Enterobacteriaceae</i>	
(Kang et al., 2012)	Cold-smoked salmon	Not stated	Four <i>L. monocytogenes</i> strains (two from RTE salmon, one from RTE meat and one from a human skin lesion)	37 °C - 18h in BHI, 16 °C - 24h in a defined minimal medium, and 16 °C - 24h in the defined medium	Spreading of the bacterial suspension to achieve a final concentration of 10 ⁴ CFU/g	Vacuum-packed samples were stored at 7 °C for 30 days	<i>L. monocytogenes</i> : spiral plating onto Oxford agar. Lactic acid bacteria	Growth
(Sant'Ana, Barbosa, et al., 2012)	RTE vegetables - escarole, collard green, spinach, watercress, arugula, grated carrot, green salad,	Not stated	Five <i>L. monocytogenes</i> strains isolated from RTE vegetables	Two subcultures at 37 °C for 24 h	Spot inoculation (0.5 mL) of portions of 25 g of each RTE vegetable. Final concentration: 10 ³ CFU/g	Packages (modified atmosphere) stored at three different conditions: I (100% of shelf-life [6 days] at 7 °C), II (30% at 7 °C and 70 % at 15 °C) and III	<i>L. monocytogenes</i> : homogenising 25 g with 225 mL of 0.1% peptone water, following decimal dilutions and inoculation on Oxford selective agar	Growth in escarole, collard green, spinach, watercress, arugula, green salad, and mix for yakisoba. No growth

	and mix for yakisoba					(100% at 15 °C)		in carrots
(Angelidis et al., 2013)	Processed cheese	Two batches.	Three <i>L. monocytogenes</i> strains (one type strain, one clinical isolate, one processed cheese isolate)	Two subcultures at 30 °C for 24 h and 30 °C for 20 h	Spreading of 40 mL of the cocktail over 25 g cheese samples to achieve three levels of inoculation: high (6×10^5 CFU/g); medium (6×10^3 CFU/g); low (10^2 CFU/g)	Modified atmosphere packed samples were stored at 4, 12 or 22 °C	Enumeration of <i>L. monocytogenes</i> according to ISO 11290-2	No growth
(Bernini et al., 2013)	Blue-veined cheeses	Not stated	Five <i>L. monocytogenes</i> strains	Two subcultures at 37 °C for 18 h	Spread inoculation of the rinds of cheese slices to a final level of between 1 log CFU/g and 2 log CFU/g	Storage at either 4 °C or 8 °C for 55 days	Enumeration of <i>L. monocytogenes</i> according to ISO 11290-2 Mesophilic bacteria, yeasts and moulds, LAB, <i>Enterobacteriaceae</i> , coliforms and <i>E. coli</i>	Growth
(Daelman et al., 2013)	Paella	Three batches	Three <i>L. monocytogenes</i> strains (one clinical isolate and two cheese isolates)	Two subcultures at 37 °C for 24 h and at 7 °C until the early stationary-	200 µl of the cocktail dispersed across the surface of the paella until an inoculum level of ca. 50	Modified atmosphere packaged paella stored at 4 °C until the end of shelf life 6 days after purchase	Enumeration of <i>L. monocytogenes</i> according to ISO 11290-2. Total psychrotrophic aerobic count, LAB, yeasts and molds,	Growth

				phase	CFU/g		and <i>B. cereus</i>	
(Everis and Betts, 2013)	Sliced cooked ham	Industry approach: one batch. AFFSA approach: three batches.	Three <i>L. monocytogenes</i> strains (one type strain, one chicken isolate, one meat factory isolate)	Industry approach: 30 °C for 24 h AFFSA approach: 37 °C for 24 h then 5 °C for 7 d	Inoculation (0.1 mL) of 90g through a double-sided foam pad on the outside of the pack Industry approach: level of 10 ³ CFU/g AFFSA approach: level of 50-100 CFU/g	Industry approach: 8 °C for 21 days AFFSA approach: 8 °C for 7 days, then 12 °C for 14 days	Enumeration of <i>L. monocytogenes</i> according to ISO 11290-2	Growth in all cases
(Grassi et al., 2013)	Cheese and mushroom sauces for pasta	One batch	Three <i>L. monocytogenes</i> strains (one from a soft cheese, one from Gorgonzola cheese, one from a meat product)	Not stated	Inoculation of the sauce with 1 mL of the cocktail in order to reach a final concentration of 10 ³ CFU/g	Storage for 31 days at two different temperatures, 4 °C and 8 °C	Enumeration of <i>L. monocytogenes</i> according to ISO 11290-2. Lactic acid bacteria	Growth
(Leong et al., 2013)	Whole and sliced mushrooms , mushroom	Three batches	Three <i>L. monocytogenes</i> strains (one	Subculture at 37 °C for 24 h	Mushrooms: 500 mL of the cocktail poured into 200 g and	Mushrooms: 10 days at 8 and 15 °C Mushroom	Enumeration of <i>L. monocytogenes</i> according to ISO 11290-2	Growth

	casing and substrate		clinical isolate, one persistent strain, one isolate from a mushroom production facility)		mixed for 15 min. Mushroom casing and substrate: 10 mL of cocktail added to 200 g and blended for 5 min	casing and substrate: 20 days at 20 °C		
(Manios et al., 2013)	Romaine lettuce and white cabbage	Two batches	One <i>L. monocytogenes</i> strain (type strain)	Two subcultures at 30 °C for 24 h	Spot-inoculation of 10 g at low (-1 to -0.4 log CFU/g) or high (2 log CFU/g) level	Storage at 8 °C	<i>L. monocytogenes</i> : enumeration on ALOA agar TVC, <i>Pseudomonas</i> spp., <i>Enterobacteriaceae</i>	Growth
(Samapundo et al., 2013)	Cooked ham and white sauce products with reduced NaCl levels	Not stated	One <i>L. monocytogenes</i> strain isolated from cooked ham	Two subcultures at 30 °C for 24 h and 30 °C for 16h followed by incubation at 7 °C for 6-8h	Ham: spreading (50 mL) of 50 g to a level of $\sim 10^2$ CFU/g. White sauce: inoculation of 200 g samples to a level of $\sim 10^3$ CFU/g	Modified atmosphere packaged ham samples and white sauce samples were stored at 7 °C	Enumeration of <i>L. monocytogenes</i> according to ISO 11290-2. Total aerobic and anaerobic plate count, yeasts and moulds, LAB	Growth
(Sant'Ana et al., 2013)	Fresh Lettuce (different varieties) and collard greens	Two batches	Three <i>L. monocytogenes</i> strains isolated from RTE vegetables	Two subcultures at 37 °C for 24 h	Vegetables were soaked in the cocktail for 15 min and then were spun in a sanitised salad	Packages (modified atmosphere) stored at four different conditions: I	<i>L. monocytogenes</i> : homogenizing 25 g with 225 mL of 0.1% peptone water, following decimal dilutions and	Growth

					spinner. Final concentration: 10^1 - 10^2 CFU/g	(100% of shelf life [6 days] at 7 °C), II (70% at 7 °C and 30% at 15 °C), III (30 % at 7 °C and 70 % at 15 °C), IV (100% at 15 °C)	inoculation on Oxford selective agar	
(Wemmenhove et al., 2013)	Gouda cheese	One batch per strain	Three <i>L. monocytogenes</i> strains (one from cheese, one from a cheese factory, and one type strain)	Not stated	<i>L. monocytogenes</i> strains were added to separate batches of milk before cheese making to a final level of approximately 10^7 CFU/ml	Storage at 12 °C for up to 52 weeks	<i>L. monocytogenes</i> : Dilutions in peptone physiological NaCl and plating on PALCAM-selective agar	Increase in numbers during curd formation due to concentration of <i>L. monocytogenes</i> , no growth during ripening

Table 4.2 Methodological approach followed in research publications published to date on challenge tests of *L. monocytogenes* in RTE foods.

4.5 Discussion and Recommendations

Determination of the ability of RTE foods to support the growth of *L. monocytogenes* under reasonably foreseeable storage conditions is very important for FBOs in order to demonstrate compliance with the criteria laid down under European Regulation (EC) No. 2073/2005. However, few of the challenge studies described in literature have strictly followed all aspects of the EURL Guidelines available at the moment of their publication, i.e. the EURL Guidelines of 2008 (Grassi et al., 2013; Kang et al., 2012; Manios et al., 2013; Sant'Ana, Barbosa, et al., 2012; Sant'Ana et al., 2013; Uyttendaele et al., 2009; Vermeulen et al., 2011). While these may be valid research studies, the failure to follow the guidelines means that they are of limited value to the competent authorities, who have the ultimate decision on which category a food fits into, and to FBOs, who cannot use the published results to infer *L. monocytogenes* growth potential and as a consequence are forced into carrying out further expensive challenge studies.

Challenge tests described in the literature so far in a range of meat, seafood, dairy, vegetable and prepared meal products have been conducted following significantly different methodologies. While cocktails of three to five strains have been normally used, a couple of studies have used a single *L. monocytogenes* isolate or have inoculated various strains individually into separate batches. Temperature and time of incubation for the inoculum preparation varied widely among studies. While some authors grew the bacterial strains at an optimum temperature, others performed an adaptation step at low temperatures of ≤ 10 °C. Surface inoculation with a low volume of inoculum was the preferred method of inoculation, but other methods such as deep inoculation, dipping or immersion into the

inoculum suspension, have been also employed. Storage time and temperature following inoculation also differed among studies and were generally agreed with the FBO. Therefore, although European regulation permits the use of scientific literature to estimate *L. monocytogenes* growth ability on particular foodstuffs, the lack of available studies carried out following a harmonised approach and conforming to the EU guidance documents impedes its utilisation with this aim. This lack of harmonisation leads to an information and knowledge void. An additional problem results from challenge tests which have been performed in an industrial context intended for regulatory purposes rather than an academic context. In this case, the information belongs to the FBO conducting the challenge testing and it is unlikely that an FBO would release information concerning testing which concludes that their foodstuffs supports growth. In this way, publically available results from challenge tests performed by industry are limited and may be skewed towards no growth results.

Although the recently published European guidelines are undefined to some extent when describing the methodology to be followed for some of the processes (e.g. inoculation of the food), their application will facilitate a more harmonised approach and will make the comparison of results among laboratories easier. Future investigations analysing the growth of *L. monocytogenes* in particular foodstuffs should therefore be carried out following these guidelines if they are to be valid from a regulatory viewpoint. Nevertheless, studies focused on comparing the proposed methodology with any alternative and simpler methodology are also very valuable (for example, Everis and Betts, 2013) and could contribute to any further revision of the guidance document in the future. For that, both the standard methodology and the alternative methodology must be followed in parallel, and the performance of both

approaches must be compared. Results obtained following an alternative methodology will not be considered valid otherwise.

The recently published (2014) EURL Lm Technical Guidance document for conducting shelf-life studies on *L. monocytogenes* in RTE foods is an improvement on the 2008 version, and is a valuable document which will give FBOs the opportunity to have challenge studies undertaken in a harmonised manner. However, FBOs willing to determine growth potential of *L. monocytogenes* on their products face several major challenges which are not yet resolved. These are mainly regarding expertise and economic constraints. The industry stakeholders (in some cases artisanal food producers and operators of small and medium enterprises) do not usually have the technical knowledge, expertise and resources required to effectively undertake challenge tests. Since they do not usually have adequate laboratory facilities and equipment and will have difficulties with understanding and strictly adhering to the European Technical Guidance document (i.e. they may not have access to scientific literature on *L. monocytogenes* growth or will have difficulties in interpreting the results; they may not be able to use predictive microbiology software or will not have the sufficient knowledge or skills in “food microbiology” to design and execute a challenge test), they need to outsource their studies to independent laboratories.

However, in some countries there are very few laboratories currently offering this service (with optimised and accredited protocols) and the associated expenses are often too high. In addition, the expenses and efforts may be intensified by the need to carry out challenge tests for all different sorts of RTE foods produced and in all the instances when a change in product formulation has occurred. Some countries have a harmonised procedure for the implementation of challenge tests. For instance, France has a network of laboratories accredited for *L. monocytogenes* challenge testing. Such laboratories are

accredited by a working group composed of agents from the competent authority and agents of the national reference laboratory (NRL) for *L. monocytogenes* after the laboratory passes an audit (conducted by the NRL) which assesses the ability of the laboratory to take into account the data from the producer and the technical competence of the laboratory, and after the laboratory gets satisfactory results to an interlaboratory assay of aptitude organized by the NRL for *L. monocytogenes*. An additional major challenge occurring in countries where a harmonised procedure is not in place is lack of coordination between regulatory authorities, FBOs and laboratories carrying out challenge studies. In cases where a flexible interpretation of the European Technical Guidance document occurs, the results of a challenge study may not be considered acceptable by regulatory authorities, which have the final say as to whether the foodstuff is categorised as a RTE food unable or able to support *L. monocytogenes* growth.

4.6 Conclusion

To sum up, some FBOs interested in categorising their RTE foods into RTE foods that do not support the growth of *L. monocytogenes* will not be able to carry out a proper challenge test due to lack of expertise and/or resources. The large variation of methodologies used in previously performed challenge tests may have a large impact on the results and therefore may not be accepted by the competent authorities. There is a clear need for training of FBOs and of independent laboratory employees on the objectives, design, execution and interpretation of results of challenge tests to determine *L. monocytogenes* growth potential on food. The coordinated implementation of national training networks and of networks of accredited laboratories would help to set up the basis for an improved application of the European Guidelines. In addition, the establishment of a dialogue with regulatory authorities prior to the execution of challenge tests is recommended in order to avoid the possibility of the results being rejected due to a misleading design of the study.

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Chapter 5

Challenge Tests Performed on Mushrooms and Smoked Salmon According to Varying Methodologies

Dara Leong Chapter Contributions:

Experimental:

- Major contributor to design of all experiments
- Major contributor to performance of all challenge tests

Results Interpretation:

- Major contributor to data analysis and performed all comparative analysis between experiments

Manuscript Preparation:

- Major contributor to the preparation of the manuscripts

**Determination of *Listeria monocytogenes* growth during mushroom production and distribution
Foods 2013**

**A note on challenge trials to determine the growth of *Listeria monocytogenes* on mushrooms (*Agaricus bisporus*)
Irish Journal of Agricultural and Food Research 2015**

**Examination of *Listeria monocytogenes* in seafood processing facilities and smoked salmon in the Republic of Ireland
Journal of Food Protection 2015**

5.1 Abstract

The common incidence of *L. monocytogenes* in both the RTE food processing environment and in food itself is cause for concern for food business processors. If an RTE food business can prove, by means of a challenge test, that their food does not support the growth of *L. monocytogenes*, then a different regulatory limit applies; 100 CFU/g at the end of shelf-life as opposed to zero incidence in 5 batches. This difference in regulations can have a large impact on food business owners so there is a large demand for challenge testing which will be accepted by the Food Safety Authority. However, currently there are no commercial laboratories offering challenge testing in the Republic of Ireland.

In this study, both mushrooms and smoked salmon were examined by two different challenge test methodologies to evaluate their ability to support the growth of *L. monocytogenes*. The different test methodologies had no impact on the results in smoked salmon; however, a difference was seen in the growth of *L. monocytogenes* in mushrooms. According to Methodology A, with inoculation by dipping, growth was supported; however, growth was not supported in tests performed with Methodology B, with inoculation by spreading. The Irish Food Safety Authority accepted the results of Methodology B (mushrooms do not support the growth of *L. monocytogenes*) and subsequently altered their regulatory testing from absence of *L. monocytogenes* in mushrooms to enumeration. This result underlines the effect that seemingly minor differences in methodologies can have on results and the importance consulting with the relevant authorities to ensure that results of challenge tests will be accepted.

Section 1: Mushrooms

5.2 Introduction

Agaricus bisporus, widely available as commercial mushrooms, are grown commercially in a substrate which is prepared in two or three phases. In phase one, the raw materials which make up the substrate (which may contain wheaten straw, horse manure, poultry manure, and gypsum) are mixed together and composted. The composted phase one substrate is then moved to undergo a further heating step (phase two) at a temperature of 58–59 °C for 8–9 h. Following phase two, *A. bisporus* spawn is added to the substrate. In a phase 3 facility, the mycelium grows through the substrate for several days (usually <19 days) after which a nutrient supplement may be added prior to its dispatch to the mushroom producer. On a mushroom production unit, a 5 cm layer of casing material (a mixture of peat with crushed limestone or spent sugar beet lime and water) is added on top of the substrate. *A. bisporus* mycelium then grows through the casing for several days before mushrooms appear on the surface of the casing and are then harvested (Viswanath et al., 2013). Mushrooms are usually hand-picked and packaged for sale either whole or sliced. Although there have been no reports of listeriosis directly attributed to consumption of mushrooms, various recent surveys have demonstrated that *L. monocytogenes* contamination of mushrooms (Venturini et al., 2011) and mushroom production facilities (Viswanath et al., 2013) can occur. In 2012, a recall was also issued by The Canadian Food Inspection Agency (CFIA) on sliced white mushrooms potentially contaminated with *L. monocytogenes* (Canadian-Food-Inspection-Agency, 2012). However, there is little information available on whether *L. monocytogenes* is capable of growing during mushroom production and distribution.

González-Fandos and co-authors have previously evaluated the potential of *L. monocytogenes* to grow in whole mushrooms packed in two sorts of polymeric films and stored at 4 °C and 10 °C, and they reported growth of between 1 and 2 log units during the first 48 h of incubation. Thus, they recognised simple challenge tests as cost effective tests for small and medium sized production facilities.

Challenge tests were performed using two different methodologies to establish whether mushrooms supported the growth of *L. monocytogenes* and to establish whether the methodology variation had an impact on the results.

5.3 Materials and Methods

5.3.1 Sample Collection and Assessment of *L. monocytogenes* Natural Contamination

According to Methodology A

Whole and sliced mushrooms (three independent batches of each) were obtained from a mushroom supplier in Ireland. All mushroom samples were transported to the laboratory in chilled containers and immediately placed in a cold room at 4 °C and inoculation was performed within 16 h.

Before inoculation, a sample from each batch was removed and tested by enrichment and enumeration for natural contamination with *L. monocytogenes* using the ISO 11290-1 and the ISO11290-2 methods, except that only Agar *Listeria* acc. to Ottavani & Agosti (ALOA) agar (Biomérieux, UK) was used. If any positive indication of *L. monocytogenes*, *i.e.*, round, green colonies with a halo was detected, analysis of that batch was terminated.

5.3.2 Bacterial Strains and Culture Conditions and Inoculum Preparation According to Methodology A

A cocktail of three *L. monocytogenes* strains obtained from the *Listeria* Strain collection at Teagasc Food Research Centre, Moorepark was used for each challenge test. The cocktail comprised a clinical isolate, obtained from University College Hospital Galway (number 757), a persistent strain, isolated from a cheese processing plant (number 6179), and a strain previously isolated from an environmental swab from a mushroom production

facility (number 958). Cultures of each strain were grown separately overnight in 10 mL Brain Heart Infusion (BHI) broth at 37 °C and mixed together to achieve equal numbers of each strain in the mix used for inoculation. Although the European guidelines indicate that overnight cultures should be incubated at similar temperatures to the test conditions (EC, 2005b), studies have shown that incubation of overnight cultures at optimum temperature gave similar results (Everis and Betts, 2013). For each batch, a separate inoculum cocktail was prepared from independent overnight cultures.

5.3.3 Sample Inoculation, Storage Conditions and Cell Enumeration According to

Methodology A

For whole and sliced mushrooms, an inoculum of $\sim 10^3$ CFU/mL was used to give an approximate contamination level of $\sim 10^2$ CFU/g. Three independent batches of mushrooms were inoculated by pouring 500 mL of the inoculum into 200 g of produce, shaking to coat the mushroom surface and leaving to stand for 15 min before pouring off the excess inoculum. Immediately following inoculation, 4 samples were taken from different areas of the batch and cells were enumerated, as described below, to ensure inoculation was even throughout the sample. Inoculated mushroom samples were placed in a plastic tray covered by a polymeric film during storage (to simulate the commercial situation).

Inoculated samples were stored at potentially abusive storage temperatures (whole and sliced mushrooms at 8 °C and 15 °C), and samples were taken at predetermined time intervals for cell enumeration, pH and moisture determination. The length of incubation

depended on the shelf-life of the product; both sliced and whole mushrooms were incubated for 10 days.

Cells were enumerated at regular time points in duplicate for triplicate batches. At each sampling point, samples were removed from each batch (5 g for whole and sliced mushrooms), mixed with Maximum Recovery Diluent (MRD) in a 1:5 dilution and blended in a stomacher for 4 min. Following this, 0.5 mL aliquots were spread in duplicate onto ALOA plates. As required, further serial dilutions were performed in MRD and plated similarly onto ALOA. Plates were incubated at 37 °C for 48 h before cells were counted and cell numbers per gram were calculated. Growth potential was calculated as the difference between the \log_{10} of the numbers at the end of tested and on Day 0.

5.3.4 Additional Analyses According to Methodology A

At each sampling point, the pH and moisture content were also determined. The pH of mushrooms was measured by homogenising a 20 g sample with 12 mL water and a food pH probe was used to measure the pH. To measure moisture content, an aluminium cup was dried for 1 h at 102 °C, then placed in a dessicator for 1 h and weighed. A sample of approximately 1 g from each batch was weighed, correct to 3 decimal places, in the aluminium cup. The sample was dried in a 102 °C oven for 5 h and then placed in a dessicator for 1 h before being weighed again. The weight loss expressed as a percentage of the original weight was calculated and represents the moisture content of the sample.

5.3.5 Sample Collection and Assessment of *L. monocytogenes* Natural Contamination According to Methodology B

Refrigerated, fresh, whole, closed-cap, pre-packaged mushrooms (*Agaricus bisporus*; 3 batches of mushrooms of c.50 mm diameter - second flush mushrooms grown on Phase III substrate) were obtained from a mushroom supplier in Ireland. All mushroom samples were transported to the laboratory by overnight refrigerated courier and tested immediately on arrival at the laboratory.

Before inoculation, a sample from each batch was removed and tested by enrichment and enumeration for natural contamination with *L. monocytogenes* using the ISO 11290- 1 and ISO 11290-2 methods (ISO 1997; 1998), except that only Agar *Listeria* acc. to Ottavani & Agosti (ALOA) agar (Biomérieux, UK) was used. Any batches which tested positive for *L. monocytogenes* were excluded for further testing.

5.3.6 Bacterial Strains and Culture Conditions and Inoculum Preparation According to Methodology B

A cocktail of three *L. monocytogenes* strains was used. The cocktail comprised of 12MOB101LM (a genoserotype II strain from the EU Reference Laboratory *L. monocytogenes* set of reference strains) (EC, 2014), a strain originally isolated from sliced mushrooms (strain 958) and a persistent strain isolated from a cheese processing plant (strain 6179). The three strains were grown independently at 37 °C in brain–heart infusion (BHI) broth for 18–20 h and from this culture were inoculated into BHI and grown to stationary phase at 10 °C for 4 days. Each strain was diluted independently in maximum

recovery diluent (MRD) and the dilutions added together to give 30 ml of inoculation solution of approximately 10^3 CFU/ml.

5.3.7 Sample Inoculation, Storage Conditions and Cell Enumeration According to

Methodology B

Inoculum (30 μ l) was spread lightly on the cap of each mushroom with a loop, not damaging the mushroom, to give approximately 100 CFU/g. The mushrooms were dried in laminar air flow for 10 min in a Petri dish. Incubation was at 8 °C for 2 days followed by 12 °C for 4 days. The mushrooms were packed in trays of about 10 mushrooms and wrapped with film as normally used for mushrooms for retail. Triplicate analysis of each batch involving analysis of an individual mushroom chosen at random from the pack at each sampling time on days 0, 2 and 6 was undertaken.

A total of 5 g of mushroom cap from where the inoculum was spread was cut and analysed. The size of the piece cut was consistent as the mushrooms were of a consistent size. *Listeria* analysis was performed by ISO 11290-1 for detection and ISO 11290-2 for enumeration (plating on ALOA only) were used. The \log_{10} of *L. monocytogenes* numbers was calculated at each sampling time. Growth potential was calculated as the difference between the \log_{10} of the numbers on day 6 and 0. If the numbers (in any of the replicates) were 0.5 log higher on day 6 than on day 0, growth was possible.

5.3.8 Additional analyses According to Methodology B

Water activity was analysed using an Aqua Lab water activity meter (Series 3 TB, Decagon Devices Inc., Pullman, WA, USA.), total bacterial count (TBC) was measured by spreading appropriate dilutions on Plate Count Agar (plates were incubated for 3 days at 30 °C) and the pH was measured at each time point by inserting a pH probe (Hanna pH 211, Woonsocket, RI, USA.) into the mushroom.

5.4 Results

5.4.1 Mushroom Challenge Tests According to Methodology A

Growth of *L. monocytogenes* was seen at 15 °C in mushrooms with no substantial difference between sliced and whole mushrooms throughout the majority of the experiment (Figure 5.1).

No lag phase was observed in either whole mushrooms or sliced mushrooms, while maximum growth rates were 0.04 and 0.06 log CFU/g/h, respectively. Final population densities were 7.3 and 9.5 log CFU/g, respectively. Challenge testing was also carried out in whole mushrooms at 8 °C, and similar increases in bacterial numbers were observed (e.g., in the first 24 h of incubation, the numbers of *L. monocytogenes* increased by more than 0.5 log₁₀, which is assumed as the boundary to define whether a food is capable of supporting the growth of *L. monocytogenes*, and final population densities reached 7.9 log CFU/g (data not shown). No remarkable differences were seen in pH or moisture content between sliced and whole mushrooms (Figure 5.2).

The pH increased from approximately 7.0 at the beginning of the experiment to approximately 7.8 at the end of the experiment in both sliced and whole mushrooms. This increase occurred after approximately 150 h of incubation. The moisture content of mushrooms increased from 92% at the initiation of the experiment to 95% at the end of the experiment (Figure 5.2).

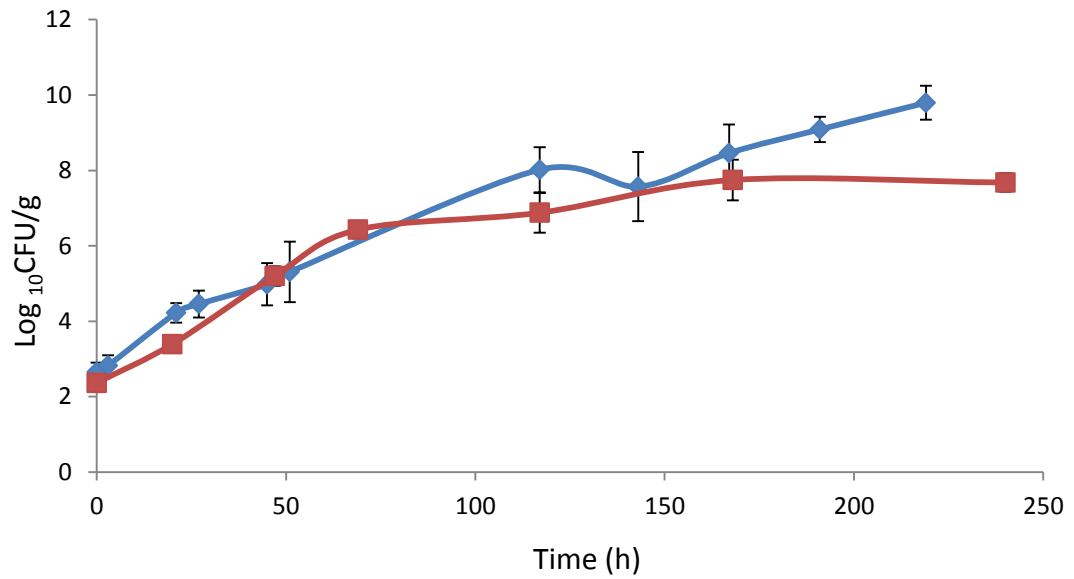


Figure 5.1: Logarithmic growth of *L. monocytogenes* in sliced and whole mushrooms in challenge tests according to Methodology A. The data points used for growth of *L. monocytogenes* observed are mean values of the duplicate analysis and triplicate batches. Growth of *L. monocytogenes* observed in sliced mushrooms (●) growth of *L. monocytogenes* observed in whole mushrooms (■).

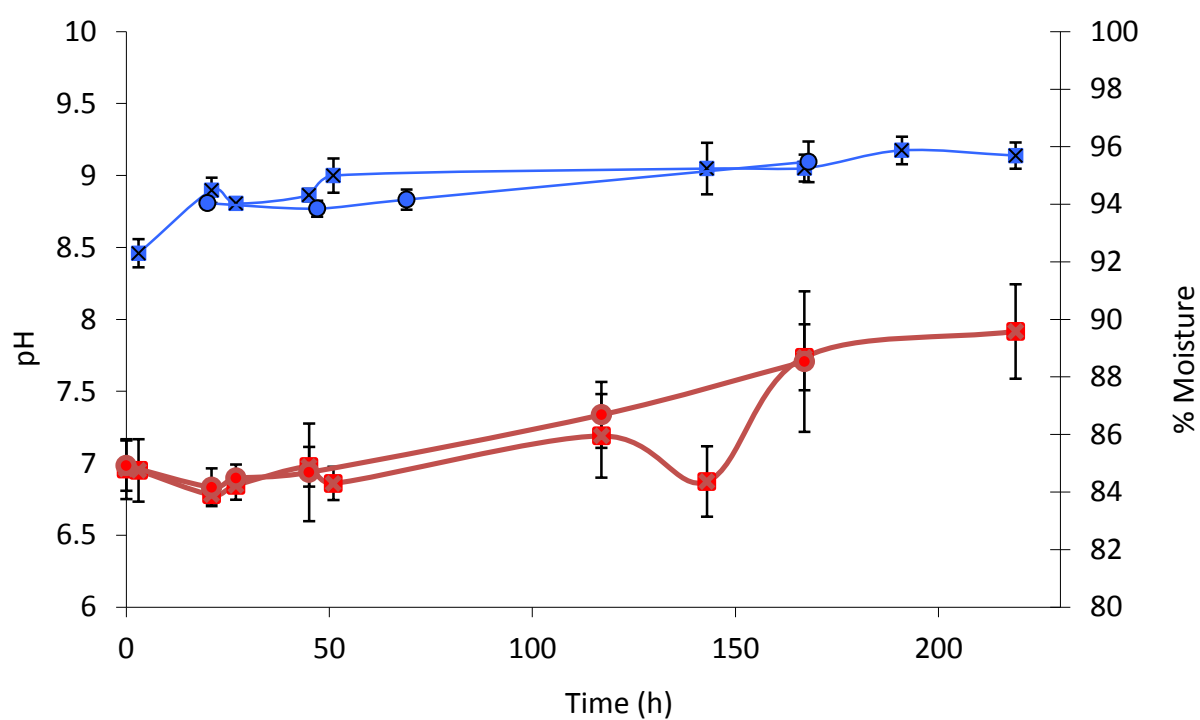


Figure 5.2: Evolution of pH and % moisture in sliced and whole mushrooms during challenge tests performed according to Methodology A. The data used are mean values of the duplicate analysis and triplicate batches. pH of sliced mushrooms (■), pH of whole mushrooms (●), % moisture of sliced mushrooms (■), % moisture of whole mushrooms (●).

5.4.2 Mushroom Challenge Test According to Methodology B

The variation in the inoculum used in each batch was <0.5 log (data not shown) and the level of inoculation was $1.26 \log \pm 0.49$, $1.06 \log \pm 0.29$ and $2.06 \log \pm 0.55$ CFU/g for each batch, respectively. For 8 of the 9 replicates, there was a decrease in the numbers of *L. monocytogenes* over the incubation time. For the 9th replicate, there was an increase, but the increase was $0.4 \log$ CFU/g on day 6, indicating no growth in any of the replicates (Figure 5.3). The TBC increased over time (Table 5.1). The addition of the inoculum had little impact on the pH or the water activity (Table 5.1).

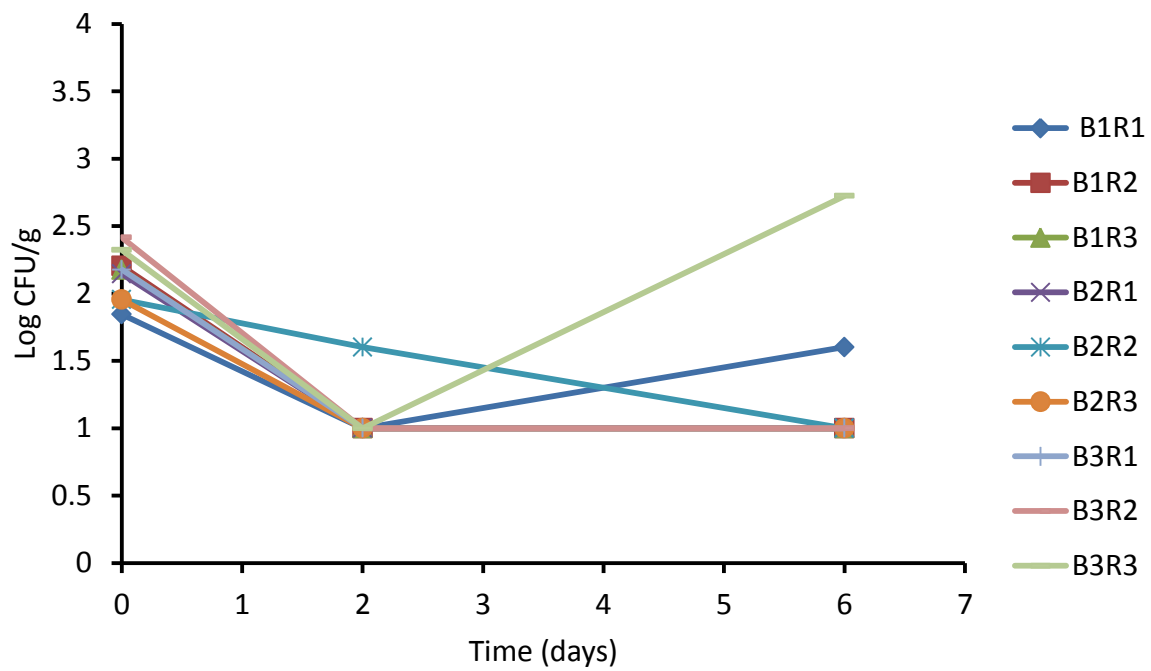


Figure 5.3. The behaviour of *L. monocytogenes* as determined on challenge tests mushrooms according to Methodology B. B1R1; batch 1 replicate 1, and so on.

Time (days)	Log ₁₀ TBC,		
	CFU/g*	pH	Water activity
Uninoculated	Not determined	6.87 ± 0.05	.996 ± .001
0	2.82 ± 0.53	6.86 ± 0.02	.994 ± .002
2	4.77 ± 0.20	6.92 ± 0.05	.993 ± .005
6	5.94 ± 0.89	6.78 ± 0.03	.996 ± .003

* TBC, CFU/g: total bacterial count, colony forming units/g

Table 5.1: Total bacteria count (TBC) in mushrooms used in challenge tests performed according to Methodology B.

5.5 Discussion

The increase in *L. monocytogenes* numbers in both whole and sliced mushrooms without an observable lag phase seen in challenge testes performed according to Methodology A is in agreement with a previous study by González-Fandos et al., who found a 1 and 2 log increase in *L. monocytogenes* numbers in mushrooms within 48 h at 4 °C and 10 °C incubation, respectively (González-Fandos et al., 2001). However, these authors observed that, after 48 h, the bacterial population remained relatively stable during incubation from 3 to 8 days, and after day 8 of incubation they reported a decline in bacterial numbers of around 1–2 log units. They linked these findings to the growth characteristics of the competitive microflora present in mushrooms.

On the contrary, the results of the challenge tests performed according to Methodology A showed a fast and progressive increase in bacterial numbers until final population densities of 7.3 and 9.5 log CFU/g were reached in whole mushrooms and sliced mushrooms, respectively. Hoelzer and co-authors have recently reviewed the available data on *L. monocytogenes* growth dynamics in produce, and it is important to note that, in terms of growth rate and maximum population density described here, fresh mushrooms would be among the commodities which support growth of *L. monocytogenes* to a higher extent (Hoelzer et al., 2012). The growth rate and maximum population densities attained in the current trials were similar to those described for broccoli and asparagus and higher than those reported for the rest of the produce analysed by Hoelzer and co-authors.

Although the growth rates observed were similar in whole mushrooms and sliced mushrooms, maximum population densities were higher in sliced mushrooms (9.5 log CFU/g vs. 7.3 log CFU/g). The differences in *L. monocytogenes* growth between sliced and whole

mushrooms can be attributed to the increased available nutrients and available attachment surface of the sliced mushrooms. In addition, breakage of tissues during slicing may also make more nutrients available for use by *L. monocytogenes*.

In contrast, it was concluded that in the challenge trial conducted by methodology B, mushrooms did not support the growth of *L. monocytogenes*. In fact, the numbers of *L. monocytogenes* decreased in most cases. The inoculation had little effect on the pH or water activity values and the TBC values were not sufficiently high enough to inhibit the growth of *L. monocytogenes*.

Hoelzer, Pouillot and Dennis (2012) suggested that fresh mushrooms would be among the commodities that support the growth of *L. monocytogenes*. On the other hand, Chikthimmah, LaBorde and Beelman (2007) showed that mushrooms do not support the growth of *L. monocytogenes*. However, in the experiment listed above, the EURL guidance document was not followed. The different inoculation and preparation methods and varying storage temperatures and conditions used may have influenced the results. The recently published EURL *Lm* Technical Guidance Document for conducting shelf-life studies on *L. monocytogenes* in ready-to-eat foods (EC, 2014) is a valuable document that will give food business operators the opportunity to have challenge studies undertaken in a timely and cost-effective manner and will guarantee a more homogeneous approach, making the comparison of results among laboratories easier.

Although a recall of sliced white mushrooms occurred in Canada in 2012 (Canadian Food Inspection Agency 2014), no illnesses were reportedly associated with the recall and the grower/producer decided to recall the product voluntarily due to the possibility of contamination. Similarly, a recall of sliced crimini mushrooms occurred in Canada in 2014 (Canadian Food Inspection Agency 2014) with no associated illnesses reported. No *L.*

monocytogenes outbreaks have historically been associated with mushrooms, although contamination occurs sporadically (FSAI 2006).

Viswanath et al. showed that mushrooms can be contaminated with *L. monocytogenes* with an occurrence of 1.2%, although there was no quantification of the level of contamination (Viswanath et al., 2013). As the regulations allow <100 CFU/g if the food cannot support growth of *L. monocytogenes*, the results of the current study demonstrate that quantification of *L. monocytogenes* on mushrooms is necessary as numbers below 100 CFU/g will not increase during the shelf-life and therefore, mushrooms with <100 CFU/g would be within European regulation.

The differences between the tests performed according to Methodologies A and B may seem relatively minor but, in tests performed on mushrooms, had a major impact on the results. The major differences in the tests performed in this study included storage temperature and method of inoculation. According to both the previous and current guidelines (EC, 2008, 2014) storage conditions should be based on national information if available or, if no data is available, a storage temperature of 8 °C for the first third of shelf-life and 12 °C for the remaining shelf life to simulate temperatures of shipping, display and storage. In Methodology A, temperatures of 8 °C and 15 °C were tested to represent abuse temperature throughout the shelf-life. However, in Methodology B, 8 °C for 2 days followed by 12 °C for 4 days was used as indicated. A method of inoculation is not specified in either set of guidelines. Instead, several methods can be considered based on the product being tested (EC, 2008, 2014). In Methodology A, a liquid inoculum was added to the product by dipping, allowed to coat the surface and then poured off. In Methodology B, an inoculum (1 % of the volume of the product as indicated in the guidelines) was surface spread on the product. It is reasonable that a combination of these two differences in methodologies

caused the differing results seen in challenge tests performed in mushrooms. The dipping method of inoculation used in Methodology A may have caused an increase in the growth of *L. monocytogenes* due to the leaching and redistribution of the nutrients present in the mushrooms or by rinsing native microflora from the mushroom surface resulting in a less competitive environment for *L. monocytogenes* growth. The higher temperature used for the second incubation stage in Methodology A compared to Methodology B may also have positively impacted *L. monocytogenes* growth and so contributed to the differing results.

The Irish Food Safety Authority accepted Methodology B as a valid challenge test which can be used for regulatory purposes i.e. the result that mushrooms do not support the growth of *L. monocytogenes*. Consequently, the Irish regulatory limits for *L. monocytogenes* in mushrooms changed from needing absence in 5 units/batches to a limit of 100 CFU/g at end of shelf-life and therefor the regulatory testing policy changed from testing for presence/absence to enumeration of *L. monocytogenes* in mushrooms.

Section 2: Smoked Salmon

5.6 Introduction

The process of cold-smoking fish varies between producers, and individual processes are frequently kept confidential. Generally, cold smoking involves the application of smoke at a low temperature, approximately 37 °C, a temperature insufficient to inactivate *L. monocytogenes* (Tang et al., 2013). The process of cold smoking of fish has been shown to reduce but not completely eliminate *L. monocytogenes* present in the raw material (Cheng et al., 2015; Porsby et al., 2008). However, additional factors, such as brining, change in pH levels and water availability, and the addition of phenolic compounds, can have a significant effect on the behaviour of *L. monocytogenes* in cold smoked salmon. The presence of *L. monocytogenes* in raw fish itself varies, with surveys on *L. monocytogenes* contamination in raw salmon showing a prevalence commonly ranging from 0 to 10% (Thimothe et al., 2004a). The smoking process also varies from producer to producer, so both the initial numbers of *L. monocytogenes* and the actual reduction of *L. monocytogenes* are impossible to calculate, and the risk to the consumer remains.

Challenge tests evaluating the growth of *L. monocytogenes* in smoked fish artificially inoculated at 50 to 100 CFU/g have been performed by Uyttendaele et al. They showed significant growth of *L. monocytogenes* in 12 of 25 samples stored for 3 to 4 weeks at 4 °C (Uyttendaele et al., 2009). This apparent ability of smoked fish to support the growth of *L. monocytogenes*, combined with the common presence of the bacterium in both the raw material itself and in the food processing environment, indicates that a potential risk to public health from the consumption of smoked fish exists and should be examined more closely.

Challenge tests were performed using two different methodologies in order to establish whether smoked salmon supported the growth of *L. monocytogenes* and to establish whether the change in methodologies had an impact on the results.

5.7 Materials and Methods

5.7.1 Sample Collection and Assessment of *L. monocytogenes* Natural Contamination According to Methodology A

Three batches of cold smoked salmon were obtained from a local supplier and refrigerated until inoculation which took place within 24 hours. Prior to inoculation, the salmon was analysed for *L. monocytogenes* by the International Organization for Standardization 11290-1 standard.

5.7.2 Bacterial Strains and Culture Conditions and Inoculum Preparation According to Methodology A

Three strains of *L. monocytogenes* from the *Listeria* Strain collection at Teagasc Food Research Centre, Moorepark were used for the inoculation cocktail. The cocktail comprised a clinical isolate, obtained from University College Hospital Galway (number 757), a persistent strain, isolated from a cheese processing plant (number 6179), and a strain previously isolated from smoked salmon sample (number 1123). Independent cultures of each strain were prepared in 10 ml Brain Heart Infusion (BHI) broth, incubated overnight at 37 °C, diluted and mixed together to achieve equal numbers of each strain in the mix used for inoculation.

5.7.3 Sample Inoculation, Storage Conditions and Cell Enumeration According to Methodology A

Inoculation was performed by adding the three-strain cocktail of inoculum to the surface of the smoked salmon, the salmon was then allowed to air dry in a laminar flow hood for 10 minutes before excess inoculum was removed. Smoked salmon pieces were inoculated at an approximate bacterial concentration of 10^2 CFU/g onto three independent batches. Samples were vacuum-packed and incubated at 8°C representing an abuse temperature.

5.7.4 Additional Analyses According to Methodology A

At time intervals *L. monocytogenes* numbers were determined as well as pH and % moisture. pH and % moisture analyses was performed as described in 5.3.4. Independent triplicate analyses of each batch were performed.

5.7.5 Sample Collection and Assessment of *L. monocytogenes* Natural Contamination

According to Methodology B

Cold-smoked salmon was obtained from two separate cold-smoked salmon producers, each using a different cold-smoking process. The salmon was collected from the producer and transported to the laboratory in a cool box at 4 °C and inoculated the following day, after overnight storage at 4 °C. Prior to inoculation, the salmon was analysed for *L. monocytogenes* by the International Organization for Standardization 11290-1 standard. Any *L. monocytogenes* positive batches were excluded from the challenge test.

5.7.6 Bacterial Strains and Culture Conditions and Inoculum Preparation According to

Methodology B

A three-strain mixture of *L. monocytogenes* was used for inoculation. The three strains used were 12MOB101LM (a genoserotype II strain from the EU Reference Laboratory *L. monocytogenes* set of reference strains) (EC, 2014), 1123, and 1319 (both of which were serotype 1/2a and isolated from smoked salmon throughout this study). Strain 1123 is a widespread strain, isolated at four different smoked salmon processing facilities. For each inoculum, cultures of each strain were independently grown overnight at 37 °C in 10 ml of BHI, and these cultures were used to inoculate a fresh 10 ml BHI volume that was incubated at the early stationary phase at 11 °C for 3 days. The three strains were then diluted independently to 10⁴ CFU/ml and mixed in equal volumes.

5.3.7 Sample Inoculation, Storage Conditions and Cell Enumeration According to

Methodology B

For each batch of smoked salmon, 10 pieces of 30 or 50 g, depending on the size of the batch, were prepared. Each piece was inoculated by surface spreading a 1% volume of the prepared inoculum (300 µl for a 30 g piece or 500 µl for a 50 g piece) to give a final contamination level of about 10^2 CFU/g. The pieces of salmon were allowed to dry for 10 min in a laminar air flow cabinet and then individually vacuum packed and incubated at 8 °C for 1 week and 12 °C for the remainder of the experiment, approximately 25 days in total, to mimic the actual shelf life of the product.

Immediately after inoculation, the salmon was tested to ensure even distribution of the inoculum. For each batch, one piece of salmon was analysed in triplicate. A 5 g sample was cut from a piece of salmon and homogenized in a sterile bag with 20 ml of maximum recovery diluent. An aliquot (0.25 ml) of this was spread plated onto each of two agar *Listeria* (according to Ottaviani and Agosti) plates that were incubated for 48 h at 37 °C. Subsequently, every 2 to 3 days during incubation, enumeration (in triplicate) of *L. monocytogenes* was undertaken, as described previously, performing further serial dilutions (1:10) of the sample in maximum recovery diluent, when necessary.

5.7.8 Additional Analyses According to Methodology B

The water activity (a_w) and pH of the salmon were also determined at all sampling points as in 5.3.8.

5.8 Results

5.8.1 Smoked Salmon Challenge Tests According to Methodology A

Growth of *L. monocytogenes* occurred on smoked salmon in all three replicate batches. The numbers increased considerably from about 10^2 CFU/g to 10^6 CFU/g after about 480 hours (Figure 5.4). The maximum growth rate was 0.010 log /g/h, with no lag time. In the first 48 hours of incubation, the numbers of *L. monocytogenes* increased in more than 0.5 log₁₀, assumed as the boundary to define whether a food is able to support the growth of *L. monocytogenes*. The pH and % moisture were slightly affected throughout incubation (Figure 5.5).

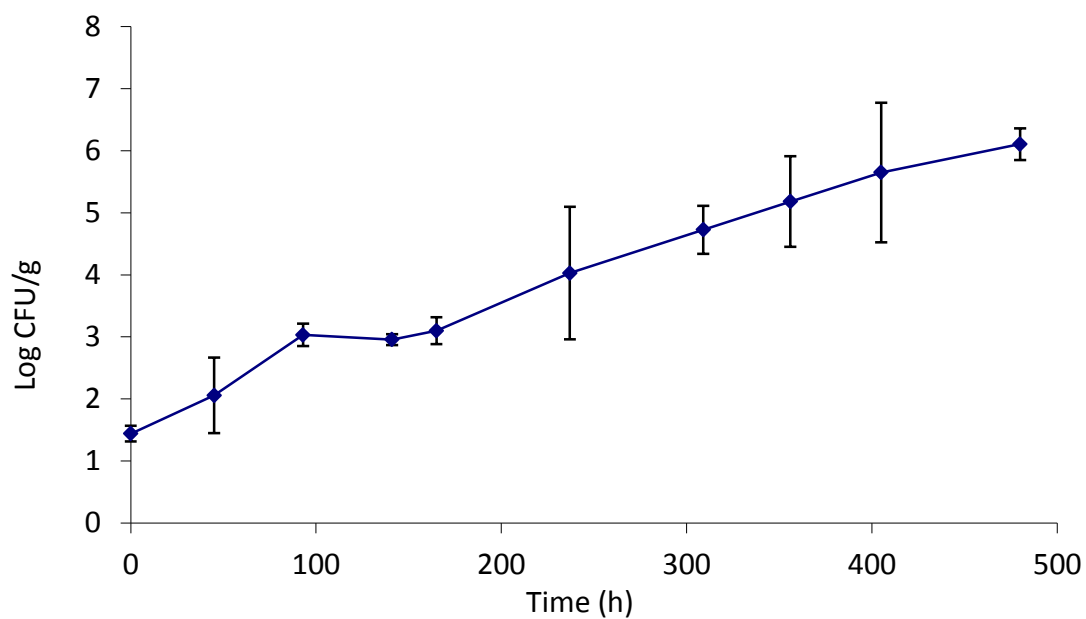


Figure 5.4: Growth of *L. monocytogenes* in smoked salmon in challenge test performed according to Methodology A. Data points represent the average of all three batches tested.

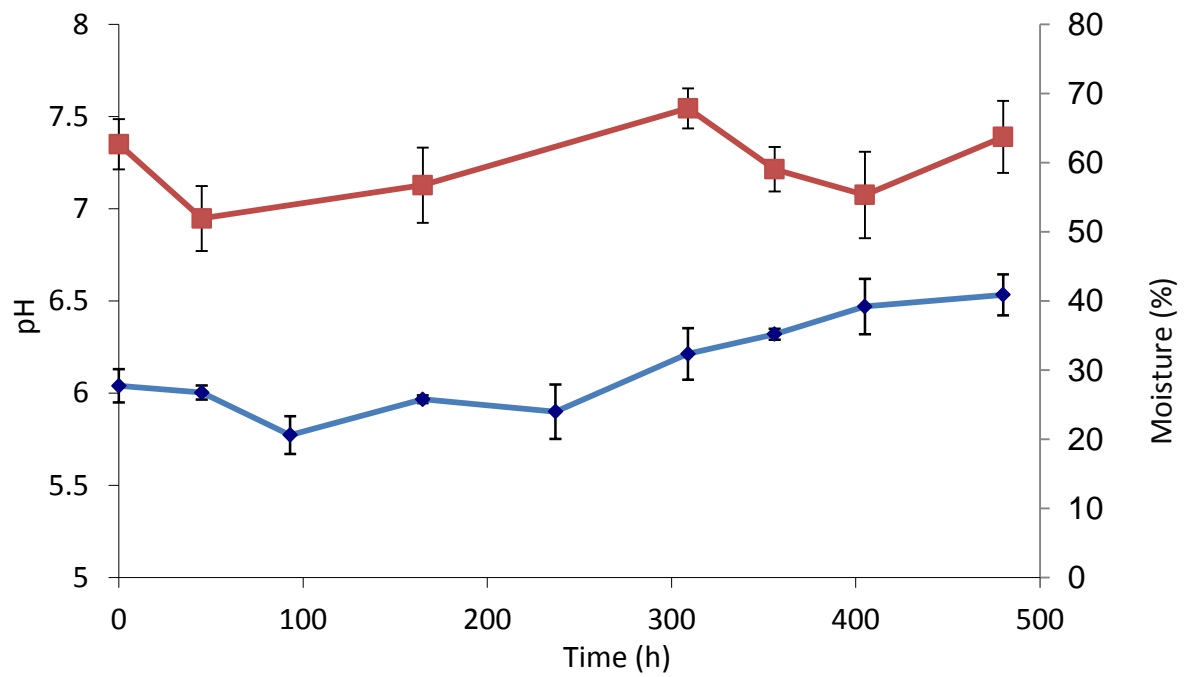


Figure 5.5: pH (●) and percentage moisture (%) (■) smoked salmon during challenge tests according to Methodology A, average of three batches.

5.8.2 Smoked Salmon Challenge Tests According to Methodology B

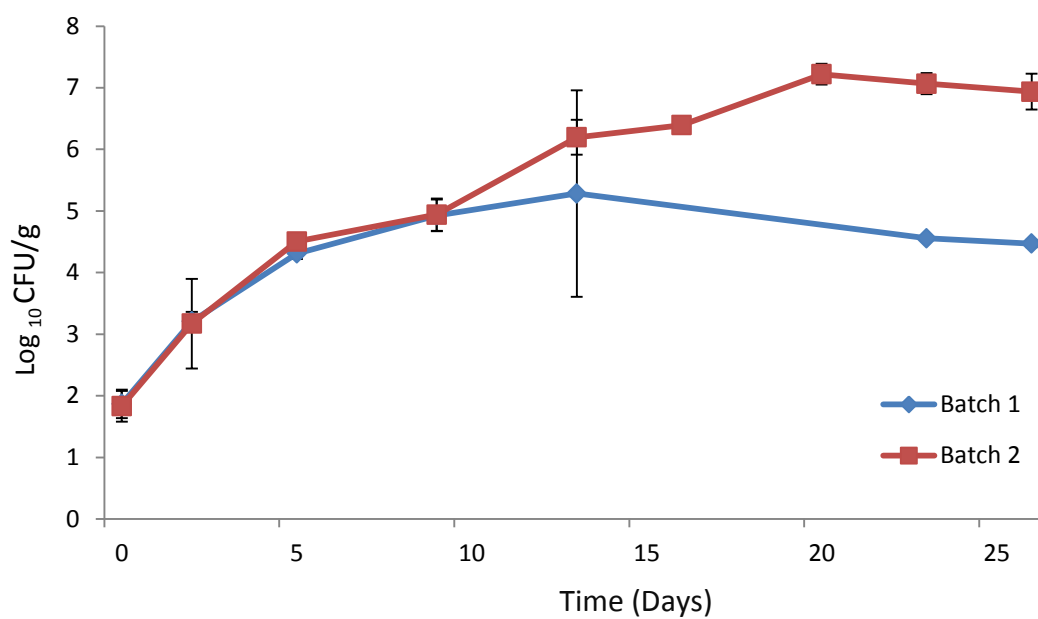
Two batches of smoked salmon were obtained from facility 1; both were free from *L. monocytogenes* and were used in challenge tests. Three batches of smoked salmon were obtained from facility 2; however, only two of these batches could be used for challenge tests as batch 1 was found to be naturally contaminated with *L. monocytogenes*. The initial numbers of *L. monocytogenes* immediately after inoculation were at $1.87 \log \text{CFU/g} \pm 0.23$ and $1.75 \log \text{CFU/g} \pm 0.36$ in batches from facility 1, and $2.15 \log \text{CFU/g} \pm 0.16$ and $2.23 \log \text{CFU/g} \pm 0.30$ in batches from facility 2. In smoked salmon from facility 1, a significant increase in *L. monocytogenes* numbers was seen in both batches (Figure 5.6A). However, in both batch 1 and 2, the initial increase was followed by a decrease.

Overall, the growth potential for each batch from Facility 1 was calculated at $2.57 \log \text{CFU/g}$ for batch 1 and $5.15 \log \text{CFU/g}$ for batch 2 over the course of the challenge test. Growth potential can be defined as the difference between *L. monocytogenes* numbers at the end of the challenge test and *L. monocytogenes* numbers at the day of inoculation to represent the potential for bacterial growth in an artificially contaminated food under foreseeable conditions of incubation, transportation, and storage at the retail and consumer level. An increase in numbers $0.5 \log \text{CFU/g}$ is taken as potential for growth (EC, 2014).

In smoked salmon from facility 2, a significant increase in *L. monocytogenes* numbers was also seen in both batches (Figure 5.6B). The growth potential for each batch was calculated at $2.94 \log \text{CFU/g}$ for batch 2 and $1.63 \log \text{CFU/g}$ for batch 3 over the course of the challenge test. For each facility, the highest growth potential value is retained for all batches tested to represent the worst-case scenario (EC, 2014): $5.15 \log \text{CFU/g}$ for facility 1

and 2.94 log CFU/g for facility 2. In all cases, the growth potential is higher than 0.5 log CFU/g, the limit over which the food is said to support the growth of *L. monocytogenes*. Over the course of the challenge testing, the pH in smoked salmon batches from facility 1 increased from 6.19 ± 0.08 to 6.24 ± 0.04 . The pH in batches from facility 2 decreased from 6.12 ± 0.05 to 6.03 ± 0.01 . The a_w in batches from facility 1 decreased from 0.967 ± 0.001 to 0.957 ± 0.001 . The a_w in batches from facility 2 increased from 0.946 ± 0.023 to 0.967 ± 0.000 . Both the pH and a_w variation was well within the limits of *L. monocytogenes* growth for the entire challenge testing period (Figure 5.7).

A



B

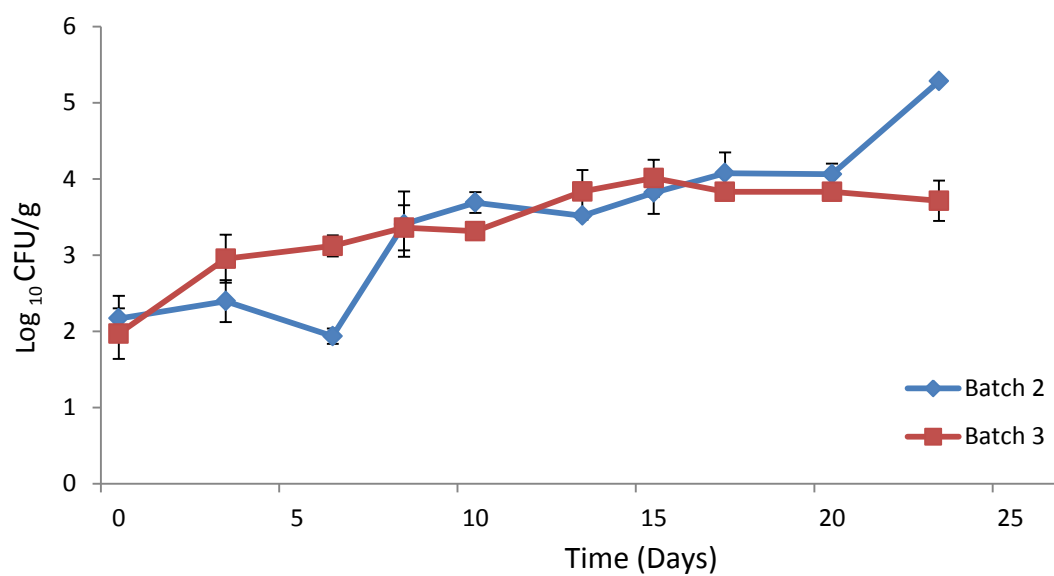


Figure 5.6: Numbers of *L. monocytogenes* in smoked salmon during challenge testing according to Methodology B with a three-strain mixture of isolates; (A) Smoked salmon obtained from Facility 1, (B) Smoked salmon obtained from Facility 2.

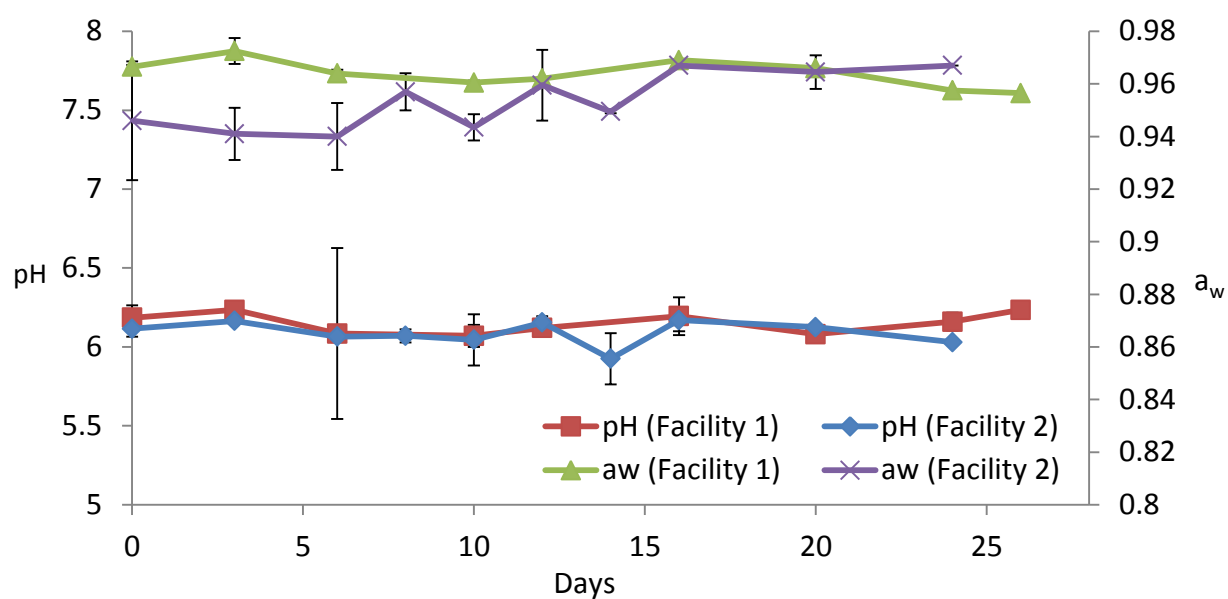


Figure 5.7: pH and a_w of smoked salmon over the course of challenge test undertaken to determine the ability of smoked salmon to support growth of *L. monocytogenes* performed according to Methodology B.

5.9 Discussion

Owing to the confidential nature of individual cold-smoking processes, previously performed challenge tests on different products cannot be used to predict whether a particular kind of cold-smoked salmon will support the growth of *L. monocytogenes*. Cold-smoked salmon obtained from two separate suppliers clearly supported the growth of *L. monocytogenes* under all the conditions tested.

In tests performed according to Methodology A, growth of *L. monocytogenes* occurred on smoked salmon. There was no lag time and the growth rate was 0.010 log increase/h, which would be considered a fast growth rate on food. The final numbers reached were high at about 10^6 CFU/g.

In tests performed according to Methodology B, the growth potential in both cases was well in excess of the cut-off (0.5 log CFU/g) listed by the EU Reference Laboratory guidance document for conducting shelf life studies on *L. monocytogenes* in RTE foods as the growth potential necessary to define growth as being supported (EC, 2014). The varying *L. monocytogenes* numbers in batch 1 and 2 from facility 1 (an initial increase in numbers followed by a decrease) underlines the importance of testing at several time points during incubation instead of only at the start and end point of the experiment. An absence of midpoint enumeration could obscure the maximum growth rate and could have hidden the highest level of *L. monocytogenes* reached. However, in this case, the decrease was not large enough to cause a major disturbance to the overall conclusion that cold-smoked salmon supports *L. monocytogenes* growth. It is notable that changes to the challenge test methodology (namely inoculation method and storage temperature, see section 5.5) had no impact on the smoked salmon results.

5.10 Conclusion

In conclusion, the changes in methodologies used to perform challenge tests can have an effect on the results. In all cases tested, smoked salmon supported the growth of *L. monocytogenes* therefore smoked salmon producers should be vigilant in attempts to monitor and prevent contamination to reduce the risk to public health. However, in challenge tests performed on mushrooms, variations in methodologies yielded different results, likely due to changes in incubation temperatures and method of inoculation. This result indicates that, even when tests are performed within the confines of the appropriate guidance documents, variations can occur and affect the result of the challenge tests. To ensure that results will be recognised by the relevant authorities, consultation should be made concerning the appropriate methodology for individual foods before conducting the testing.

5.11 References

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Chapter 6

Risk Factors Associated with the Occurrence and Persistence of *Listeria monocytogenes*

Dara Leong Chapter Contributions:

Experimental:

- Performed sample analysis and PFGE on samples from 32 food businesses
- Performed PFGE analysis on isolates from 32 food businesses
- Performed data collection from 32 food businesses

Manuscript Preparation:

- Major contributor to the manuscript preparation

Manuscript in preparation for submission in Food Control

6.1 Abstract

Although it is generally accepted that management practices can have an effect on *L. monocytogenes* occurrence and persistence, there has been little work done to directly align and statistically analyse this link. This study aligned the *L. monocytogenes* occurrence and persistence in 32 food processing facilities with a survey on management practices. Facilities were monitored bimonthly by ISO-11290-1 for the presence of *L. monocytogenes*. The isolates from this sampling were further analysed by Pulsed Field Gel Electrophoresis (PFGE) in order to identify persistence, i.e. the continued presence of an indistinguishable pulsotype for a period of 6 months or more. Food processors completed a questionnaire which included fifty-eight questions distributed into four major sections: general data on the food business; HACCP plan, food safety and quality management; manufacturing environment; and cleaning and disinfection procedures. Statistical analysis was performed to correlate the survey answers with the occurrence and persistence seen. Two factors were seen to significantly affect *L. monocytogenes* occurrence following both the univariate analysis and a final multivariate logistic regression model: (1) separation of Personal Protection Equipment (PPE) in high and low risk areas and (2) training performed by other workers. One factor was shown to have a greater effect on *L. monocytogenes* persistence, although not significantly so, following a final multivariate logistic regression model: use of a power hose. In order to help prevent and control contamination, this study recommends the separation of PPE in high and low risk areas, training to be performed by management and discontinuing the use of power hoses.

6.2 Introduction

Listeria monocytogenes is the causative agent of the foodborne illness listeriosis which is generally contracted by ingestion of a contaminated food. The mild form of listeriosis presents with typical food poisoning symptoms which can include nausea, diarrhoea, vomiting and fever. The severe form of listeriosis occurs when *L. monocytogenes* crosses the epithelial barrier of the gastrointestinal tract and causes further infection in the body. Although the incidence of listeriosis is relatively low (1,642 reported cases in the EU in the year 2012), the severe form of listeriosis is particularly dangerous to the immunocompromised and can have a mortality rate of up to 30% (EFSA, 2014). Many varieties of food (including cheeses, meats and fruits) have been implicated in listeriosis outbreaks and ready-to-eat (RTE) foods are commonly the cause of outbreaks as there is no cooking step (or other antimicrobial step) which would kill any *L. monocytogenes* present (Cartwright et al., 2013; McCollum et al., 2013; Rychli, Muller, et al., 2014). Therefore, RTE food producers need to ensure that any food produced is free of *L. monocytogenes* and EU regulations stipulate the need for sampling of both the food produced and the production environment in order to monitor the presence of the bacterium (EC, 2005a).

L. monocytogenes is a particularly resilient bacterium and can survive many stresses which would be encountered in a food processing facility, including low pH, high salt and low (even refrigeration) temperatures. It can also form biofilms so can be very difficult to completely remove from a facility (Nakamura et al., 2013). As *L. monocytogenes* is commonly present in the environment (soil, water, silage etc.) and can be carried by both humans and animals, it may be almost impossible to keep a facility completely *L.*

monocytogenes-free. Food producers need to be vigilant in order to maintain as low an occurrence of *L. monocytogenes* as possible in the facility, and to reduce further contamination events in the food production facility which may endanger the food being produced.

Persistent contamination may occur as a result of insufficient cleaning/sanitising as sporadic contamination is not properly removed and so *L. monocytogenes* strains remain in the facility and become persistent. The presence of persistent *L. monocytogenes* strains poses a more serious threat than that of sporadic contamination as the likelihood of a food becoming contaminated increases significantly due to the constant presence of the bacterium. The presence of persistent *L. monocytogenes* strains have been well documented in several food processing facilities (Leong et al., 2014; Vongkamjan et al., 2013).

Although it is likely that some *L. monocytogenes* strains may have developed some genetic advantages which help them to persist in the food processing environment (Ryan et al., 2010), it is widely accepted that the action (or inaction) of food business operators has a major influence on the occurrence of contamination (Aury et al., 2011; Pouillot et al., 2015). Many factors, which are in the control of the food business operator, can influence *L. monocytogenes* occurrence including correct cleaning/sanitation regimes, the presence of a correctly audited HACCP plan, the correct training of workers, and the separation of different production areas in the facility and the creation of critical care areas. This study aims to align the management practices, especially hygiene management and cleaning and disinfection practices, of RTE food processing facilities with two years of *L. monocytogenes*

occurrence data in order to assess whether any particular practices increase or reduce the incidence and persistence of *L. monocytogenes*.

6.3 Materials and Methods

6.3.1 *L. monocytogenes* Monitoring Programme

From January 2013 to December 2014, samples from a total of 32 food processing facilities from various food sectors were analysed bimonthly for the presence of *L. monocytogenes*. The Food Business Organisations (FBOs) were located throughout the Republic of Ireland and represented various food sectors, i.e. dairy (10 facilities), meat (4 facilities), seafood (11 facilities), fresh-cut vegetable (5 facilities) and miscellaneous (2 facilities). Sampling was carried out as described by Leong et al. (2014). Briefly, every second month, FBOs sent approximately 8 samples by overnight courier to the laboratory using the sampling packs provided. Samples consisted of 6 environmental swabs, taken from areas around the food processing facility, and 2 food samples.

6.3.2 Isolation of *L. monocytogenes* from Environmental and Food Samples

Samples were analysed for the presence of *L. monocytogenes* by the ISO11290-1 method. 100 ml of half-Fraser broth (VWR, Ireland), was added to the bags containing the swabs and were incubated at 30 °C for 24 h. Then, a 0.1 ml aliquot was transferred to 10 ml of full Fraser broth, which was further incubated at 37 °C for 48 h. In addition, a 0.02 ml aliquot of the 1st enrichment broth was plated on to Agar *Listeria* acc. to Ottavani & Agosti (ALOA) agar plates (Biomérieux, UK), which were incubated at 37°C for 48 h. After incubation, the 2nd enrichment broths were streaked onto ALOA agar plates, which were

again incubated at 37 °C for 48 h. For liquid or food samples, 225ml of half-Fraser broth was added to 25 ml or 25 g of the food samples. Samples were then homogenised in a stomacher (Colworth Stomacher 400) for 4 min, and incubated at 30 °C for 24 h. Subsequently, analysis of samples was continued by following the same approach used for environmental samples. After incubation, ALOA agar plates were examined for typical *L. monocytogenes* colonies (blue-green colonies with halo), and, if present, two characteristic *L. monocytogenes* colonies for each positive enrichment were purified by streaking first onto Brilliance *Listeria* Agar (BLA) plates, which were incubated at 37 °C for 48 h, and then onto Brain Heart Infusion (BHI) agar plates, which were incubated at 37 °C for 24 h. Isolates were then stored in cryoinstant tubes (VWR, Ireland) and kept at -20 °C prior to use.

6.3.3 PFGE Typing

PFGE analysis was carried out according to the International Standard Pulse Net protocol (PulseNetUSA, 2009). Two restriction enzymes, *Apal* and *Ascl*, were used for the analysis. Isolate similarity dendrograms were generated using Bionumerics version 5.10 software (AppliedMaths, Belgium), by the unweighted pair group method with arithmetic mean (UPGMA) with tolerance and optimization settings of 1%, as previously described (Leong et al. 2014). Facilities where the isolation of *L. monocytogenes* strains with indistinguishable PFGE profiles were obtained for more than 6 months apart during the two-years sampling period were considered to have persistence.

6.3.4 Survey of Food Business Organisations

Correlation between risk factors and occurrence of *L. monocytogenes* was evaluated with the help of a questionnaire which was completed by the FBOs. A group of scientists and advisors, with a general expertise in food safety/microbiology, contributed to the design and implementation of the questionnaire. Each of the thirty-two participating food businesses was provided with a questionnaire that was completed by the management or quality control team. This questionnaire included fifty-eight questions distributed into four major sections: general data on the food business; HACCP plan, food safety and quality management; manufacturing environment; and cleaning and disinfection procedures. Sixty-nine percent of the questions were closed (i.e. only a “Yes” or “No” answer was possible), and thirty-one percent were open-ended.

6.3.5 Survey Analysis

In a first step, open ended answers were converted to yes/no responses. Descriptive analysis was performed in order to identify variables with a large number of missing observations or a low variability that might be of little value for further investigations. This resulted in the selection of thirty-seven variables that were included in the regression analyses (Table 5.1).

Subject	Factors
General data on food businesses (n = 2)	Production sector: dairy, meat, seafood, vegetable, miscellaneous Number of employees: >20, ≤20
HACCP plan, food safety and quality management (n = 8)	Monitoring of Critical Control Points: yes, no Own testing of food for presence of <i>L. monocytogenes</i> : yes, no Positive results in foods tested for presence of <i>L. monocytogenes</i> : yes, no Frequency of food testing for presence of <i>L. monocytogenes</i> : intense, no intense Own testing of swab samples for presence of <i>L. monocytogenes</i> : yes, no Positive results in swab samples tested for presence of <i>L. monocytogenes</i> : yes, no Frequency of swab testing for presence of <i>L. monocytogenes</i> : intense, no intense Hand/glove swabs carried out on staff: yes, no
Manufacturing environment (n = 8)	Close to a farm: yes, no Building work in the last year: yes, no Segregation of high risk and low risk areas: yes, no Separate Personal Protective Equipment (PPE) worn in high and low risk areas: yes, no Junctions between walls and floor sloped: yes, no Openings in the walls/ceilings: yes, no Floors made of alkali and acid resistant material: yes, no Floors sloped to avoid water stagnation: yes, no
Cleaning and disinfection procedures (n = 19)	Frequency of drain cleaning: intense, no intense Deep cleaning: yes, no Who cleans: business personnel, external C&D training by the chemical provider: yes, no C&D training by the management team: yes, no C&D training by other workers: yes, no 6-steps C&D process: yes, no Preclean step: properly done, not done or improperly done Use of power hose: yes, no Use of chlorinated compounds: yes, no

Use of Quaternary ammonium compounds: yes, no
Use of alkaline compounds: yes, no
Use of acid compounds: yes, no
Use of peracetic acid: yes, no
Rinsing with hot water/solutions: yes, no
Drying step: done, not done
Testing of sanitiser concentration before use: yes, no
Testing of swab samples to evaluate efficacy of the C&D process: yes, no
Frequency of swab testing to evaluate efficacy of the C&D process: intense, no intense

Table 6.1: Summary of variables derived from the questionnaire and assessed as potential risk factors for *L. monocytogenes* occurrence and persistence in food businesses. Frequency of swab testing converted to intense (at least bimonthly) and no intense (less than bimonthly).

For the logistic regression analysis, a food business was considered positive for *L. monocytogenes* occurrence when more than 1.5% of food and environmental samples tested during the two-years sampling period were positive for *L. monocytogenes* detection. *L. monocytogenes* ubiquitous nature makes common the sporadic detection of *L. monocytogenes* in food processing environments during a long-term study. For that reason, food businesses with less than 1.5% positive samples were considered negative for *L. monocytogenes* occurrence. The outcome variable “occurrence” was thus dichotomous: positive business vs. negative business. PASW Statistics 18 (SPSS Inc., Chicago, USA) was used to perform the logistic regression analysis in a two-stage procedure. In the first stage, the *L. monocytogenes* status of the food businesses was related to each explanatory variable by means of a univariate analysis (Chi-square test). Variables associated with the outcome variable (Pearson χ^2 -test, $p < 0.25$) were selected for further analysis in a multivariate logistic model. When two of the selected explanatory variables were highly correlated (Pearson correlation coefficients with a $p < 0.05$), only one was used in the multivariate analysis (i.e. the one with a lower p -value in the univariate analysis). A multivariate logistic model was constructed in PASW Statistics 18 using a backward elimination approach based on the Wald test until a model with all variables significant at $p < 0.05$ was obtained. Once the main effects model was obtained, two-way interactions amongst independent variables remaining in the model were tested by addition into the model and retained if they were significant ($p < 0.05$). Goodness of fit of the final model was assessed using the following tests: Pearson χ^2 , deviance and Hosmer-Lemeshow (Hosmer Jr et al., 2013).

The same approach was also followed to assess through multivariate logistic regression, the risk factors associated with persistence of *L. monocytogenes* in the processing environment of collaborating food businesses. In this case, a food business was considered positive for *L. monocytogenes* persistence when *L. monocytogenes* strains with undistinguishable PFGE profiles were isolated for more than 6 months apart during the two-years sampling period. On the contrary, food businesses free of *L. monocytogenes* or for which strains with undistinguishable PFGE profiles were not isolated for more than 6 months apart were considered negative for *L. monocytogenes* persistence. The outcome variable “persistence” was therefore also dichotomous: business with persistence vs. business without persistence.

In a final stage, a multiple linear regression analysis was carried out using the square root of the actual occurrence of *L. monocytogenes* at each food business as the dependent variable. Explanatory variables associated with the outcome variable (Pearson χ^2 -test, $p < 0.25$) were included in the analysis. A stepwise approach was used to include only significant ($p < 0.05$) variables in the final model. Predictive values of independent variables were analysed by computing the coefficient estimates (B values), p -values for the B values and 95% confidence intervals for the B values.

6.4 Results

The occurrence of *L. monocytogenes* among the thirty-two food business operators for 2013 and 2014 are shown in Table 6.2. Occurrence varied from 0% in many companies to as high as 24.3% in some of the FBOs tested. In terms of persistence, ten of the food businesses tested showed isolates of *L. monocytogenes* strains that had undistinguishable PFGE profile for more than 6 months apart during the two-years sampling period. These results were used to determine a correlation between the risk factors identified and either occurrence or persistence.

A questionnaire consisting of fifty-eight questions, mainly focused on hygiene management and cleaning and disinfection practices, was distributed among collaborating food business operators. Several of the explanatory variables derived from the questionnaire were initially discarded due to their low variability (n=21). For instance, the majority of food businesses have a certified and audited HACCP program (29/32), a pest control system implemented (32/32), specifications available for raw materials and finished products (32/32), changing rooms for workers, staff, visitors or contractors for changing into working clothes before entering the production area (31/32), visible "Wash Hands" instructions available before entering the production areas (31/32), washbasins available and suitably located (32/32), Personal Protection Equipment (PPE) available (32/32), suitable footwear available for workers (31/32), hand sanitiser provided at critical areas (31/32), and clean and maintain drains regularly (32/32).

A first univariate analysis (Pearson χ^2) of risk factors associated with occurrence of *L. monocytogenes*, defined as the isolation of *L. monocytogenes* in >1.5% of the samples

analysed at the food business during the two-years sampling scheme, revealed eight variables with $p < 0.25$ (Table 6.2). These included frequency of internal swab testing, existence of openings, floors sloped, wearing of separate PPE for low and high risk areas, training on cleaning and disinfection by other workers, training on cleaning and disinfection by members of the management team, use of chlorine agents and rinsing with hot water or solutions. The percentage of businesses with *L. monocytogenes* occurrence as a function of these explanatory variables and the distributions of occurrence for each of them are shown in Table 5.3 and Figure 5.1 respectively.

A correlation analysis showed that the variables floor sloped and hot rinsing (Pearson correlation coefficient of -0.455), and training by workers and training by management (Pearson correlation coefficient of -0.429) were correlated. Therefore, the variables floor sloped and training by management (the ones with a higher p -value in the univariate analysis) were not included in the multivariate logistic regression model.

Multivariate logistic regression with backward elimination of non-significant variables retained only two of the tested variables (with $p < 0.05$) in the final model (Table 5.4). The Hosmer and Lemeshow goodness-of-fit test showed that the model fitted the data adequately (Hosmer and Lemeshow Chi-square = 0.812 with 2d.f., $p = 0.666$). The probability of a food business being contaminated by *L. monocytogenes* increased when training on cleaning and disinfection was carried out by other fellow workers (O.R. = 8.4; 95% CI: 1.32-53.44; $p = 0.024$) instead of by the chemical providers or members of the management team. On the other hand, the probability decreased when food businesses had separate PPE for low and high risk areas (O.R. = 0.099; 95% CI: 0.013-0.754; $p = 0.026$). Final checks on the model showed no significant interactions between variables in the model.

Facility No:	Type:	% Positive (2013)	% Positive (2014)	Persistence
1	Seafood	5	0	No
2	Meat	3.3	6	Yes
3	Dairy	2.6	0	No
4	Seafood	0	0	No
5	Dairy	2.6	0	No
6	Seafood	0	3.3	No
7	Seafood	6.25	7.5	No
8	Seafood	0	0	No
9	Vegetable	10.8	22.2	Yes
10	Vegetable	2.5	0	No
11	Seafood	0	0	No
12	Dairy	0	0	No
13	Dairy	2.6	3.1	No
14	Dairy	0	1.7	No
15	Seafood	10	3.1	Yes
16	Seafood	0	0	No
17	Dairy	5.6	9.5	No
18	Meat	0	2.1	No
19	Vegetable	3.1	4.1	No
20	Meat	15	6.25	Yes
21	Miscellaneous	0	2.1	No
22	Dairy	5.6	24.3	Yes
23	Dairy	0	0	No
24	Dairy	10	8.3	Yes

25	Seafood	0	0	No
26	Vegetable	5	2.2	No
27	Vegetable	20.5	12.5	Yes
28	Meat	n/a	13.6	No
29	Seafood	12	1.8	Yes
30	Dairy	24.5	0	Yes
31	Miscellaneous	35.2	21	Yes
32	Seafood	n/a	23.3	No

Table 6.2: Occurrence and persistence of *L. monocytogenes* according to facility and year.

n/a = not applicable, FBOs did not participate in testing in 2013.

Definition of variables	Level	No. of businesses	% of positive businesses*	p-value
Frequency of swab testing	Intense	15	73.33333333	0.131
	No intense	17	47.05882353	
Openings	Yes	6	83.33333333	0.185
	No	26	53.84615385	
Floor slope	Yes	26	53.84615385	0.185
	No	6	83.33333333	
PPE for high & low risk areas	Yes	19	42.10526316	0.016
	No	13	84.61538462	
Training by other workers	Yes	18	77.77777778	0.016
	No	14	35.71428571	
Training by management	Yes	4	25	0.135
	No	28	64.28571429	
Use of chlorine agents	Yes	14	78.57142857	0.051
	No	18	44.44444444	
Hot rinsing	Yes	5	100	0.044
	No	27	51.85185185	

Table 6.3: Variables identified as being significantly associated ($p < 0.25$) in the univariate analysis of risk factors for *L. monocytogenes* occurrence in food businesses. * A positive business is one with >1.5% positive samples during the two-years sampling.

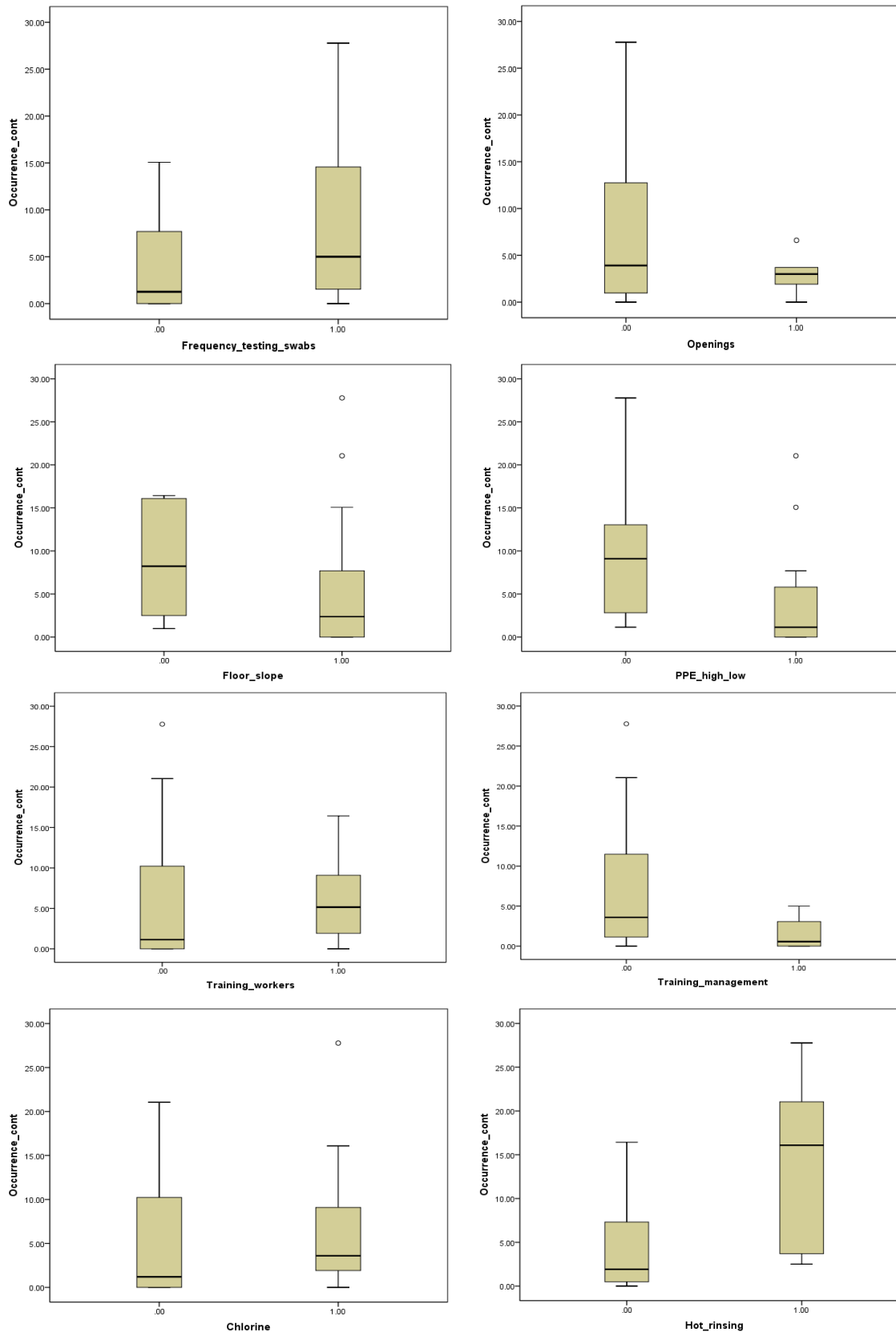


Figure 6.1: The distribution of occurrence for each of 8 explanatory variables devised by a first univariate analysis (Pearson χ^2). .00= No, 1.00= Yes except in frequency of swabbing, 1.00= Intense, at least bimonthly, .00= Not intense, less than bimonthly.

Definition of variables	Level	Odds ratio	95% CI (O.R.)	p-value
PPE for high & low risk areas	Yes	0.099	0.013-0.754	0.026
	No	-	-	
Training by other workers	Yes	8.4	1.32-53.44	0.024
	No	-	-	
Model: p = 0.002; Nagelkerke R ² = 0.430				

Table 6.4 Final multivariate logistic regression model for factors associated with *L. monocytogenes* occurrence in food businesses.

Univariate analysis (Pearson χ^2) of risk factors associated with persistence of *L. monocytogenes*, defined as the isolation of *L. monocytogenes* strains with undistinguishable PFGE profile for more than 6 months apart during the two-years sampling period, revealed seven variables with $p < 0.25$ (Table 6.5). These included monitoring of critical control points (CCPs), segregation of low and high risk areas, wearing of separate PPE for low and high risk areas, use of power hose in cleaning, use of quaternary ammonium compounds (QACs), inclusion of a dry step in a six-steps cleaning process, and testing of sanitiser's concentration before application. The percentage of businesses with *L. monocytogenes* persistence as a function of these explanatory variables is shown in Table 6.2.

A correlation analysis showed that the variables monitoring of CCPs, segregation of low and high risk areas and wearing of separate PPE in low and high risk areas were correlated (Pearson correlation coefficients: 0.444 for monitoring of CCPs and segregation of low and high risk areas; 0.389 for monitoring of CCPs and wearing of separate PPE in low and high risk areas; 0.473 for segregation of low and high risk areas and wearing of separate PPE in low and high risk areas). Variable segregation of low and high risk areas was also correlated to variable dry step (Pearson correlation coefficient of 0.385). Variables monitoring of CCPs and segregation of low and high risk areas (correlated to variable wearing of separate PPE in low and high risk areas and with higher p -values in the univariate analysis) were not included in the multivariate logistic regression model. Multivariate logistic regression with backward elimination of non-significant variables did not retain any variable at $p < 0.05$ in the final model. However, information on the most accurate model is provided in Table 5.6. The Hosmer and Lemeshow goodness-of-fit parameters were: Hosmer and Lemeshow Chi-square = 2.448 with 5d.f., $p = 0.784$. According to that model the

probability of a food business showing *L. monocytogenes* persistence increased when a power hose was used for cleaning (O.R. = 6.663; 95% CI: 0.783-56.733; $p = 0.083$). On the other hand, the probability decreased when a dry step was included in a six-steps cleaning process (O.R. = 0.254; 95% CI: 0.029-2.182; $p = 0.212$) and when testing of sanitiser's concentration was carried out before application (O.R. = 0.193; 95% CI: 0.025-1.478; $p = 0.113$).

L. monocytogenes occurrence (% of positive samples) did not follow a normal distribution, as shown by the Kolmogorov-Smirnov test statistic ($p = 0.003$), the Shapiro-Wilk test statistic ($p = 0.000$), and by visual inspection of the histogram (Figure 6.2(A)). However, square root of the occurrence was closer to normality as shown by visual inspection of the histogram (Figure 6.2(B)). Indeed, the Kolmogorov-Smirnov test statistic ($p = 0.200$) and the Shapiro-Wilk test statistic ($p = 0.063$) indicated that the square root of the occurrence followed a normal distribution. A multiple linear regression analysis using the square root of the actual occurrence of *L. monocytogenes* as the dependent variable provided a model with R^2 of 0.345 where the variables wearing of separate PPE in low and high risk areas and rinsing with hot water or solutions were included at $p = 0.013$ and $p = 0.024$, respectively (Table 6.7). According to the linear model, *L. monocytogenes* occurrence increased when rinsing with hot water or hot solutions was carried out ($B = 1.506$; 95% CI: 0.214 – 2.799) and decreased when separate PPE was worn in low and high risk areas ($B = -1.237$; 95% CI: [-2.192]–[-0.281]).

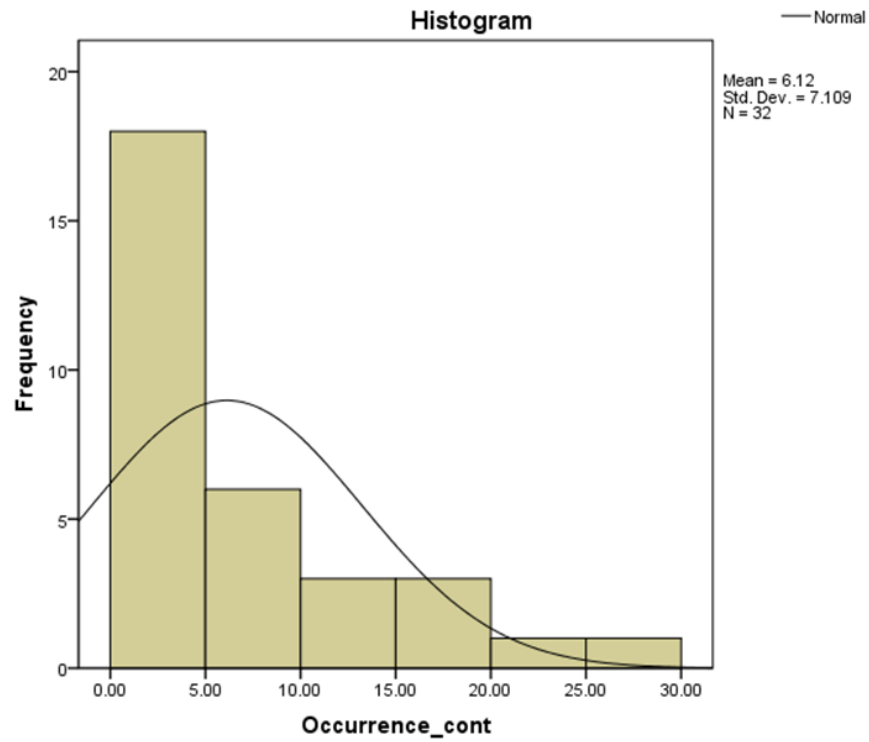
Definition of variables	Level	No. of businesses	% of businesses with persistence*	p-value
Monitoring of CCPs	Yes	29	27.5862069	0.16447
	No	3	66.66666667	
Segregation of high risk & low risk areas	Yes	21	23.80952381	0.2096
	No	11	45.45454545	
PPE for high & low risk areas	Yes	19	21.05263158	0.13244
	No	13	46.15384615	
Use of power hose	Yes	8	50	0.18645
	No	24	25	
Use of QACs	Yes	11	18.18181818	0.24838
	No	21	38.0952381	
Dry step in C&D	Yes	11	18.18181818	0.24838
	No	21	38.0952381	
Testing of sanitiser concentrations	Yes	13	15.38461538	0.10924
	No	19	42.10526316	

Table 6.5 Variables identified as being significantly associated ($p < 0.25$) in the univariate analysis of risk factors for *L. monocytogenes* persistence in food businesses.* Persistence is defined as the identification of the same PFGE type >6 months apart.

Definition of variables	Level	Odds ratio	95% CI (O.R.)	p-value
Use of power hose	Yes	6.663	0.783-56.733	0.083
	No	-	-	
Dry step in C&D	Yes	0.254	0.029-2.182	0.212
	No	-	-	
Testing of sanitiser concentrations	Yes	0.193	0.025-1.478	0.113
	No	-	-	
Model: p = 0.071; Nagelkerke R ² = 0.277				

Table 6.6 Final multivariate logistic regression model for factors associated with *L. monocytogenes* persistence in food businesses.

A



B

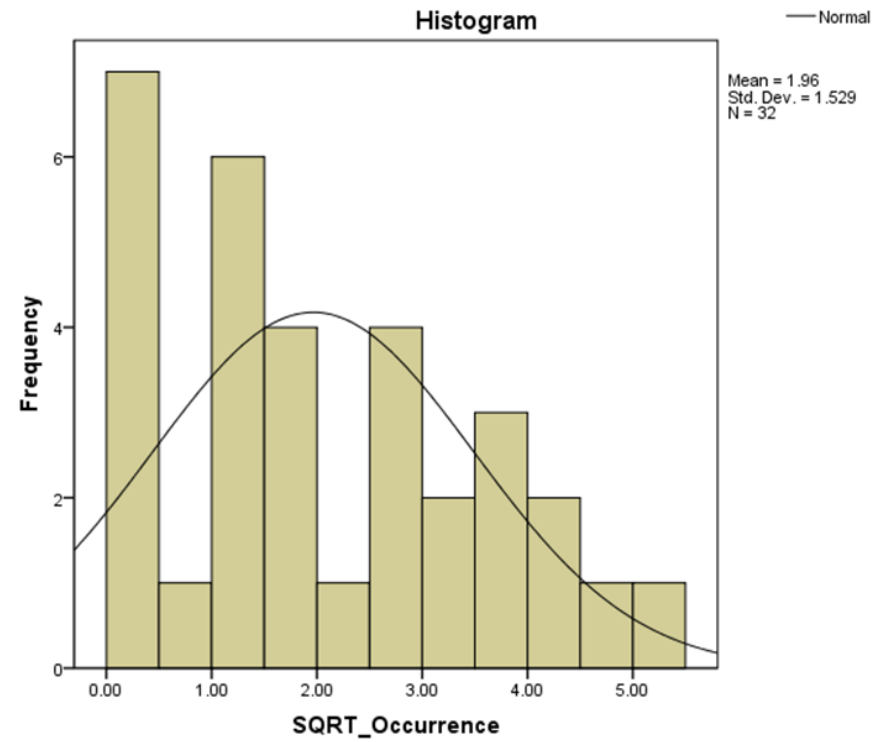


Figure 6.2 Histogram depicting *L. monocytogenes* occurrence (% of positive samples).

Definition of variables	Standard coefficients (Beta)	B (SE)	95% CI (B)	t-score	p-value
PPE for high & low risk areas	-0.404	-1.237 (0.467)	(-2.192) – (-0.281)	-2.648	0.013
Hot rinsing	0.364	1.506 (0.632)	0.214 – 2.799	2.384	0.024
Model: F = 7.638; p = 0.002; R ² = 0.345					

Table 6.7 Final multiple linear regression model for factors associated with *L. monocytogenes* occurrence in food businesses (square root of the occurrence was used as dependent variable).

6.5 Discussion

The general prevalence of *L. monocytogenes* seen in this study (6.0%) is in agreement with the latest E.U. survey data (EFSA, 2014). The fact that many of the questions included in the survey yielded a harmonious answer from all facilities involved indicated that previous regulatory advice and education has been successful in many areas. Where the answers to the questions were harmonious, such factors were omitted from the analysis as they clearly had no impact on *L. monocytogenes* occurrence. There were still some aspects of food production facility management which could be improved to help prevent and control contamination. By correlating *L. monocytogenes* occurrence and persistence data with management practices, several risk factors were identified.

Although several factors were identified as being correlated with *L. monocytogenes* occurrence following the univariate analysis of risk factors (Table 6.3), the majority of these factors were not statistically significant ($P > 0.05$). Only two factors, separation of PPE in high and low risk areas and training being performed by management, were both shown to be significant ($P = 0.016$) factors in the reduction of *L. monocytogenes* occurrence. Further analysis with a final multivariate logistic regression model correlated with occurrence of *L. monocytogenes* was performed and similarly, the same two risk factors were the only factors shown to be statistically significant, $P = 0.026$ for separation of PPE in high and low risk areas and $P = 0.024$ for training being performed by management (Table 6.4). The separation of PPE in high and low risk areas is a well-established factor in contributing to the prevention of contamination and was shown in this study to decrease occurrence. As several of the facilities involved in this study are small farm-adjacent facilities, some

facilities may be housed in converted farm buildings. Therefore, these buildings were not designed for the purpose of food production and may not have the option of fully separating high and low risk areas which is an important hurdle in the spread of contamination, although in light of this study, greater efforts towards separation would help. Training of workers being performed by management, rather than by other workers, was shown to decrease occurrence of *L. monocytogenes*. This result is not surprising when you consider the more vested interest management/owners have in ensuring training is performed to a higher standard in order to comply with regulations and prevent contamination which can lead to large losses for business owners.

Using a univariate analysis for factors associated with *L. monocytogenes* persistence, no factors were shown to be statistically significant. However, following analysis using a final multivariate logistic regression model with *L. monocytogenes* persistence, one factor was seen to have a greater effect than the others analysed; the use of a power hose, $P = 0.083$ (Table 6.7). Again, this is a previously recognised risk area in terms of *L. monocytogenes* occurrence. The use of a power hose is generally thought by food producers to be effective due to the obvious visible cleaning effect. However, the use of a power hose creates aerosols which can take a long time to dissipate and settle. This frequently redistributes *L. monocytogenes* from lower, more commonly contaminated areas such as floors and drains, and allows it to become airborne and then reach much higher areas including food preparation and storage areas such as tables and shelves (Kang and Frank, 1990). In this way, power hoses can also contribute to persistence as this redistribution of strains can allow *L. monocytogenes* strains to access areas which may not usually be subject to *L. monocytogenes* contamination and so may not be cleaned with the same intensity or

frequency as other areas. This can allow strains to create an ecological niche from which the strain cannot be easily removed. Although food production facilities have been advised for many years against the use of power hoses for cleaning, this study shows their use in many facilities is still prevalent and should be eliminated.

6.6 Conclusion

Several risk factors were identified which correlated with *L. monocytogenes* occurrence and persistence including separation of PPE for low and high risk areas, training being performed by other workers and use of a power hose. These are areas which should be examined by food processing facilities in order to prevent and control contamination.

6.7 References

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Chapter 7

Comparative Analysis of two serotype 1/2a *Listeria monocytogenes* genomes isolated from smoked salmon

Dara Leong Chapter Contributions:

- Performed all experimental work, data analysis and manuscript preparation

7.1 Abstract

Although Pulsed Field Gel Electrophoresis (PFGE) has previously been considered the “gold standard” in *L. monocytogenes* subtyping, in more recent years the use of whole genome sequencing (WGS) has increased significantly. WGS offers a much more detailed examination of strains and has been especially useful in outbreak investigations. In this study, WGS was used to examine two strains of 1/2a *L. monocytogenes* which were isolated from smoked salmon samples obtained from different food processing facilities within the Republic of Ireland. A possible epidemiological link exists between the two smoked salmon producing facilities as there are a limited number of salmon suppliers available to producers in the Republic of Ireland. Therefore the same supplier is frequently used by many smoked salmon producers. Isolates displaying similar PFGE profiles, including P58 and the persistent pulsotype P59, have been previously seen in several food processing facilities and foods, see Chapter 2.

The two strains here displayed similar PFGE profiles but contained a one band differences in both the *Sgs1* and *Apa1* digestion profiles. Previous studies have found significant differences in strains displaying similar PFGE profiles. However, comparative genomic analysis revealed these two strains to be very similar, including in their virulence and multidrug, heavy metal, antibiotic and sanitiser resistance genes. Both strains appeared to have reduced virulence potential and identical resistance profiles. Small differences that were seen between the genomes are thought to be due to minor prophage insertions which did not have a significant effect on the behaviour of the strains. Therefore, this research demonstrated that PFGE, when combined with epidemiological information, can still be a useful tool in *L. monocytogenes* contamination examination studies.

7.2 Introduction

The subtyping of *L. monocytogenes* strains is important in many areas. *L. monocytogenes* is the causative agent of listeriosis, a foodborne illness which, although rare, can have a mortality rate as high as 20-30% in its severe form (EFSA, 2015; Vázquez-Boland et al., 2001). Outbreak investigations of listeriosis can be difficult to perform due to several factors including; 1) the common presence of *L. monocytogenes* in the environment, 2) the prolonged and varied incubation time of *L. monocytogenes* (Goulet et al., 2013) and 3) the potentially wide geographical spread of outbreaks (Laksanalamai et al., 2012). Subtyping is vital in outbreak investigations as the ability to differentiate between strains of *L. monocytogenes* allows the identification of which cases of listeriosis belong to the outbreak and can help to identify the putative source of the outbreak (Chen et al., 2016; Rychli et al., 2014). Subtyping is also important in food production as it allows the tracking of strains throughout a facility and therefore can help to identify sources and routes of contamination which can then be addressed. Subtyping can also show the presence of persistent strains which is vital in preventing food contamination as the constant presence of persistent strains poses a much higher risk of contamination than the presence of a sporadic strain which will be removed by regular cleaning (Stasiewicz et al., 2015).

Traditionally, PFGE has been held as the “gold standard” in *L. monocytogenes* subtyping (PulseNetUSA, 2009) as it is regarded as the most discriminatory method of subtyping (Graves and Swaminathan, 2001). However, WGS offers a much higher discriminatory power than PFGE as it uses the entire genome rather than obtaining a profile by cutting the genome with restriction enzymes and using the resulting pattern of bands to identify the strain as in PFGE. Whole genome sequencing (WGS) also offers a major

advantage over other forms of subtyping as it allows the examination of all genes in the genome and so the characteristics of the strain can also be examined.

WGS used in outbreak investigations has previously been seen to be able to differentiate between strains with indistinguishable PFGE profiles and so can facilitate enhanced resolution in outbreak investigations, for example, separating a larger outbreak in Austria between 2011 and 2013 into two separate outbreaks caused by differing 1/2b serotype strains (Schmid et al. 2014). Advances in WGS, especially the cost reduction in recent years, have allowed WGS to become a viable alternative subtyping technique. In 2013, the Centre for Disease Control and Prevention (CDC) in the United States moved to using WGS as their primary subtyping technique for listeriosis outbreaks which has facilitated the solving of more outbreaks and a reduction in the number of cases within outbreaks (CDC, 2016). The implementation of this nationwide real-time WGS system in the U.S.A. has also allowed regulators to take act based on lower levels of epidemiological evidence (previously not possible through the use of PFGE) and identify listeriosis clusters more quickly and accurately which has facilitated the solving of more outbreaks (Jackson et al., 2016). The use of WGS in outbreak investigation has also been used in the UK since 2014 and in Denmark, following an outbreaks in smoked fish in 2013-2015 and in ready-to-eat meats in 2014 (Gillesberg Lassen et al., 2016; Jensen et al., 2016; PublicHealthEngland, 2014).

Although the movement of subtyping away from PFGE towards WGS has advanced in many countries in recent years, there are still many obstacles to this move, particularly in developing countries, see section 1.5.2. Even in areas where infrastructure is well-developed and sufficient sampling programmes are in place, the data analysis of whole genome can prove a significant problem. The large variety and continuing development of software and

programmes used in genome analysis can make it difficult for even experienced bioinformaticians to identify the best and most up-to-date pipeline to use for analysis (FAO, 2016). There is also no worldwide consensus on how to evaluate and quantify differences between genomes to identify genomes as being distinct from each other. Systems currently being used include but are not limited to; Kmer content, high-quality single nucleotide polymorphism (hqSNP) and whole-genome Multilocus sequence typing (wgMLST) (Jackson, 2015; CDC, 2016).

The aim of this study to was investigate any genomic differences between two serotype 1/2a *L. monocytogenes* strains which displayed closely related PFGE profiles; to examine the properties of the strains as well as evaluate the use of PFGE for strain subtyping.

7.3 Materials and Methods

7.3.1 PFGE

PFGE was performed according to the International Standard PulseNet protocol (PulseNetUSA, 2009) with the restriction enzymes *Sgs1* (formerly *Asc1*) and *Apa1*, in two separate experiments. An isolate similarity dendrogram was generated using Bionumerics version 7.5 software (Applied Maths, Belgium), by the unweighted pair group method with arithmetic mean (UPGMA) with tolerance and optimisation settings of 1%.

7.3.2 Genome Sequencing

DNA from *L. monocytogenes* strains 1123 and 1439 was extracted using the UltraClean Microbial DNA isolation kit (MoBio Laboratories Inc., USA) as per the manufacturer's protocol. Isolated DNA was shipped on ice by overnight courier and sequenced by MicrobesNG, Birmingham. 300bp Paired-end sequencing was performed using the Illumina MiSeq platform. Raw reads were pre-processed to remove adapter sequences and low quality reads using Trimmomatic software (version 0.32.1) on the Galaxy platform (Bolger et al., 2014). Overlapping reads were detected and joined using Flash. *De novo* assembly of the strains were performed using the DNASTar Lasergene SeqMan NGen software (DNASTar Inc., Madison, USA). Open reading frames (ORFs) were predicted using RAST (Aziz et al., 2008). Annotations were verified and curated using GLIMMER and BLASTp (Altschul et al., 1997) and Artemis (Rutherford et al., 2000).

7.3.3 Multilocus Sequence Typing (MLST)

The sequence type of each strain was determined as previously described (Ragon et al., 2008). Gene fragments of seven housekeeping genes, namely *abcZ* (ABC transporter), *bglA* (beta-glucosidase), *cat* (catalase), *dapE* (succinyl diaminopimelate desuccinylase), *dat* (D-alanine aminotransferase), *ldh* (lactate dehydrogenase), and *lhkA* (histidine kinase), were queried against the *L. monocytogenes* MLST database hosted by the Pasteur institute (http://bigsdbs.pasteur.fr/perl/bigsdbs/bigsdbs.pl?db=pubmlst_listeria_seqdef_public&page=sequenceQuery) to determine their respective allele numbers. The corresponding allele numbers identified from the database searches were subsequently combined to generate a specific sequence type and clonal complex for each of the *L. monocytogenes* strains (http://bigsdbs.pasteur.fr/perl/bigsdbs/bigsdbs.pl?db=pubmlst_listeria_seqdef_public&page=profiles&scheme_id=2) (Moura et al., 2016).

7.3.4 Whole Genome Analysis

Comparative genomic analysis was performed using Mauve (Darling et al., 2004), Artemis (Rutherford et al., 2000) and BRIG (BLAST Ring Image Generator) (Alikhan et al., 2011). The presence of virulence and resistance genes was examined using Artemis and confirmed using BLASTp comparisons against the well-annotated *L. monocytogenes* EDGe. Each of the genomes were scanned for the presence of prophage DNA using the PHAST software tool (Zhou et al., 2011). SnapGene was used to predict the location of *Sgs1* and *Apa1* restriction sites in the assembled genomes.

7.4 Results

7.4.1 Strain information

In this study, two *L. monocytogenes* strains (1123 and 1439) were isolated from smoked salmon samples obtained from two separate production facilities. Strain 1123 was isolated in July 2013 and strain 1439 was isolated in May 2014. Both strains belong to the serotype 1/2a and PFGE fingerprint analysis revealed them to have 94% similarity as calculated by Bionumerics. The major differences observed between the two strains' PFGE patterns was the presence of additional bands in strain 1123, one band in the *Sgs1* digestion and one band in the *Apa1* digestion, both of which are absent in strain 1439 (Figure 7.1).

7.4.2 Genome Assembly and Multilocus Sequence Typing (MLST)

L. monocytogenes strain 1123 was assembled at a length of 3,099,588 bp in 33 contigs. *L. monocytogenes* strain 1439 was assembled at a length of 3,121,972 bp in 23 contigs (Table 7.1). *In silico* MLST analysis determined that both isolates belonged to the same sequence type (ST) and clonal complex (CC), ST121 and CC121 (Table 7.2). Neither of the strains contained plasmids and five prophages (either intact or incomplete) were identified in each genome (Table 7.3).

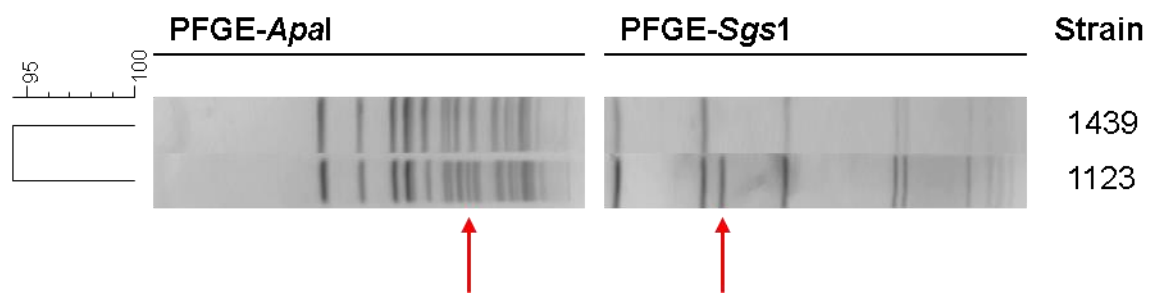


Figure 7.1: PFGE restriction profile comparisons of *L. monocytogenes* strains 1123 and 1439, with *Apa1* and *Sgs1* restriction profiles. Arrows denote band differences between the two strains.

<i>L. monocytogenes</i> strain	1123	1439
Source	Smoked Salmon	Smoked Salmon
Date isolated	July 2013	May 2014
Serotype	1/2a	1/2a
Genome length (bp)*	3,099,588	3,121,972
No. of contigs	33	23
G+C Content (%)	37.85	37.8
No of coding sequences (CDS)	3,108	3,135
No. of plasmids	0	0

*Genomes not closed

Table 7.1: General Features of *L. monocytogenes* strains 1123 and 1439.

Strain	Multi Locus Sequence Type							Allelic Profile	Clonal Complex	Lineage
	abcZ	bglA	cat	dapE	dat	ldh	lhkA			
1123	7	6	8	8	6	37	1	121	CC121	II
1349	7	6	8	8	6	37	1	121	CC121	II

Table 7.2: Sequence Type and Clonal Complex of *L. monocytogenes* strains 1123 and 1439.

7.4.3. Whole Genome Analysis

Visual comparisons performed in Mauve and Brig indicated a high degree of similarity between the two strains (Figure 7.2 and 7.3). Five prophages were identified in each genome by PHACTS software, including two intact prophages identified in strain 1123 and three intact prophages identified in strain 1439 (Table 7.3). No *Sgs1* restriction site was predicted to occur within these prophages. One *Apa1* site was predicted to occur within the incomplete prophage 1123_4 at 1,265,687 bp.

The examination of virulence genes showed the presence of intact *Listeria* pathogenicity island 1 (LIPI-1), the absence of *Listeria* pathogenicity island 3 (LIPI-3) and the presence of a truncated Internalin A (*inlA*) gene in both genomes (Table 7.4).

The examination of multidrug, heavy metal, antibiotic and sanitiser resistance genes revealed the presence of identical genes in both 1123 and 1439 genomes encoding for multi-drug resistance genes, β -lactamase & metallo- β -lactamase proteins, fosfomycin/fosmidomycin resistance, lincomycin resistance, aminoglycoside N3-acetyltransferase, aminoglycoside N3-acetyltransferase, aluminium resistance, copper resistance, lead/cadmium/zinc resistance, cobalt/zinc/cadmium resistance and quaternary ammonium compound resistance (Table 7.5).

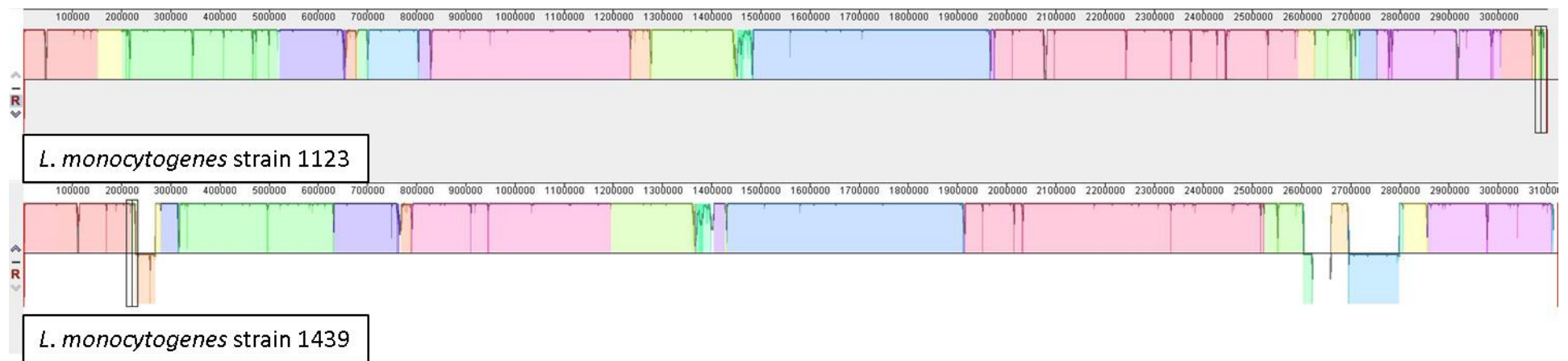


Figure 7.2: Linear comparison of between *L. monocytogenes* strains 1123 and 1439 performed in Mauve.

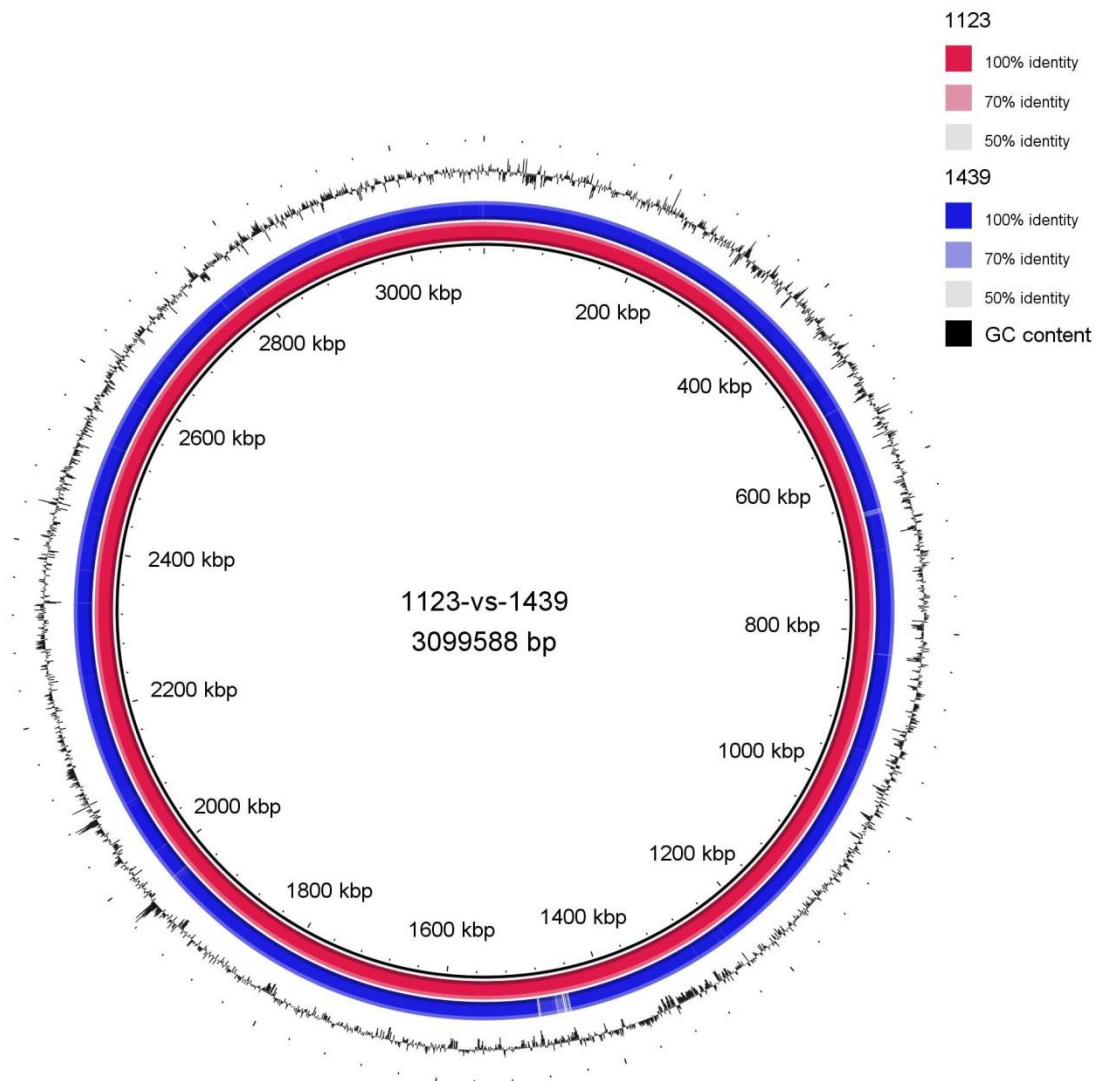


Figure 7.3: Whole genome comparisons between strains 1123 and 1439 visualised using BRIG.

Strain	Prophage No.	Status	Size (Kb)	Number of CDS	% GC	Location	Possible Phage
1123	1	Incomplete	22.9	27	38.62	93499-116474	PHAGE_Lister_A118_NC_003216
	2	Intact	48	47	36.73	633600-681673	PHAGE_Lister_A006_NC_009815
	3	Intact	43.8	63	36.25	785594-829456	PHAGE_Lister_A118_NC_003216
	4	Incomplete	17.5	13	35.39	1258880-1276421	PHAGE_Cronob_vB_CsaM_GAP32_NC_019401
	5	Intact	43.2	65	35.40	1440817-1484096	PHAGE_Lister_LP_101_NC_024387
1439	1	Incomplete	23.2	26	38.56	159865-183083	PHAGE_Lister_A118_NC_003216
	2	Intact	32.2	110	37.02	757849-790088	PHAGE_Lister_A006_NC_009815
	3	Intact	81.4	97	36.29	1348905-1430387	PHAGE_Lister_LP_101_NC_024387
	4	Incomplete	38.9	56	35.20	2619994-2658992	PHAGE_Lister_A006_NC_009815
	5	Incomplete	18.4	26	35.79	2693473-2711937	PHAGE_Lister_A118_NC_003216

Table 7.3: Prophages identified in *L. monocytogenes* strains 1123 and 1439.

		1123		1439	
Gene Family	Gene	Gene locus	Location	Gene locus	Location
Internalin Family	Internalin A	0418	428400-429875	0534	543772-545247
		0419	429909-430802	0535	545281-546174
	Internalin B	0420	430887-432779	0536	546259-548151
<i>Listeria</i> Pathogenicity Island 1 (LIPI-1)	prfA	0183c	182161-181448	2855c	2836779-2836066
	plcA	0184c	183385-182432	2856c	2838003-2837050
	hly	0185	183627-185216	2857	2838245-2839834
	mpl	0186	185547-187079	2858	2840165-2841697
	actA	0187	187278-189092	2859	2841896-2843710
	plcB	0188	189129-189998	2860	2843747-2844616
<i>Listeria</i> Pathogenicity Island 3 (LIPI-3)		-	-	-	-

Table 7.4: Virulence genes present in *L. monocytogenes* strains 1123 and 1439.

Function	Strain 1123 Locus Tag and Gene Description		Strain 1439 Locus Tag and Gene Description	
Multidrug Resistance	0595c	Na ⁺ driven multidrug efflux pump	0708c	Na ⁺ driven multidrug efflux pump
	0953	Multidrug resistance ABC transporter ATP-binding and permease protein	0897	Multidrug resistance ABC transporter ATP-binding and permease protein
	1048c	Multidrug-efflux transporter, major facilitator superfamily (MFS)	0992c	Multidrug-efflux transporter, major facilitator superfamily (MFS)
	1715	Multidrug resistance protein, putative	1646	Multidrug resistance protein, putative
	1929c	Membrane component of multidrug resistance system	1861c	Membrane component of multidrug resistance system
	2617c	Multidrug resistance protein B	2701c	Multidrug resistance protein B
	2912c	Multidrug-efflux transporter, major facilitator superfamily (MFS)	3030c	Multidrug-efflux transporter, major facilitator superfamily (MFS)
	2923	Multidrug resistance ABC transporter ATP-binding and permease protein	3041	Multidrug resistance ABC transporter ATP-binding and permease protein
	0509	Drug resistance transporter, EmrB/QacA family	0622	Drug resistance transporter, EmrB/QacA family
	1193c	Drug resistance transporter, EmrB/QacA family	1136c	Drug resistance transporter, EmrB/QacA family
β-lactamase & Metallo-β-lactamase Proteins	2953	Drug resistance transporter, Bcr/CflA family	3071	Drug resistance transporter, Bcr/CflA family
	1140	Metal-dependent hydrolases of the beta-lactamase superfamily I; PhnP protein	1084	Metal-dependent hydrolases of the beta-lactamase superfamily I; PhnP protein
	1926c	Metallo-beta-lactamase family protein	1858c	Metallo-beta-lactamase family protein
	2106	Metallo-beta-lactamase family protein	2033	Metallo-beta-lactamase family protein
	2233	Beta-lactamase class C and other penicillin binding proteins	2159	Beta-lactamase class C and other penicillin binding proteins
Fosfomycin/ Fosmidomycin Resistance	2018c	Fosfomycin resistance protein FosX	1945c	Fosfomycin resistance protein FosX
Lincomycin Resistance	2754c	Lincomycin resistance protein LmrB	0313c	Lincomycin resistance protein LmrB
Aminoglycoside N3-acetyltransferase	2024c	Aminoglycoside N3-acetyltransferase	1951c	Aminoglycoside N3-acetyltransferase

Aluminium Resistance	1603	Aluminium resistance protein	1533	Aluminium resistance protein
Copper Resistance	2381c	Copper resistance protein CopC	2307c	Copper resistance protein CopC
Lead/Cadmium/Zinc Resistance	0848	Lead, cadmium, zinc and mercury transporting ATPase; Copper-translocating P-type ATPase	0791	Lead, cadmium, zinc and mercury transporting ATPase; Copper-translocating P-type ATPase
Cobalt/ Zinc /Cadmium Resistance	2740c	Cobalt-zinc-cadmium resistance protein CzcD	0299c	Cobalt-zinc-cadmium resistance protein CzcD
	2684c	Cobalt-zinc-cadmium resistance protein	2578c	Cobalt-zinc-cadmium resistance protein
	2556	Cobalt-zinc-cadmium resistance protein	2482	Cobalt-zinc-cadmium resistance protein
	0549	Magnesium and cobalt transport protein CorA	0662	Magnesium and cobalt transport protein CorA
Quaternary Ammonium Compound Resistance	1062	Quaternary ammonium compound-resistance protein SugE	1006	Quaternary ammonium compound-resistance protein SugE
	1063	Quaternary ammonium compound-resistance protein SugE	1007	Quaternary ammonium compound-resistance protein SugE

Table 7.5: Multidrug, heavy metal, antibiotic and sanitiser resistance genes present in *L. monocytogenes* strains 1123 and 1439.

7.5 Discussion

The two *L. monocytogenes* strains examined in this study were serotype 1/2a and were obtained from smoked salmon samples from separate smoked salmon producers at different times, 1123 in July 2013 and 1439 in May 2014. PFGE analysis showed that they displayed highly similar PFGE profiles, with a noticeable difference of only one band in the *Sgs1* digestion and one band in the *Apa1* digestion. Due to this similarity, the strains underwent whole genome sequencing in order to assess their relatedness.

Comparing their respective draft genomes showed that these two strains differed in length by 22,757 base pairs (bp). However, as neither genome was closed, this difference may be negligible. Both genomes shared a similar GC content; 37.85% in 1123 and 37.8% in 1439 and neither contained plasmids. The number of open reading frames (ORFs) varied between the two annotated genomes; 3,108 ORFs in 1123 and 3,135 ORFs in 1439. Similarly, some of these differences may occur due to the unclosed genomes.

Following *in silico* analysis, both strains were found to belong to the same MLST type, ST121 and Clonal Complex, CC121. This finding is unsurprising as PFGE performed with two enzymes has been shown to have higher discriminatory powers than MLST (PulseNetUSA, 2009). Therefore, two strains with highly similar PFGE patterns, as seen here, are likely to be of the same MLST type. MLST type 121 is also a commonly found MLST type globally and has previously been identified in food processing facilities in several countries including but not limited to Austria, Ireland, Denmark, Italy and Spain (Schmitz-Esser et al., 2015).

The genomes were visualised linearly using MAUVE and the general organisation of both genomes appears similar (Figure 7.2). Differences in the genomes were visualised using BRIG and the genomes appeared very similar (Figure 7.3). Some differences were seen at approximately 640,000 bp, 1,440,000 bp and 1,480,000 bp. Each of these areas was seen to correspond to intact prophages, 1123_2, 1439_3 and 1123_5, respectively (Table 7.3). Five prophages were identified in each genome by PHACTS software. PHACTS also gives a possible phage source for these prophages and the same three phages were predicted in both genomes; PHAGE_Lister_A118_NC_003216, PHAGE_Lister_A006_NC_00981 and PHAGE_Lister_LP_101_NC_024387. These three phages were previously recognised siphoviridae *Listeria* phages (Denes et al., 2014; Klumpp and Loessner, 2013) and account for four of the five prophages identified in 1123 and all five of the prophages identified in 1439. Although the majority of these prophages have likely originated from the same phages, they may represent minor differences between the genomes as the length of the prophages was varied and the prophages did not occur in the same places in the genome. Although no *Sgs1* restriction site was predicted to occur within any of these prophages, the insertion of the prophages may have caused a shift in the surrounding area of the genome which could account for the difference seen in the *Sgs1* PFGE profiles. One *Apa1* restriction site was predicted to occur within the incomplete prophage 1123_4 which may account for the difference seen in the *Apa1* restriction profiles.

The occurrence of virulence genes in both genomes was examined in order to evaluate their pathogenic potential. Some of the main virulence factors were examined including, LIPI-1, LIPI-3 and the internalin gene family using the well-annotated *L. monocytogenes* EDGe as a reference genome. An intact LIPI-1 was identified in both genomes (Table 7.4). The production of lysteriolysin O (LLO) is encoded on LIPI-1 and is

responsible for the haemolytic activity of *L. monocytogenes* (Gedde et al., 2000). LIPI-3 is absent in both strains as expected. Both strains belong to Lineage II, which has been seen to lack LIPI-3. LIPI-3 has only been seen to occur in Lineage I strains and encodes an additional lysteriolysin, LLS, which increases the virulence of *L. monocytogenes*. This is thought to account for the overrepresentation of Lineage 1 strains in human isolates (Cotter et al., 2008). Truncated *inlA* genes were identified in both genomes; genes 418 and 419 in 1123 and genes 534 and 535 in 1439 (Table 7.3). InlA has been shown to be an important cell-surface internalin with a role in early invasive disease, InlA helps to facilitate the crossing of *L. monocytogenes* across the intestinal epithelium through interaction with the host-cell-receptor E-cadherin (Bierne and Cossart, 2007). Due to this truncation, it is likely that these two strains could have greatly reduced virulence potential as has been seen previously in strains with truncated internalin genes (Nightingale et al., 2005; Olier et al., 2005; Ragon et al., 2008). One current theory hypothesises that a truncated *inlA* is better adapted for survival in food and food environments and a full-length *inlA* is better adapted for survival in mammalian hosts (Manuel et al., 2015). This information concerning the reduced virulence potential, due to the lack of LIPI-3 and the truncated *inlA*, could prove significant when evaluating these strains and their relevance to public health.

Under examination of multidrug, heavy metal, antibiotic and sanitiser resistance genes, both strains contained identical genes (Table 7.5); therefore, it is likely that these two strains are phenotypically identical. The presence of quaternary ammonium compound resistance genes, (gene loci 1123_1062, 1123_1063, 1439_1006 and 1439_1007) may indicate that these strains are adapted to survival in the food processing environment where sanitisers such as quaternary ammonium compounds are commonly used and resistance to them offers a distinct advantage (Müller et al., 2013). This apparent adaptation

to environmental conditions is congruent with the lowered virulence capabilities of the strains indicated by the lack of LIPI-3 and the truncated *inlA*.

Casey (2015) previously examined the whole genomes of two pairs of strains with indistinguishable PFGE profiles where, in each strain pair, one strain was isolated in the Republic of Ireland and one strain was isolated in Australia. One pair of strains was seen to contain high genetic diversity while the other strain pair was seen to only differ slightly. Due to the geographic distance associated with each strain pair, WGS of these strains was necessary to obtain sufficient strain information in order to examine strain relatedness. When examining contamination patterns, PFGE profiles combined with the knowledge of epidemiological information can facilitate investigations where clear links can be seen between strain sources. However, in the absence of clear epidemiological links, WGS represents a major advance over PFGE analysis in strain subtyping.

The PFGE profile of strains 1123 and 1439 has also been seen in food and swab environmental samples isolated from four separate Irish seafood processing facilities in 2013 and 2014 (Leong et al., 2015). When we consider the close relatedness of the two strains examined in this study, we can categorise these strains together and consider the prevalence of this strain type across the industry sector. The band differences seen are likely due to a recent prophage insertions and do not appear to have any effect on the strains phenotypic behaviour; therefore strains with or without these bands can be categorised together when assessing the strains prevalence. Although, there are many seafood processing facilities operating in Ireland, the vast majority of these processors produce smoked salmon and there are relatively few suppliers of salmon to Irish processors. It is therefore possible that a shared supplier could be the source of this widespread strain or that another unknown link exists between these food processors which allowed for the

proliferation of this strain across the Irish seafood processing sector. The widespread presence of this strain in the seafood sector emphasises the importance of *L. monocytogenes* monitoring and control of both the production facility and the raw materials used, especially as the process of smoking salmon has been shown to reduce but not eliminate *L. monocytogenes* present in the raw salmon (Cheng et al., 2015; Porsby et al., 2008).

7.6 Conclusion

As *L. monocytogenes* strains 1123 and 1439 were isolated from the same food type within the same country, an epidemiological link between the strains can clearly be seen. When this information is combined with the very similar PFGE profiles, it is reasonable to categorise these two strains together when examining contamination patterns in food processing environments.

Previous studies have displayed the advantage of WGS over PFGE and has seen significant differences in the genomes of *L. monocytogenes* strains which have displayed very similar PFGE profiles (Casey, 2015; Gilmour et al., 2010). However, in this study, strains 1123 and 1439, which differ slightly in their PFGE profiles, appear to have very similar genomes. Although some small differences can be seen, they do not seem to have affected the virulence or phenotypic behaviour of the strains. The small differences seen between the strains are likely due to recent prophage integrations into the genome and do not appear to have had a significant effect on the strains.

Although WGS did not reveal substantial differences between these two strains in this case, the use of WGS offers a significant advantage over previous subtyping techniques. Here, WGS allowed the examination of resistance and virulence genomic characteristics of the strains which would not have been possible otherwise.

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Chapter 8

General Discussion and Conclusions

8.1 General Discussion

The objective of this work was to monitor, examine and help to control *Listeria* in the food processing environment. Although the incidence of listeriosis is low in comparison with other foodborne illnesses (de Noordhout et al., 2014), the high mortality rate makes it a major concern for public health (EFSA, 2015).

In Chapter 2, a 3-year study examined *L. monocytogenes* in 54 food production facilities by analysing over 5,000 samples. The overall rates found were broadly in line with the reports from other countries (EFSA, 2013). As there are relatively few long-term studies assessing the presence of *L. monocytogenes* in food processing facilities, this study offered an overview of contamination occurring in Irish facilities at present. A reduction in occurrence was seen over the 3-year study which may indicate that the heightened awareness created by the sampling, combined with information given to food processors, contributed to the reduction of *L. monocytogenes*. The finding of a large number of diverse pulsotypes indicates the high diversity of strains present in Ireland and the finding that 11/86 pulsotypes share high similarity with 11 clinical pulsotypes re-enforces the importance of food processors being vigilant against food contamination. This work has also expounded the usefulness of performing a large monitoring programme in the food processing industry, similar to that which is carried out in the cheese industry in Austria, as a means of understanding and controlling *Listeria*. By utilising comparisons with strains which have been isolated from the food processing environment during routine sampling, it is possible that any food recalls/outbreak investigations in the future could trace and identify sources of contamination more efficiently and accurately.

Examination of the retail environment, which is a known source of *L. monocytogenes* contamination, was unfortunately absent from this work (Simmons et al., 2014). Although post processing contamination has been recognised as an area of concern, this study focused generally on foods which were packaged on site at the production facility and would not be further processed at a retail site.

Future aspirations of this work include the characterisation of the large bank of strains which was generated during the sampling project. Several hundred strains have been isolated over the course of the study. Thus far, these strains have been subject to PFGE and serotyping but many of their characteristics remain unexplored. With the advances in WGS, there is large potential for an in-depth study relating to the characteristics of these strains and their genomes including examination of any genomic indicators of persistence and virulence.

A significant factor which affects how *L. monocytogenes* contamination is controlled is the fact that currently, all strains must be considered virulent, regardless of their actual virulence capacity. By further examination of these strains, isolated from the food processing environment, a better understanding of the relationship between virulence and survival may be elucidated. One current theory suggests that strains will evolve either towards virulence (survival and proliferation in a mammalian host) or towards persistence (survival and proliferation in a seemingly inhospitable environment e.g. a food processing facility). According to this theory, it is possible that strains which persist in a processing facility may have lost their virulence capacity and may no longer be capable of causing disease, therefore they may not be a threat even if present in a food product. A better understanding of *L. monocytogenes* strains isolated from the food processing environment,

especially persistent strains, may provide more information concerning whether all *L. monocytogenes* should truly be considered virulent.

In Chapter 3, *L. ivanovii* in foods and food processing environments in the Republic of Ireland was examined over a 12-month period. Although *L. ivanovii* is more commonly a pathogen to ruminants, infection in humans does occur (Guillet et al., 2010; Snapir et al., 2006). Patterns of *L. ivanovii* contamination can also be indicative of *L. monocytogenes* contamination. The occurrence of *L. ivanovii* was seen to be of concern particularly in the dairy sector where prevalence of 1.7% was found. Isolated *L. ivanovii* strains were examined further and some strains were seen to be capable of invasion of human epithelial cells in vitro. These findings emphasise the need for dairy processors to be vigilant against *L. ivanovii* contamination especially if they are located on/adjacent to a farm.

Chapter 4 examined variations in methodology used for conducting challenge test studies on *L. monocytogenes* in RTE foods, especially in relation to the guidance documents published in June 2014 and the previous version of the document published in 2008 (EC, 2008, 2014). It was concluded that there is a clear need for further training of both food business operators and independent laboratories in order to perform challenge tests of a high enough quality to be accepted by the competent regulatory authorities. Further dialogue with regulatory authorities should be undertaken in order to ensure that results of challenge tests performed will be accepted. The absence of an accredited lab which will perform challenge tests in the Republic of Ireland is a major hindrance to the RTE food processing industry and may result in the occurrence of unnecessary recalls due to the fact that foods for which no challenge test data is available must abide by regulations pertaining to foods which support *L. monocytogenes* growth.

In Chapter 5, challenge tests were performed according to two different methodologies on both mushrooms and smoked salmon in line with the European guidance documents (EC, 2008, 2014). It was found that seemingly small changes made to the methodology can have an effect on the result. Smoked salmon was seen to support the growth of *L. monocytogenes* under all conditions tested, highlighting the need for smoked salmon producers to carefully monitor their facilities and products for *L. monocytogenes*. In challenge tests performed on mushrooms, a difference in growth was seen depending on the methodology used. In tests performed according to methodology B with inoculation by spreading and incubation temperatures of 8 °C for 1/3 and 12 °C for 2/3 of shelf-life, mushrooms did not support the growth of *L. monocytogenes*.

One of the major findings of this work with relevance to the food business industry concerns the results from challenge tests performed on mushrooms. The Irish food safety authority accepted the challenge test results (performed using challenge test methodology B) which stated that mushrooms did not support the growth of *L. monocytogenes*. Previously, regulatory testing procedure involved testing for presence/absence of *L. monocytogenes* as the authorities were required to abide by the regulations pertaining to foods for which challenge test information is not available i.e. the assumption that a food will support the growth of *L. monocytogenes*. The regulatory testing procedure has now been altered to enumeration and a limit of 100 CFU/g *L. monocytogenes* is permitted at the end of shelf-life. It is likely that this change in regulations will decrease the number of recalls triggered and will therefore prevent loss to the mushroom industry.

In Chapter 6, the risk factors associated with *L. monocytogenes* occurrence and persistence were examined. Separation of PPE for low and high risk areas, training being performed by other workers and use of a power hose were seen to correlate with *L.*

monocytogenes occurrence and persistence. These are areas which should be focused on by food business operators in order to help prevent and control contamination. Many of the highlighted areas of concern include areas which would already have been included in advice given to food business operators. However, it is hoped that this study, which utilises real-time occurrence/persistence data, would offer more concrete evidence to back up this advice and so would offer more reason for FBOs to alter their practices in accordance with this advice.

In Chapter 7, two 1/2a *L. monocytogenes* strains isolated from smoked salmon from two different processing facilities were compared by WGS. Similar PFGE profiles had been seen previously to occur in several processing facilities in all four industry sectors and to persist in one facility for a period of 2 years. Although a slight difference was seen in their PFGE profiles, following whole genome comparison, the two strains were seen to be very similar. Both strains were seen to have identical reduced virulence potential and identical multidrug, heavy metal, antibiotic and sanitiser resistance profiles. Previous studies have seen large genomic differences in strains with similar PFGE profiles (Casey, 2015; Gilmour et al., 2010). However, this study indicates that, when combined with epidemiological data, PFGE is still a valuable tool for contamination investigations despite recent advancements in the use of WGS for subtyping.

It would be expected in the future that strain subtyping and examination will be heavily influenced by WGS. Although the use of PFGE remains prevalent in contamination studies, outbreak investigations and academic studies are increasingly using WGS for subtyping and strain analysis. The much more comprehensive nature of WGS will allow for a better understanding of strains and why certain strains persist or cause disease. However,

there are still several limitations attached to WGS which may hinder this advancement. *Listeria* monitoring in many developing countries has not yet reached the level of monitoring programmes and strain collection so the advances in WGS will not apply until basic systems are first put in place. Even if such systems are in place, WGS may be prohibitively expensive due to necessities, e.g. high speed broadband and trained bioinformaticians, which may not be internationally available. The sharing of data across borders may also act as a limitation as, to facilitate worldwide comparisons, genome sequences would need to be made publicly available and it is common for trust issues to hamper the sharing of data.

8.2 Conclusion

In conclusion, monitoring and examination of *Listeria* in the food processing environment is an extremely valuable endeavour. Further understanding of persistence, contamination routes, strain subtyping, the effects of management practices, the behaviour of *Listeria* within certain foods, and genomic characteristics of strains all contribute to the further understanding and therefore better control of *Listeria* contamination and so help reduce the risk to public health.

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