Control and monitoring of *Listeria monocytogenes* with the use of natural antimicrobial techniques including the bacteriocin nisin.

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2016

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Thesis submitted as full requirement for the degree of Master of Science (Microbiology) at the University of Limerick, November 2016
Acknowledgements

I must express my eternal appreciation to my supervisor Dr. Achim Schmalenberger for his guidance, dedication, wisdom and for allowing me to be a member of his research laboratory over this past two years.

I would also like to thank Dr. Johann Scollard for showing me the ins and outs of all thing Listeria related.

I must show gratitude Prof. Dr. Matthias Noll for allowing me to spend time in his laboratory last year and for providing L. lactis strains and instructions regards nisin and its uses.

I would like to thanks all those in the Life Sciences Department, particularly those in the Food Lab and Microbiology Lab, with a special shout-out to Israel, Jessica, Jacinta, Aaron, Paul and Ronan.

I would finally like to be grateful to all of my family and friends for their support and for putting up with me over years.
Declaration

The substance of this thesis is the original work of the author and due reference and acknowledgement has been made, where necessary, to the work of others. No part of this thesis has already been submitted for any degree and is not being concurrently submitted in candidature for any degree.

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Abstract

According to the Department of Agriculture, Food and the Marine (DAFM) the agri-food industry contributes €24 billion to the Irish economy. One of DAFM’s chief concerns is the maintenance of Ireland’s reputation for high food quality and food safety. *Listeria monocytogenes* is of a particular risk to this reputation especially for food businesses within the ready to eat (RTE) food sector as *L. monocytogenes* is extraordinarily well adapted to the harsh conditions employed for food preservation. This thesis, through the literature review discusses the risk factors associated with the bacteria, the preventative measures taken to reduce occurrence and their effectiveness, outbreaks and potential solutions to the problem of *L. monocytogenes* within the ready-to-eat food industry specifically the fresh-cut fruit and vegetable produce sector. Experimentally, three individual experiments were conducted with the aims of: a) to establish if the effects of inoculation density induces any experimental biases on studies of growth of *L. monocytogenes* b) to test the effect of nisin A and *L. lactis* as potential viable antilisterial agents without the negative organoleptic effects of other antimicrobials currently used on fresh-cut lettuce such as essential oils c) to partake in a national sampling program for *L. monocytogenes* occurrence and persistence rates in Irish food businesses. Results from these experiments it was shown that a) low initial cell densities can lead to higher growth rates when compared to higher inoculation densities potentially leading to a need to reclassify high inoculation based *L. monocytogenes* growth experiments. b) demonstrated that the introduction of low amounts of purified commercial nisin A can reduce *L. monocytogenes* counts by a significant amount without a substantial negative effect of other antilisterial compounds on the sensory quality of the lettuce. c) Provided prevalence data from dairy, meat and vegetable producing industries as part of a 3-year national *L. monocytogenes* sampling program and displayed that vigilance and awareness of a potential pathogen can lead to a reduction in its occurrence.
List of abbreviations

µ: Micron (10^-6)

aw: Water activity

Bp: Base pairs

°C: Degree Celsius

CFU: Colony forming units

dH_2O: Distilled/ Deionized Water

DNA: Deoxyribose nucleic acid

dNTP: Deoxyribose nucleic triphosphate

EDTA: Ethylene diamine tetra-acetic acid

e.g.: for example

Eh: Redox potential

E.O: Essential Oils

FBO(s): Food Business Operators

g: Gram

GRAS: Generally Recognised As Safe

HACCP: Hazard Analysis Critical Control Point

h(s): Hour(s)

i.e.: That is/ Such as
IU: international units

Kb: kilo base

LAB: Lactic acid bacteria

L: litre

MAP: Modified atmosphere packaging

mg: Milligram

mg/mL: Milligram/millilitre

M: Molarity

mM: Millimolar

Min(s): Minute(s)

nm: Nanometre

OPP: Orientated polypropylene

PCR: Polymerase chain reaction

PBS: Phosphate buffer saline

PFGE: Pulse field gel electrophoresis

ppm: Parts per million

RTE: Ready to eat

RPM: Rotations per minute

SDS: Sodium dodecyl sulphate
**TAE:** Tris-acetic-acid EDTA

**UV:** Ultraviolet
Objectives

This thesis sought to study the consequence of *Listeria monocytogenes* and its growth under the following reference points:

1. To establish if the effects of inoculation density induces any experimental biases on studies of growth of *L. monocytogenes*.

2. To test the effect of nisin A and *L. lactis* as potential viable antilisterial agents without the negative organoleptic effects of other antimicrobials currently used on fresh-cut lettuce such as essential oils.

3. To sample for *L. monocytogenes* occurrence and persistence rates in Irish food business over a year period as part of a larger 3-year national study.
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Chapter 1: Literature Review

1.1: Abstract

*Listeria monocytogenes* is of a substantial risk to the ready-to-eat food industry due to its exceptional physiological abilities, ubiquitous nature and potential pathogenicity. This literature review shall discuss the risk factors associated with the bacteria, the preventative measures taken to reduce occurrence and their effectiveness, outbreaks and cases of the bacterium within Ireland and globally and potential solutions to the problem of *L. monocytogenes* within the ready-to-eat food industry specifically the fresh-cut fruit and vegetable produce sector.

1.2: Introduction

The global fruit and vegetable industry has rapidly increased production by approximately 47 percent between the years 1990 and 2002. This growth has been attributed to consumer demand for convenient, healthy and fresh foods. With this rise in consumption also come as rise in food related illnesses especially in foods that are designed and demanded to be as minimally processed as possible. The majority of these foods have such a nature that they comprise of large amounts of natural microbiota some for which have the potential to be pathogenic. *Listeria monocytogenes* is an example of one such microorganism.

While procedures are put in place in order to minimise the potential of contamination by various foodborne organisms regularly occur, with approximately 15,000 reported foodborne illnesses occurring in the United States alone (CDC 2016). Broadly speaking, the minimally processed food industry, particularly fresh cut products rely on techniques such as Hazard Analysis Critical Control Point (HACCP) procedures in order to prevent contamination. Examples of this include the use of chlorine washes, low storage temperatures, and modified atmospheres. However, contamination by *L. monocytogenes* it is still a common occurrence, with 98 *Listeria* related deaths occurring from the year 2000 to 2015 in the USA alone (CDC 2016).

The reported cause of these outbreaks is twofold either failure of the HACCP processes or a lack of sufficient hurdles in to contain the metabolic processes of the pathogenic organisms.
This thesis examines some of the current methods employed in order to control the proliferation of *L. monocytogenes* on fresh-cut produce under certain conditions and suggests potential alternatives, specifically the use of the natural bacteriocin nisin when combined with other antimicrobial techniques currently employed. This review of the literature inspects these issues with regards to the current issues in the scientific field.

1.3: **Listeria species and Listeria monocytogenes**

*Listeria monocytogenes* is a Gram-positive, facultative anaerobic, non-sporeforming rod which displays flagellar motility at temperatures below 30 °C. A psychrotrophic organism which has been shown to grow at temperatures ranging from -0.5 °C to 55 °C with an optimum at 37 °C. It has been shown to grow at pH levels between 4.4 and 9.4 and at water activities in excess of 0.92 with NaCl as the solute. A member of the genus *Listeria* which as of 2015 consists of 17 published species. *L. monocytogenes* (Pirie, 1940), *L. grayi* (Larsen & Seeliger, 1966), *L. innocua* (Seeliger, 1981), *L. welshimeri*, *L. seeligeri* (Rocourt and Grimont 1983), *Livanovii*, *L. marthii* (Graves et al. 2010), *L. rocourtiae* (Leclercq et al. 2010), *L. fleischmannii* (Bertsch et al. 2013), *L. weihenstephanensis* (Lang Halter et al. 2013), *L.floridensis*, *L. aquatica*, *L. cornellensis*, *L. riparia*, *L. grandensis* (den Bakker et al. 2014), *L. booriae* and *L. newyorkensis* (Weller et al. 2015).

Currently *L. monocytogenes* and *L. ivanovii* are considered pathogens of warm blooded mammals. While *L. ivanovii* infections are quite rare in humans they have been shown to be fatal, however it is primarily associated with sheep and cattle infections. *L. monocytogenes* is chief pathogen of the *Listeria* species in humans, with most infections associated with the serotypes 4b, 1/2a and 1/2b of Lineages I and II (Vazquez-Boland et al. 2001).

*L. monocytogenes* developed its reputation as an important foodborne pathogen due to its ability to grow at refrigeration temperatures, in high salt foods, in highly acidic foods all while being found through various environments such as within soil, water, vegetation animal, processing and storage facilities. This ubiquity allows easy entry into the food processing chain at multiple positions. In combination with its ability to grow under adverse conditions for long periods and its ease of penetration into the food chain contributes to its persistence, thus allowing *L. monocytogenes* to persist for long periods despite sanitation procedures. It has been shown that *L. monocytogenes* persistence is predominantly due to the lack of ability to dislodge the bacterium from harbourage sites in combination with its own
growth abilities and not due to any resistance to disinfectants (Carpentier and Cerf 2011). However it has been shown that removal of these harbourage sites can be next to impossible in the modern food process industries in particular the minimally processed sector due to several interacting factors i.e. acid and heat tolerance and adherence to surfaces (Lundén et al. 2007).

*L. monocytogenes* is frequently found in raw and minimally processed “Ready to Eat” (RTE) foods of both plant and animal sources. It is also commonly isolated in cooked food that has undergone post-processing contamination. *L. monocytogenes* has been isolated from a wide variety of food products such as fermented and cooked pork, raw and cooked poultry, raw and smoked seafood, milk both raw and pasteurised, cheeses and raw vegetables (Buchanan et al. 1989, Ercolini et al. 2003, WHO and FAO 2004, Koseki and Isobe 2005a, Koseki and Isobe 2005b, Weiler et al. 2013). Even the presence of low levels of *L. monocytogenes* allows for the potential of growth at refrigeration temperature storage and thus the potential of infection increases in foods that can support the growth of the pathogen over the course of the storage. A 2010 European Union study established a 10.4% prevalence rate among fish samples, a 2.07% prevalence rate among meat samples and a 0.47% among cheese samples at the time of sampling (EFSA 2013).

**Table 1**: Reported hospitalisation and case-fatality rates due to *Listeria* in confirmed human cases in the EU, 2014. Adapted (EFSA 2015).

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number of confirmed human cases</th>
<th>Hospitalisation</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number of reporting Member States</td>
<td>Number of reporting Member States</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reported hospitalised cases</td>
<td>Proportion hospitalised (%)</td>
</tr>
<tr>
<td>Listeriosis</td>
<td>2,161</td>
<td>16</td>
<td>812</td>
</tr>
</tbody>
</table>

The potential of *L. monocytogenes* to cause severe illness, its prevalence within natural reservoirs and its access to the food supply systems has lead it be become a concern of both national and international public health associations.
Chapter 1: Literature Review

1.4: Listeriosis

Listeriosis is a name for the symptoms caused by the infection of *Listeria* spp. namely *L. monocytogenes* and *L. ivanovii* in humans. Initial described in 1926 when Murray, Webb and Swann isolated the then named “*Bacterium monocytogenes*” from a epizootic outbreak among guinea pigs and rabbits (WHO and FAO 2004). However it wasn’t until large common source listeriosis outbreaks occurred during the 1980s that showed that the primary exposure route for humans was via foodborne transfer (Bibb *et al*. 1990, Swaminathan and Gerner-Smidt 2007). It is a relatively rare disease (0.52 notifications per 100,000 population), when compared to other foodborne infections such as campylobacteriosis (71.0 notifications per 100,000 population), salmonellosis (23.4 notifications per 100,000 population) and verocytoxigenic *E. coli* (1.56 notifications per 100,000 population) (EFSA 2015). However, listeriosis’ severe effects allow it to be a “reportable” disease permitting it to be accurately represented when compared to other foodborne illnesses (Scallan *et al*. 2011).

The primary individuals at risk from listeriosis are the immunocompromised, elderly, pregnant women and the subsequent neonates. In general, most reported cases display themselves as one of three clinical syndromes: maternofoetal listeriosis or neonatal listeriosis (resulting result in spontaneous abortions or stillbirths), bloodstream infection, and meningoencephalitis. In combination with these syndromes the listerial infection can spread via the blood stream to present secondary infections most commonly located within peritoneum, joints, the endocardium, or the eyes. (Swaminathan and Gerner-Smidt 2007). Listeriosis can have a mortality of up to 20-30% in high risk groups despite appropriate antimicrobial treatment as certain isolates have been shown to be vulnerable to penicillins, aminoglycosides, trimethoprim, tetracycline, macrolides, and vancomycin (Troxler *et al*. 2000).

*L. monocytogenes* is primarily a soil microorganism, which has evolved the ability to invade and travel within eukaryotic cells possibly due to interfere with a multicellular eukaryotic soil organism, thus *L. monocytogenes* is not host adapted to humans allowing it to be an opportunistic or incidental pathogen only (Mc Laughlin 1997, McLauchlin *et al*. 2004). In combination with the foodborne contamination being the chief route of infection and *L. monocytogenes*’ opportunistic nature has led to an uncertainty about the minimum infectious dose, mainly depending on the health, age and sex of the host. This can range from $10^7$ to $10^9$. 

Chapter 1: Literature Review
in healthy individuals down to $10^5$ to $10^7$ in potential risk groups (Vazquez-Boland et al. 2001, Goldfine and Shen 2007). However according to the Food Safety Authority of Ireland $10^5$ cells per gram of food “represent a very low risk of listeriosis for all population groups” (FSAI 2011).

In Ireland, listeriosis cases have been notified by the Health Protection Surveillance Centre since 2004 providing an average rate of 11.5 cases per year. In the year 2014, 15 cases of listeriosis were observed the highest occurrence since 2007. This specifies an occurrence rate of 0.3 notifications per 100,000 population which is below the EU wide incidence rate of 0.52 notifications per 100,000 population. Of the 15 cases eight were reported as adult/juvenille with five being over the age of 65, two as neonatal, four as pregnancy related and one was not specified (HPSC 2015).

![Number of listeriosis notifications, Ireland 2004-2014](image)

**Figure 1**: Number of listeriosis notifications, Ireland 2004-2010. Adapted from (HPSC 2015).

From the 15 cases reported in Ireland in 2014, 10 were serotyped by the National *Salmonella, Shigella* and *Listeria* Reference Laboratory (NSSLRL) Galway, Ireland. Of these clinical isolates six were from blood cultures, 2 were from ear swabs, one from cerebrospinal fluid and one from placental surface swab (Delappe et al. 2015).
Table 2: Number of listeriosis notifications, Ireland 2004-2010. Adapted from (HPSC 2015) and (Delappe et al. 2015).

<table>
<thead>
<tr>
<th>Type</th>
<th>Serotype 1/2a</th>
<th>Serotype 1/2b</th>
<th>Serotype 4b</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult/Juvenile</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Neonatal</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Pregnancy related</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>0</td>
<td>4</td>
<td>10</td>
</tr>
</tbody>
</table>

Globally, listeriosis outbreaks are still a cause for concern with three multistate outbreaks occurring within the United States in the first six months of 2016 alone (CDC 2016). These outbreaks involved frozen vegetables, raw milk and packaged RTE salads. This shows that despite efforts by the United States Centers for Disease Control and Prevention (CDC), European Food Safety Authority (EFSA), PulseNet and other food safety authorities worldwide to investigate, track, subtype and recall if necessary that listeriosis is a major problem. A potential reason for this increase is the ever growing demand for minimal processed goods and produce. Such goods which may lack any antimicrobial treatment step can allow the respective goods to carry microbial loads of which potentially may harbour microorganisms hazardous to human health.

1.4: The market for fresh-cut produce

The agri-food industry is one of the foremost economic contributors to the Irish national economy. As of 2014 the industry accounted for 8.4% of the total employment of the country predominantly in rural areas. Total agri-food exports accounted for €10.5 billion, the highest of all time, which represented 12% of all goods exported from the Republic of Ireland (DAFM 2015). The demand for fruit and vegetables has led to global fruit and vegetable production more than doubling in the years between 1990 and 2014 (FAO 2015). Multiple countries have run media campaigns showing the benefits for fresh and minimally processed fruit and vegetables. Most now recommend five servings per day of fruit and vegetables from all sources. However, consumer demand has continued to demand high quality, low cost
produce with no to little amount of preparation time. In turn the market has adapted to the change in consumer attitudes allowing for regular and easy access to high quality minimally processed fruits and vegetables, namely fresh cut and pre-packaged produce.

On a global level China produces the most fresh produce unsurprisingly due to 56% of the average diet consisting of fruits, vegetables and starch roots (FAOSTAT 2015). In Europe consumption of fresh-cut fruits and vegetables varies from 1–1.5 kg per person per year in Spain compared with the UK, 12 kg; France, 6 kg; Italy, 4 kg, Germany, Belgium and Netherlands consume more than 3 kg respectively, meanwhile the USA consume 30 kg per person per year (Abadias et al. 2008). In Ireland the retail market for fresh cut produce is said to be one of the most important segments for the entire grocery market with a market share of 14%, currently valued at €1.2 billion annually. The market for prepared fruit and vegetables is not quite as large, currently valued at €84 million per annum (Bia 2014).

**1.5: Fresh Cut Produce**

According to the International Fresh-Cut Produce Association (IFPA) fresh cut produce is defined as “any fresh fruit or vegetable or any combination thereof that has been physically altered from its original form, but remains in a fresh state”. These products may consist of peeled, trimmed, shredded, sliced and/or washed fruit or vegetables. These products are then usually packaged and stored at refrigeration temperatures.
Most fresh cut produce goes through an initial step of removing outer leaves, damaged areas, stalks, and dirt. Depending on the produce it may undergo slicing, shredding, cutting, grating or dicing, which induces physiological changes in the produce which can reduce the shelf life, increase the microbial load, reduce the quality and appearance of the produce (Allende et al. 2006). To counter this many fresh cut and minimally processed produce may undergo antimicrobial treatment such as chlorine solution dipping or other sanitising chemicals such as organic acids (lactic acids, citric acids, acetic acids), hydrogen peroxide, calcium-based solutions, ozone, electrolysed water and natural preservatives/antimicrobials such as essential oils and bacteriocins (Allende et al. 2006). Other techniques include the use of modified atmospheres, thermal treatments, irradiation, ultraviolet (UV) light, ultrasound decontamination, high pressure processing and combination of preservation techniques used together called hurdle technology (Rico et al. 2007). The wide variety of processing and

Figure 2: A flow diagram for the production of minimally processed fruits and vegetables. Adapted from (Francis et al. 1999).
treatment techniques has allowed of development of new market opportunities among the concern about the potential harmful effects of chemical treatments which in turn has expanded the demand for natural antimicrobials or chemical free treatments.

1.6: **Microbial safety of fresh cut produce**

It is well known that fresh cut produce can harbour a large and diverse microbial community. It has been shown that counts of $10^5 - 10^8$ CFU/g can be present in leafy green vegetables, carrots and sprouts. Fresh cut fruits such as apple, orange, pineapple etc. have been also shown to hold lower counts of indigenous microflora as they tend to not proliferate due to the low pH of the fruit and the low temperature storage conditions used for fruit (G. A. Francis and D. O’Beirne 1998, Francis *et al.* 1999, Abadias *et al.* 2008). While the majority of these bacteria are Gram- negative rods, mainly *Pseudomonas, Enterobacter* or *Erwinia* species, large numbers of lactic acid bacteria have also been found and are generally regarded as part of the normal flora of fresh cut produce. However coliforms, yeasts and moulds have been shown to arise from the raw material or from contamination during the processing procedure (Abadias *et al.* 2008). The potential of fresh cut produce to be a vector for transmission of bacterial, viral or parasitic pathogens is common knowledge. Some human pathogens may form part of this natural microbiota these may include *L. monocytogenes*, enterohaemorrhagicic *E. coli, Salmonella spp., Campylobacter* spp., *Yersinia enterocolitica, Bacillus cereus, Vibrio cholerae, Clostridium* spp. and the virus hepatitis A (Francis *et al.* 1999).

The processing of fresh cut produce can also introduce conditions that allow for survival and growth of pathogenic microorganisms. This can arise from the cutting or slicing step. Cut produce allows for both chemical and microbial degradation which with the release of the contents of the cells can provide compounds which provide a nourishing environment for the growth of microorganisms (Abadias *et al.* 2008). To counter this, microbial conscious agricultural, processing and manufacturing techniques must be employed at every level from “farm to fork”.

1.7: **Listeria prevalence in fresh cut produce**

The pervasive presence of *Listeria* species within the natural environment generally connected with soil, sewage, degrading vegetation and manure allows fresh cut produce to become contaminated with relative ease. Thus it can be regarded that *L. monocytogenes* is
naturally present on both fruit and vegetables (Francis et al. 1999, Beuchat 2002, Jablasone et al. 2005). Numerous studies have demonstrated that fresh cut produce can contain \textit{L. monocytogenes} and the potential of its growth during the shelf life of the product can represent a substantial health risk to consumers.

Studies have shown that \textit{Listeria} species can be found in up to 32% of RTE leafy green vegetables, 21% in Iceberg lettuce, 22% in salads containing a mix of different vegetables, corn, beans, cheese, ham, tuna or chicken. \textit{L. monocytogenes} was detected in 1% of samples (1/100) however this fell below the EU limit of 100 CFU/g (Kovačević et al. 2012). Other studies such as Abadias et al. (2008) exhibited that 0.7% (2/300) of the tested minimally process fruits and vegetables from Spanish retail displayed \textit{L. monocytogenes} activity. A Canadian study carried out by Denis et al. (2016) demonstrated that a prevalence rate for \textit{L. monocytogenes} of 0.32% (14/4435) in leafy vegetables (leaf lettuce, head lettuce, mixed greens, spinach, etc.) and 1.43% (2/140) in whole and fresh cut cantaloupes. In Brazil a study revealed that 3.03% (4/132) of samples were contaminated with \textit{L. monocytogenes}, comprising 2.22% (1/45) of raw vegetables and 5.56% (3/54) of ready-to-eat salad vegetables. In conjunction with this; antimicrobial resistance profiling showed that two isolates from RTE vegetables exhibited resistance to penicillin G, in contrast to a previous study by (Troxler et al. 2000) and tetracycline (de Vasconcelos-Byrne et al. 2016). A Swiss study, demonstrated a 3.5% (5/142) \textit{L. monocytogenes} prevalence rate among RTE lettuce samples from a national producer of fresh-cut and washed RTE salads and fresh-cut fruit (Althaus et al. 2012).

Over the decades a number of outbreaks of listeriosis have been linked to the consumption of fresh cut produce (Harris et al. 2003, CDC 2016). It is regarded that most contamination of fresh cut produce can occur on the field where soil, water, pesticides and fertiliser can be a natural haven for \textit{Listeria} species (Ng et al. 2005, De Keuckelaere et al. 2015, EFSA 2015). Other studies have shown that harbourage sites can be found within the food processing premises on equipment and in the water supply sources (Carpentier and Cerf 2011). The increased amount of processing that the produce goes through adds further risk of contamination. Washing in water removes the soil and other unwanted material but can introduce cross contamination of produce, while also the operations of peeling, shredding and slicing cause a removal of the plants natural barrier allowing the produce to become more susceptible to decay and bacterial contamination (Francis et al. 2012).
L. monocytogenes has been implicated in numerous outbreaks of listeriosis due to consumption of fresh cut produce such as whole cantaloupes, packaged salads, mung bean sprouts, coleslaw and pre-packaged mixed fruits and vegetables (Harris et al. 2003, CDC 2016). In early 2016 as part of a routine sampling program the Ohio Department of Agriculture collected Dole brand Field Greens pre-packaged salads from a commercial retail location and isolated L. monocytogenes. The salad products were processed from a Dole plant in Springfield, Ohio where both the CDC and the Canadian Food Inspection Agency (CFIA) confirmed the presence of L. monocytogenes, both the CDC’s and CFIA’s isolates being closely genetically related. The outbreak spanned across 5 provinces in Canada and 9 states in the USA. A total of 33 cases of listeriosis caused by L. monocytogenes were reported with one confirmed death from listeriosis and three other fatalities, however it has not been determined if Listeria contributed to the cause of these deaths. In January of 2016 the CDC had stopped production of the processing plant and in the same month Dole voluntarily recalled all salad mixes produced in the processing plant, effecting products in 30 states and provinces in both countries. As of March 2016 the CDC and Public Health Agency of Canada declared the outbreak to be over (CDC 2016, Public Health Agency of Canada 2016).

Table 3: Multistate Outbreak of Listeriosis from Springfield, Ohio Dole Processing Plant.
*Not been determined if Listeria contributed to the cause of these deaths.

<table>
<thead>
<tr>
<th>Country</th>
<th>States/Provinces Effected</th>
<th>Male</th>
<th>Female</th>
<th>Total Cases</th>
<th>Average Age</th>
<th>Reported Deaths*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canada</td>
<td>5</td>
<td>5</td>
<td>9</td>
<td>14</td>
<td>64</td>
<td>3*</td>
</tr>
<tr>
<td>USA</td>
<td>9</td>
<td>4</td>
<td>14</td>
<td>19</td>
<td>78</td>
<td>1</td>
</tr>
</tbody>
</table>

In Ireland and subsequently Europe the limit for presence of L. monocytogenes in fresh cut produce is 100 CFU/g over the course of the entire shelf-life of the respective product, falling under EU regulation (EC) No. 2073/2005. The manufacturer must have the ability to demonstrate this with regards to their product as is or employ techniques that have the
capacity to induce this limit. Testing for this method is conducted under EN/ISO 11290-2 (FSAI 2011) (E.U.). The United States employs a limit of “zero detection in one or more subsamples” of 25g of produce when tested using ISO 11290-2 (U.S. Food and Drug Administration 2015). Products that have properties such as low pH (4.4 or lower), have a low water activity (aw of 0.92 or lower) or are frozen have been well established that L. monocytogenes doesn’t grow upon them and are thus are generally regarded as unnecessary to test. However, these limits ensure that the majority of fresh cut produce must undergo antimicrobial treatments to safeguard from the growth of L. monocytogenes over the course of its shelf life.

1.8: Modified Atmosphere Packaging (MAP)

Modified atmosphere packaging is a technique that has been employed for over 50 years to increase the shelf life of fresh cut and whole fruits and vegetables. The process involves altering the gases surrounding particular product in order to create a gas composition that allows for extension of the shelf life of a product. MAP creates a predetermined gas composition which can change of the products life time due to variables such as physiological and physical factors or both the product and the environment. MAP is employed either passively or actively (Lamikanra 2002).

Passive MAP consists of hermetically sealing the produce in a semi-permeable vessel. Both the respiration of the product and the microbial activity combine to alter the gas composition. The effect of respiration consumes the surrounding O2 and produces CO2 eventually forming an equilibrium between the permeation and respiration rate (Heard 2002). Depending on the type of packaging used O2 levels can be reduced from 21% down to levels close to 0% while CO2 levels can be increased from 0.04% to 20% or higher. Active MAP consists of flushing out the resident atmospheric air within the packaging a replacing it with an accurate mixture of initial gases (Wiley 1994).

Primarily nitrogen, carbon dioxide, oxygen and the noble gases are the main gases used in MAP (Sandhya 2010). N2 is generally employed as a filler gas with other gases such as CO2 and O2 being employed as precisely as possible. The gases are used in different concentrations depending on the product and the producer’s needs. It has been shown that the greatest extension of shelf life can occur at the lowest concentration of oxygen before anaerobic respiration is initiated. The optimum percentage of O2 for both produce quality and
microbial safety is generally regarded as between 1 and 5%. CO$_2$ is usually the only gas employed that has a direct antimicrobial activity allowing for an increased lag phase and reduction in total growth, however this depends on concentration. Levels above 20% CO$_2$ has shown to induce widespread antimicrobial properties, while lower levels in combination with O$_2$ can selectively inhibit various foodborne pathogens (Francis et al. 1999, Caleb et al. 2012). Other gases including various nitrous and nitric oxides, sulphur dioxides, ethylene, chlorine and ozone have been investigated however they have not been applied due to cost, regulatory and safety concerns (Sandhya 2010).

Various forms of packaging are also used to safeguard and improve the modified atmosphere (MA). Usually MAP consists of the enclosure of the fresh cut produce in different types of polymeric films depending on the product and the envisioned effects. Examples of the types of packaging include oriented polypropylene (OPP), low density polyethylene (LDPE), medium density polyethylene (MDPE), polyvinyl chloride (PVC) and cellulose film. Each offer abilities such as strength; attractive appearance; low permeability to water vapour or gases, odours and greases, printability and durability (Lamikanra 2002).

In general, extensive knowledge of gas concentrations, respiration rates, microbial activity, intended shelf life, storage conditions and packaging types need to be engaged in order to develop optimum conditions for microbial suppression and produce shelf life. However, it has also been shown that use of unsuitable packaging materials for the produce or the physical conditions endured throughout the entire shelf life can lead to unsuitable atmospheres which can have detrimental effects. Cliffe-Byrnes et al. (2003) demonstrated that high product respiration in combination with low film permeability of OPP can lead to an increase of CO$_2$ and a reduction of O$_2$ leading to the formation of anaerobic conditions within the packaging and induced detrimental effects on the quality, with loss of firmness, high cell permeability and exudate, high surface moisture, poor acceptability of aroma and reduced pH all being recorded. Conversely the little modification of the internal atmosphere by the use of micro-perforated films resulted in an insufficiently modified atmosphere to be technically useful. This demonstrates the potential problems that can arise depending on the many types of fresh cut produce, packaging and potential modified atmospheric conditions.

1.9: MAP and Listeria
Modified atmospheres have been shown to be an effective preservation technique for maintenance of the quality and appearance of fresh cut fruits and vegetables throughout the shelf life of the respective products. This comes through the effect of the gases on the natural microbiota, control of the humidity during storage and protection from contamination or further damage via the use of correct packaging (Lamikanra 2002). However the increase in shelf life, longer preservation of appearance and an effectively sealed environment can allow for growth of ubiquitous pathogens and increase the microbial count in excess of $10^8$ CFU/g with the visual quality still being regarded as fit for sale (Allende et al. 2004).

The ability of *Listeria* spp. to survive and grow under the anaerobic conditions that are commonly used in the modified atmosphere packaging steps of the production of fresh cut fruits and vegetables. In combination with its ability to grow at low temperatures can allow for *Listeria* to gain a foothold against a technique that was designed to control and provide a hurdle against further microbial spoilage. This has been shown by several authors Beuchat and Brackett (1991), Oliveira et al. (2010) Gillian A. Francis and David O’Beirne (1998). For example, Francis and O’Beirne (1997) showed that on shredded lettuce a flushed packaged atmosphere of Nitrogen at 3 °C extended the survival of *Listeria* species and at 8 °C extended their growth. However, there are conflicting reports on the behaviour of *L. monocytogenes* on fresh cut produce under MAP conditions at refrigeration temperatures (Oliveira et al. 2015b). For instance Siro et al. (2006) demonstrated that *L. monocytogenes* was slightly inactivated when stored under an equilibrium modified atmosphere of 3% O$_2$/5% CO$_2$ however under an atmosphere high oxygen atmosphere (95% O$_2$/5% N$_2$) it could survive. This was also recorded using strawberries as a growth medium, where the same high oxygen atmosphere and an air atmosphere provided favourable conditions for the growth of the pathogen.

The ability of CO$_2$ to inhibit the growth of bacteria is four fold. 1. Directly affecting cellular enzymes thus decreasing the rate of metabolic reactions. 2. Increasing the production of carboxylates and decarboxylases. 3. Disruption of the cell membrane by interfering with specific structures or functions. 4. The overall decrease in pH in the substrate. In order to counter the low pH that *L. monocytogenes* encounters on its infectious cycle the bacterium possess a stress adaption. Specifically *L. monocytogenes* possess a glutamate decarboxylase dependant (GAD) stress response system (Hill et al. 2001). This system plays a major role in its acid tolerance as when the cell is exposed to low pH GAD catalyses the conversion of
glutamate to γ-amino butyrate and CO₂, thus removing a proton from the cytoplasm and resulting in an increase in cytoplasmic pH (Francis et al. 2007). The stress adaption was shown to have a significant role in the ability of L. monocytogenes to survive in storage while under a modified atmosphere of up to 35% CO₂ (Francis et al. 2007).

According to Wiley (1994) there are only two main safety concerns when it comes to MAP of produce. Firstly, is the potential of the physical packaging introducing the potential for harmful or toxic chemicals onto the produce. Secondly, and more relevant, is the potential increase in microbial load. The increase in shelf life, allowing for more time for the propagation of potential microorganisms added with the somewhat selective nature of MAP when product quality and appearance is the primary concern increases the need for the principles of good agriculture practice (GAP), good manufacturing practice (GMP) and hazard analysis critical control point (HACCP) throughout the entire chain of production, storage and consumption. This also enhances the use of specific natural antilisterial compounds to be used in conjunction with MAP and low refrigeration temperatures.

1.10: Natural antimicrobials

Antimicrobials can be defined as any substance that kills or inhibits the growth of microorganisms causing little or no damage to the host (MSU 2011). “Natural” states that the source of the compound is from plants, animals or microflora. Antimicrobials have many uses ranging from antibiotics, antifungals, disinfectants, antiseptics, sanitizers and an antiviral medicine.

A major use of natural antimicrobials is for food preservation. The spoilage and contamination of various types of foods has been a problem that humanity has had to deal with since time immemorial and is not yet under sufficient control, nonetheless a wide range of preservation techniques has been developed included sterilization, dehydration, freezing and use or synthetic and natural preservatives. With the rise of minimal processing and RTE produce, food producers and retailers have had to rely on more mild processing techniques to maintain the natural appearance, quality and nutritional values of the produce. In order to meet these criteria new natural alternatives have been developed with natural antimicrobials taking a major role (Rahman 1999, Tiwari et al. 2009).

Plants have been used for hundreds if not thousands of years for their antimicrobial properties, with herbs and spices being generally recognised as safe (GRAS) for their
traditional use and because of dedicated toxicological studies. Plant antimicrobials mostly fall in to these categories: phytoalexins, organic acids, phenolic compounds, essential oils (EOs) and their subsequent components (Smid and Grorris 1999). EOs are considered to be secondary metabolites of plants and have an important function in defence of the plant from microbial infection (Tajkarimi et al. 2010). The antimicrobial compounds in EOs and their secondary constituents have been shown to exhibit strong antimicrobial affects against a broad range of pathogenic foodborne microorganisms including Listeria monocytogenes, Salmonella typhimurium, Escherichia coli O157:H7, Shigella dysenteria, Bacillus cereus and Staphylococcus aureus (Burt 2004). The mode of action of EOs is as diverse as the group itself but it has been demonstrated that the effects of the EOs act by causing structural and functional damage to the bacterial cell membrane. This has been linked to the hydrophobicity of the EOs and the characteristic accumulation in the lipid rich environments of the cell membrane (Goñi et al. 2009). Combined with their antimicrobial properties EOs are also used as flavourings which proving an interesting “in” for use on food products but this can also be a negative factor as a negative organoleptic effect limits can be easily reached with minimal application of the EO (Hyldgaard et al. 2012).

In recent decades, the use of antimicrobials from animal sources has increased mainly driven by the emergence of numerous food pathogens and the developed understanding of the molecular mechanisms of defensive bioactive substances derived from animals (Satyanarayan Naidu 2003, Tiwari et al. 2009). Satyanarayan Naidu (2003) describes three broad categories of animal antimicrobials: immunoglobulins, iron-chelators and enzymes. Immunoglobulins or as they are more commonly called antibodies can act on both the disease causing pathogen and the toxins produced from the pathogen. However, the high costs of immunoglobulins such as ovoglobulins (IgY) and lactoglobulins (IgG) have primarily led to its use in the medical fields and not specifically food safety (Satyanarayan Naidu 2003).

Lactoferrin is an example of an iron-chelator. A glycoprotein most commonly found in milk but also can be found in animal saliva and other bodily fluids. Lactoferrin displays antimicrobial activity over a wide range of bacteria and viruses, its functionality, depends on the protein confirmation, with its main mode of action being the seizing of iron from the environment and thus the inhibition of bacterial growth or biofilm formation (Jenssen and Hancock 2009). Lactoferrin is commonly used in infant food formulations, mainly in South
East Asia, however it is cost prohibitive as large doses of lactoferrin are required to obtain a preservative effect (Satyanarayan Naidu 2003).

Enzymes such as lysozyme play an important role in the natural defence systems of many plants and animals. Commonly found in the egg white of chicken eggs, with each egg containing approximately 0.3-0.4g of lysozyme (Satyanarayan Naidu 2003). Lysozyme displays broad spectrum activity against Gram positive bacteria by catalytic hydrolysis of the cell wall peptidoglycan allowing for the build-up of internal osmotic pressure and thus the lysis of the cell. It has also been shown to display catalysis independent antimicrobial properties (Satyanarayan Naidu 2003, Nash et al. 2006, Kluter et al. 2014). Lysozyme has been used in several different types of foods including meat, fish, beer but most commonly in cheese. The specificity of lysozyme, commonly used against Clostridium species, allows it to be effective without effecting the starter culture within the cheese vats (Satyanarayan Naidu 2003).

Microorganisms have also been used for hundreds of years to produce a wide variety of compounds that can impact the growth of other microorganism. Primarily these compounds have been used and found naturally in fermentation based foods to provide microbial security from pathogens to products such as cheeses, sausages meats and fermented vegetables (Sauerkraut etc.). Lactic acid bacteria (LAB) are described a significant source of bio-preservatives and are commonly found in various food types and form part of the natural human microbiota allowing the Lactobacilli and Lactococcus species to be considered GRAS (Pessione 2012). LAB have the ability to produce organic acids (lactic acid, acetic acid, carbonic acid) and inorganic acids (hydrogen peroxide) both of which have a wide spectrum of antimicrobial activity. In addition, LAB can produce antimicrobial compounds which have a relatively narrow spectrum (bacteriocins).

According to Alvarez-Sieiro et al. (2016) bacteriocins from LAB can be classified into 3 classes. Class I consists of ribosomally produced and post translationally modified peptides (RiPPs) of less than 10 kDa. Examples include lanthipeptides (Nisin), Glycocins, LAPs (Streptolysin S). Class II comprises of unmodified bacteriocins of less than 10 kDa. Examples include Pediocin-like bacteriocins (Pediocin PA-1) and two-peptide bacteriocins (Lactococcin Q). Class III encompasses unmodified bacteriocins greater than 10 kDa with bacteriolytic or non-lytic mechanism of action. Examples include bacteriolysins and non-lytic
bacteriocins. Classes I and II can be further subdivided into 6 (Ia-Ie) and 4 (IIa-IIe) subclasses each (Alvarez-Sieiro et al. 2016).

Cystibiotics are an example of a group of bacteriocins from the IIa category which have been shown to be highly bactericidal towards *Listeria* species and *L. monocytogenes* specifically (Ray and Miller 2003). Pediocin PA-1/AcH is an example of a cystibiotic that displayed antilisterial activity. A study by Mattila (2003) showed that a preparation of pediocin had an antilisterial effect, reduction of *L. monocytogenes* cells to <2 log CFU/g without having an effect on the growth of LAB nor the pH or flavour of sliced sausage. However, to date nisin remains the only approved antimicrobial worldwide for food applications (Kouakou et al. 2016). Nevertheless, commercial preparations of pediocin PA1-AcH are available in certain countries.

1.11: Nisin

Nisin was first identified in 1928 in milk cultures that had been stored overnight prior to cheese making (Rogers and Whittier 1928). In 1953 it was first marketed as an antimicrobial agent for sale in the United Kingdom and subsequently approved by the FAO/WHO in 1969 and in the United States by the food and Drug Administration (FDA) in 1988 as a generally regarded safe food additive (Delves-Broughton et al. 1996, Cotter et al. 2005). Nisin when used as a treatment is most stable in acidic conditions, and is soluble in aqueous environments.

Nisin interacts electrostatically with the phospholipids of the cytoplasmic membrane with particular affinity for the Lipid II peptidoglycan while also interfering with the cell wall biosynthesis. This two fold interaction allows for the formation of pores which permits the uncontrollable flow of the molecular cellular constituents out of the cell resulting in the cessation of the transmembrane proton motive force and ultimately cell death (Breukink et al. 1999, de Arauz et al. 2009). This method of effect is usually present when nisin effects Gram-positive bacteria. Gram-negative cells with a lipopolysaccharide layer obtain tolerance to the action of nisin as the layer blocks the interaction of nisin and the cytoplasmic membrane. However, to counter this, chelating agents such as EDTA can be employed to destabilise the lipopolysaccharide layer allowing for nisin to gain access to the cytoplasmic membrane thus inducing the formation of pores (de Arauz et al. 2009).
Nisin A, the first described variant of nisin, is a 34 amino acid ribosomally synthesised peptide produced by specific strains of the bacterium *Lactococcus lactis*. Initially synthesised as a precursor peptide it then undergoes post translational modification to produce lanthionine and β-methyllanthionine groups, finally the modified peptide is exported from the cell and the C-terminal leader peptide cleaved resulting in mature nisin (Adams and Smid 2003). It is part of the lantibiotic Class Ia group of bacteriocins due to the modified peptide and having a molecular weight of 3354.07 g/mol (3.3 kDa).

To date eight natural variants of nisin have been discovered, with nisin A, Z, F and Q isolated from *L. lactis* sub species and nisin H, U, U2 and P isolated from various members of the *Streptococcus* species.

**Table 4:** Natural variants of nisin. Adapted from Shin et al. (2016)

<table>
<thead>
<tr>
<th>Variant</th>
<th>Unmodified amino acid sequence</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin A</td>
<td>ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK</td>
<td>(Gross and Morell 1971)</td>
</tr>
<tr>
<td>Nisin Z</td>
<td>ITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK</td>
<td>(Mulders et al. 1991)</td>
</tr>
<tr>
<td>Nisin F</td>
<td>ITSISLCTPGCKTGALMGCNMKTATCNCSVHVSK</td>
<td>(de Kwaadsteniet et al. 2008)</td>
</tr>
<tr>
<td>Nisin Q</td>
<td>ITSISLCTPGCKTGVLMGNCNLKTATCNCSVHVSK</td>
<td>(Zendo et al. 2003)</td>
</tr>
<tr>
<td>Nisin H</td>
<td>FTSISMCTPGCKTGALMTCNYKTATCHCSIKVSK</td>
<td>(O'Connor et al. 2015)</td>
</tr>
<tr>
<td>Nisin U</td>
<td>ITSKSLCTPGCKTGILMTCPLKTATCGCHFG</td>
<td>(Wirawan et al. 2006)</td>
</tr>
<tr>
<td>Nisin U2</td>
<td>VTSKSLCTPGCKTGILMTCPLKTATCGCHFG</td>
<td>(Wirawan et al. 2006)</td>
</tr>
<tr>
<td>Nisin P</td>
<td>VTSKSLCTPGCKTGILMCAIKTATCGCHFG</td>
<td>(Zhang et al. 2012)</td>
</tr>
</tbody>
</table>
Bioengineered variants have also been developed in order to increase the efficiency and stability under the various conditions that the antimicrobial may be used. Benefits of the bioengineered variants include increased thermal stability, enhanced activity against specific bacteria including Gram-positive drug-resistant pathogens, Gram-negative food-associated pathogens and greater solubility at alkaline pH (Yuan et al. 2004, Field et al. 2012). The development of the new nisin bioengineered variants, and the natural nisin variants have also shown promise as an effective alternative in the treatment of infectious diseases, particularly antibiotic resistant skin infections and biofilm associated infections (Shin et al. 2016). These biomedical uses have expanded the use of nisin beyond its use in food processing technologies and into areas such as cancer therapy, oral health, gastrointestinal and respiratory infections (Shin et al. 2016).

Current uses of nisin in the minimal processing of foods can be divided into two specific areas, the traditional use of nisin as a food preservative and the use of nisin combination or “hurdle” antimicrobial technologies. Traditional preservative use consists of adding nisin or nisin infused salt directly to the food product allowing for the antimicrobial action to be the main source of antimicrobial control. This is commonly used cheese, milk, dairy products, dressing, sauces and post fermented meats (Adams and Smid 2003). The hurdle concept consists of using multiple inhibitory and/or preservative techniques and processes together in order to provide a product with a sustainable shelf life without impacting on its sensory qualities (Zhou et al. 2010). Combinations of nisin and numerous physical and chemical techniques have been described within the literature. Currently the combination of nisin plus heat is commonly used and has been proven to be quite effective, especially when used against spores that may survive the heat treatment stage (Boziaris et al. 1998). Even sub-lethal heat has been shown to increase the efficiency of nisin which may allow for lower temperatures to be used (Galvez et al. 2007). This has also been observed with L. monocytogenes, where nisin was shown to decrease the organism’s resistance to thermal treatments allowing for a milder heat treatment process (Budu-Amoako et al. 1999).

Other physical treatments include the use of high hydrostatic pressure. The introduction of high pressures (>100 MPa) induces a physical change in the cell membrane as well as protein degradation due to compression and other sub-lethal effects. These distresses in combination with nisin has been shown to induce a synergistic effect to reduce L. innocua counts by 5-6 log CFU/ml in liquid egg (Ponce et al. 1998). Offering the possibilities of a technique to be
an alternative to thermal based pasteurisation techniques which allow for the conservation of the products physical characteristics.

The use of nisin in combination with other chemical treatments is another area of interest. Particular interest has been the synergistic effects of nisin when combined with other “green” or preservatives perceived as natural. Examples include the use of plant derived antimicrobials such as garlic shoot juice, grapefruit seed extract (GSE) and animal derived antimicrobials such as lysozyme and lactoferrin (Murdock et al. 2007, Xu et al. 2007, Kim et al. 2008, Bhatia and Bharti 2015). Xu et al. (2007) showed that a combination of 50 μg/mL nisin, 0.5% citric acid and 0.02% GSE contributed to a 2.5 log CFU/g reduction of *L. monocytogenes* on whole lettuce without a negative effect on the organoleptic properties. Murdock et al. (2007) displayed that when nisin is used in combination with lactoferrin it can be an effective control of the growth of *L. monocytogenes* allowing for a synergistic inhibition for as low as 10 IU ml⁻¹ of nisin when used in combination with 250 μg ml⁻¹ of lactoferrin.

Commercial nisin is generally available as a 2.5% w/w fermentation product in sodium chloride, the majority, and residue protein and carbohydrate. Pure nisin has an assigned activity of 40x10⁶ international units (IU) with most commercially available nisin having a activity of 1 x10⁶ IU (Handary 2015). However, the application of commercially available nisin shows differences in activity when compared to pure nisin. de Arauz et al. (2009) states that it is thought that the milk proteins in the preparations bind to the nisin molecule limiting its antimicrobial activity, thus partial or complete purification can increase its activity and thus its performance.

**Table 4**: International regulation on Nisin A (2.5% w/w). Adapted from Handary (2015).

<table>
<thead>
<tr>
<th>Country</th>
<th>Application</th>
<th>Limit (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>European Union</td>
<td>Clotted Cream</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>Cheese</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>Semolina, Tapioca and Similar Puddings</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>Mascarpone</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>Liquid Eggs Products</td>
<td>500</td>
</tr>
<tr>
<td>Russia</td>
<td>Processed Cheese</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Canned Vegetables</td>
<td>100</td>
</tr>
<tr>
<td>Country</td>
<td>Category</td>
<td>Limit</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>Ukraine</td>
<td>Preserved Vegetables</td>
<td>4000</td>
</tr>
<tr>
<td></td>
<td>Cheese</td>
<td>500</td>
</tr>
<tr>
<td>United States</td>
<td>Processed Cheese Spreads</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Sauces and Nonstandard Salad Dressings</td>
<td>600</td>
</tr>
<tr>
<td></td>
<td>Pasteurised and Chilled Soups</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Liquid Egg Products</td>
<td>600</td>
</tr>
<tr>
<td>Mexico</td>
<td>Permitted Additive</td>
<td>No Limit</td>
</tr>
<tr>
<td></td>
<td>Processed Cheese</td>
<td>500</td>
</tr>
<tr>
<td>Australia</td>
<td>Crumpets, Flapjacks &amp; Pikelets</td>
<td>1000</td>
</tr>
<tr>
<td>New Zealand</td>
<td>Processed Cheese and Cheese Spread</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>Beer</td>
<td>200</td>
</tr>
<tr>
<td>Turkey</td>
<td>Clotted Cream</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>Semolina, Tapioca and Similar Puddings</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>Ripened and Processed Cheese</td>
<td>500</td>
</tr>
<tr>
<td>South Africa</td>
<td>Ripened and Processed Cheese</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>Semolina, Tapioca and Similar Puddings</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>Cheeses, Soft Cheese and Cheese Spread</td>
<td>500</td>
</tr>
<tr>
<td>China</td>
<td>Milk and Dairy Products</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>Canned edible Fungi, Seaweeds and Cereal Porridge</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Pre-processed meat products</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>Beverages</td>
<td>200</td>
</tr>
<tr>
<td>India</td>
<td>Cheese and Processed Cheese</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Coconut Water</td>
<td>125</td>
</tr>
</tbody>
</table>

**Table 5:** European Union regulations on adding E 234 (Nisin) to foodstuffs (E.U. 1995).

<table>
<thead>
<tr>
<th>European Union</th>
<th>Semolina and tapioca puddings and similar products</th>
<th>Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ripened cheese and</td>
<td>12.5 ppm</td>
</tr>
</tbody>
</table>

Chapter 1: Literature Review
processed cheese

Clotted cream 10 ppm

Mascarpone 10 ppm

1.12: Inoculation densities

Inoculation density is described as the number of a specific target organism per unit of area or volume, more specifically, in terms of microbiology, it is the amount of a microorganism that is applied to a substrate in order to study its effect on the substrate or surrounding microbiota. Generally, current studies establishing the effects of microbial exposure to minimally processed fresh cut produce are conducted under conditions using laboratory media, high initial inoculation densities and broth based assays (Presser et al. 1998, Tienungoon et al. 2000, Koutsoumanis and Sofos 2005, Gysemans et al. 2007). However, in more realistic situations pathogens are more likely to contaminate food products at low densities and grow over the course of the shelf-life to a level upon which they are a hazard to human health (Samelis and Metaxopoulos 1999, Uyttendaele et al. 1999, Vermeulen et al. 2009). It has been shown that the lower the inoculum levels can lead to an increase in variability in the observed population lag phase (Robinson et al. 2001, Francois et al. 2006). While low levels (<10² CFU/g) of pathogenic bacteria such as L. monocytogenes are not of concern to human health, the potential to outgrow the non-pathogenic microflora, which should provide competition for resources, thus naturally limiting the effect of the pathogenic bacterium. Other environmental effects which influence the inoculum, include water activity (a_w), redox potential (Eh), pH, environmental structure or matrix and temperature (FDA 2001, Koutsoumanis and Sofos 2005, Vermeulen et al. 2009, Hereu et al. 2014).

Numerous studies into the effect of inoculum size on the growth of L. monocytogenes have been carried out with the vast majority being conducted under in vitro 24 or 96-well microtiter plate based assays, where variables such water activity, pH, environmental stresses and temperature can be easily controlled (Tienungoon et al. 2000, Robinson et al. 2001, Koutsoumanis and Sofos 2005, Gysemans et al. 2007, Vermeulen et al. 2009). These studies have allowed for the development of mathematical models which under applied
circumstances can accurately predict bacterial growth. However, many of these models have inadequacies when cell densities are below a certain limit (Vermeulen et al. 2009), particularly when under environmental stresses which can lead to cell variability (Aguirre and Koutsoumanis 2016). These models have also shown that an increase in the inoculum size can result in a change of the normal growth limits to more extreme inhibitory conditions (Robinson et al. 2001, Koutsoumanis and Sofos 2005, Dupont and Augustin 2009, Aguirre and Koutsoumanis 2016). While the information gain from these models is of great use there is an increase trend to move away from the broth-based Buchanan model and its successor, as Koseki and Isobe (2005a) describe can for *L. monocytogenes* deviate markedly from the observed data when grown under solid media and lettuce.

As several factors can influence the growth of *L. monocytogenes* deterministic and stochastic models provide more valuable insight as they can describe and account for variability and uncertainty (Mejlholm et al. 2015). While some deterministic (Mejlholm et al. 2010) and stochastic (WHO and FAO 2004) models have been employed, they are generally used in quantitative microbiological risk assessments. However according to Mejlholm et al. (2015) many of these remain “incomplete and insufficiently validated” for *L. monocytogenes*. The development on models for the predictive growth of *L. monocytogenes* such as (Koseki and Isobe 2005b, Koseki and Isobe 2005a, Ding et al. 2009) have been shown to be based on higher temperature for up to 25 °C than have been reported in studies carried out in the storage and transport of lettuce (O’Beirne et al. 2015, Brown et al. 2016). Even recently developed models for the growth of *L. monocytogenes* on leafy greens have shown that more studies need to be conducted into the effects of cut and uncut leaves on the growth of the pathogens and that these results should impact the effect of future quantitative microbial risk assessments and trials (Mishra et al. 2017).

To compound the lack of complete predictive microbiology models for *L. monocytogenes* there is an apparent lack of studies which can provide information on the effect of low to high inoculation cell densities *in situ* on various food matrices in which *L. monocytogenes* has been shown to grow. Many of the current experiments focus on fixed starting cell densities examining the effects of atmosphere, temperature, food matrix, antimicrobial treatment, pH and *a*_w on the microorganisms (Barmpalia et al. 2005, Koseki and Isobe 2005a, Hereu et al. 2014, Mejlholm et al. 2015, Mejlholm and Dalgaard 2015, Ostergaard et al. 2015), however some such as Quinto et al. (2016) and (Omac et al. 2015) did use multiple inoculation
densities but their experiment were not specifically designed to study the effects on inoculation density on *L. monocytogenes* growth.

Thus there is somewhat of a void between experiments using low inoculation densities, similar to the effect of a contamination and those using high inoculation densities for the investigation of antimicrobial properties of various natural and man-made chemical treatments. Many of these treatment use cell densities greater than $10^4$ log CFU/g (Oliveira et al. 2010, Bae et al. 2011, O’Beirne et al. 2015, Oliveira et al. 2015b). While not substantial the filling this knowledge gap could provide useful information for models that are tailored for specific food matrices, continuing the trend away from liquid media growth and towards food based growth experiments.

1.13: *L. lactis*

*Lactococcus lactis* is a species of bacteria that form part of the Lactic Acid Bacteria (LAB) or *Lactobacillales* clade consisting of the following genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, *Weisella*. LAB are Gram-positive, non–sporeforming rods, cocci or coccibacilli, normally are non-respiratory, lack the enzyme catalase and unified by the production of lactic acid from glucose, be they homofermenters or heterofermenters (Samaržija et al. 2001, Todar 2012). Commonly found on plants, in meats and dairy products. LAB are most notably known for their use as starter cultures of various dairy products including yogurt, buttermilk, many different cheese types. Other beneficial uses encompass their inclusion in the processing of fermented meats, alcoholic drinks, fermented vegetables such as sauerkraut, olives and as probiotic organisms (Carr et al. 2002, Bachmann 2009). LAB also can provide negative effects such as producing unsavoury flavours, excess lactic acid production and with some species becoming an opportunistic pathogen in patients with compromised immune systems (Carr et al. 2002).

*L. lactis* itself is a facultative anaerobic Gram-positive spherical shaped species which is further broken down into two major subspecies *Lactococcus lactis* spp. *lactis* formerly known as *Lactobacillus lactis* and *Lactococcus lactis* spp. *cremoris* and one biovar *L. lactis* subsp. *lactis* biovar diacetylactis (Kim et al. 1998, Rademaker et al. 2007, Karaaslan et al. 2016). *L. lactis* is recognised as a good model organism for low GC content Gram positive bacteria (Wegmann et al. 2007). As a species *L. lactis* has an optimal growth temperature of between
30 °C to 42 °C with only a slight reduction in viable cell when exposed to 42 °C. At low temperatures the species has a doubling time of 7 days at 4 °C (Sanders et al. 1999). It has also been demonstrated that L. lactis can resist a pH as low as 2.5 to 3.0. The main reported differences being the ability of L. lactis spp. lactis to respond better to external stresses such as acid, bile-salts and freezing stresses thus L. lactis spp. cremoris is seen as being more delicate and effected by environmental conditions (Kim et al. 1998).

Numerous bacteriocins are produced by members of the LAB, with various strains of L. lactis producing several including nisin A, nisin Z nisin F, nisin Q, lacticin 481, lacticin 3147 and lacticin J46 (Mozzi et al. 2010). These ribosomally synthesised antimicrobial peptides have been shown to target to bacteria that are similar to the producer i.e. Gram positive bacterium (Cotter et al. 2005). These bacteriocins are generally growth associated with production occurring during the growth phase and concluding near the end of the exponential phase. The bacteria can be grown on various types of media in order to produce the required bacteriocin for example nisin Z can be grown from glucose, sucrose and xylose (Parente and Ricciardi 1999). Commonly Man, Rogosa and Sharpe (MRS) media is used however this can be expensive. Some studies have shown that a MRS-Skimmed milk can allow better antibacterial activity as compared to other medium formulations (Dussault et al. 2016). The bacteriocins are then extracted from the medium on a laboratory scale using methods such as ion exchange, hydrophobicity interactions and reverse phase chromatography (de Arauz et al. 2009). These methods require expensive machinery and are quite time consuming. Other alternative methods such as liquid-liquid and two-phase micellar systems have been shown to be successful (Jozala et al. 2008, Xiao et al. 2010).

Numerous species of LAB have been shown to be effective in reducing the viable count of several pathogenic organisms, particularly L. monocytogenes on various different food matrices, including fresh and minimally processed products (Stecchini et al. 1995, Leriche et al. 1999, Allende et al. 2007, Liu et al. 2008, Trias et al. 2008, Randazzo et al. 2009, Unlu et al. 2016). With regards to all food products, the bacteriocinogenic strains are generally added as starter cultures, most commonly with dairy products or as protective cultures (Allende et al. 2007) however the production of bacteriocin are generally growth associated and thus the yield of production is affected by the conditions in which the strain is allowed to grow (Parente and Ricciardi 1999). A recent study by Gomez et al. (2016) showed that the use of probiotic LAB from a food source can form protective biofilms that can lower the incidence
of pathogenic bacteria such as *L. monocytogenes*, *S. Typhimurium* and *E. coli* O157:H7. While the experiment was carried out on a plastic surface it provides useful and positive information on the use of LAB as protective biofilms, with potential to be used in foods.

The main problem with the use of *L. lactis* and LAB produced bacteriocins *in situ* with RTE fresh or minimally processed produce is the due the fact that bacteriocin production is linked with the growth of the bacteriocin producing bacteria (Parente and Ricciardi 1999). Other effects such as autochthonous bacteria, the binding of the bacteriocin to the food, inactivation of the bacteriocin by proteases and the general physical properties (pH, fats, starch, proteins) of the foods (Perin et al. 2015). Thus as recommended by Woraprayote et al. (2016) direct addition of bacteriocins in recommended and is seen as more feasible from both an economic and legal point of view. However, in certain foods, especially cheeses, the direct addition of LAB can be beneficial. A study by Perin et al. (2015) displayed by the use of PCR-DGGE means that the addition of a specific *L. lactis* subsp. *lactis* strain on a starting culture of cheese compared to a control cheese had a clear difference in the autochthonous microbiota while also controlling the growth of coagulase-positive cocci and influencing the production of biogenic amines (which can cause several toxicological problems) in the cheeses to a level acceptable for human consumption.

### 1.14: Hurdle Technology

Hurdle technology is the use of multiple preservation techniques in order to achieve an acceptable level of microbial safety and security. The correct application of hurdle technology ensures that any microorganisms present during the life time of the storage of the food don’t have the ability to overcome all of the antimicrobials techniques in order to cause food spoilage or food poisoning. According to Leistner (2000) the aim of hurdle technology is to “deliberately via an intelligent mix” combine hurdles which will “improve the microbial safety and the sensory quality of foods as well as their nutritional and economic properties”. Hurdle technology is of great importance to minimally processed foods as consumers’ demand products that are quick and easy to prepare while also have the appearance of being “fresh”, “natural” and “green” (Abadias et al. 2008).

When applied to lettuce and other fresh-cut produce, examples of hurdle technology consist of using techniques such as low and consistently controlled temperatures, MAP, washing in water, chlorine dips and washes, bio-preservatives and more rarely used irradiation.
(Hagenmaier and Baker 1997, Leistner 2000, Abadias et al. 2008, Scollard et al. 2013, Oliveira et al. 2015b, Scollard et al. 2016). In general, when applied together these techniques have the ability to provide enough of a barrier to stop most if not all pathogenic organisms. However, as has been shown by the numerous outbreaks of L. monocytogenes and other pathogenic bacteria with the ability to survive under the extreme conditions the hurdle technology induces, more individual hurdles or the implementation of harsher “upgrades” of the current hurdles need to be deployed. The mechanisms of hurdle technology to disrupt the microorganisms is applied by the several “hurdles”. Each hurdle simultaneously and intentionally disrupts a homeostasis mechanism of the microorganism the combination of which induces bactericidal or bacteriostatic effects. Thus according to Lee (2004) the success of hurdle technology depends on “ensuring metabolic exhaustion”.

Table 6: Examples of some hurdles used to preserve foods. Adapted (Ohlsson and Bengtsson 2002, Lee 2004).

<table>
<thead>
<tr>
<th>Type of Hurdle</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical Hurdle</td>
<td>Aseptic packaging, electromagnetic energy, high/low temperatures, ionic radiation, MAP, ultra-high pressures, ultrasound, UV radiation</td>
</tr>
<tr>
<td>Physico-Chemical Hurdle</td>
<td>Carbon dioxide, ethanol, lactic acid, low pH, low water activity, organic acids, ozone, salts, essential oils, surface treatment agents</td>
</tr>
<tr>
<td>Microbial Derived</td>
<td>Antibiotics, bacteriocins, competitive microflora, protective/starter cultures</td>
</tr>
</tbody>
</table>

However, problems can arise from the stresses put on the respective microorganisms as some bacteria can become more resistant and some can enhance their virulence via the generation of stress shock proteins (Rahman 2015). Other problems can arise though failures of certain hurdles, a common example is the lack of continuously low temperatures over the life time of the products storage (Oliveira et al. 2010). As many of today’s minimally processed food products are based on more than one hurdle treatment, it allows for a “safety net” in case of
failure of one of the hurdles. While this is true for almost all bacteria, certain bacteria such as *L. monocytogenes* and its variations have the ability to operate at the harshest conditions that hurdle technology can provide. Thus extra hurdles must be introduced to specifically counter these bacteria (Oliveira *et al.* 2010). Commonly combined hurdles used in various industries include temperature control, washing, modified atmosphere, specific packaging, chemical control of ripeness, use of products from fermentation such as LAB and their respective by-products (Zhou *et al.* 2010, Perin *et al.* 2015, Scollard *et al.* 2016).

Normally these hurdles are only added as a result of the method of the products production or necessity in order to supply a fresh product. This includes the use of starter/protective cultures in cheese or other fermentation products but not in products where fermentation doesn’t normally happen i.e. fresh cut produce. This provides an opportunity to use already proven methods of bacterial control as hurdles in other disparate products. Examples of extra hurdles which have been developed in one capacity but not used widespread within other industries include use of plant extracts including essential oils, bacteriocins and other products of fermentation and animal based antimicrobials (Burt 2004, Galvez *et al.* 2007, Scollard *et al.* 2013, Siroli *et al.* 2015, Scollard *et al.* 2016, Siroli *et al.* 2016). This provides openings for research in order to study the effects and effectiveness of these hurdles on differing products be they fresh cut, fermented, minimally or fully processed.
Chapter 2: Research Methodology

2.1: Investigation into the influence of inoculation density, temperature and atmosphere on the growth and enumeration of *Listeria monocytogenes*.

2.1.1: Preparation of fresh-cut vegetable (Iceberg Lettuce)

Iceberg Lettuce (Class I Spain) were obtained locally from a fruit and vegetable supplier (Supervalu Castletroy, Limerick) the day of testing and stored at 4 °C prior to processing. Outer layers and stem were manually removed using a disinfected sharp stainless steel knife. Any damaged leaves and the core of the heads were excluded. The remaining inner leaves were sliced with a disinfected sharp stainless steel knife and cut into 20 mm pieces. The cut lettuce (10 g) was transferred into 35 μm think orientated polypropylene (OPP) packaging bags (18 x 10 cm) which had a permeability to O₂ of 5.7 nmol m⁻² s⁻¹ kPa⁻¹ and to CO₂ of 19 nmol m⁻² s⁻¹ kPa⁻¹ (Scollard *et al.* 2016) (Amcor Flexibles, Gloucester, UK).

2.1.2: Preparation of *Listeria* cultures and inoculation of fruits/vegetable

For all inhibition tests a combination of three *L. monocytogenes* strains from the *Listeria* strain collection at Teagasc Food Research Centre (Moorepark, Ireland) were used in order to follow the European Guidelines for challenge tests on ready-to-eat foods (EUR *Lm*, 2014). The mix comprised of a EUR*Lm* reference strain (number 1382), a persistent strain isolated from a food processing plant (number 6179) and a strain isolated from a vegetable production facility (number 959). The cultures were grown separately in tryptone soya broth (50 mL TSB, Oxoid CM129, Fannin Healthcare, Cork, Ireland) at either 4 or 8 °C in accordance with European Guidelines for challenge tests, which specify that cultures should be incubated at similar temperatures to test conditions. Cultures were then mixed together to achieve equal numbers (CFU) of each strain and then diluted in phosphate buffered saline (PBS, Oxoid BR014, Fannin Healthcare) to allow inoculation of fresh-cut produce at \(10^2, 10^3, 10^4\) and \(10^5\) CFU/g. Aliquots of 0.1 mL of *L. monocytogenes* suspension were distributed uniformly over the lettuce contained within each of the packages.
2.1.3: Atmospheric treatments, package sealing and storage conditions

Following inoculation, packs were flushed with the following gas atmospheres: (a) air, (b) 8kPa CO₂, 4kPa O₂, 88kPa N₂ (optimum) or (c) 15kPa CO₂, 1kPa O₂, 84kPa N₂ (sub-optimum modified atmosphere packaging, MAP) and heat sealed using a vacuum packer (Multivac, UK). The packs were then stored (incubated) at either 4 or 8 °C for up to 7 days.

2.1.4: Enumeration of *Listeria*

Bacterial cell counts were carried out on day 0 (day of inoculation) and days 2, 5, 7 (throughout storage) from three replicate packs. The lettuce samples from each package were homogenised for 120 s at high speed in phosphate buffer saline (PBS, Oxoid BR0014) in a 2-fold dilution (20 mL) for inoculum density $10^2$ CFU/g, a 5-fold dilution (50 mL) for inoculum density $10^3$ CFU/g and a 10-fold dilution (100 mL) for inoculum density $10^4$ and $10^5$ CFU/g, using a Seward laboratory stomacher (Stomacher Model 400, AGB Scientific, Ireland). Following this, depending on cell count, 1 mL of sample was centrifuged at 4000 g for 240 s (centrifugation had no effect on cell viability, data not shown). Supernatant was removed and 0.2 mL of PBS added before pellets were re-suspended and 0.1 mL aliquots were plated in duplicate on *Listeria* selective agar (LSA, Oxoid CM856) containing a modified *Listeria* selective supplement (Oxoid SR0206). The resulting detection limit was therefore 0.5 CFU/g lettuce. As required, further serial dilutions were performed in PBS and plated on LSA during the testing period. CFU of *Listeria monocytogenes* (black colony with black zones around colony) were determined after incubation at 37 °C for 48 hours.

2.1.5: Analysis of the gaseous atmospheres

On each sampling day (Day 0, 2, 5, 7) gases within the packages were analysed using a gas analyser (PBI-Dansensor, PBI Development, Denmark, Model TIA-III LV).

2.1.6: Enumeration of Total Cell Count

Bacterial cell counts were carried out on day 0 (day of inoculation) and days 2, 5, 7 (throughout storage) from three replicate packs. The lettuce samples from each package were homogenised for 120 s at high speed in phosphate buffer saline (PBS, Oxoid BR0014) in a 2-fold dilution (20 mL) for inoculum density $10^2$ CFU/g, a 5-fold dilution (50 mL) for inoculum density $10^3$ CFU/g and a 10-fold dilution (100 mL) for inoculum density $10^4$ and $10^5$ CFU/g using a Seward laboratory stomacher (Stomacher Model 400, AGB Scientific, Ireland).
Following this, depending on cell count, 1 mL of sample was centrifuged at 4000 g for 240 s (centrifugation had no effect on cell viability, data not shown). Supernatant was removed and 0.2 mL of PBS added before pellets were re-suspended and 0.1 mL aliquots were plated in duplicate on Tryptone Soya Agar (TSA, Oxoid CM0131). As required, further serial dilutions were performed in PBS and plated on TSA during the testing period. CFU of total bacteria counts were determined after incubation at 37 °C for 48 hours.

2.1.7: Isolate confirmation and serogrouping

All isolates were confirmed as *L. monocytogenes* and serogrouped by multiplex PCR as described in Doumith *et al.* (2004). DNA used in multiple PCR analyses was extracted from presumptive *L. monocytogenes* isolates, with 50 colonies taken from each of the Day 7 inoculation densities in order to obtain the spread of the three serogroups over the 7 days.

Table 7: PCR Primers. Adapted from Doumith *et al.* (2004)

<table>
<thead>
<tr>
<th>Gene Target</th>
<th>Primer sequence (5’-3’)</th>
<th>Product size</th>
<th>Serovar specificity</th>
<th>Protein encoded by target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>lmo0737</em></td>
<td>F: AGGGCTTCAAGGACTTACCC</td>
<td>691</td>
<td><em>L. monocytogenes</em> serovars 1/2a, 1/2c, 3a, and 3c</td>
<td>Unknown, no similarity</td>
</tr>
<tr>
<td></td>
<td>R: ACGATTTCTGCTTGCCATTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>lmo1118</em></td>
<td>F: AGGGGTCTTAATCCTGGAA</td>
<td>906</td>
<td><em>L. monocytogenes</em> serovars 1/2c and 3e</td>
<td>Unknown, no similarity</td>
</tr>
<tr>
<td></td>
<td>R: CGGCTTGTTCGGCATCTTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORF2819</td>
<td>F: AGCAAATGCCAAAATCGCTG</td>
<td>471</td>
<td><em>L. monocytogenes</em> serovars 1/2b, 3b, 4b, 4d, and 4e</td>
<td>Putative transcriptional regulator</td>
</tr>
<tr>
<td></td>
<td>R: CATCACTAAAGCCTCCCATTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORF2110</td>
<td>F: AGTGGACAATTGATTGGTGA</td>
<td>597</td>
<td><em>L. monocytogenes</em> serovars 4b, 4d, and 4e</td>
<td>Putative secreted protein</td>
</tr>
<tr>
<td></td>
<td>R: CATCCATCCCTACTTGGAC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.1.8: PCR Conditions

Total reaction volume of 25 µL containing 2U of Taq DNA polymerase (Thermo Scientific EPO702), 0.2 mM deoxynucleoside triphosphates mix (Thermo Scientific EP0681), and 1X Buffer (Thermo Scientific EPO768). The five primer sets were added at the following final concentrations: 1 µM for lmo0737, ORF2819, and ORF2110; 3.0 µM for lmo1118; and 0.2 µM for prs (Table 8) (Integrated DNA Technologies Inc.). PCR was performed with an initial denaturation step at 94 °C for 5 minutes; 35 cycles of 94 °C for 0.40 min, 53 °C for 1.15 minutes, and 72 °C for 1.5 minutes; and one final cycle of 72 °C for 7 minutes. Post PCR product were electrophoresed (Cleaver Scientific Ltd., UK) with Gene Ruler 100bp Plus (Thermo Scientific) as a molecular marker on Safe-View (NBS Biologicals Ltd. USA. 5 µL of the reaction mixture was mixed with 2 µL of loading dye and separated on a 2% agarose gel in a 1X TAE buffer (242 g of trisaminomethane, 57.1 mL of glacial acetic acid and 100 mL 0.5 M EDTA per litre, pH 8). The electrophoresed PCR product was visualized using the gel documentation system Gbox (Syngene).

2.2: Inhibition of Listeria monocytogenes growth on lettuce through application of the bacteriocin Nisin.

2.2.1: Preparation of fresh-cut vegetable (Iceberg Lettuce)

Iceberg Lettuce (Class I Spain) was obtained locally from a fruit and vegetable supplier (Supervalu Castletroy, Limerick) the day of testing and stored at 4 °C prior to processing. Outer layers and stem were manually removed using a disinfected sharp stainless steel knife. Any damaged leaves and the core of the heads were excluded. The remaining inner leaves were sliced with a disinfected sharp stainless steel knife and cut into 20 mm pieces. The cut lettuce (10 g) were transferred into 35 µm think orientated polypropylene (OPP) packaging bags (18 x 10 cm) which had a permeability to O₂ of 5.7 nmol m⁻² s⁻¹ kPa⁻¹ and to CO₂ of 19 nmol m⁻² s⁻¹ kPa⁻¹ (Scollard et al. 2016) (Amcor Flexibles, Gloucester, UK).
2.2.2: Preparation of Listeria cultures and inoculation of fresh-cut vegetable (Iceberg Lettuce)

For all inhibition tests a combination of three L. monocytogenes strains from the Listeria strain collection at Teagasc Food Research Centre (Moorepark, Ireland) were used in order to follow the European Guidelines for challenge tests on ready-to-eat foods (EUR Lm, 2014). The mix was grown as in section 2.1.2. Cultures were then mixed together to achieve equal numbers (CFU) of each strain and then diluted in phosphate buffered saline (PBS, Oxoid BR014, Fannin Healthcare) to allow inoculation of fresh-cut produce at \(10^2\) CFU/g. Aliquots of 0.1 mL of L. monocytogenes suspension were distributed uniformly over the lettuce contained within each of the packages.

2.2.3: Antimicrobial treatments

The effectiveness of the antimicrobial treatments on the survival and growth of Listeria were tested as follows:

2.2.4: L. lactis application

Lactococcus lactis subsp. lactis DSM 20729 (ATCC 11454) was grown in tryptone soya broth (10 mL TSB, Oxoid CM129, Fannin Healthcare, Cork, Ireland) at either 4 or 8 °C in accordance to the temperatures being used in the test conditions. The culture was then diluted in phosphate buffered saline (PBS, Oxoid BR014, Fannin Healthcare) to allow inoculation of fresh-cut produce at \(10^2\) CFU/g. Aliquots of 1 mL of L. lactis suspension were distributed uniformly over the lettuce contained within each of the packages.

2.2.5: Nisin A application

Commercially available Nisin A (2.5% w/w in salt) (Handary SA, Brussels, Belgium) was suspended in 10 ml of distilled water in Float-A-Lyzer membranes of 5 kDa (Spectrum Laboratories, Inc. California, USA) for 24 hours the remaining solution was extracted and autoclaved at 121 °C for 15 minutes. The solution was then diluted in phosphate buffered saline (PBS, Oxoid BR014, Fannin Healthcare) to allow inoculation of fresh-cut produce at 2.5 ppm and 5 ppm. Aliquots of 1 mL of the Nisin A suspension were distributed uniformly over the lettuce contained within each of the packages.
2.2.6: Atmospheric treatments, package sealing and storage conditions

Following inoculation and antimicrobial treatment, packs were flushed with the following gas atmospheres: (a) air, (b) 8kPa CO₂, 4kPa O₂, 88kPa N₂ (optimum) or (c) 15kPa CO₂, 1kPa O₂, 84kPa N₂ (sub-optimum modified atmosphere packaging, MAP) and heat sealed using a vacuum packer (Multivac, UK). The packs were then stored (incubated) at either 4 or 8 °C for up to 7 days.

2.2.7: Enumeration of Listeria

Bacterial cell counts were carried out on day 0 (day of inoculation) and days 2, 5, 7 (throughout storage) from three replicate packs. The lettuce samples from each package were homogenised for 120 s at high speed in phosphate buffer saline (PBS, Oxoid BR0014) in a 2-fold dilution using a Seward laboratory stomacher (Stomacher Model 400, AGB Scientific, Ireland). Following this, depending on cell count, 1 mL of sample was centrifuged at 4000 g for 240 s (centrifugation had no effect on cell viability, data not shown). Supernatant was removed and 0.2 mL of PBS added before pellets were re-suspended and 0.1 mL aliquots were plated in duplicate on Listeria selective agar (LSA, Oxoid CM856) containing a modified Listeria selective supplement (Oxoid SR0206). The resulting detection limit was therefore 0.5 CFU/g of lettuce. As required, further serial dilutions were performed in PBS and plated on LSA during the testing period. CFU of Listeria monocytogenes (black colony with black zones around colony) were determined after incubation at 37 °C for 48 hours.

2.2.8: Enumeration of Total Cell Count

Bacterial cell counts were carried out on day 0 (day of inoculation) and days 2, 5, 7 (throughout storage) from three replicate packs. The lettuce samples we treated as in the enumeration of Listeria section (2.2.9), however Tryptone Soya Agar (TSA, Oxoid CM0131) was used instead of LSA. As required, further serial dilutions were performed in PBS and plated on TSA during the testing period. CFU of total bacteria counts were determined after incubation at 37 °C for 48 hours.

2.2.9: Evaluation of sensory quality

Evaluation of appearance was performed on the fresh-cut produce packages during storage (days 0, 2, 5 and 7) by an untrained sensory panel (Scollard et al. 2013). The panel consisted of 5 evaluators (4 males and 1 female) who were members of our laboratory with experience...
in sensory evaluation of fresh-cut produce. Prior to analysis, panellists were familiarised with the product and scoring procedure. The panellists were asked to score the appearance of samples, on an 11-point scale ranging from 10 to 0, where 10 represented excellent appearance and 0 represented very poor appearance. A combined score of 6 was also considered the lowest acceptable commercial score. The samples were coded (three random digits) and randomly offered to panellists. Panellists were given one sample and asked to evaluate colour and appearance against that of an untreated sample (control). The evaluations were carried out under typical indoor daylight conditions and at a temperature of 18–20 °C (Scollard et al. 2016).

2.2.10: 24-well plate assay

A basic 24-well plate assay was conducted in order to establish the effectiveness of the purified nisin A at various quantities (0, 5, 10 and 25 ppm) \textit{in vitro} under 3 different atmospheric conditions: (a) air, (b) 8kPa CO$_2$, 4kPa O$_2$, 88kPa N$_2$ (optimum) or (c) 15kPa CO$_2$, 1kPa O$_2$, 84kPa N$_2$ (sub-optimum modified atmosphere packaging, MAP). Again, the \textit{L. monocytogenes} mix comprised of a EURL \textit{Lm} reference strain (number 1382), a persistent strain isolated from a food processing plant (number 6179) and a strain isolated from a vegetable production facility (number 959). The cultures were grown separately in tryptone soya broth (50 mL TSB, Oxoid CM129, Fannin Healthcare, Cork, Ireland) at 37 °C. Cultures were then mixed together to achieve equal numbers (CFU) of each strain and then diluted in phosphate buffered saline (PBS, Oxoid BR014, Fannin Healthcare) to allow inoculation at $10^6$ CFU mL$^{-1}$. Into each well 100 µL of TSA broth, 100 µL of PBS containing the appropriate amount of purified nisin and 10 µL of the \textit{L. monocytogenes} 3 strain mix. This was carried out aseptically in triplicate per purified nisin A amount. The 24-well plate was then placed into a 35 μm think orientated polypropylene (OPP) packaging bags (18 x 10 cm) and sealed using heat sealed using a vacuum packer (Multivac, UK) at the selected atmosphere. The packs were then stored (incubated) at 37 °C for 48 hours. At the selected time point the plates were removed from the 37 °C incubator and analysed for cell density using a spectrophotometer (VWR, USA) at 600 nm. Sterile 10 µL loops were used to streak the broth on LSA to confirm for the presence on \textit{L. monocytogenes}. Individual 24-well plates were used for each of the sampling points and then discarded after reading.

2.3: Multi-food sampling study of the occurrence and persistence of \textit{Listeria monocytogenes} in 12 food business operators
2.3.1: *L. monocytogenes* sampling programme

From September 2014 to October 2015, 12 food processing facilities submitted both food and swab samples for detection of *L. monocytogenes*. These included 6 meat, 2 seafood, 3 vegetable and 1 dairy food producers. All these food processors were ready-to-eat food producers. Every six months, Teagasc, Moorepark sent sample kits 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.

Every two months from September 2014 each food producer submitted samples that generally consisted 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, apple juice etc. Following sampling, the sample kit and samples was sent by overnight courier (Nightline, Limerick) to the laboratory for testing (Leong *et al.* 2016) submitted manuscript under review.

2.3.2: Analysis of samples

Analysis of samples for the presence of *L. monocytogenes* was performed according to the ISO 11290-1 Horizontal method for the detection and enumeration of *L. monocytogenes* and other *Listeria* spp.

2.3.3: Environmental swab samples

Enrichment for *Listeria* from environmental swab samples consisted of the addition of 100 ml of Half-Fraser broth (VWR, USA) into the sampling bag containing the respective swab. The bags were incubated at 30 °C for 24 hours. Post incubation 100 µL of the incubated Half-Fraser broth into a tube containing 10 mL Full-Frazer broth (VWR, USA) and incubated 30 °C for 48 hours. 20 µL was also taken from the Half-Fraser broth and plated onto a chromogenic agar plate Agar *Listeria* acc. to Ottavani & Agosti (ALOA) (VWR, USA) and incubated @ 37 °C for 48 hours and then examined for typical *L. monocytogenes* colonies (blue/green colonies with a halo).
2.3.4: Food samples

Food samples were tested following their “best before date” where possible to avoid causing recalls which would have prevented food processors from engaging with the project. Enrichment for *Listeria* from food samples consisted of the aseptic addition of 25 g of food sample into a stomacher bag with 225 ml Half-Frazer broth and homogenised for 4 minutes using a Seward laboratory stomacher (high setting) (Stomacher Model 400, AGB Scientific, Ireland). The contents were then poured into a sterile 250 mL Duran bottle and incubated at 30 °C for 24 hours. Post incubation 100 µL of the incubated Half-Fraser broth food sample mix into a tube containing 10 mL Full-Frazer broth (VWR, USA) and incubated 30 °C for 48 hours. 20µL was also taken from the Half-Fraser broth and plated onto a chromogenic agar plate Agar *Listeria* acc. to Ottavani & Agosti (ALOA) (VWR, USA) and incubated @ 37 °C for 48 hours and then examined for typical *L. monocytogenes* colonies (blue/green colonies with a halo).

2.3.5: Liquid samples

Enrichment for *Listeria* from liquid samples consisted of the aseptic addition of 25ml of liquid sample into a Duran bottle with 225 ml Half-Frazer broth and incubated at 30˚ C for 24 hours. Post incubation 100 µl of the incubated Half-Fraser broth food sample mix into a tube containing 10 ml Full-Frazer broth (VWR, USA) and incubated 30˚C for 48 hours. 20µl was also taken from the Half-Fraser broth and plated onto a chromogenic agar plate Agar *Listeria* acc. to Ottavani & Agosti (ALOA) (VWR, USA) and incubated @ 37 °C for 48 hours and then examined for typical *L. monocytogenes* colonies (blue/green colonies with a halo).

2.3.6: Positive samples

From each positive plate, two presumptive positive colonies were restreaked to a second chromogenic agar plate (ALOA 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; 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.
2.3.7: Isolate confirmation and serogrouping

All isolates were confirmed as *L. monocytogenes* and serogrouped by multiplex PCR as described in Doumith *et al.* (2004). DNA used in multiple PCR analyses was extracted from presumptive *L. monocytogenes* isolates. This extraction consisted of obtaining genomic DNA of a presumptive *L. monocytogenes* isolate via a sterile toothpick, placing it into 50 µL of lysis buffer (0.05 M NaOH (Fisher Scientific) 0.25% Sodium dodecyl sulphate (SDS) (Fisher Scientific)) with a 1.5 mL micro-centrifuge tube and incubating it at 95 °C for 20 minutes in a shaker incubator (Medical Supply Company, Ireland), shaking at 750 RPM. 450 µL of distilled water was then added to the micro-centrifuge tube after the solution had cooled to room temperature. Next, the micro-centrifuge tube was centrifuged at 6000 RPM for 4 minutes. 100 µL aliquots of the supernatant were placed into separate 1.5 mL micro-centrifuge tube in order to minimise cell debris (Schmalenberger *et al.* 2001).

2.3.8: PCR Conditions

PCR conditions, procedure and primers used as in section 2.1.8.

2.3.9: PFGE

PFGE was performed by Teagasc, Moorepark according to the International Standard PulseNet protocol (PulseNetUSA 2009) with the restriction enzymes Sgs1 (formerly Asc1) and Apa1, in two separate experiments.

2.3.10: Statistical analysis

All counts were reported as the means of 3 independent variables ± standard deviations. The respective data from the experiments at different atmospheres and temperatures were tested for homoscedasticity (Levene’s test) and normality (Shapiro-Wilk test). Where conditions of homoscedasticity and normality were met, possibly after a log transformation experiment dependent, an ANOVA with Tukey posthoc test was conducted to identify significant differences (for all tests, P≤0.05).
Chapter 3: Results

3.1: Investigation into the influence of inoculation density, temperature and atmosphere on the growth and enumeration of *Listeria monocytogenes*.

Fresh-cut Iceberg Lettuce was inoculated with *L. monocytogenes* at four different inoculation densities (approximately $10^2$, $10^3$, $10^4$, $10^5$ log CFU/g Lettuce) and examined for growth at 8 °C and 4 °C under 3 different atmospheres: (a) air, (b) 8kPa CO$_2$, 4kPa O$_2$, 88kPa N$_2$ and (c) 15kPa CO$_2$, 1kPa O$_2$, 84kPa N$_2$ and sampled on days 0, 2, 5 and 7.

3.1.1: 8 °C Air Atmosphere

*Figure 3:* Effect of four different inoculation densities and subsequent growth at 8 °C in an Air atmosphere. Including oxygen content (right) and *L. monocytogenes* plate (left) counts over the 7-day trial. Error bars represent ± standard deviation.

Figure 3 displays the outcome of the different starting inoculum densities on the rates of *L. monocytogenes* growth over a period of 7 days in an air atmosphere at 8 °C showing that growth rates decrease with increasing inoculation density. The lowest inoculum approximately of $10^2$ CFU/g displayed a growth rate of 590 times (Significant increase of 2.7 log CFU/g $P \leq 0.05$) its day 0 *L. monocytogenes* LSA plate count compared to the day 7 plate count. The $10^3$ CFU/g inoculum exhibited a growth rate of 32 times (Significant increase of 1.5 log CFU/g $P \leq 0.05$) its day 0 *L. monocytogenes* LSA plate count respective to the day 7 plate count. The $10^4$ CFU/g inoculum showed a growth rate of 10 times (Significant increase of 1.3 log CFU/g $P \leq 0.05$) its day 0 *L. monocytogenes* LSA plate count compared to the day 7 plate count. The highest starting inoculum $10^5$ CFU/g indicated a growth rate of 1.6 times...
(Insignificant increase of 0.18 log CFU/g) its day 0 L. monocytogenes LSA plate count when compared to the day 7 plate count. The O₂ content of the lowest inoculum approximately of 10² CFU/g decreased from a mean of 20.56 % to 13.13 % over the course of the trial. The 10³ CFU/g inoculum’s O₂ content reduced from 20.49 % to 13.5 %. Starting inoculum of 10⁴ CFU/g had an initial O₂ content of 20.6 % diminished to 13.32 % by day 7. The highest starting inoculum density of 10⁵ CFU/g displayed a reduction in its starting O₂ content of 20.63 % to 12.72 % by day 7. The control (no L. monocytogenes) had an opening O₂ content of 20.69 % which declined to 13.38 % by day 7.

3.1.2: 8 °C Modified Atmosphere (15kPa CO₂, 1kPa O₂, 84kPa N₂)

**Oxygen Gas Analysis 8 °C**

1kPa O₂

**Figure 4:** Effect of four different inoculation densities and subsequent growth at 8 °C in a modified atmosphere of 15kPa CO₂, 1kPa O₂. Including oxygen content (right) and L. monocytogenes plate counts (left) over the 7-day trial. Error bars represent ± standard deviation.

Figure 4 displays the outcome of the different starting inoculum densities on the rates of L. monocytogenes growth over a period of 7 days in a 15kPa CO₂, 1kPa O₂ atmosphere at 8 °C. The lowest inoculum approximately of 10² CFU/g showed a growth rate of 269 times (Significant increase of 2.43 log CFU/g P≤0.05) its day 0 L. monocytogenes LSA plate count compared to its day 7 plate count. The 10³ CFU/g inoculum displayed a growth rate of 15.1 times (Significant increase of 1.06 log CFU/g P≤0.05) its day 0 L. monocytogenes LSA plate count when compared to its respective day 7 plate count. The 10⁴ CFU/g inoculum presented a growth rate of 13.3 times (Significant increase of 0.94 log CFU/g P≤0.05) its day 0 L. monocytogenes LSA plate count compared to its particular day 7 plate count. The highest starting inoculum 10⁵ CFU/g showed a growth rate of 2.6 times (insignificant increase of 0.39 log CFU/g) its day 0 L. monocytogenes LSA plate count compared to its respective day 7

Chapter 3: Results
plate count. The O₂ content of the lowest inoculum approximately of 10² CFU/g increased from a mean of 1.003 % to 9.81 % over the course of the trial. The 10³ CFU/g inoculum’s O₂ content enlarged from 1.02 % to 9.14 %. Starting inoculum of 10⁴ CFU/g had an initial O₂ content of 1.06 % that rose to 9.09 % by day 7. The highest starting inoculum density of 10⁵ CFU/g displayed an enlargement in its starting O₂ content of 1.05 % to 9.15 % by day 7. The control (no *L. monocytogenes*) had an opening O₂ content of 1.06 % which rose to 9.02 % by day 7.

3.1.3: 8 °C Modified Atmosphere (8kPa CO₂, 4kPa O₂, 88kPa N₂)

![Oxygen Gas Analysis 8 °C](4kPa O₂)

**Figure 5:** Effect of four different inoculation densities and subsequent growth at 8 °C in a modified atmosphere of 8kPa CO₂, 4kPa O₂. Including oxygen content (right) and *L. monocytogenes* plate counts (left) over the 7-day trial. Error bars represent ± standard deviation.

Figure 5 displays the outcome of the different starting inoculum densities on the rates of *L. monocytogenes* growth over a period of 7 days in an 8kPa CO₂, 4kPa O₂ atmosphere at 8 °C. The lowest inoculum approximately of 10² CFU/g displayed a growth rate of 92.4 times (Significant increase of 1.85 log CFU/g *P*≤0.05) of its day 0 *L. monocytogenes* LSA plate count compared to particular to day 7 plate count. The 10³ CFU/g inoculum exhibited a growth rate of 44.1 times (Significant increase of 1.60 log CFU/g *P*≤0.05) its day 0 *L. monocytogenes* LSA plate count compared to is respective day 7 plate count. The 10⁴ CFU/g inoculum displayed a growth rate of 12.9 times (Significant increase of 1.10 log CFU/g *P*≤0.05) its day 0 *L. monocytogenes* LSA plate count compared to its corresponding day 7 plate count. The highest starting inoculum 10⁵ CFU/g presented a growth rate of 3 times (Significant increase of 0.48 log CFU/g *P*≤0.05) its day 0 *L. monocytogenes* LSA plate count compared to day 7 plate count. The O₂ content of the lowest inoculum approximately of 10²
CFU/g increased from a mean of 4.09 % to 9.35 % over the course of the trial. The $10^3$ CFU/g inoculum’s O$_2$ content enlarged from 4.12 % to 10.01 %. Starting inoculum of $10^4$ CFU/g had an initial O$_2$ content of 4.10 % rise to 11.7 % by day 7. The highest starting inoculum density of $10^5$ CFU/g displayed an enlargement in its starting O$_2$ content of 4.18 % to 10.1 % by day 7. The control (no L. monocytogenes) had an opening O$_2$ content of 4.03 % which rose to 13.85 % by day 7.

3.1.4: 4 °C Air Atmosphere

**Figure 6:** Effect of four different inoculation densities and subsequent growth at 4 °C in an Air atmosphere. Including oxygen content (right) and L. monocytogenes plate counts (left) over the 7-day trial. Error bars represent ± standard deviation.

Figure 6 displays the outcome of the different starting inoculum densities on the rates of L. monocytogenes growth over a period of 7 days in an Air atmosphere at 4 °C. The lowest inoculum approximately of $10^2$ CFU/g showed a growth rate of 61.7 times (Significant increase of 1.77 log CFU/g  $P<0.05$) its day 0 L. monocytogenes LSA plate count when compared to the day 7 plate count. The $10^3$ CFU/g inoculum displayed a growth rate of 14.2 times (increase of 1.13 log CFU/g) its day 0 L. monocytogenes LSA plate count when compared to the respective day 7 plate count. The $10^4$ CFU/g inoculum showed a growth rate of 9.6 times (increase of 0.91 log CFU/g) its day 0 L. monocytogenes LSA plate count when compared to the corresponding day 7 plate count. The highest starting inoculum $10^5$ CFU/g presented a growth rate of 3 times (insignificant increase of 0.44 log CFU/g ) its day 0 L. monocytogenes LSA plate count when compared to the respective day 7 plate count. The O$_2$ content of the lowest inoculum approximately of $10^2$ CFU/g decreased from a mean of 20.63 % to 13.23 % over the course of the trial. The $10^5$ CFU/g inoculum’s O$_2$ content reduced from
20.36 % to 13.2 %. Starting inoculum of $10^4$ CFU/g had an initial O₂ content of 20.53 % diminished to 13.03 % by day 7. The highest starting inoculum density of $10^5$ CFU/g displayed a reduction in its starting O₂ content of 20.63 % to 11.3 % by day 7. The control (no *L. monocytogenes*) had an opening O₂ content of 20.6 % which declined to 13.73 % by day 7.

### 3.1.5: 4 °C Modified Atmosphere (15kPa CO₂, 1kPa O₂, 84kPa N₂)

**Figure 7:** Effect of four different inoculation densities and subsequent growth at 4 °C in a modified atmosphere of 15kPa CO₂, 1kPa O₂. Including oxygen content (right) and *L. monocytogenes* plate counts (left) over the 7-day trial. Error bars represent ± standard deviation.

Figure 7 displays the outcome of the different starting inoculum densities on the rates of *L. monocytogenes* growth over a period of 7 days in a 15kPa CO₂, 1kPa O₂ atmosphere at 4 °C. The lowest inoculum approximately of $10^2$ CFU/g exhibited a growth rate of 11.3 times (Significant increase of 1.01 log CFU/g *P*≤0.05) its day 0 *L. monocytogenes* LSA plate count when compared to the day 7 plate count. The $10^3$ CFU/g inoculum displayed a growth rate of 11.3 times (increase of 1.06 log CFU/g) its day 0 *L. monocytogenes* LSA plate count when compared to its respective day 7 plate count. The $10^4$ CFU/g inoculum presented a growth rate of 5.2 times (increase of 0.72 log CFU/g) its day 0 *L. monocytogenes* LSA plate count when compared to the corresponding day 7 plate count. The highest starting inoculum $10^5$ CFU/g had a growth rate of 3.7 times (Significant increase of 0.55 log CFU/g *P*≤0.05) its day 0 *L. monocytogenes* LSA plate count when compared to the respective day 7 plate count. The O₂ content of the lowest inoculum approximately of $10^2$ CFU/g increased from a mean of 0.96 % to 9.48 % over the course of the trial. The $10^3$ CFU/g inoculum’s O₂ content enlarged
from 1.02 % to 9.51 %. Starting inoculum of $10^4$ CFU/g had an initial O$_2$ content of 1.06 % rise to 9.40 % by day 7. The highest starting inoculum density of $10^5$ CFU/g displayed an enlargement in its starting O$_2$ content of 1.00 % to 9.10 % by day 7. The control (no *L. monocytogenes*) had an opening O$_2$ content of 1.00 % which rose to 9.11 % by day 7.

3.1.6: 4 °C Modified Atmosphere (8kPa CO$_2$, 4kPa O$_2$, 88kPa N$_2$)

Figure 8: Effect of four different inoculation densities and subsequent growth at 4 °C in a modified atmosphere of 8kPa CO$_2$, 4kPa O$_2$. Including oxygen content (right) and *L. monocytogenes* plate counts (left) over the 7-day trial. Error bars represent ± standard deviation.

Figure 8 displays the outcome of the different starting inoculum densities on the rates of *L. monocytogenes* growth over a period of 7 days in an 8kPa CO$_2$, 4kPa O$_2$ atmosphere at 4 °C. The lowest inoculum approximately of $10^2$ CFU/g presented a growth rate of 51.1 times (Significant increase of 1.68 log CFU/g $P\leq 0.05$) its day 0 *L. monocytogenes* LSA plate count when compared to the day 7 plate count. The $10^3$ CFU/g inoculum displayed a growth rate of 13.7 times (increase of 1.14 log CFU/g) its day 0 *L. monocytogenes* LSA plate count when compared to the respective day 7 plate count. The $10^4$ CFU/g inoculum exhibited a growth rate of 10.2 times (increase of 0.93 log CFU/g) its day 0 *L. monocytogenes* LSA plate count when compared to the corresponding day 7 plate count. The highest starting inoculum $10^5$ CFU/g had a growth rate of 5.7 times (increase of 0.73 log CFU/g) its day 0 *L. monocytogenes* LSA plate count when compared to the day 7 plate count. The O$_2$ content of the lowest inoculum approximately of $10^2$ CFU/g increased from a mean of 4.01 % to 9.73 % over the course of the trial. The $10^3$ CFU/g inoculum’s O$_2$ content enlarged from 4.00 % to 9.31 %. Starting inoculum of $10^4$ CFU/g had an initial O$_2$ content of 4.11 % rise to 10.7 % by day 7. The highest starting inoculum density of $10^5$ CFU/g displayed an enlargement in its
starting O₂ content of 4.00 % to 10.02 % by day 7. The control (no L. monocytogenes) had an opening O₂ content of 4.10 %, which rose to 10.14 % by day 7.

**Table 8:** Inoculation density growth rates summary and changes over the 7-day trial.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Atmosphere</th>
<th>Starting inoculum density</th>
<th>Final CFU/g vs starting CFU/g</th>
<th>Increase log CFU/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 °C</td>
<td>Air</td>
<td>10⁶ CFU/g</td>
<td>590</td>
<td>2.7 log CFU/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10⁵ CFU/g</td>
<td>32</td>
<td>1.5 log CFU/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10⁴ CFU/g</td>
<td>10</td>
<td>1.3 log CFU/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10³ CFU/g</td>
<td>1.6</td>
<td>0.18 log CFU/g</td>
</tr>
<tr>
<td>8 °C</td>
<td>15kPa CO₂, 1kPa O₂, 84kPa N₂</td>
<td>10⁶ CFU/g</td>
<td>269</td>
<td>2.43 log CFU/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10⁵ CFU/g</td>
<td>15.1</td>
<td>1.06 log CFU/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10⁴ CFU/g</td>
<td>13.3</td>
<td>0.94 log CFU/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10³ CFU/g</td>
<td>2.6</td>
<td>0.39 log CFU/g</td>
</tr>
<tr>
<td>8 °C</td>
<td>8kPa CO₂, 4kPa O₂, 88kPa N₂</td>
<td>10⁶ CFU/g</td>
<td>92.4</td>
<td>1.85 log CFU/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10⁵ CFU/g</td>
<td>44.1</td>
<td>1.60 log CFU/g</td>
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<td>10⁴ CFU/g</td>
<td>12.9</td>
<td>1.10 log CFU/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10³ CFU/g</td>
<td>3</td>
<td>0.48 log CFU/g</td>
</tr>
<tr>
<td>4 °C</td>
<td>Air</td>
<td>10⁶ CFU/g</td>
<td>61.7</td>
<td>1.77 log CFU/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10⁵ CFU/g</td>
<td>14.2</td>
<td>1.13 log CFU/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10⁴ CFU/g</td>
<td>9.6</td>
<td>0.91 log CFU/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10³ CFU/g</td>
<td>3</td>
<td>0.44 log CFU/g</td>
</tr>
<tr>
<td>4 °C</td>
<td>15kPa CO₂, 1kPa O₂, 84kPa N₂</td>
<td>10⁶ CFU/g</td>
<td>11.3</td>
<td>1.01 log CFU/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10⁵ CFU/g</td>
<td>11.3</td>
<td>1.06 log CFU/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10⁴ CFU/g</td>
<td>5.2</td>
<td>0.72 log CFU/g</td>
</tr>
</tbody>
</table>

Chapter 3: Results
Table 2 presents the growth of *L. monocytogenes* over the whole experiment. Displaying the reported increase in growth in CFU/g and also the amount of growth relative to the day 0 counts. This data displays that the lower initial inoculation density of *L. monocytogenes* results in a greater rate and amount of growth when compared with higher starting inoculation densities across all temperatures and gas atmospheres. However, this is displayed to be much greater at 8 °C where the increased temperature allows for more incidences of significant increases in relative growth compared to that of 4 °C.

### 3.1.7: Serogroup Identification Assay

To achieve a picture of the spread of the individual *L. monocytogenes* strains within the 3 strain mix a multiplex PCR was used to differentiate between the 3 inoculated strains.

![Figure 9: Safe-View stained agarose gel (2%) displaying electrophoresed products of multiplex PCR. L: Molecular marker GeneRuler 100Plus. Lane 26-28: Serogroup Controls. Remaining lane are sample lanes.](image-url)
Figure 9 describes a typical agarose gel following the methodology of Doumith et al. (2004). Well 26 shows the primers ORF2110 (597 bp) and ORF2819 (471 bp) displaying specificity for *L. monocytogenes* serovars 4b, 4d, and 4e (Strain 1382). Well 27 displays primer *lmo0737* (691 bp) presenting serovar specificity for *L. monocytogenes* serovars 1/2a, 1/2c, 3a, and 3c (Strain 6179). Well 28 demonstrates primers *lmo118* (906 bp) and *lmo0737* (691 bp) presenting serovar specificity for *L. monocytogenes* serovars 1/2c and 3c (Strain 959). Wells 8,9,12,32,33,43,48 show no reaction.

![Detection Rates at 8 °C](image)

**Figure 10:** Day 7 detection rates at 8 °C.

![Detection Rates at 4 °C](image)

**Figure 10:** Day 7 serotype detection at 4 °C.
The above figures (10 and 11) display the relative detection rates of the various serogroups of *L. monocytogenes* used in the trial on Day 7 at both 4 °C (Figure 11) and 8 °C (Figure 10). No significant differences were observed thus continuation of the serotyping for the remaining days was not carried out.

### 3.2: Inhibition of *Listeria monocytogenes* growth on lettuce through application of the bacteriocin Nisin.

#### 3.2.1: Effect of nisin and modified atmosphere on the growth of *L. monocytogenes* *in vitro*

Initially, the effect of various amounts of nisin was measured *in vitro* in 24-well plates sealed in an atmosphere of: (a) air, (b) 8kPa CO₂, 4kPa O₂, 88kPa N₂ and (c) 15kPa CO₂, 1kPa O₂, 84kPa N₂ using an OPP bag at 37 °C. Measurements of the optical density were conducted using a spectrophotometer at 600 nm.

![24-Well Assay 37 °C Air](image.png)

**Figure 11**: *In vitro* assay of the effect various quantities (0, 5, 10, 25 ppm) of nisin on *L. monocytogenes* in an unmodified air atmosphere at 37 °C.
Figure 12: *In vitro* assay of the effect various quantities (0, 5, 10, 25 ppm) of nisin on *L. monocytogenes* in a modified atmosphere of 8kPa CO$_2$, 4kPa O$_2$, 88kPa N$_2$ at 37 °C.

Each of the modified atmosphere *in vitro* assays showed that 5 ppm of nisin was sufficient to completely retard growth from reaching detectable levels. Upon streaking of samples from the respective wells on LSA it was established that *L. monocytogenes* was present in the wells thus suggesting a bacteriostatic rather than a bactericidal effect.
3.2.2: 8 °C Air Atmosphere

**Figure 14:** Effect of three different nisin (2.5 and 5 ppm) and *L. lactis* treatments at 8 °C in an Air atmosphere. Including sensory analysis (top right), oxygen content (bottom left), *L. monocytogenes* (top left) and aerobic plate counts (bottom right) over the 7-day trial. Error bars represent ± standard deviation.

Figure 15 displays the outcome of the three different nisin (2.5 and 5 ppm) and *L. lactis* treatments rates of *L. monocytogenes* growth over a period of 7 days in an air atmosphere at 8 °C. From a starting inoculum approximately of 10^3 CFU/g the day 0 *L. monocytogenes* LSA plate count differed between the treatments applied. The application of 5 ppm nisin contributed to an initial 1.2 log reduction in *L. monocytogenes* growth compared to the control (no treatment) of which was maintained until day 7 where a 1.04 log reduction was recorded. The application of 2.5 ppm nisin contributed to a 0.71 log reduction in *L. monocytogenes* growth compared to the control (no treatment) lettuce bags which was maintained until day 7 where a 0.71 log reduction was also recorded. The application of *L. lactis* contributed to no reduction in *L. monocytogenes* growth compared to the control (no treatment) lettuce bags. The aerobic plate counts showed a day 2 reduction of 0.90 log for the 5 ppm nisin treated lettuce however this reduction is diminished from day 5 onwards. A 1.6 log reduction was recorded in the aerobic counts of lettuce treated with 2.5 ppm nisin again this reduction is lessened from day 5 onwards. Application of *L. lactis* displays no substantial change in the aerobic counts over the 7 days.
The $O_2$ content of the 5 ppm nisin treatments decreased from a mean of 20.8 % to 13.06 % over the course of the trial. The 2.5 ppm treatment’s $O_2$ content reduced from 20.9 % to 13.16 %. The *L. lactis* treatment had an initial $O_2$ content of 20.7 % diminished to 15.8 % by day 7. The control (no treatment) lettuce bags had an opening $O_2$ content of 20.76 % which declined to 15.76 % by day 7. The overall sensory effects displayed acceptable scoring for the nisin 5 ppm treatment from day 0 to day 5 and on day 7 it fell below the satisfactory level (a combined sensory score of 6). The nisin 2.5 ppm treatment displayed acceptable scoring from day 0 to day 2, from day 5 onward it fell below an acceptable level. The *L. lactis* treatment displayed adequate satisfactory levels until day 7 upon which it fell below the acceptable level.

### 3.2.3: 8 °C Modified Atmosphere (8kPa CO$_2$, 4kPa O$_2$, 88kPa N$_2$)

![Sensory Analysis 8 °C 4kPa O$_2$](image1)

![Aerobic Plate Count 8 °C 4kPa O$_2$](image2)

**Figure 15:** Effect of three different nisin (2.5 and 5ppm) and *L. lactis* treatments at 8 °C in a 8kPa CO$_2$, 4kPa O$_2$, 88kPa N$_2$ atmosphere. Including sensory analysis (top right), oxygen content (bottom left), *L. monocytogenes* (top left) and aerobic plate counts (bottom right) over the 7-day trial. Error bars represent ± standard deviation.

Figure 16 displays the outcome of the different nisin (2.5 and 5ppm) and the *L. lactis* treatments on *L. monocytogenes* growth over a period of 7 days in an 8kPa CO$_2$, 4kPa O$_2$, 88kPa atmosphere at 8 °C. From a starting inoculum approximately of $10^3$ CFU/g the day 0 *L. monocytogenes* LSA plate count differed between the treatments applied. The application of...
5 ppm nisin contributed to an initial 1.17 log reduction in *L. monocytogenes* growth compared to the control (no treatment) lettuce bags of which was maintained until day 7 where a 0.95 log reduction was recorded. The application of 2.5 ppm nisin contributed to a 0.92 log reduction in *L. monocytogenes* growth compared to the control (no treatment) lettuce bags of which was only to some extent maintained until day 7 where a 0.42 log difference was recorded. The application of *L. lactis* contributed to no reduction in *L. monocytogenes* growth compared to the control (no treatment). The aerobic plate counts showed a day 2 reduction of 0.89 log for the 5 ppm nisin treated lettuce however this reduction is diminished from day 5 onwards. A 1.57 log reduction was recorded in the aerobic counts of lettuce treated with 2.5 ppm nisin again this reduction is lessened from day 5 onwards. Application of *L. lactis* displayed a 0.79 log reduction was recorded in the aerobic counts on day 2 however this effect did not appear from day 5 onwards.

The O₂ content of the 5 ppm nisin treatments increased from a mean of 4.07% to 10.43 % over the course of the trial. The 2.5 ppm treatment’s O₂ content expanded from 4.07 % to 10.18 %. The *L. lactis* treatment had an initial O₂ content of 4.12 % enlarged to 13.27 % by day 7. The control (no treatment) had an opening O₂ content of 4.13 % which increased to 13.83 % by day 7. The overall sensory effects displayed acceptable scoring for the nisin 5 ppm treatment from day 0 to day 2 and on day 5 it fell below the satisfactory level, however it was notably below both the 2.5 ppm and *L. lactis* treatments from day 2. The nisin 2.5 ppm treatment displayed acceptable scoring from day 0 to day 2, from day 5 onward it fell below an acceptable level. The *L. lactis* treatment displayed adequate satisfactory levels until day 5 upon which it fell below the acceptable level.
3.2.4: 8 °C Modified Atmosphere (15kPa CO₂, 1kPa O₂, 84kPa N₂)

Figure 16: Effect of three different nisin (2.5 and 5ppm) and *L. lactis* treatments at 8 °C in a 15kPa CO₂, 1kPa O₂, 84kPa N₂ atmosphere. Including sensory analysis (top right), oxygen content (bottom left), *L. monocytogenes* (top left) and aerobic plate counts (bottom right) over the 7-day trial. Error bars represent ± standard deviation.

Figure 17 displays the outcome of the different nisin (2.5 and 5ppm) and *L. lactis* treatments on *L. monocytogenes* growth over a period of 7 days in a 15kPa CO₂, 1kPa O₂, 84kPa N₂ atmosphere at 8 °C. From a starting inoculum approximately of 10³ CFU/g the day 0 *L. monocytogenes* LSA plate count differed between the treatments applied. The application of 5 ppm nisin contributed to an initial 0.84 log reduction in *L. monocytogenes* growth compared to the control (no treatment) lettuce bags of which was maintained until day 7 where a 1.22 log reduction was recorded. The application of 2.5 ppm nisin contributed to a 0.78 log reduction in *L. monocytogenes* growth compared to the control (no treatment) of which was maintained until day 7 where a 1.66 log difference was recorded. The application of *L. lactis* contributed to no reduction in *L. monocytogenes* growth compared control (no treatment) lettuce bags. The aerobic plate counts showed a day 2 reduction of 0.53 log for the 5 ppm nisin treated lettuce however this reduction is diminished from day 5 onwards. A 1.4 log reduction was recorded in the aerobic counts of lettuce treated with 2.5 ppm nisin again this reduction is lessened from day 5 onwards. Application of *L. lactis* displayed a 0.66 log reduction was recorded in the aerobic counts on day 2 however this effect did not appear from day 5 onwards.

Chapter 3: Results
The O₂ content of the 5 ppm nisin treatments increased from a mean of 1.14% to 9.23 % over the course of the trial. The 2.5 ppm treatment’s O₂ content expanded from 1.23 % to 9.0 %.

The *L. lactis* treatment had an initial O₂ content of 1.12 % enlarged to 9.60 % by day 7. The control (no treatment) had an opening O₂ content of 1.13 % which increased to 9.86 % by day 7. The overall sensory effects displayed acceptable scoring for the nisin 5 ppm treatment from day 0 to day 2 and on day 5 it fell below the satisfactory level, however it was notably below both the 2.5 ppm and *L. lactis* treatments from day 2. The nisin 2.5 ppm treatment displayed acceptable scoring from day 0 to day 2, from day 5 onward it fell below an acceptable level. The *L. lactis* treatment displayed adequate satisfactory levels on days 0, 2 and 7 only falling slightly below the acceptable level on day 5.

### 3.2.5: 4 °C Air Atmosphere

![Graph](image.png)

**Figure 17:** Effect of three different nisin (2.5 and 5ppm) and *L. lactis* treatments at 4 °C in an Air atmosphere. Including sensory analysis (bottom right), oxygen content (bottom left), *L. monocytogenes* (top left) and aerobic plate counts (top right) over the 7-day trial. Error bars represent ± standard deviation.
Figure 18 displays the outcome of the different nisin (2.5 and 5ppm) and *L. lactis* treatments on *L. monocytogenes* growth over a period of 7 days in an air atmosphere at 4 °C. From a starting inoculum approximately of 10^3 CFU/g the day 0 *L. monocytogenes* LSA plate count differed between the treatments applied. The application of 5 ppm nisin contributed to an initial 0.46 log reduction in *L. monocytogenes* growth compared to the control (no treatment) of which increased to 1.52 log on day 2, decreased to 1.48 log on day 5 and decreased on day 7 where a 1.04 log reduction was recorded. The application of 2.5 ppm nisin contributed to a 0.14 log reduction in *L. monocytogenes* growth compared to the control (no treatment) lettuce bags which was increased to 1.90 log on day 2, decreased to 1.40 log on day 5 and decreased on day 7 where a 0.48 log reduction was recorded. The application of *L. lactis* contributed to no substantial reduction in *L. monocytogenes* growth compared to the control (no treatment). The aerobic plate counts showed an initial reduction of 0.17 log for the 5 ppm nisin treated lettuce however this reduction is diminished from day 2 onwards. A 0.70 log reduction was recorded in the aerobic counts of lettuce treated with 2.5 ppm nisin again this reduction is lessened from day 2 onwards. Application of *L. lactis* displays no substantial change in the aerobic counts over the 7 days.

The O₂ content of the 5 ppm nisin treatments decreased from a mean of 20.8 % to 12.03 % over the course of the trial. The 2.5 ppm treatment’s O₂ content reduced from 20.9 % to 12.60 %. The *L. lactis* treatment had an initial O₂ content of 20.7 % diminished to 17.0 % by day 7. The control (no treatment) lettuce bags had an opening O₂ content of 20.7 % which declined to 14.3 % by day 7. The overall sensory effects displayed acceptable scoring for the nisin 5 ppm treatment from day 0 to day 2 and on day 5 and onwards it fell below the satisfactory level. The nisin 2.5 ppm treatment displayed acceptable scoring from day 0 to day 5, from day 7 onward it fell below an acceptable level. The *L. lactis* treatment displayed adequate satisfactory levels until day 5 upon which it fell below the acceptable level.
3.2.6: 4 °C Modified Atmosphere (8kPa CO₂, 4kPa O₂, 88kPa N₂)

Figure 18: Effect of three different nisin (2.5 and 5ppm) and L. lactis treatments at 4 °C in a 8kPa CO₂, 4kPa O₂, 88kPa N₂ atmosphere. Including sensory analysis (bottom right), oxygen content (bottom left), L. monocytogenes (top left) and aerobic plate counts (top right) over the 7-day trial. Error bars represent ± standard deviation.

Figure 19 displays the outcome of the different nisin (2.5 and 5ppm) and L. lactis treatments on L. monocytogenes growth over a period of 7 days in an 8kPa CO₂, 4kPa O₂, 88kPa atmosphere at 8 °C. From a starting inoculum approximately of 10³ CFU/g the day 0 L. monocytogenes LSA plate count differed between the treatments applied. The application of 5 ppm nisin contributed to an initial 1.81 log reduction in L. monocytogenes growth compared to the control (no treatment) lettuce bags of which was maintained until day 7 where only a 0.40 log reduction was recorded. The application of 2.5 ppm nisin contributed to an initial 0.36 log reduction in L. monocytogenes growth compared to the control (no treatment) of which increased to 1.4 log on day 2 but decreased on day 5 to 1.4 log and decreased again until day 7 where a 0.22 log difference was recorded. The application of L. lactis contributed to no reduction in L. monocytogenes growth compared control (no treatment) lettuce bags. The aerobic plate counts showed an initial reduction of 0.37 log for the 5 ppm nisin treated lettuce however this reduction is diminished from day 2 onwards. A 0.24 log reduction was recorded in the aerobic counts of lettuce treated with 2.5 ppm nisin however the counts decreased when compared to the control (no treatment) from day 2
onwards to a final reduction of 0.58 log on day 7. Application of *L. lactis* displays no substantial reduction in the aerobic counts over the 7 days.

The O₂ content of the 5 ppm nisin treatments increased from a mean of 4.07 % to 9.15 % over the course of the trial. The 2.5 ppm treatment’s O₂ content expanded from 4.07 % to 7.90 %. The *L. lactis* treatment had an initial O₂ content of 4.12 % enlarged to 20.4 % by day 7. The control (no treatment) had an opening O₂ content of 4.12 % which increased to 8.92 % by day 7. The overall sensory effects displayed acceptable scoring for the nisin 5 ppm treatment from day 0 to day 2 and on day 5 it fell below the satisfactory level. The nisin 2.5 ppm treatment displayed acceptable scoring from day 0 to day 7. The *L. lactis* treatment displayed adequate satisfactory levels throughout the experiment.

**3.2.7: 4 °C Modified Atmosphere (15kPa CO₂, 1kPa O₂, 84kPa N₂)**

![Figure 19](image1)

**Figure 19:** Effect of three different nisin (2.5 and 5ppm) and *L. lactis* treatments at 4 °C in a 15kPa CO₂, 1kPa O₂, 84kPa N₂ atmosphere. Including sensory analysis (bottom right), oxygen content (bottom left), *L. monocytogenes* (top left) and aerobic plate counts (top right) over the 7-day trial. Error bars represent ± standard deviation.

Figure 20 displays the outcome of the different nisin (2.5 and 5ppm) and *L. lactis* treatments on *L. monocytogenes* growth over a period of 7 days in a 15kPa CO₂, 1kPa O₂, 84kPa N₂ atmosphere at 8 °C. From a starting inoculum approximately of 10³ CFU/g the day 0 *L. monocytogenes* LSA plate count differed between the treatments applied. The application of
5 ppm nisin contributed to an initial 1.17 log reduction in *L. monocytogenes* growth compared to the control (no treatment) of which maintained until day 7 where a 1.02 log reduction was recorded. The application of 2.5 ppm nisin contributed to a 0.53 log reduction in *L. monocytogenes* growth compared to the control (no treatment) of which increased and peaked on day 5 with a 1.13 log reduction however decreased on day 7 where a 0.71 log difference was recorded. The application of *L. lactis* contributed to no reduction in *L. monocytogenes* growth compared to the control (no treatment) lettuce bags. The aerobic plate counts showed an initial reduction of 0.20 log for the 5 ppm nisin treated lettuce however this reduction is diminished from day 2 onwards. A 0.04 log reduction was recorded in the aerobic counts of lettuce treated with 2.5 ppm nisin however this the counts decreased when compared to the control (no treatment) from day 5 onwards to a final reduction of 0.78 log on day 7. Application of *L. lactis* displays no substantial reduction in the aerobic counts over the 7 days.

The O$_2$ content of the 5 ppm nisin treatments increased from a mean of 1.14% to 5.56 % over the course of the trial. The 2.5 ppm treatment’s O$_2$ content expanded from 1.23 % to 15.4 %.

The *L. lactis* treatment had an initial O$_2$ content of 1.12 % enlarged to 20.43 % by day 7. The control (no treatment) had an opening O$_2$ content of 1.12 % which increased to 8.39 % by day 7. The overall sensory effects displayed acceptable scoring for the nisin 5 ppm treatment from day 0 to day 2 and on day 5 it fell below the satisfactory level. The nisin 2.5 ppm treatment displayed acceptable scoring from day 0 to day 2, on day 5 it was borderline on the acceptable level and on day 7 it definitely fell below the acceptable level. The *L. lactis* treatment displayed adequate satisfactory levels on days 0 and day 2, falling below that of both the acceptable level and that of nisin 2.5 ppm treatment on day 5, however, it displays an acceptable level on day 7.

### 3.3: Multi-food sampling study of the occurrence and persistence of *Listeria monocytogenes* in 12 food business operators

#### 3.3.1: *L. monocytogenes* occurrence

In total, 583 samples from 12 food processing facilities were analysed for the presence of *L. monocytogenes* from September 2014 until October 2015. This included 123 food based samples and 460 environmental swab or water samples. The average number of samples submitted by each food processing facility was 48.6 (Standard Deviation 19.9). Seven food
processing facilities maintained a 0 % *L. monocytogenes* prevalence over the year of sampling; these included one dairy facility, two vegetable processors, three meat facilities and one seafood facilities. The five remaining food processing facilities had an overall occurrence between 0-5%.

**Table 9:** Total samples from FBO sampling program 2014.

<table>
<thead>
<tr>
<th>2014 Samples</th>
<th>Total Samples</th>
<th>Food Samples</th>
<th>Environmental Samples</th>
<th>Positive Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>September</td>
<td>77</td>
<td>16</td>
<td>61</td>
<td>1</td>
</tr>
<tr>
<td>December</td>
<td>47</td>
<td>9</td>
<td>38</td>
<td>2</td>
</tr>
<tr>
<td><strong>2014 Total</strong></td>
<td><strong>124</strong></td>
<td><strong>25</strong></td>
<td><strong>99</strong></td>
<td><strong>3</strong></td>
</tr>
</tbody>
</table>

**Table 10:** Total samples from FBO sampling program 2015.

<table>
<thead>
<tr>
<th>2015 Samples</th>
<th>Total Samples</th>
<th>Food Samples</th>
<th>Environmental Samples</th>
<th>Positive Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>February</td>
<td>70</td>
<td>15</td>
<td>55</td>
<td>2</td>
</tr>
<tr>
<td>April</td>
<td>79</td>
<td>18</td>
<td>61</td>
<td>1</td>
</tr>
</tbody>
</table>
Chapter 3: Results

June

<table>
<thead>
<tr>
<th>June</th>
<th>July</th>
<th>August</th>
<th>September</th>
<th>October</th>
</tr>
</thead>
<tbody>
<tr>
<td>77</td>
<td>16</td>
<td>61</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>17</td>
<td>63</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>17</td>
<td>63</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>73</td>
<td>15</td>
<td>58</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2015 Total</td>
<td>459</td>
<td>98</td>
<td>361</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 11: Total samples from FBO sampling program 2014-2015.

<table>
<thead>
<tr>
<th>Total Samples</th>
<th>Food Samples</th>
<th>Environmental Samples</th>
<th>Positive Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>583</td>
<td>123</td>
<td>460</td>
<td>6</td>
</tr>
</tbody>
</table>

The general prevalence of *L. monocytogenes* decreased from 2.42% in 2014 to 0.65% in 2015. The prevalence of *L. monocytogenes* in food samples decreased from 8% in 2014 and 1% in 2015. Overall, 3 companies showed a decrease in occurrence between 2014 and 2015, 7 had 0% occurrence during both sampling years with 1 showing an increase in occurrence from 2014 to 2015 and 1 only being sampled in 2015.

3.3.2: *L. monocytogenes* occurrence per industry

Table 12: Samples from FBO sampling program 2014-2015 by industry.

<table>
<thead>
<tr>
<th>FBO Industry</th>
<th>Number of FBOs</th>
<th>Total Samples</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meat</td>
<td>6</td>
<td>225</td>
<td>3</td>
</tr>
<tr>
<td>Dairy</td>
<td>1</td>
<td>55</td>
<td>0</td>
</tr>
</tbody>
</table>
Chapter 3: Results

Different industry sectors had differing rates of occurrence in samples. Including food and processing environment samples, the industry with the highest prevalence was the seafood industry, in which 1.82% of 110 samples were positive for *L. monocytogenes*. The meat industry had 1.33% *L. monocytogenes* positives from 225 samples. The vegetable industry with 0.5% of 193 samples positive for *L. monocytogenes*. The dairy industry had no *L. monocytogenes* positives from 55 samples. No persistent strains, that is strains consistently present over a six-month time period, were detected (Leong et al. 2014).

3.3.3: *L. monocytogenes* positive samples

Table 13: Positive *L. monocytogenes* detections from food sampling program, confirmed via multiplex PCR (Doumith et al. 2004).

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Isolate source</th>
<th>Method of confirmation</th>
<th>Serogroup</th>
<th>Persistent</th>
</tr>
</thead>
<tbody>
<tr>
<td>G4</td>
<td>Swab from High Risk Drain</td>
<td>Not tested in Limerick</td>
<td>1/2a-3a</td>
<td>No</td>
</tr>
<tr>
<td>F8</td>
<td>Oak/Peat Smoked Salmon</td>
<td>Species PCR (Doumith <em>et al.</em> 2004)</td>
<td>1/2a-3a</td>
<td>No</td>
</tr>
<tr>
<td>E5</td>
<td>Pickle Water liquid</td>
<td>Species PCR (Doumith <em>et al.</em> 2004)</td>
<td>1/2c-3c</td>
<td>No</td>
</tr>
<tr>
<td>A8</td>
<td>Water from</td>
<td>Species PCR</td>
<td>1/2c-3c</td>
<td>No</td>
</tr>
</tbody>
</table>
From the six confirmed positive samples obtaining from the FBO sampling program four of the six were from serogroup 1/2c-3c while the remaining two were from serogroup 1/2a-3a. Five of the samples were obtained from environmental swabs with the remaining one obtained from a sample of smoked salmon. No persistent strains were observed.
**Chapter 4: Discussion**

**4.1: Investigation into the influence of inoculation density, temperature and atmosphere on the growth and enumeration of *Listeria monocytogenes*.**

The volume of contamination has been shown to significantly affect the growth of *L. monocytogenes* (Robinson *et al.* 2001, Koutsoumanis and Sofos 2005). Thus the initial inoculation density of *L. monocytogenes* when conducting experiments into its growth or response under different physical conditions or in the presence of various chemicals is of equal importance. Although predictive modelling data accounting for the effect of initial inoculation density exists it is generally derived from 24/96-well based experiments. While information of growth assays of *L. monocytogenes* under a wide range of growth conditions and media are relatively common no specific trials have been conducted measuring the effect of the starting inoculum density on growth rates at refrigeration temperatures.

This experiment demonstrates the effect of inoculation density at various temperatures (4 °C and 8 °C) under both an optimum and suboptimum modified atmosphere and an air atmosphere on the rate of *L. monocytogenes* growth. At 8 °C significant differences (*P*≤0.05) could be seen between each of the Day 0 and Day 7 sampling days under all atmospheres and inoculation densities $10^2$, $10^3$ and $10^4$ and under a 4kPa O$_2$ atmosphere also at $10^5$ CFU/g$^{-1}$. This demonstrates that initial amounts of *L. monocytogenes* can under temperature abuse conditions influence or even negate the combination effect of the various employed hurdles such as modified gas atmospheres. Numerous studies have been conducted into the effects of MAP on the growth of *L. monocytogenes* on fruits and vegetables showing that its growth is neither reduced nor affected by the modified atmospheres (Beuchat and Brackett 1991, Francis and O’Beirne 1997, Bourke and O’Beirne 2004, Oliveira *et al.* 2010). However, on fresh cut fruits and vegetables there are more disparities in the results with Siro *et al.* (2006) presenting reductions of *L. monocytogenes* growth on strawberries in an active 3% O$_2$ 5% CO$_2$ packaging and Kakiomenou *et al.* (1998) showing reductions on lettuce and carrots under a modified atmosphere of approximately 5% CO$_2$ and varying O$_2$ of 2% or 5%. A study by Francis and O’Beirne (2005) showed that all of the 15 tested *L. monocytogenes* strains grew on lettuce reducing the O$_2$ levels to 2-4% over the 10-day trial however, differences between the strains were much more pronounced when grown in an environment of coleslaw...
with the O₂ levels reaching 0-1% and CO₂ levels reaching twice to triple the levels (25-30%) of the lettuce packs after the experimental period.

At the lower temperature (4 °C) the lowest inoculation density 10² CFU/g still maintained significant differences ($P \leq 0.05$) across the three atmospheres, however at 10⁵ CFU/g only the 1kPa O₂ atmosphere showed any significant ($P \leq 0.05$) difference between day 0 and day 7. Also at the lower temperature the growth rates are expectantly lower. Within the literature the effect of temperature on the growth of *L. monocytogenes* is much more uniform with many studies showing a reduction in growth at lower temperatures (4 °C) compared to that of higher temperatures (>8 °C) (Francis and O’Beirne 1997, Corbo *et al.* 2005, Uhlich *et al.* 2006, Scollard *et al.* 2016).

As described in the literature review section recent experiments focus on fixed starting cell densities inspecting the effects of atmosphere, temperature, food matrix, antimicrobial treatment, pH and $a_w$ on the microorganisms (Barmpalia *et al.* 2005, Koseki and Isobe 2005a, Hereu *et al.* 2014, Mejilholm *et al.* 2015, Mejilholm and Dalgaard 2015, Ostergaard *et al.* 2015). While multiple inoculation densities have been conducted (Omac *et al.* 2015, Quinto *et al.* 2016) these experiment were not specifically designed to study the effects on inoculation density on *L. monocytogenes* growth. Thus direct comparisons are difficult and data derived from predictive modelling can be unreliable at low inoculation densities (Gysemans *et al.* 2007, Vermeulen *et al.* 2009).

What also can be derived from the results shown is that the lower the starting inoculation density the higher the growth potentials of *L. monocytogenes* are. At the highest inoculation density 10⁵ CFU/g both at 4 °C and 8 °C day 7 count where indistinguishable from day 0 counts in an air atmosphere ($P \geq 0.05$). When contrasted to lowest inoculation density of 10² CFU/g both at 4 °C and 8 °C day 0 and 7 counts where significantly larger with a minimum 1 log to a maximum of 2.7 log of growth occurring ($P \leq 0.05$) from each other this shows the effect that starting inoculation can have. While low levels (<10² CFU/g) of pathogenic bacteria such as *L. monocytogenes* are not of concern to human health, the potential of rapid growth under refrigeration conditions and under modified atmosphere shows the potential dangers of *L. monocytogenes*.

From the serogroup identification assay, similar results have been shown to that of previous experiments conducted Francis and O’Beirne (2005) showing that specific *L. monocytogenes*
serogroups used in this experiment have not been considerably effected by the conditions of their growth on lettuce at both 4 °C and 8 °C. Possible explanations for these effects may be that Iceberg Lettuce and the conditions used are not a stressful enough environment for the development of significant differences to appear between the individual serogroups used. The mix comprised of a EURL Lm reference strain (number 1382), a persistent strain isolated from a food processing plant (number 6179) and a strain isolated from a vegetable production facility (number 959) all displayed adequate ability to be detected via PCR after 7 days of growth on the lettuce medium. Francis and O’Beirne (2005) showed that under harsher conditions such as that which develop when L. monocytogenes is grown on a coleslaw medium can lead to significant differences between the serogroups, where both lower O₂ and a very high CO₂ atmosphere was used.

These experiments displayed in this thesis have shown that L. monocytogenes can readily survive and grow beyond acceptable regulatory levels at low and abuse temperatures that can be found within the food processing and transport systems. Displaying the ability to overcome specific hurdles designed to halt the growth of pathogenic bacteria. Thus the data within this study demonstrates a larger potential growth rate at lower inoculation densities under both the abuse and standard storage temperatures and specific modified atmospheric conditions from these experiments may be useful for the development of predictive models where the effect of contamination at low cell densities from a persistent source within a cold storage chain. Another result of this experiment is the lack of significant growth at the highest inoculation density suggesting that an inherent bias could exist in growth experiments where only high inoculation densities are used. Potentially leading to a need to reclassify growth based L. monocytogenes experiments.

**4.2: Inhibition of Listeria monocytogenes growth on lettuce through application of the bacteriocin Nisin.**

The use of natural antimicrobial compounds has been gaining increasing attention over the recent decades leading to the inclusion of numerous different types in a wide variety of products. Examples of such include the use of the plant derived essential oils, animal derived immunoglobulins and microbial derived bacteriocins (Cotter et al. 2013). While some of these have been used for thousands of years only recently studies have been carried out in order to establish if these constituents could be feasibly used in other areas of food preservation as potential additional hurdles in the current set hurdle technology. One of the
main drivers of this is the GRAS status of these compounds, combined with demand by consumers to move away from chemical sanitizers has led to the development and increased study of these antimicrobials, with particular interest towards nisin (Burt 2004, Cotter et al. 2005, Rico et al. 2007, Scollard et al. 2016).

The goal of this experiment was to establish if two purified commercial nisin treatments (2.5 and 5 ppm) and a nisin producing strain of *L. lactis* can inhibit or reduce *L. monocytogenes* on Iceberg Lettuce under two modified atmospheres and an air atmosphere at both 4 °C and 8 °C with little to a moderate effect on the appearance of the lettuce. Individually the application of both amounts of purified nisin showed a significant (*P*≤0.05) maximum reduction of 1.6 log CFU/g with application on 5 ppm of the nisin treatment at 4 °C when compared to the control (no treatment) trial. Application of 2.5 ppm of the purified nisin showed a significant (*P*≤0.05) maximum reduction of 1.3 log CFU/g under the same conditions. At 8 °C a significant (*P*≤0.05) maximum reduction of 1.07 log CFU/g with application on 5 ppm of the nisin when compared to the no treatment trial. While with application of the purified 2.5 ppm nisin treatment showed a significant (*P*≤0.05) maximum reduction of 0.93 log CFU/g again when compared to the control (untreated) trial. The effect of the *L. lactis* treatment in reducing the count of *L. monocytogenes* was negligible to minimal at both 4 °C and 8 °C.

The effect of the nisin treatments seemed to be directly tied to the application with the majority of the reductions occurring on the initial 2 days of the trial. However, once the effect of the nisin has “worn off” the *L. monocytogenes* appears to return to normal growth rates while maintaining the reduction that the treatment induced. With the development of food grade films with encapsulated nisin and greater understanding of the release kinetics of encapsulated nisin as a whole, this shows the potential that nisin can be applied as an antibacterial compound throughout the entire shelf life of RTE produce (da Silva-Malheiros et al. 2012, Boelter and Brandelli 2016, Wu et al. 2016). However, studies into the effectiveness of these new developments and their potential antilisterial and sensory effects on fresh cut produce would need to be conducted.

When compared with the non-treated lettuce the application of the nisin producing bacteria *L. lactis* showed little to no effect on the inhibition of *L. monocytogenes* growth, a trend seen in a similar study (Allende et al. 2007). A possible reason for this may be due to the reported production of the antimicrobials during its exponential growth phase, with it completely
stoppage when cells enter the stationary growth phase (Pongtharangkul and Demirci 2004, de Arauz et al. 2009). Thus production of the antimicrobial nisin A molecule by *L. lactis* 20729 may have been affected by numerous conditions such as the natural microbiota of the lettuce, the physical and chemical environment of the lettuce and the relative low temperatures of the experimental conditions. This has been shown in a study conducted by Allende et al. (2007) where at temperatures under 10 °C only a nisin Z producer actually produced detectable levels of nisin while other *L. lactis* still grew without production. Also this study was conducted on washed lettuce which also may contribute to the growth of the nisin producing bacteria. A recent study by Siroli et al. (2016) showed that again with the use of a nisin Z producing strain of *L. lactis* on fresh cut apples combined with a biocontrol agent hexanal/2-(E)-hexenal could significantly reduce the counts of *L. monocytogenes* at 6 °C over a period of 28 days. This suggests that the increase of time in combination with an effective nisin producing strain over a longer period may produce adequate results in other fresh-cut produce, however, due to the physical characteristics these techniques may be of little use on fresh-cut lettuce and other short shelf life RTE products.

The effect of the nisin treatments on the natural microbiota did not appear to affect the survival of the non-*Listeria* aerobic microbial populations. These natural microbial populations can form part of a protective competitive “shield” which may play a significant role in the quality and microbial safely of the fresh cut produce (Allende et al. 2007, Oliveira et al. 2015a). Thus the continuation of the maintenance of this microbiota is important in order to prevent the rise of other pathogenic organisms which may be unaffected by the bacteriocin application. Further studies would need to be conducted into the effect of nisin and its interactions with the microbiota of fresh-cut produce on a community-wide level.

A primary reason towards the push towards natural antimicrobials is due to the concerns over is decreasing consumer acceptance of chemical “man-made” preservatives and sanitizers (Rico et al. 2007). Still, the effects of natural antimicrobials, such as essentials oils, can have substantial negative organoleptic effects. While techniques such as dipping and ample dilution have been developed these can still be destructive to certain fresh-cut produce such as iceberg lettuce (Scollard et al. 2016). The use of nisin as an alternative to these harsh antimicrobials has been shown frequently within in the literature. In these experiments nisin has shown to be effective in reducing *L. monocytogenes* counts while still maintaining an acceptable sensory value over 5-7 days under certain MAP conditions. These is especially
true when compared to a similarly designed experiment by Scollard et al. (2016) where application of essential oils to fresh cut lettuce proved detrimental to its sensory appearance within 0-2 days.

The information gained from this experiment should be of value as it displays data on the effects of the antilisterial agents and its subsequent effects on the sensory value and the consumer appointed use by condition. While experiments including this data have been common place in areas dealing with sensory practices and evaluations, they become rarer when dealing with the direct effects of studies into the control agents on pathogenic organisms. However recently sensory analysis of the effects of antimicrobials have started to become more commonplace (Tajkarimi et al. 2010, Scollard et al. 2013, Scollard et al. 2016, Siroli et al. 2016).

Future work within the area of L. lactis and nisin as a potential antilisterial agent should focus on the areas of optimisation of the conditions used during the storage of the respective food matrix. As these conditions, particularly the surface pH can affect the actual nisin activity. Thus an understanding of these conditions could lead to a potential increase in the effectiveness of nisin. Another potential optimisation area would be selecting a strain or strains of L. lactis that can produce nisin consistently under conditions found within the selected food matrix. A final area in which nisin’s antilisterial properties could be improved is a re-evaluation of the purification and application of nisin. Potential techniques which are commonly used such as dipping or washing in a nisin solution, or application of nisin via spray could add to its effectiveness.

4.3: Multi-food sampling study of the occurrence and persistence of Listeria monocytogenes in 12 food business operators

The aim of this experiment was to obtain data of the overall occurrence and prevalence rate of L. monocytogenes of 12 FBOs forming part of a national sampling program (Leong et al. 2016). Over the length of the sampling period the overall occurrence rate of L. monocytogenes dropped considerably, with the 6 positive L. monocytogenes occurring within the first four sampling periods. This suggests that the attitude of surveillance and awareness developed via the regular sampling sessions combined with reporting of results back to the FBO leads to a top down impact of good management practices which can propagate throughout the business to create practices that can reduce occurrence of L. monocytogenes.
Similar results were reported by the larger 3-year *L. monocytogenes* sampling program that these results where a part of. That study reported a 1.3% decrease in in the overall rate of *L. monocytogenes* occurrences over the 3 years in environmental samples, while a 2.4% reduction was recorded in food samples (Leong et al. 2016). Serogroups 1/2a-3a and 1/2c-3c made up all of the positive *L. monocytogenes* samples when confirmed by a serogroup PCR. This is of a similar nature to that of the national study upon which was found that serogroups 1/2a-3a and 1/2c-3c making up well over half of the total serogrouped samples (Leong et al. 2016); possibly suggesting that these serogroups may be prolific when dealing with environmental derived *Listeria* species. Thus these species should be included as default in all environmental *Listeria* simulation experiments.

The processing environment of the different food industries also contributed to the rate of occurrence with the meat industry showing both the highest amount positive of *L. monocytogenes* occurrences in this study and in the national study however this may be due to its overrepresentation making up 6 of the 12 FBOs in the experiment. While seafood displayed the highest occurrence rate in this study, the national study contrasts this with a considerably lower occurrence rate of 1.7 % where compared to other industries such as meat (5.5%), dairy (3.2%) and vegetables (7.65 %). A potential reason for the seafood industry’s low occurrence especially when compared to the EU wide level of 10% (EFSA 2013), is due to numerous studies and awareness into *L. monocytogenes* in the seafood industry as there has been several *L. monocytogenes* outbreaks (Uyttendaele et al. 2009, Gonzalez et al. 2013). With only one dairy based FBO providing both environmental samples and food samples comparisons between the national trial and this trial are difficult. However, while no positive *L. monocytogenes* samples were obtained this could be attributed to the techniques employed by the FBO to counter *L. monocytogenes* contamination as dairy processors are generally well aware of the potential hazards leading to *L. monocytogenes* contamination of cheese, milk and other dairy-based products. The vegetable FBOs had a total of 1 positive *L. monocytogenes* sample for approximately 193 provided samples. This relatively low rate is in contrast with the national study where an occurrence rate of 7.65% was obtained, however, this was with approximately 60% less samples then the other FBO sectors. Potential reasons for the high incidence within certain food sectors may be due to the relationship between *L. monocytogenes* and its ubiquitous nature within the soil environment and the relative potential for a FBO to acquire contamination from the soil (Uyttendaele et al. 1999).
Seven food processing facilities maintained a 0% *L. monocytogenes* prevalence over the sampling period; these included one dairy facility, two vegetable processors, three meat facilities and one seafood facilities. This again suggests that management and the various hygiene practices that occur at each of the specific FBOs facilities may have a large impact on the occurrence of the bacteria and thus more studies should be directed towards why these facilities, management practices and bacterial control procedures effect the occurrence of *L. monocytogenes*.

The combination of an effective hygiene practice with the heightened awareness that comes with regular and continuous sampling for suspect pathogens suggests a potential method for control, reduction and potential elimination or minimisation of *L. monocytogenes* outbreaks at an industrial level. While not a new development, (Uyttendaele *et al.* 2009) these methods if correctly and habitually employed combined with increased public awareness from recent outbreaks over a wide variety of foodstuffs (CDC 2016) should ensure the minimum number of causalities from *L. monocytogenes*.

### 4.4: Conclusions

This study displayed the effects of inoculation densities on the growth of *L. monocytogenes* at refrigeration temperatures under both modified and unmodified atmospheres on fresh cut Iceberg Lettuce. Low initial cell densities can lead to higher growth rates when compared to higher inoculation densities. This potentially leads to a need to reinvestigate experiments where high inoculation density was used in order to establish if growth was viable in a specific foodstuff.

The effect of nisin as an alternative hurdle to other more product damaging antilisterial compounds commonly used in “hurdle technology” on fresh cut Iceberg lettuce. It was demonstrated that the introduction of low amounts of purified commercial nisin A can reduce *L. monocytogenes* counts by a significant amount without a substantial negative effect on the sensory quality of the lettuce. This displays that nisin is a viable natural antilisterial compound that can be used on fragile food matrices, specifically lettuce where other antilisterial compounds such as essential oils degrade the quality beyond an acceptable amount.

Sampling data was obtained as part of a 3-year national *L. monocytogenes* sampling program of various food business operators. The data displayed that awareness and vigilance of *L.
*monocytogenes* can contribute to a reduction in the pathogenic bacteria in food processing and food production environments, thus decreasing the risk to public health. This information can be employed on a national and international level to ensure that policies and programs can be engaged to allow for the minimisation of *L. monocytogenes* outbreaks and illness and thus reduce deaths.
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Chapter 6: Appendix

6.1: Investigation into the influence of inoculation density, temperature and atmosphere on the growth and enumeration of *Listeria monocytogenes*.

*Figure 20:* Oxygen content analysis of 4 °C Air atmosphere over the 7-day trial. Error bars represent ± standard deviation.
Figure 21: Oxygen content analysis of 4 °C 4kPa Oxygen atmosphere over the 7-day trial. Error bars represent ± standard deviation.

Figure 22: Oxygen content analysis of 4 °C 1kPa Oxygen atmosphere over the 7-day trial. Error bars represent ± standard deviation.
Figure 23: Oxygen content analysis of 8 °C Air atmosphere over the 7-day trial. Error bars represent ± standard deviation.

Figure 24: Oxygen content analysis of 8 °C 4kPa Oxygen atmosphere over the 7-day trial. Error bars represent ± standard deviation.
**Figure 25:** Oxygen content analysis of 8 °C 1kPa Oxygen atmosphere over the 7-day trial. Error bars represent ± standard deviation.

**Figure 26:** Effect of four different inoculation densities and subsequent growth at 4 °C in an Air atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.
Figure 27: Effect of four different inoculation densities and subsequent growth at 4 °C in an 4kPa Oxygen atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.

Figure 28: Effect of four different inoculation densities and subsequent growth at 4 °C in an 1kPa Oxygen atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.
Figure 29: Effect of four different inoculation densities and subsequent growth at 8 °C in an Air atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.

Figure 30: Effect of four different inoculation densities and subsequent growth at 8 °C in an 4kPa Oxygen atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.
**Figure 31**: Effect of four different inoculation densities and subsequent growth at 8 °C in an 1kPa Oxygen atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.

### 6.2: Inhibition of *Listeria monocytogenes* growth on lettuce through application of the bacteriocin *Nisin*.

**Listeria Specific Count 8 °C 1kPa O₂**

![Graph showing Listeria Specific Count 8 °C 1kPa O₂](image)

**Listeria Specific Count 4 °C Air**

![Graph showing Listeria Specific Count 4 °C Air](image)
Figure 32: Effect of three different nisin (2.5 and 5 ppm) and *L. lactis* treatments on *L. monocytogenes* growth at 4 °C in an Air atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.

![Listeria Specific Count 4 °C 1kPa O2](image)

Figure 33: Effect of three different nisin (2.5 and 5 ppm) and *L. lactis* treatments on *L. monocytogenes* growth at 4 °C in a 1 kPa Oxygen atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.

![Listeria Specific Count 4 °C 4kPa O2](image)

Figure 34: Effect of three different nisin (2.5 and 5 ppm) and *L. lactis* treatments on *L. monocytogenes* growth at 4 °C in a 4 kPa Oxygen atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.
Figure 35: Effect of three different nisin (2.5 and 5ppm) and *L. lactis* treatments on *L. monocytogenes* growth at 8 °C in an Air atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.

Figure 36: Effect of three different nisin (2.5 and 5ppm) and *L. lactis* treatments on *L. monocytogenes* growth at 8 °C in a 1kPa Oxygen atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.
**Figure 37:** Effect of three different nisin (2.5 and 5ppm) and *L. lactis* treatments on *L. monocytogenes* growth at 8 °C in a 4kPa Oxygen atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.

**Figure 38:** Effect of three different nisin (2.5 and 5ppm) and *L. lactis* treatments on the sensory analysis of fresh-cut Iceberg Lettuce at 4 °C in an Air atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.
Figure 39: Effect of three different nisin (2.5 and 5ppm) and *L. lactis* treatments on the sensory analysis of fresh-cut Iceberg Lettuce at 4 °C in a 1kPa Oxygen atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.

Figure 40: Effect of three different nisin (2.5 and 5ppm) and *L. lactis* treatments on the sensory analysis of fresh-cut Iceberg Lettuce at 4 °C in a 4kPa Oxygen atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.
**Figure 41:** Effect of three different nisin (2.5 and 5ppm) and *L. lactis* treatments on the sensory analysis of fresh-cut Iceberg Lettuce at 8 °C in an Air atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.

**Figure 42:** Effect of three different nisin (2.5 and 5ppm) and *L. lactis* treatments on the sensory analysis of fresh-cut Iceberg Lettuce at 8 °C in a 1kPa Oxygen atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.
Figure 43: Effect of three different nisin (2.5 and 5ppm) and *L. lactis* treatments on the sensory analysis of fresh-cut Iceberg Lettuce at 8 °C in a 4kPa Oxygen atmosphere. Different letters identify significant differences within sampling days. Error bars represent ± standard deviation.

Figure 44: Oxygen content analysis of 4 °C Air atmosphere over the 7-day trial. Error bars represent ± standard deviation.
Figure 45: Oxygen content analysis of 4 °C 1kPa Oxygen atmosphere over the 7-day trial. Error bars represent ± standard deviation.

Figure 46: Oxygen content analysis of 4 °C 4kPa Oxygen atmosphere over the 7-day trial. Error bars represent ± standard deviation.
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Figure 47: Oxygen content analysis of 8 °C Air atmosphere over the 7-day trial. Error bars represent ± standard deviation.

Figure 48: Oxygen content analysis of 8 °C 1kPa Oxygen atmosphere over the 7-day trial. Error bars represent ± standard deviation.
Figure 49: Oxygen content analysis of 8 °C 4kPa Oxygen atmosphere over the 7-day trial. Error bars represent ± standard deviation.
Figure 50: Aerobic plate count of 8 °C air atmosphere over the 7-day trial. Error bars represent ± standard deviation.

Figure 51: Aerobic plate count of 8 °C 1kPa Oxygen atmosphere over the 7-day trial. Error bars represent ± standard deviation.
Figure 52: Aerobic plate count of 8 °C 4kPa Oxygen atmosphere over the 7-day trial. Error bars represent ± standard deviation.

Figure 53: Aerobic plate count of 4 °C air atmosphere over the 7-day trial. Error bars represent ± standard deviation.
Figure 54: Aerobic plate count of 4 °C 1kPa Oxygen atmosphere over the 7-day trial. Error bars represent ± standard deviation.

Figure 55: Aerobic plate count of 4 °C 4kPa Oxygen atmosphere over the 7-day trial. Error bars represent ± standard deviation.
6.3: Submitted Abstracts

Investigation into the influence of inoculation density, temperature and atmosphere on the growth and enumeration of *Listeria monocytogenes*.

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*Presented at IUFoST Dublin August 2016*

*Listeria monocytogenes* is a particular risk for the ready-to-eat food sector because it’s extraordinarily well adapted to various environmental conditions including low temperatures and modified atmospheres. Currently in foods that cannot support growth of *L. monocytogenes* EU regulation allow up to $10^2$ colony forming units per gram (CFU/g) food. To be consistent with EU regulations, growth conditions at low inoculation densities of $10^2$ CFU/g need to be investigated instead of common practice experiments with $10^5$ CFU/g of food. In this study, inoculation densities from $10^2$ to $10^5$ were tested under different storage temperatures and atmospheres to identify any potential inoculation density effects on growth of *L. monocytogenes*. $10^3$ to $10^6$ CFU of *L. monocytogenes* were applied onto 10 g of Iceberg lettuce (*Lactuca sativa*) into sealed bags with an atmosphere of i) air, ii) 8% CO₂, 4% O₂, 88% N₂ iii) 15% CO₂, 1% O₂, 84% N₂ at 4 °C or 8 °C. On days 0, 2, 5 and 7, *L. monocytogenes* was extracted from the lettuce surface and enumerated via selective media. Two separate multiplex assays were used for the confirmation of *L. monocytogenes* abundance. The resulting growth curves clearly identified an inoculation density effect at abuse temperature (8 °C) for both atmospheres with 10-100 times higher growth rates when lettuce was inoculated with $10^2$ instead of $10^5$ CFU/g. Thus, past inoculation experiments using high inoculation densities may have underestimated the growth potentials of *L. monocytogenes*. Therefore, future growth studies of *Listeria* on food products should consider the application of lower cell densities.
Inhibition of Listeria monocytogenes growth on lettuce through application of the bacteriocin Nisin.

Oisin McManamon and Achim Schmalenberger

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Presented at IUFoST Dublin August 2016

The bacteriocin nisin, produced naturally by various Lactococcus lactis strains, is commonly used in the cheese industry as it displays broad-spectrum activity against many Gram-positive bacteria including Listeria monocytogenes. In the ready-to-eat food sector L. monocytogenes poses a risk to minimally processed foods due to its ability to grow under refrigeration conditions. Many natural antilisterial products render Iceberg lettuce unsuitable for consumption within 2 days of storage and thus this study investigated the efficacy of nisin as antilisterial agent and its sensory impact on lettuce. In-vitro assays confirmed the efficacy of nisin to inhibit growth of a three strain mix of L. monocytogenes. Subsequently, this L. monocytogenes strain mix (10^3 CFU) was added to lettuce (10g) that was treated either with nisin (25 or 50 ppm), L. lactis DSM20729 or was kept treatment-free. Incubation took place at 4 and 8°C under various atmospheres. On days 0, 2, 5 and 7, Listeria was extracted from the food surface and enumerated on selective agar and a sensory panel graded the lettuce on product appearance. At 4 and 8°C a 10-100-fold reduction of Listeria growth was achieved with 50 ppm nisin over a seven-day period, while lettuce kept an acceptable sensory appearance over the first 5 days. Direct application of nisin producing L. lactis had no detectable effect on Listeria growth in vitro and in situ. Overall results demonstrate that nisin has the potential to be used on fresh-cut produce lettuce as antilisterial agent.
Inhibition of *Listeria monocytogenes* on fresh-cut produce with extracts of essential oils

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*Presented at SGM Galway June 2015*

The anti-listerial effectiveness of thyme essential oil (EO), verbenone and camphor (EO extracts) was examined on a range of modified atmosphere packaged fresh-cut fruits and vegetables (lettuce, cantaloupe melon and pineapple). The effects of commercial modified atmospheres was evaluated by comparing survival in air with optimum (4% O\(_2\)/8% CO\(_2\)/88% N\(_2\)) and sub-optimum (1% O\(_2\)/15% CO\(_2\)/84% N\(_2\)) atmospheres in model packages at 4 °C. *L. monocytogenes* was found to be able to survive and grow in both atmospheres with pineapple experiencing the lowest and melon the highest growth rates, presumably due to product pH that is low for the former and neutral for the latter product. Thyme EO demonstrated the best anti-listerial effect, although direct application of the EO damaged product appearance, particularly when used on lettuce. While Camphor showed no anti-listerial effects, verbenone was found to have good anti-listerial properties and maintained high sensory acceptance in fresh-cut fruit. The high growth rates of *L. monocytogenes* on melon could be reduced with the application of verbenone while completely being eliminated on pineapple. The use of thyme EO and verbenone as an antimicrobial dip (150 ppm) was successfully applied to reduce growth of *Listeria* on fresh-cut melon; however, reductions in growth were higher when compared to a traditional chlorine dip (150 ppm). Further research will be necessary to optimise conditions in fresh-cut produce treatments with natural products like verbenone to replace current chlorine treatments for improved food safety.
6.4: Submitted and Published Papers
Postharvest Biology and Technology
2016, 120, pp. 61-68

Inhibition of Listeria monocytogenes growth on fresh-cut produce with thyme essential oil and essential oil compound verbenone

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The published version is available at:
http://dx.doi.org/10.1016/j.postharvbio.2016.05.005

The author accepted version is available at:
http://hdl.handle.net/10344/5320
A 3-year multi-food sector study of the presence and persistence of *Listeria monocytogenes* in 54 small food businesses in Ireland.


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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 pulsortypes 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 pulsortypes detected were unique to a particular food group (63/86), while only three pulsortypes 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 pulsortypes 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 pulsortypes 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 pulsortypes 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 strains are also found in clinical settings highlights the need for continued vigilance by food producers, including frequent sampling and typing of isolates detected.

Keywords: Listeria monocytogenes; persistence; food safety; hygiene; control
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* suffer 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 psychrotrophic 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 Chilindras, 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 et al., 2016). Due to its psychrotrophic 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 5 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; Luber 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, 2002). 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 determine if routine surveillance and sub-typing (by PFGE) of *L. monocytogenes*, following a sampling plan carried out over a three-year period, in 34 Irish food processing facilities helped reduce the occurrence and persistence of *L. monocytogenes* at those facilities. *L. monocytogenes* isolates obtained were characterised by molecular methods and compared to other food processing isolates 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.
1. Materials and Methods

2.1. *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.

2.2. Analysis of samples
At all four sites (TIFR, UoN, 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 spp.* to Ottaviani & 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 cryoinstant 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. 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 QIAamp Mini kit (QIAGen, Ireland).

2.4. 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).

2.5. PFGE

PFGE was performed according to the International Standard PulseNet protocol (PulseNetUSA, 2009) with the restriction enzymes SgrI (formerly Ascl) and Apal, 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'.

2.6. Statistical analysis

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

2. Results

3.1. L. monocytogenes occurrence

In total, 3869 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 1). The average number of samples

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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 S1).

The general prevalence of *L. monocytogenes* decreased from 4.8% in 2013 to 3.7% in 2014 and 3.2% in 2015. The prevalence of *L. monocytogenes* in food samples decreased from 5.1% in 2013 to 4.7% in 2014 and 2.7% in 2015 (Table 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.

There was an uneven distribution of participants in the surveillance programme across the country (Figure S1). 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 1.73% 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 4.28% *L. monocytogenes* positives from 1681 samples. The highest processing environment prevalence occurred in the vegetable industry with 8.5% of 647 samples positive for *L. monocytogenes*. Positive food samples were obtained from all industry sectors (Table S1, Table 2).
3.2. Selection of isolates for characterisation

Ten percent of the positive samples yielded more than one PFGE pulsortype. When all
four isolates from the same sample belonged to the same PFGE pulsortype, only one isolate
was carried forward for further study. If differing pulsortypes were seen from the same
positive sample, a representative isolate of each pulsortype 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 227 positive samples.

3.3. 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 3). Serotyping was also performed on 110 of these isolates; all isolates in each
serogroup belonged to a single serotype (see Table 3). 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).

3.4. PFGE

Pulsotype numbers (P numbers) were assigned to PFGE pulsortypes based on their
relatedness. The 255 isolates were assigned to eighty-six different pulsortypes. Several
pulsotypes occurred in multiple industry sectors, but only three pulsortypes, P44, P46 and P59
occurred in all industry sectors (Figure 1).
Overall, there was great diversity in the isolates obtained, as seen in the minimum spanning tree (Figure 2). The majority of pulsortypes (59/86) 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 pulsortype belonged to the same serogroup.

The distribution of the pulsortypes around the country can be seen in Figure 3. From these data it is clear that certain pulsortypes are prevalent on the island of Ireland and persistent isolates are found in each of the 4 food groups included in the study.

3.5. 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 4). Sixteen processing facilities had at least one persistent L. monocytogenes strain over the three-year period. Seventeen different pulsortypes were observed as being persistent. Five pulsortypes 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 S1).

3.6. Comparison with pulsortypes 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 pulsortypes showed close similarity with the clinical pulsortypes (Figure 5). These 11 pulsortypes were identified in 26 facilities and were found in both processing environment and food samples from all sectors. Seven of these 11 pulsortypes were identified as persistent in one (P2, P31, P45 and P48) or several (P6, P32, P44) facilities, and one of them (P44) occurred in all industry sectors.

3.7. Comparison with pulsortypes identified internationally

The 86 pulsortypes obtained were compared with pulsortypes obtained in 5 other countries, including United States of America, Australia, United Kingdom, France and Romania. Of the 86 pulsortypes obtained in Ireland, 32 were seen internationally, with 11 of the pulsortypes 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.

3.8. Evidence of cross-contamination from the processing environment to food

There was evidence of cross-contamination between the processing environment and food (indistinguishable pulsortypes found in processing environment samples and food samples) at 12 facilities, representing all food sectors (Table S1). In two cases, transfer of more than one pulsortype occurred. The cross-contamination included seven different persistent pulsortypes (P6, P31, P32, P33, P44, P45 and P59) and five sporadic pulsortypes (P10, P17, P21, P61 and P67).
3.9. 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, there was an increase from 5% occurrence in 2013 to 25% occurrence in 2014 (Table S1). This increase coincided with installation of new equipment. In a second facility, there was a decrease from 15% occurrence in 2013 to 0% occurrence in 2014 (Table S1). This decrease coincided with inclusion of a peracetic acid final rinse in the cleaning and disinfection scheme.

3. Discussion

During the course of this study, the overall occurrence of *L. monocytogenes* in the processing environment of the 34 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 2012 and 2015), a trend towards a reduction in occurrence over the sampling period was observed. 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) show 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 be successful in 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 resulted in a reduced risk of cross-contamination to food, which was reflected in the reduced occurrence in food observed from 2013 to 2015. Further statistical analysis of this data was not appropriate as there would be no meaningful output; the samples analysed were not necessarily from the same sampling points, and each company did not submit the same number of samples or the same type at each time point. Such limitations, in addition to human factors, and 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 produces 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 (Uyttendaal 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., 2004). 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 (Fornebech Vogel et al., 2001). In this study 1.8% of 272 food samples from seafood processors were positive for L. monocytogenes. This was the industry sector with the lowest overall occurrence which may be due to the seafood sector’s already heightened awareness of the problems associated with L. monocytogenes, partly due to past outbreaks in the sector, in comparison to other sectors in this study. The occurrence is
considerably lower than that reported in the recent EU baseline survey, where the average EU occurrence was about 10% (EU, 2013).

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. Indeed, studies have shown varying degrees of contamination of dairy products, from 0.47% to 7% (EFSA, 2013; 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 (1990-1999) 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 (Laksanalam et al., 2011)
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 (Arunugaswamy 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 pulotypes. 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. This suggests that management and hygiene practices implemented at each individual facility may have an impact on the *L. monocytogenes* status and warrants the execution of further studies focused on the identification of risk factors and practices associated with a *Listeria*-free status. Indeed, large changes in occurrence could be associated with particular changes in management practices in some individual facilities. In one facility where there was a large increase in occurrence from 2013 to 2014, the increase coincided with the installation of new equipment. This emphasises the need for vigilance during installation of equipment and construction, activities that facilitate the transit of people unaccustomed to hygiene practices. In another facility, a decrease in occurrence coincided with the introduction of a peracetic acid rinse in the cleaning protocols, suggesting that management practices can influence occurrence.
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.

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 pulsortypes isolated along the food chain were classified as non-persistent indicates the diversity of strains that exists in the food processing environment. A high level of sporadic contamination in a facility may be symptomatic of a breakdown in hygiene barriers and should be addressed in order to prevent the contamination of food products. This was particularly the case for industries from the vegetable sector, where different pulsortypes were continuously identified throughout the 3-year period.

To address persistent contamination requires a different approach than that required to address sporadic contamination. The identification of persistent strains is 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. sanitizer resistance,
ability to use different carbon sources, ability to form biofilms, etc. (Carpentier and Cerf,
2011). 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 colonization abilities in comparison to strains from other pulsotypes. Whole
gene 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 (McCullum et al.,

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Evidence of cross-contamination was seen in 12 of the 54 facilities in this study where indistinguishable pulsortypes 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 56 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; Osi 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, 1/2b-3b and 4b-4d-4e belonged to serotypes 1/2a, 1/2b, 1/2c and 4b-4e, respectively. Similar results were obtained by Murugesan et al., indicating validity in serogroup rather than serotype analysis as a single serotype is significantly overrepresented in each serogroup (Murugesan et al., 2015).

*Listeria 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 *Listeria 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 pulsortypes from the food processing
environment surveillance matched 11 of the pulotypes of clinical isolates. Seven of these 11
pulotypes were persistent, and eight of them were found in several facilities, among these
was pulotype, 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 pulotypes 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 pulotypes
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 pulotypes 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.

4. Conclusions

In conclusion, this 3-year study has highlighted the diversity of L. monocytogenes
strains that exist in the food sector in Ireland, 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

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and vigilance created by a 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.

Acknowledgements

The authors acknowledge the contribution of the food business operators who contributed samples for this study. The contribution of Prof. Martin Cormican (UCHG), Dr. Niall De Lappe (UCHG), Dr. Peter Germer-Smidt (CDC), Dr. Benjamin Felix (ANSES) and Dr. Edward Fox (CSIRO) with PFGE profile comparisons is appreciated. This work was supported by the Department of Agriculture, Food and the Marine under the Food Institutional Research Measure, project number 11F008.
References


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Chapter 6: Appendix
Table 1. Summary of results of the annual occurrence of *L. monocytogenes* in processing environments and food obtained from 54 food businesses in Ireland.

<table>
<thead>
<tr>
<th></th>
<th>2013</th>
<th>2014</th>
<th>2015</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of samples taken</td>
<td>1696</td>
<td>2102</td>
<td>2071</td>
<td>5869</td>
</tr>
<tr>
<td>% of positive samples</td>
<td>4.78%</td>
<td>3.71%</td>
<td>3.24%</td>
<td>3.85%</td>
</tr>
<tr>
<td>No of environmental samples taken</td>
<td>1345</td>
<td>1654</td>
<td>1668</td>
<td>4667</td>
</tr>
<tr>
<td>% of positive environmental samples</td>
<td>4.68%</td>
<td>3.45%</td>
<td>3.36%</td>
<td>3.77%</td>
</tr>
<tr>
<td>No of food samples taken</td>
<td>351</td>
<td>448</td>
<td>403</td>
<td>1202</td>
</tr>
<tr>
<td>% of positive food samples</td>
<td>5.13%</td>
<td>4.73%</td>
<td>2.73%</td>
<td>4.17%</td>
</tr>
</tbody>
</table>
Table 2: 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.

<table>
<thead>
<tr>
<th>Food Category</th>
<th>No. Process Environment Samples</th>
<th>% Positive</th>
<th>No. Food Samples</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy</td>
<td>1512</td>
<td>4.2</td>
<td>408</td>
<td>2.2</td>
</tr>
<tr>
<td>Meat</td>
<td>1322</td>
<td>3.5</td>
<td>349</td>
<td>7.5</td>
</tr>
<tr>
<td>Seafood</td>
<td>1549</td>
<td>1.6</td>
<td>272</td>
<td>1.8</td>
</tr>
<tr>
<td>Vegetables</td>
<td>474</td>
<td>5.5</td>
<td>173</td>
<td>5.8</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>466</strong></td>
<td><strong>3.8</strong></td>
<td><strong>1262</strong></td>
<td><strong>4.2</strong></td>
</tr>
</tbody>
</table>
Table 3. 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.

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>No. of isolates</th>
<th>Serotype</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2a-3a</td>
<td>112</td>
<td>1/2a</td>
<td>50</td>
</tr>
<tr>
<td>1.2b-3b-7</td>
<td>41</td>
<td>1/2b</td>
<td>15</td>
</tr>
<tr>
<td>1.2c-3c</td>
<td>31</td>
<td>1/2c</td>
<td>15</td>
</tr>
<tr>
<td>4b-4d-4e</td>
<td>70</td>
<td>4b/4e</td>
<td>28</td>
</tr>
<tr>
<td>Untypeable</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number of isolates tested</td>
<td>255</td>
<td>Total number of isolates tested</td>
<td>110</td>
</tr>
</tbody>
</table>

1 Serogroup testing by the method of Dowith et al. 2004

2 Serotype testing using antisera from Denka Seiken UK Ltd, Coventry, UK
Table S1. Complete results of the occurrence of L. monocytogenes in processing environments and food obtained from 54 food businesses in Ireland. Persistent subtypes are indicated in bold.

<table>
<thead>
<tr>
<th>Facility no</th>
<th>2013</th>
<th>2014</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overall</td>
<td>Dairy</td>
</tr>
<tr>
<td></td>
<td>Number positives (%)</td>
<td>Environment</td>
</tr>
<tr>
<td>D1</td>
<td>0.74%</td>
<td>0.6%</td>
</tr>
<tr>
<td>D2</td>
<td>2.26%</td>
<td>0.0%</td>
</tr>
<tr>
<td>D3</td>
<td>3.21%</td>
<td>4.37%</td>
</tr>
<tr>
<td>D4</td>
<td>4.45%</td>
<td>0.0%</td>
</tr>
<tr>
<td>D5</td>
<td>7.77%</td>
<td>0.0%</td>
</tr>
<tr>
<td>D6</td>
<td>0.00%</td>
<td>0.0%</td>
</tr>
<tr>
<td>D7</td>
<td>0.00%</td>
<td>0.0%</td>
</tr>
<tr>
<td>D8</td>
<td>5.36%</td>
<td>5.36%</td>
</tr>
<tr>
<td>D9</td>
<td>15.55%</td>
<td>17.13%</td>
</tr>
<tr>
<td>D10</td>
<td>0.78%</td>
<td>0.0%</td>
</tr>
<tr>
<td>D11</td>
<td>7.09%</td>
<td>3.96%</td>
</tr>
<tr>
<td>D12</td>
<td>0.32%</td>
<td>0.0%</td>
</tr>
<tr>
<td>D13</td>
<td>3.44%</td>
<td>0.0%</td>
</tr>
<tr>
<td>D14</td>
<td>2.37%</td>
<td>0.0%</td>
</tr>
<tr>
<td>D15</td>
<td>5.66%</td>
<td>3.37%</td>
</tr>
<tr>
<td>D16</td>
<td>7.30%</td>
<td>3.25%</td>
</tr>
<tr>
<td>M3</td>
<td>2.28%</td>
<td>0.6%</td>
</tr>
</tbody>
</table>

*Table continued...*
<table>
<thead>
<tr>
<th>Year</th>
<th>P1, P2</th>
<th>P1, P3</th>
<th>P1, P4</th>
<th>P1, P5</th>
<th>P1, P6</th>
<th>P1, P7</th>
</tr>
</thead>
<tbody>
<tr>
<td>2017</td>
<td>1.43%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td>2018</td>
<td>2.31%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td>2019</td>
<td>2.30%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td>2020</td>
<td>0.69%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
</tbody>
</table>

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**Figure 1**

*These pulotypes were persistent (isolated more than once at least 6 months apart in a single facility)*
Figure 2
<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Source</th>
<th>Facility</th>
<th>Date</th>
<th>Pulsortype</th>
</tr>
</thead>
<tbody>
<tr>
<td>976</td>
<td>Food</td>
<td>M16</td>
<td>Apr 2013</td>
<td>P01</td>
</tr>
<tr>
<td>1401</td>
<td>Food</td>
<td>M16</td>
<td>Apr 2014</td>
<td>P32</td>
</tr>
<tr>
<td>1385</td>
<td>FC</td>
<td>D16</td>
<td>Mar 2014</td>
<td>P99</td>
</tr>
<tr>
<td>694</td>
<td>NRC</td>
<td>D16</td>
<td>Mar 2013</td>
<td>P128</td>
</tr>
<tr>
<td>1816</td>
<td>FC</td>
<td>M16</td>
<td>Mar 2015</td>
<td>P20</td>
</tr>
<tr>
<td>987</td>
<td>FC</td>
<td>M18</td>
<td>Apr 2013</td>
<td>P02</td>
</tr>
<tr>
<td>1691</td>
<td>NFC</td>
<td>V1</td>
<td>Dec 2014</td>
<td>P10</td>
</tr>
<tr>
<td>2140</td>
<td>NFC</td>
<td>V1</td>
<td>Aug 2015</td>
<td>P14</td>
</tr>
<tr>
<td>1433</td>
<td>NFC</td>
<td>D9</td>
<td>May 2014</td>
<td>P16</td>
</tr>
<tr>
<td>1665</td>
<td>Raw material</td>
<td>D9</td>
<td>Nov 2014</td>
<td>P18</td>
</tr>
<tr>
<td>1900</td>
<td>NFC</td>
<td>V1</td>
<td>May 2015</td>
<td>P2</td>
</tr>
<tr>
<td>1291</td>
<td>NFC</td>
<td>V1</td>
<td>Dec 2013</td>
<td>P20</td>
</tr>
<tr>
<td>1395</td>
<td>NFC</td>
<td>V5</td>
<td>Apr 2014</td>
<td>P05</td>
</tr>
<tr>
<td>1805</td>
<td>NFC</td>
<td>V5</td>
<td>Mar 2015</td>
<td>P05</td>
</tr>
</tbody>
</table>

Figure 4

FC= Food contact area, NFC= Non-food contact area
Figure 5
Figure S1. Summary of sample number and % *L. monocytogenes* positive at different locations and in different food sectors throughout Ireland. For each county, the food sector is shown (D – dairy; M – meat; S – seafood; V – vegetable), followed by the number of processing facilities sampled and the number of samples, followed by the percentage positives at those facilities.
Figure 1. Distribution of the different *L. monocytogenes* pulsortypes obtained from different food sectors in processing environments and food obtained from 54 food businesses in Ireland.

Figure 2. Minimum spanning tree of *L. monocytogenes* pulsortypes obtained from processing environments and food from multiple food sectors from 54 food businesses in Ireland. This was created in Biosnumerics (version 7.5) using default settings except maximum distance between nodes in the same position of 12. Pulsortypes containing 10 or more strains are identified.

Figure 3. Summary of the *L. monocytogenes* pulsortypes detected in each county throughout Ireland. Unique pulsortypes are in black, persistent pulsortypes are in red, persistent pulsortypes found at multiple locations are in red and underlined, pulsortypes found in multiple sites are in blue and D – dairy, M – meat, S – seafood, V – vegetable. U- untypeable strain.

Figure 4. Examples of *L. monocytogenes* pulsortypes, persistent for at least 6 months within a single facility, isolated from processing environments and food obtained from 54 food businesses in Ireland.

Figure 5: Dendrogram showing pulsortypes of clinical isolates in common with strains isolated from food and food processing facilities.