Detection, subtyping and control of *Listeria monocytogenes* in food processing environments

A thesis submitted for the degree of

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by

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Abstract

Listeria monocytogenes is the causative agent of the disease 'listeriosis' which can have severe manifestations in susceptible populations. It is one of the leading causes of death occurring due to foodborne illnesses in Australia. Stringent regulations exist for monitoring and controlling the prevalence of this pathogen in the food industry. This in turn highlights the need for rapid and reliable techniques for detecting, subtyping and controlling this pathogen in processing environments.

In the current study the application of MALDI-TOF MS as a single tool for detecting and subtyping *L. monocytogenes* was investigated. Overall, MALDI-TOF MS was found to a rapid, simple and reliable approach. In the case of direct detections from solid culture media using MALDI-TOF MS, alterations in culture media and the time of incubation greatly affected species level identifications. A successful and rapid scheme of detection (30 h) was developed for detecting the pathogen directly from selective enriched broths containing spiked food samples using MALDI-TOF MS. As few as 1 colony-forming unit (cfu) of *L. monocytogenes* per mL of initial selective broth could be detected in spiked UHT milk and cantaloupe samples, while in the case of Camembert cheese and chicken pâté, initial inoculation levels of 10 cfu/mL were required. In order to investigate if MALDI-TOF MS could be used to source-track *L. monocytogenes* isolates obtained from dairy sources within Australia, two data analysis approaches SPECLUST and SIMCA were explored. The latter was found to be more suited for discriminating the isolates based on their source. Similar to species level detections, source-tracking was also dependent on culture conditions. Analysis of data obtained by culturing the isolates on HBA for 24 h (37 °C) showed maximum congruence with the gold standard pulsed-field gel electrophoresis technique.

Use of essential oils as a control strategy for the pathogen in processing environments was investigated in this research. Essential oils of yarrow (*Achillea millefolium*) were found to exhibit anti-listerial and biofilm inhibitory effects. The essential oil inhibited the metabolic activity and initial attachment of listerial cells to polystyrene, stainless steel and high density polyethylene surfaces but was less efficient towards preformed biofilms.

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Declaration

I hereby declare, that to the best of my knowledge, this thesis contains neither material which has been accepted for the award of any other degree or diploma, or any material previously published or written by another person, except where due reference is made in the text of the thesis.

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Publications arising from this thesis

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List of Abbreviations

AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
ALOA	Agar *Listeria* Ottaviani Agar
ATCC	American Type Culture collection
BHIA	Brain heart infusion agar
cfu	Colony forming units
CHCA	α-cyano-4-hydroxy cinnamic acid
CV	Crystal Violet
dH$_2$O	Distilled water
Da	Dalton
DMEM	Dulbeco's Modifed Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribosenucleic acid
EDTA	Ethylenediaminetetraacetic acid
ERIC	Enterobacterial repetitive intergenic consensus sequence
FSANZ	Food Standards Australia and New Zealand
FTIR	Fourier transform infrared
g	Gravitational acceleration
HACCP	Hazard analysis and critical control points
HBA	Horse blood agar
HDPE	High Density Polethylene
MALDI-TOF MS	Matrix assisted laser desorption/ionisation-time of flight mass spectrometry
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration

MLST	Multilocus sequence typing
MLVA	Multilocus variable number of tandem repeat analysis
OA	Oxford agar
OBL	One Broth *Listeria*
OD	Optical density
PA	Palcam agar
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
rpm	Revolutions per minute
RAPD	Random amplified polymorphic DNA
REP	Repititive extragenic pallindromic
SARAMIS	Spectral ARchiving and Microbial Identification System
SD	Standard Deviation
SS	Stainless steel
TSB	Tryptone soya broth
TTO	Tea tree oil
UHT	Ultra high temperarure
USFDA	United States food and drug administration
WGS	Whole genome sequencing
YEO	Yarrow essential oil

Chapter 1

Literature Review

1.1   General Introduction

Listeria monocytogenes is a Gram-positive, intracellular human pathogen responsible for the disease 'listeriosis', which is one of the most severe foodborne diseases in humans. Although the disease has an overall low incidence, it has a high mortality rate of 20-30 %. A diverse range of food products have been implicated with sporadic and epidemic incidents of listeriosis, linked to the consumption of ready-to-eat (RTE) foods, dairy products, seafood, pre-cooked or frozen meat, pork and fresh produce. The pathogen is a major threat to the food industry since it can survive and proliferate in harsh conditions such as high salinity, acidity and refrigeration temperatures. Further, it has the ability to attach to abiotic surfaces and form biofilms that can frequently contaminate food products and the production environment. Since the invasive form of this disease can have severe manifestations in pregnant women, neonates, the elderly and immunocompromised populations, many countries have a zero tolerance policy towards the presence of this bacterium in certain high risk foods (such as dairy and RTE foods) having a long shelf-life that can support its growth. Hence, there is a compelling need for rapid and reliable detection, subtyping and control measures to monitor and minimise the presence of *L. monocytogenes* in food and food processing environments. This chapter will review the prevalence of *L. monocytogenes* in food processing environments (particularly dairy environments) and the currently available methods for its detection and subtyping. A section of the review will also highlight the role biofilms play in persistence of this pathogen in production environments and the use of essential oils as suitable alternatives to currently used disinfectants.

1.2   Introduction to the genus *Listeria*

The genus *Listeria* consists of Gram-positive, facultatively anaerobic, non-spore forming bacteria (Wong and Freitag, 2004). The genus is comprised of seventeen species; *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri, L. grayi,* and the more recently characterised *L marthii, L. rocourtiae, L weihenstephanensis and L. fleischmannii, L. floridensis, L. aquatica, L. cornellensis, L. riparia, L. grandensis, L. booriae* and *L. newyorkensis* (Bell and Kyriakides 2005; Graves *et al.,* 2010; Leclercq *et al.,* 2010; Lang Halter *et al.,* 2013; Weller *et al.,* 2015). *Listeria* species are generally rod-shaped bacteria that are catalase positive, indole and oxidase negative. They can

hydrolyse esculin but not urea, and are generally motile in the temperature range 20-25 °C. They exhibit peritrichous flagella with a tumbling motility and can grow over a wide temperature range from 4-38 °C (Bell and Kyriakides, 2005). *L. monocytogenes* is primarily the pathogenic species that causes the life-threatening disease listeriosis in humans. However, very rarely *L. ivanovii* (predominantly a ruminant pathogen) and *L. seeligeri* have also been associated with disease in humans (Wong and Freitag, 2004; Lopez, 2008).

According to FoodNet USA, human listeriosis was responsible for 30% of foodborne deaths in USA from 1996 to 2005 (Barton *et al.,* 2011). Although this disease has an overall low incidence compared to other foodborne illnesses such as campylobacteriosis and salmonellosis, it is still considered as major threat due to its higher case fatality rate (Allerberger and Wagner, 2010). Serological techniques that detect interactions between somatic (O) and flagellar (H) antigens with the corresponding antisera are used to classify *L. monocytogenes* into 13 serotypes. Based on the virulence potential in mammalian hosts, it has been noted that *L. monocytogenes* serovars 4b, 1/2a, 1/2b, and 1/2c are responsible for over 98% of the isolations from clinical cases of human listeriosis (Viswanath *et al.,* 2013).

1.3   Listeriosis

Listeriosis was first described in 1926 as the cause of an epizootic outbreak in guinea pigs and rabbits. In 1929, the first case of human listeriosis was reported (Swaminathan and Garner-Smidt, 2007). Listeriosis occurs mainly in the elderly, pregnant women, neonates, immunocompromised populations (such as people suffering from AIDS or cancer or organ transplant recipients) and people working in direct contact with animals with listeriosis (e.g. cows having mastitis). Listeriosis has also been observed in a much milder non-invasive version in healthy individuals (Farber and Peterkin, 1991; Bell and Kyriakides, 2005; Lorber, 2007).

In animals, contamination of stored forage, silage or the farm environment is responsible for the spread of listeriosis (Lopez, 2008). However, infection in humans occurs mainly due to consumption of food contaminated by *L. monocytogenes* (Minea *et al.,* 2005). Transmission from mother to infant via the placenta or an infected birth canal

has also been reported (Lorber, 2007). The foodborne route of transmission of *Listeria* was first documented in 1981 (Lundén *et al.,* 2004). Since then listeriosis outbreaks have been found to be linked to the consumption of various contaminated foods such as fresh soft cheese, cooked and frozen poultry products, RTE foods, meat products and smoked fish (Almeida and Almeida, 2000; Alessandria *et al.,* 2010). Although relatively rare, transmission from animals to humans can occur as a skin infection after exposure to an infected animal (McLauchlin and Low, 1994; Wojciech *et al.,* 2004).

1.3.1   Manifestations of listeriosis

Listeriosis exists in either invasive or non-invasive forms. The invasive form is recognized as a more serious manifestation of the disease due to a longer incubation period, severity of symptoms and the associated high case-fatality rate. The invasive form can result in stillbirths, abortions and premature deliveries. In neonates, it can lead to septicaemia or meningitis and is often fatal, whereas in immunocompromised populations it can cause meningitis, encephalitis, meningoencephalitis and septicaemia (Bell and Kyriakides, 2005; Garrido *et al.,* 2008). In contrast, non-invasive listeriosis results in a milder gastroenteritis-type illness (Berrada *et al.,* 2006). These clinical conditions have non-specific symptoms (including fever, diarrhoea and vomiting) and therefore remain vastly underdiagnosed. In particular, febrile gastroenteritis may affect perfectly healthy individuals following ingestion of heavily contaminated food. Cutaneous listeriosis is another manifestation that can result in an eczematous skin infection caused by direct exposure of intact skin to *L. monocytogenes*. This form is typically observed in veterinarians exposed to a diseased animal often after a listerial abortion (McLauchlin and Low, 1994; Godshall *et al.,* 2013). Rare manifestations of listeriosis are sometimes observed in the form of endocarditis, hepatitis, myocarditis, arteritis, pneumonia, sinusitis, conjunctivitis, ophthalmitis and joint infections (Wong and Freitag, 2004; Lorber, 2007). Currently, combined administration of ampicillin and gentamicin is a standard therapy for listeriosis. Combinations of trimethoprim-sulfamethoxazole and vancomycin-erythromycin are also used to treat bacteraemia and listeriosis in pregnant women (Yan *et al.,* 2010).

1.3.2   Pathogenesis of listeriosis

Consumption of foods contaminated by *L. monocytogenes* is considered to be the major source of infection in humans. The infectious cycle of *L. monocytogenes* can be divided into multiple stages: bacterial colonisation of host cell, internalisation of the bacteria by phagocytic or non–phagocytic host cells, release of the bacterium from the phagosome, bacterial growth and actin polymerisation in the cytosol of the host cell and spread to the adjacent cell and escape from a double membrane vacuole. After adhering to the epithelial tissue of the gastrointestinal tract, the bacterium is internalised by the macrophages in a primary phagosomal vacuole. Here it secretes a cytolysin called listeriolysin O (LLO) which helps it escape the phagosomal vacuole. The bacterium then multiplies within the cytosol where it obtains nutrients from the host. This is followed by host cell actin polymerisation which creates a tail-like structure that allows the organism's movement across the cytoplasm to the adjacent cell to begin a new infection cycle, without being exposed to the environment outside the cell. In actin polymerisation, a cloud of host F–actin surrounds the microbes present in the cytosol and then forms the tail like structure around one end of the bacterium. When it escapes from one cell to infect the adjacent cell, it forms a pseudopodium-like structure using the plasma membrane of the originally infected cell. This structure is thus recognised by the adjacent cell without evoking an immune response. During this period, the bacterium is enclosed in a secondary double membrane vacuole which is lysed again in the new cell leading to a new infection cycle (Wong and Freitag, 2004; Bell and Kyriakides, 2005). This entire cycle of pathogenesis has been depicted in Figure 1.1.

1.3.3   Virulence genes involved in listeriosis

The entire infection cycle of *L. monocytogenes* is governed by multiple proteins such as internalin A and internalin B (encoded by *inlA* and *inlB* genes respectively), haemolysin (encoded by *hly* gene; its product in *L. monocytogenes* is known as listeriolysin O or LLO), phosphatidylinositol–specific phospholipase C (PI–PLC, encoded by *plcA* gene), phosphatidylcholine–specific phospholipase C (PC–PLC, encoded by *plcB* gene) and actin polymerization protein (ActA, encoded by *actA* gene) (Jaradat *et al.,* 2002). The internalins are the key proteins involved in listerial adherence and invasion of host cells. *L. monocytogenes* protein InlA is involved in invasion of human epithelial cells (Wong

and Freitag, 2004; Lorber, 2007). In contrast, InlB interacts with the hepatocyte growth factor Met in order to invade hepatocyte cells (Sabet *et al.,* 2008). ActA and p60 protein (encoded by *iap* gene) and fibronectin binding protein FBP (encoded by *fbp* gene) have also been associated with the adherence of *L. monocytogenes* to host cells. Upon entry in phagocytic or non–phagocytic host cells, *L. monocytogenes* cells first reside in primary vacuoles. LLO (58 kDa pore-forming thiol-activated toxin) is primarily involved in escaping these vacuoles. This protein is more active in acidic environments. PI– PLC, in synergy with lecithinase–PC–PLC, is also involved in the lysis of the primary vacuole. Mpl protein (a metalloprotease encoded by *mpl* gene) is required for the activation of the inactive proenzyme form of PlcB. Cell-to-cell spread requires surface protein ActA which facilitates the formation of the actin tails which propel the bacteria towards the cytoplasmic membrane. At the cytoplasmic membrane the bacteria are enveloped in structures resembling pseudopodia and these become engulfed by the neighbouring cells forming secondary vacuoles. This secondary vacuole is then lysed to begin a new cycle of infection by the synergistic action of *hly, plcA* and *plcB* gene products (Vázquez–Boland *et al.,* 2001b). Figure 1.1 outlines the genes involved at these different stages.

The 9.6 kb virulence cluster of *L. monocytogenes*, referred to as the *Listeria* pathogenicity island 1 (LIPI-1, Figure 1.2), consists of genes encoding PI–PLC, LLO, Mpl, ActA and PC–PLC and is positively regulated by PrfA (encoded by *prfA* gene). LIPI-1 is located between the *prs* and *ldh* housekeeping genes encoding phosphoribosyl–pyrophosphate synthetase and lactate dehydrogenase proteins, respectively (Jung *et al.,* 2009). LIPI-1 has been found to be expressed optimally at 37 °C. Figure 1.2 highlights that the *prfA- plcA* operon and the lecithinase operon in this cluster are in the reverse orientation. This LIPI-1 cluster is also present in *L. ivanovii* and *L. seeligeri*, although it is non–functional in the latter due to insertions between *plcA* and *prfA*. In *L. ivanovii*, although the cluster is similar to the one in *L. monocytogenes*, the individual genes show a substantial amount of divergence (Vázquez-Boland *et al.,* 2001a).
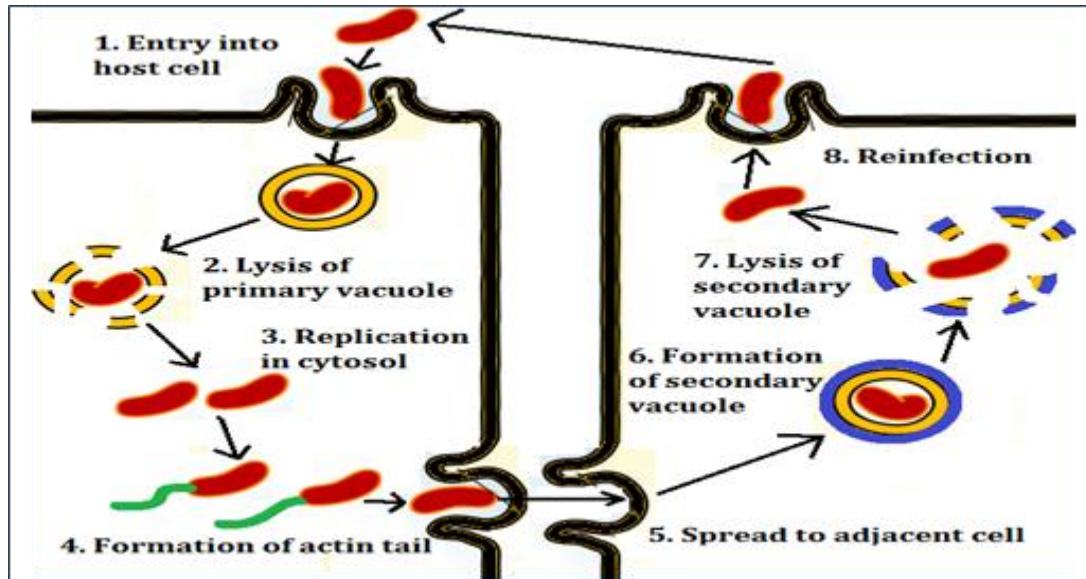
Figure 1.1 Pathogenesis and virulence genes involved in listeriosis infection in human cells. Genes involved at different stages; Stage 1 – *inlA, inlB, actA, iap, fbp*; Stage 2 – *hly, plcA, plcB, mpl*; Stage 4 and 5 – *actA*; Stage 7 – *plcA, plcB, hly*
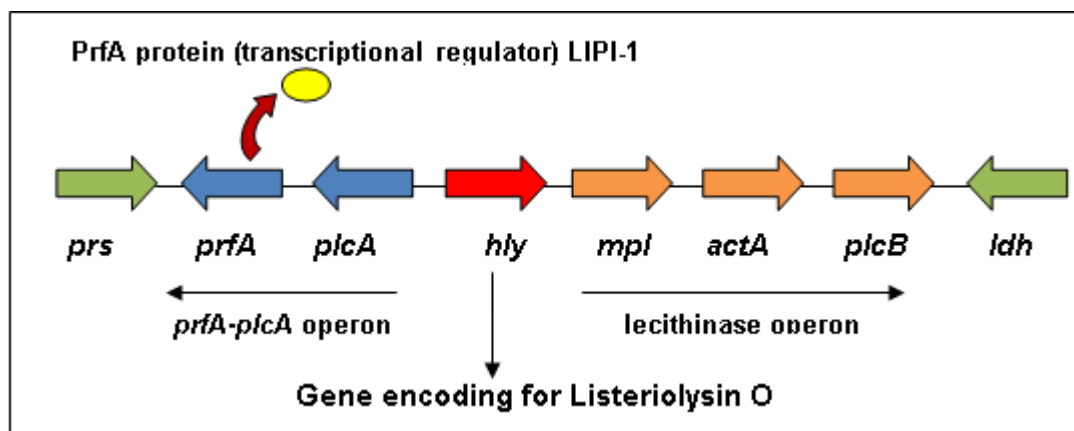
Figure 1.2 Organisation of LIPI-1 (*Listeria* pathogenicity island), as adapted from Vázquez-Boland *et al.* (2001a) and Jung *et al.* (2009)

1.4   Foodborne outbreaks of listeriosis

The first documented foodborne listeriosis outbreak in 1981 was linked to contaminated coleslaw (Linnan *et al.,* 1988). Subsequent to this, a common source of pasteurized milk was found to be associated with an outbreak in Massachusetts in 1983 (Wesley and Ashton, 1991). In 1985, the outbreak in California resulting from consumption of

inadequately pasteurized cheese made listeriosis a concern for the food industry (Linnan *et al.,* 1988). Since then, listeriosis outbreaks have been linked to the consumption of various contaminated foods that are generally consumed without heating (Gravesen *et al.,* 2000). Epidemiological surveillance studies have clearly indicated that listeriosis is an important issue affecting many countries such as USA, Canada, Japan, New Zealand, Germany, England, France and other European countries (Warriner and Namvar*,* 2009). Table 1.1 provides the overall incidence of listeriosis per 100,000 people in different countires and Table 1.2 gives an overview of the outbreaks during 2005-2013 and the associated serotypes.

Table 1.1 Incidences of listeriosis in different countries/regions

Countries/regions	Period	Incidence of listeriosis per 100,000 people	Reference
Canada	1990-1998	0.18-0.34	Todd and Notermans, (2011)
England-Wales	1990-2000; 2001-2009	0.21; 0.36	Mook *et al.,* (2011)
European Union	2000-2005	0.10-0.30	Denny and McLauchlin, (2008)
Belgium	2000-2005	0.43-0.86	Goulet *et al.,* (2008)
Finland	2000-2005	0.35-0.79	Goulet *et al.,* (2008)
Sweden	2000-2005	0.44-0.75	Goulet *et al.,* (2008)
Germany	2001; 2005	0.26; 0.62	Koch and Stark, (2006)
Switzerland	2001-2005	0.38-0.98	Goulet *et al.,* (2008)
Netherlands	2002-2005	0.20-0.56	Doorduyn *et al.,* (2006); Goulet *et al.,* (2008)
USA	2004-2009	0.25-0.32	Silk *et al.,* (2012)
Denmark	2005-2008	0.52-0.85	Goulet *et al.,* (2008)
Australia	2005-2009	0.30-0.40	OzFoodNet, (2012)
New Zealand	2009-2010	0.50-0.60	Lim *et al.,* (2012)

Table 1.2 Foodborne outbreaks of listeriosis (2005-2013)

Year	Location	Infected Cases	Food vehicle	Serotype	References
2005	Switzerland	10	Tomme cheese	1/2a	Garrido *et al.,* (2010)
2006-2007	Germany	189	Cheese (pasteurised milk)	4b	Koch *et al.,* (2010)
2006-2007	Belgium	11	NA[*]	4b	Valderrama and Cutter, (2013)
2006	Czech Republic	75	Cheese and salad	1/2b	Garrido *et al.,* (2010)
2006-2007	Germany	16	Scalded sausages	4b	Garrido *et al.,* (2010)
2007	Norway	21	Cheese	NA	Todd and Notermans, (2011)
2007	USA	5	Pasteurised milk	NA	Garrido *et al.,* (2010)
2008	Canada	57	Ready-to-eat meats	1/2a	Garrido *et al.*, (2010)
2008	Chile	119	Brie and Camembert cheese	NA	Garrido *et al.*, (2010)
2008	Austria	19	Jelly pork	4b	Garrido *et al.*, (2010)
2008	USA	20	Sprouts	1/2a	Cartwright *et al.,* (2013)
2009	Austria and Germany	14	Curd cheese	1/2a	Fretz *et al.,* (2010)
2009	Denmark	8	Beef	NA	Garrido *et al.,* (2010)
2009-2010	Germany, Austria and Czech republic	34	Curd cheese	1/2a	Garrido *et al.,* (2010)
2010	USA	15	Celery	NA	Valderrama and Cutter , (2013)
2011	USA	147	Cantaloupe	1/2a, 1/2b	Lomanaco *et al.,* 2011
2012	USA	22	Soft ricotta cheese	NA	CDC, (2012)
2012	Spain	10	Latin style fresh cheese	1/2a	de Castro *et al.,* (2012)
2013	USA	6	Cheese	NA	CDC, (2013)

NA- not available

1.5 Detection of Listeria monocytogenes

Most countries have a zero tolerance policy towards the presence of *L. monocytogenes* in certain high risk foods (Berrada *et al.,* 2006). For some other RTE foods, levels

exceeding 100 cfu/g during the shelf-life of the product are considered as a health risk (EFSA 2012). Regulatory bodies such as Food and Drug Administration (USFDA), US Department of Agriculture (USDA) and Food Standards Australia and New Zealand (FSANZ) do not distinguish between *Listeria* spp. as even the presence of a non-pathogenic *Listeria* spp. suggests possible involvement of pathogenic species (Gasanov *et al.,* 2005). In Australia, the presence of *L. monocytogenes* in certain high risk foods that have been previously implicated in listeriosis can lead to a food recall (Popovic *et al.,* 2014). This can lead to considerable economic loss to the industry. Since *L. monocytogenes* is closely-related to the other *Listeria* species, and does not produce specific symptoms, its diagnosis is difficult. In addition, linking specific foods with outbreaks is particularly challenging since the incubation period can often vary between 3-70 days (Dawson *et al.,* 2006). Some of the techniques currently employed are discussed in the following section.

1.5.1   Culture-based techniques

Food laboratories currently rely on conventional microbiological methods for detection of *Listeria* spp. Some of the standard methods of isolation from food and environmental samples (which include swabs collected from food production plants and work surfaces) used by regulatory agencies have been previously reviewed in Gasanov *et al.* (2005). ISO 11290 standards and FDA–BAM methods are used for isolation of *Listeria* spp. from dairy food items, seafood and vegetables, and USDA Standard method is used for its isolation from meat and poultry food items and environmental swabs (Kim *et al.,* 2005; Jeyaletchumi *et al.,* 2010).

Culture-based methods generally involve a series of primary (24 h) and secondary (48 h) enrichments of food samples in selective media such as *Listeria* enrichment broth (LEB), University of Vermont broths (UVM1 and UVM2), Fraser broth and Half Fraser broth. These media contain selective agents including cycloheximide, colistin, cefotetan, fosfomycin, lithium chloride, nalidixic acid, acriflavine, phenylethanol, ceftazidime, polymyxin B and moxalactam (Bell and Kyriakides, 2005). As *Listeria* can be found in very low numbers in foods, enrichment is required to inhibit any other bacteria present in the sample. Following primary and secondary enrichment, the broth is generally plated onto selective/differential media which include Oxford and

PALCAM agar or chromogenic media such as Agar *Listeria* Ottaviani and Agosti (ALOA) and RAPID'L.mono (Kabuki *et al.,* 2004). The detection of *Listeria* spp. using the chromogenic medium ALOA is typically dependent on the β-glucosidase activity of *Listeria,* which cleaves the chromogenic substrate producing blue/green colonies. Lecithin present in the agar is hydrolysed by phospholipase enzyme synthesized only by *L. monocytogenes* and *L. ivanovii*, leading to formation of opaque halos around their colonies (Jeyaletchumi *et al.,* 2010). Occasionally, the enriched sample is inoculated on a non-selective agar and presumptive listerial colonies are confirmed based on biochemical properties such as Gram stain, catalase test, motility test, ability to produce haemolysis on blood agar plates, Christie, Atkins and Much-Peterson (CAMP) test with *Rhodococcus equi* and *Staphylococcus aureus* and carbohydrate utilization tests (Bell and Kyriakides, 2005).

This entire conventional approach of *Listeria* spp. detection generally takes 5-10 days and is also laborious as it requires randomly testing a number of colonies from a single sample, and often results in false presumptives (Frece *et al.,* 2010). In addition, *L. monocytogenes* is a low cell number bacterium and the non-pathogenic *L. innocua* has occasionally been found to outgrow *L. monocytogenes* in food enrichment broths (Aznar and Alarcon, 2003). Other disadvantages associated with conventional methods include extensive reliance on phenotype which is subject to changes under different environmental conditions, requirement for different chemicals, media and reagents, interference due to contaminating bacteria which can mask the presence of the target organism and atypical reactions given by atypical strains.

1.5.2   Enzyme-linked immunosorbent assays (ELISA)-based techniques

The specificity of antibody-antigen reactions makes immunobased techniques such as ELISA suitable for detection of pathogens. In ELISA, anti-*Listeria* antibodies are immobilised to a microtitre well for capturing *Listeria* antigens, along with secondary antibodies linked to an enzyme. These tests are used in food testing as they are simple and easy to interpret, and require minimal treatment of the sample. They produce results in approximately 30-50 h which is significantly faster compared to culture-based techniques (Bell and Kyriakides, 2005); however, they are not as sensitive as molecular methods. Sensitivity for ELISA ranges between 10^5 and 10^6 cfu/mL of enriched sample

(all approved tests require enrichment of food samples) (Brehm-Stecher and Johnson, 2007) hence requiring a prior enrichment. Secondly, cross reactivity can occur with other closely-related species (e.g. other *Listeria* spp.) and differential antigenic expression under different cell environments leads to variability in reactions (Brehm-Stecher and Johnson, 2007). Thirdly, antibody preparation is much more time consuming and expensive and less specific in comparison to the preparation of nucleic acid primers and probes.

In most cases, *L. monocytogenes* screening is not performed directly in enrichment media, as virulence proteins, which are the general targets for immunological methods, are produced in much lower quantities in the extracellular environment. Immunoassays can also be carried out in multiplex formats, further reducing the analysis time, reagent and sample costs involved. One such assay for simultaneous detection of *Escherichia coli* O157:H7, *Yersinia enterocolitica*, *Salmonella enterica* and *L. monocytogenes* was developed based on the sandwich ELISA method by Magliulo *et al.* (2007). In this assay, highly specific monoclonal antibodies targeting each bacterium were immobilised in a 96-well microtitre plate. Once the target bacterium from the sample was bound to the monoclonal antibody, a horseradish labelled polyclonal antibody was added and the enzyme activity was measured by measuring the chemiluminescence of a luminol-based substrate. This assay had a limit of quantification of 10^4-10^5 cfu/mL for all bacteria which clearly indicates the requirement for an initial enrichment. Currently, the most widely used commercial test, VIDAS® LMO2 assay (BioMérieux), is based upon an enzyme-linked fluorescent assay for *L. monocytogenes* for testing food samples. The technique is observed to be reliable and requires about 70 min to obtain a result post the initial enrichment period. However, the presumptive *L. monocytogenes* detected by using these techniques must be confirmed by other culture-based or molecular techniques (Kim *et al.,* 2005).

1.5.3   Molecular methods of detection

Conventional culture techniques and immuno-based methods have the disadvantage of being time consuming with the possibility of detecting false positives (Zhang *et al.,* 2009). Frece *et al.* (2010) suggested that more reliance on genetic methods can improve

the reliability of *Listeria* detection. An overview of some of these methods is discussed here.

1.5.3.1 Conventional PCR

The polymerase chain reaction (PCR) method has been used extensively for the detection of *L. monocytogenes*. Various genes such as *hly* (the most common target)*, inlA, inlB, iap*, intergenic spacer regions, 16S and 23S rRNA genes, or the genes encoding invasion-associated protein p60, aminopeptidase C, phospholipase C protein, a fibronectin-binding protein and *dth-18* delayed-type hypersensitivity protein have been targeted for PCR (Aznar and Alarcon, 2002, 2003; Brehm-Stecher and Johnson, 2007). PCR methods can be useful only if the cell count in the given samples is in the scope of detection by the PCR assay, and the technique cannot distinguish between DNA from dead or live cells (Lei *et al.,* 2008). Additionally, the detection of *L. monocytogenes* in food samples is difficult, since they generally contain inhibitory substances. Many studies, thus, use internal amplification control (IAC) DNA fragment to verify the absence of PCR inhibitors (Rodriguez-Lazaro *et al.,* 2005).

Some PCR assays that are in compliance with standard isolation methods have been developed. In one such study, a three day PCR-based assay was developed by Kaclíková *et al.,* (2003) which gave detection limits of 10^0 cfu per 25 g of food, equivalent to the standard EN ISO 11290-1 or ISO 10560 methods of *Listeria* detection. By incorporating a simple boiling lysis method, the *inlB* gene was detected along with an IAC in artificially and naturally-contaminated foods. Aznar and Alarcon, (2003) performed a PCR-based assay to detect *L. monocytogenes* from 217 natural samples. While culture-based methods detected 17 positives the PCR assay detected 56 positives. Culture-based methods however, do not account for the presence of dead cells, viable but non-culturable cells (VBNC) or metabolically injured and stressed cells. This may explain the difference in positives between the PCR-based and traditional techniques. Hence, DNA amplification-based methods can lead to an overestimation of the health risk associated with a food sample.

For a low-count organism such as *L. monocytogenes*, an estimate of the concentration of the cells in the given sample is essential along with distinction between dead and live

cells. To address this issue, mRNA can be targeted instead of DNA using reverse transcriptase PCR (RT-PCR), as it has a shorter half-life compared to DNA and can thus be a useful target molecule to detect the presence of viable cells. However, it is crucial to recognise that mRNA molecules are sometimes only expressed under certain environmental conditions such as particular temperature ranges. Also, the extraction of mRNA without interference due to DNA can be a tedious procedure (Brehm-Stecher and Johnson, 2007). Alternatively, the use of propidium monoazide and ethidium monoazide dyes that are not taken up by viable cells but can stain dead cells with a damaged cell membrane should be considered. Inside the cell, after exposure to light, the dyes bind to double-stranded DNA or RNA and form a permanent covalent linkage with the nucleic acids which inhibits their amplification in PCR (Pan and Breidt, 2007).

1.5.3.2 Multiplex PCR

Multiplex PCR involving simultaneous detection of different foodborne pathogens has evolved as an area of great potential, as it is more cost-effective and time saving than conventional PCR. Zhang *et al.* (2009) developed a highly sensitive multiplex PCR assay which could detect as low as ten copies of the genome by targeting *hly* gene of *L. monocytogenes, nuc* gene of *S. aureus, invA* gene of *Salmonella enterica,* and *stx* and *eae* genes of *E. coli* O157:H7. Eight fresh food samples of meat, beef, pork, fish, shrimp, cheese, canola leaf and cabbage were washed with contaminated water containing 10^{-1} to 10^4 cfu/mL of each pathogen, and it was observed that the individual genes in each pathogen gave the desired amplified product at 1 cfu/mL (and above). The only drawback of using multiplex PCR is that this technique cannot be used when the target amplicons are similar in size if the results have to be viewed by agarose gel electrophoresis. The method also requires a considerable amount of optimisation to adjust the primer pair concentrations and the concentrations of Mg^{+2}, dNTPs and polymerase as well as the annealing temperatures of every primer to obtain amplification of each target sequence. Once optimised, however, it is a sensitive and selective technique (Mustapha and Li, 2006).

1.5.4 Real-Time or Quantitative PCR

In real-time PCR, the PCR amplicon is observed as it accumulates, in contrast to conventional PCR where the resulting product is observed at the end of the reaction. There are different types of real-time PCR systems available which include the TaqMan real-time PCR, SYBR Green, molecular beacons and scorpions. The progress of the PCR is measured by monitoring the change in fluorescence levels, which in turn depend on amount of accumulated PCR product. The fluorescent molecule is either a target specific probe labelled with a fluorescent dye along with a quencher or it can be a non-specific DNA binding dye such as SYBR Green I. Rodriguez-Lazaro *et al.,* (2005) developed a quantitative PCR (qPCR) assay for detection of *L. monocytogenes* in artificially inoculated cooked ham, raw pork meat, raw salmon and smoked salmon by using *hly* gene as the target along with an IAC. The assay gave positive results only for the ham samples, while in the other samples it failed to give positive amplification due to the presence of PCR inhibitors. Thus, it is also important to test the method on naturally-contaminated food samples which differ in their composition.

In a study performed by Bhagwat (2003), a modified Association of Official Analytical Chemists (AOAC)-approved PCR method was used for the real-time detection of three pathogens (*E. coli* O157: H7, *Salmonella* species and *L. monocytogenes*) simultaneously from freshly cut vegetables. In comparison to other pathogens, the assay was less sensitive for *L. monocytogenes,* probably due to certain inhibitory compounds in the food samples. Thus, it is important to verify that the proposed multiplex real-time PCR assay detects all the pathogens efficiently and within the standard limits of detection for approved methods of isolation. Real-time PCR has certain advantages which include increased sensitivity, ability to detect trace amounts of target DNA, possible automation and ability to quantify bacterial load without any post-PCR handling (Berrada *et al.,* 2006). However, it has some disadvantages as well. Using species-specific probes makes the assay more expensive, SYBR green can bind to non-target DNA or even primer-dimers and show fluorescence, primer concentration and primer design can influence the fluorescence ability of the probe used (Fairchild *et al.,* 2006).

1.5.5   Biosensor-based techniques

A biosensor contains a biomolecule which interacts with the target organism and gives a signal which is then used to analyse the presence of the organism in the given samples. Many studies have focussed on developing sensitive biosensors for *L. monocytogenes*. The biosensor BIA3000 was used to detect *L. monocytogenes* cells in solution (Leonard, 2004). This technique involved incubating the cells for 30 min with anti-*L. monocytogenes* polyclonal rabbit antibodies followed by removal of dead cells and bound antibodies. The solution containing free antibodies was passed over the biosensor chip which contained immobilised polyclonal goat anti-rabbit antibodies. Hence, when more cells were present in solution, less free antibodies were detected by the biosensor.

Surface Plasmon Resonance (SPR) is an another biosensor-based optical technique that produces detection signals with changes in the refractive index that occur in the proximity of the sensor surface due to binding between an analyte and a ligand. SPR biosensors contain a gold electrode bound to anti-bacterial antibodies and have been found to be suitable for pathogen detection in water and environmental samples (Poltronieri *et al.,* 2009) but whether they would work for detection of *L. monocytogenes* directly from food samples needs to be investigated. A mammalian cell-based biosensor (detection level of 10^2 - 10^4 cfu/g) that had the ability to differentiate between pathogen and non-pathogen on basis of cytotoxicity was successfully tested for detection of *L. monocytogenes* from artificially-inoculated food samples by Banerjee and Bhunia, (2010).

1.5.6 Loop-mediated isothermal amplification (LAMP)

LAMP is a method of DNA amplification which, unlike PCR, involves an auto-cyclic isothermal amplification of target DNA by *Bst* DNA polymerase (large fragment of *Bacillus stearothermophilus* DNA polymerase) and can be completed within an hour in a regular water bath or heating block. The *Bst* polymerase lacks the exonuclease activity of *Taq* polymerase and instead has a strand displacement activity. The primer pairs used are specific for six-eight regions on the target sequence and form stem-loop structures during amplification (Prasad and Vidyarthi, 2009). Tang *et al.* (2011) developed a highly specific and sensitive LAMP assay targeting the *hly* gene. The products were

observed by visualising a colour change by utilising calcein and manganous ion; thus saving time compared to gel electrophoresis. The LAMP assay was sensitive enough to detect 2 cfu *L. monocytogenes* per reaction and was 100-fold more sensitive compared to conventional PCR. However, this study only used poultry samples, it is important to evaluate this assay over a wide variety of food samples (Wang *et al.,* 2008).

1.5.7 Spectroscopy-based techniques

Fourier transform infrared (FTIR) spectroscopy, Raman spectroscopy and matrix-assisted laser desorption/ionization time-of-flight coupled with mass spectroscopy (MALDI-TOF MS) have been used recently to detect foodborne pathogens. These methods are advantageous because they are rapid, reliable, require limited sample preparation and have low running costs. They give a unique spectrum or fingerprint of a bacterium that helps identify it at the genus, species or sometimes at the strains level, generally by targeting the bacterium's lipids, proteins, carbohydrates or nucleic acid compositions without the reliance on dyes or labels (Al-Qadiri *et al.,* 2008).

1.5.7.1 FTIR and Raman spectroscopy

FTIR spectroscopy of microbes involves generating fingerprint spectra that provide information about the biochemical composition of microbes. FTIR has been used previously for *Listeria* detection at the species level by Holt *et al.* (1995), Rebuffo *et al.* (2006), Al-Qadiri *et al.* (2008), Janbu *et al.* (2008), and Davis and Mauer, (2011). A major advantage of this technique is that it does not involve destruction of the sample; hence minimal sample preparation is required (Wenning and Sherer, 2013). However, availability of limited database that often contains few reference spectra is a major weakness in application of this technique for detection of microbes. Since the FTIR spectra can change with alterations in culture conditions, standardisation of conditions is required (Davis and Mauer, 2010).

In recent years, there has been much interest in surface-enhanced Raman spectroscopy (SERS), which is a specific type of Raman spectroscopy that results in enhanced Raman signals obtained from samples deposited on SERS-active nanosubstates such as gold or silver (Fan *et al.,* 2011). Grow *et al.* (2003) used a biochip containing immobilised

capture ligand biomolecules on an SERS-active surface. The binding of the biological analyte to the ligand on the chip was followed by analysis using Raman spectroscopy and it was observed that *L. monocytogenes* incubated at different temperatures produced different Raman spectral profiles. In another study, Liu *et al.* (2009) concluded that a colloidal silver SERS technique could be used to differentiate between *E. coli, Listeria* and *Salmonella* species. The only drawback of these techniques is the need for pure cultures during analysis, thus limiting the detection of pathogens directly from complex food matrices.

1.5.7.2 Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS was developed in the late 1980's and since then it has been applied in various areas of research such as polymer analysis, protein and proteome analysis, DNA sequencing, recombinant protein studies, analysis of body fluid and tissues, and investigation of glycans and glycoproteins (Bucknall *et al.,* 2002; Hejernø and Jensen, 2014). However, only in the last decade has this technology been explored as a diagnostic tool for microorganisms (Wieser *et al.,* 2012). This technique mainly uses the soft ionisation method to detect large unfragmented molecules from a sample that is overlayed with a matrix which absorbs energy from a nitrogen laser and causes the simultaneous desorption and soft ionisation of intact molecules (Figure 1.3). Generally, for bacterial detection using MALDI-TOF MS, the term 'whole cell' mass spectrometry is used, since the entire cell is analysed with minimal or no pre-extraction. A single bacterial colony from solid culture media or a small amount of culture from a broth sample is spotted on a target plate and overlaid with a suitable matrix solution. The solvents used in the matrix such as water, ethanol, acetonitrile and trifluroacetic acid, invade the cells wall and make the inner proteins available for analysis. The energy from the laser is used to ionise the proteins (mainly ribosomal proteins). These ions travel through the time-of-flight under vacuum until they reach the detector to generate fingerprint spectra (generally in the range 2 to 20 kDa) (Welker and Moore, 2011; Croxatto *et al.,* 2012). These spectra are then compared to commercially available reference databases such as MALDI Biotyper, SPECLUST or SARAMIS, which include reference spectra for identification of numerous microorganisms.

Since the advent of this technology, it has been used to detect a range of bacteria including *Staphylococcus, Streptococcus, Neisseria, Salmonella, Aeromonas, Campylobacter* and *Helicobacter* (Murray, 2010). Recently, this technique has been used to detect bacteria from urine (Ferreira *et al.,* 2010) and positive blood cultures (Drancourt, 2010) and to detect *Salmonella* species directly from selective enrichment broth samples containing clinical stool samples (Sparbier *et al.,* 2012). This encourages more investigation in the application of this technique to detect pathogens from other complex matrices, such as food (Welker and Moore, 2011). Another crucial application of MALDI-TOF MS in microbiology is the detection of antibiotic resistance in bacteria, where it has been applied for detecting resistance in methicillin-resistant *S. aureus* (MRSA)*,* vancomycin-resistant *Enterococcus* (VRE), beta-lactamase producing *Bacteroides fragilis* and carbapenemase-producing *Enterobacteriaceae* (CPE) (Kostrzewa, 2014).

In case of *Listeria,* Barbuddhe *et al.* (2008) were the first to use whole cell MALDI-TOF MS to identify *Listeria* isolates to the species level. The *L. monocytogenes* isolates in this study were separated at the lineage level by observing the presence or absence of particular peaks and this lineage distribution was also confirmed using the standard technique of pulsed-field gel electrophoresis (PFGE). Other studies that have used MALDI-TOF MS to detect *Listeria* isolates include Böhme *et al.* (2011), Guo *et al.* (2014) and Hsueh *et al.* (2014).

The main advantages of using MALDI-TOF MS in routine diagnostics is that is it a simple, rapid and reliable technique that can be automated and incorporated in the workflow of a high throughput diagnostic laboratory. Although the instrument costs are high, the running costs are comparatively lower than some of the currently available detections kits. The majority of the research has focussed on using MALDI-TOF MS in combination with culture-based methods and it is difficult to infer any quantitative information from the data (Lavigne *et al.,* 2012). In addition, the type of growth medium, the age of the culture, sample preparation, the type of matrix used and the matrix to solvent ratio used are known to greatly affect the mass spectra obtained, which emphasizes the need for standardisation of this technology (Murray, 2010). Additionally, the detection of bacteria using MALDI-TOF MS is highly dependent on

up-to-date commercial databases. While the databases have extensive reference spectra for clinical isolates, the representation of food or environmental isolates is comparatively lower. As more and more reference spectra get added to these databases, better identification results will be obtained for foodborne pathogens.

Fingerprint mass spectra

Detector

Time-of-flight tube

Ions with lower m/z ratio travel faster to detector

Protonated analyte ions

Ions with higher m/z ratio travel slower to detector

Nitrogen laser beam

Protonated matrix ions

Sample well containing the analyte-matrix mixture
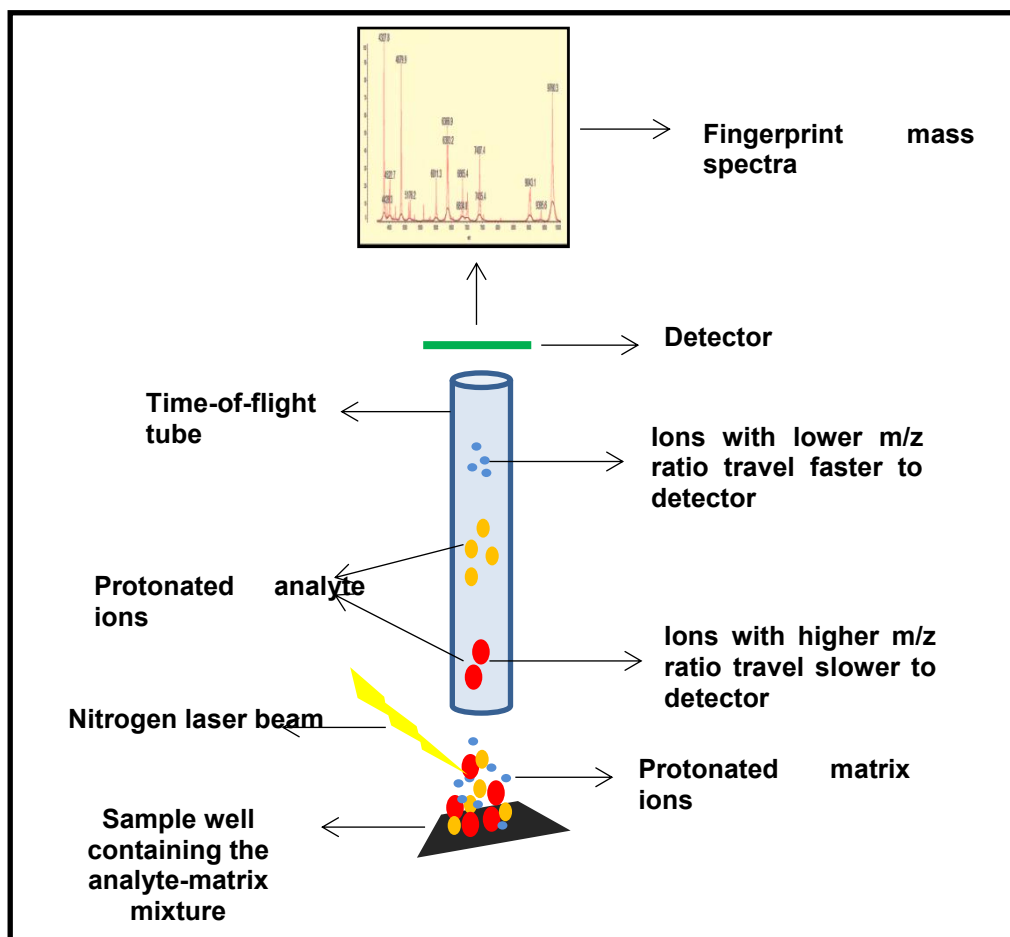
Figure 1.3 Schematic representation of MALDI-TOF MS mechanism. The analyte-matrix mixture is bombarded with a nitrogen laser beam (337 nm) which ionises the analyte (red and orange circles) and the matrix ions (blue circles). These ions travel to the detector based on their relative mass to charge (m/z) ratio.

In general, an ideal detection method should be specific, sensitive, fast, simple, reproducible, cost-effective, be able to detect the bacteria from complex food environments, have the ability to distinguish between dead and live cells, and most importantly, be validated (Brehm-Stecher and Johnson, 2007). After reviewing the

above literature it is evident that although a range of techniques are available for detecting *L. monocytogenes*, no single technique satisfies all criteria. Thus, depending on the type of sample and the final requirement, a single technique or a combination of techniques may be adopted.

1. 6   Prevalence of *Listeria monocytogenes* in dairy processing environments

L. monocytogenes has been isolated previously from poultry, meat, fish and dairy processing plants (Vogel *et al.,* 2001). Many dairy products such as cheese (soft cheese in particular), pasteurised milk (contaminated post-pasteurisation) and creams have been associated with some of the major listeriosis outbreaks (Kabuki *et al.,* 2004; Borucki *et al.,* 2005). With respect to the dairy industry, farm environments (contamination of raw milk in bulk tanks or milk filters, silage, drinking water, or mammary contamination) can be major sources of *Listeria* in the processing environment (Borucki *et al.,* 2005; Latorre *et al.,* 2009; Fox *et al.,* 2011a). Healthy and infected ruminants routinely shed the organism in their faeces, which can result in contamination of the raw milk (Borucki *et al.* 2005). Hence products made from unpasteurised milk are often at high risk. In contrast, products made from pasteurised milk are mainly contaminated during post-pasteurisation processing (Cagri-Mehmetoglu *et al.,* 2011). Soft, semi-soft, or brined cheeses that can be consumed fresh or after ripening are more prone to contamination compared to hard cheeses. Generally, the addition of starter cultures to milk during cheese production initially lowers the pH of the milk, which helps in controlling contaminating bacteria such as *Listeria*. However, in soft ripened cheeses, secondary cultures are inoculated on the exterior surfaces of the cheese to develop the special organoleptic characteristics, such as texture, smell or flavour and these cultures increase the surface pH making it more alkaline. During this period, *Listeria* is most likely to contaminate the cheese (Bell and Kyriakides, 2005).

Other than incoming raw material, the highly likely sources of contamination in processing environments are product-contact surfaces such as plastic tube connectors, ripening room racks, sinks, crates, tables, plastic milk packages, floor drains, and water on floor (Viswanath *et al.,* 2013). Some sampling points considered important for monitoring the presence of *L. monocytogenes* are cutting boards, utensils, raw material, drains, floors, conveyor belts, slicers, skinners, work tables, tanks, vats, centrifuges,

strainers, sieves, knives, walls, personnel hands, gloves, aprons and boots (Dass *et al.,* 2011; Fallah *et al.,* 2013). In summary, it is essential to use clean raw materials, appropriate materials (which can be easily sanitised) for surfaces and have suitable workflows, effective cleaning regimes and hygienic environments (Kabuki *et al.* 2004; Fox *et al.* 2011b).

One of the major reasons for *L. monocytogenes* posing a serious threat to the food industry is its ability to adhere to various abiotic surfaces in the processing environments, forming biofilms that can continuously contaminate the food products. Contamination by *L. monocytogenes* can either be due to sporadic strains or persistent strains. The sporadic strains may frequently be detected but are not able to establish their niche, while the persistent strains can exist in a particular processing stage for a long period of time, sometimes over a decade (Swaminathan and Gerner-Smidt, 2007; Takahashi *et al.*, 2010). This persistent type of contamination can be attributed to the ability of microbes to tolerate extreme conditions such as vacuum, freezing temperatures, high salinity, desiccation and extreme acidic and alkaline environments (Vogel *et al.,* 2010). *L. monocytogenes* can also exhibit resistance to one or more antimicrobial agents and disinfectants, allowing it to survive in food processing environments (Carpentier and Cerf, 2011; Luz *et al.,* 2012).

Due to above reasons, most jurisdictions expect high risk food processing environments to have their own hazard analysis and critical control point (HACCP) program for controlling *Listeria* contamination in the food product as well as the entire facility. These programs require the industries to have systematic and periodic sampling regimes. Subsequent *L. monocytogenes* detections in the product are generally notified to the relevant regulatory authority and may eventually lead to product recalls. This may not only have serious economic impacts, but can also destroy the confidence of customers in a particular brand. Hence, it is important to perform environmental sampling in order to trace the source of contamination (Fox *et al.,* 2011a). The prevalence of *Listeria* in food processing environments is investigated using different subtyping techniques, for outbreak investigations and for 'source-tracking' or 'source-attribution' studies, wherein the probable origins of contamination in the food processing environments are estimated. By comparing the subtyping profiles of all the

isolates collected post-sampling, correlations with their origins can be made and used to associate individual strains with particular areas of the processing environment. Source-tracking studies are very important for developing suitable control strategies for the processing environment (Palumbo *et al.,* 2003). In case of outbreak investigations, molecular subtyping helps in timely tracing of the source of the original contamination and its elimination (Wiedmann, 2002).

Various phenotypic and genotypic subtyping methods have been developed for *L. monocytogenes* with the former known to be less sensitive, with a low differentiation ability and reproducibility (Shuckken *et al.,* 2003). These methods are summarised in Figure 1.4 and a detailed description of these techniques, with the associated advantages and disadvantages, are discussed below. They have been classified into five major types: conventional subtyping techniques which are generally used in combination with other recently developed techniques; restriction digestion-based techniques which rely on polymorphisms in DNA banding patterns; PCR-based techniques which generally rely on the amplification of certain loci in the genome; sequence-based techniques which rely on differences in nucleotide sequences at various loci in the genome; and typing based on proteomic data.
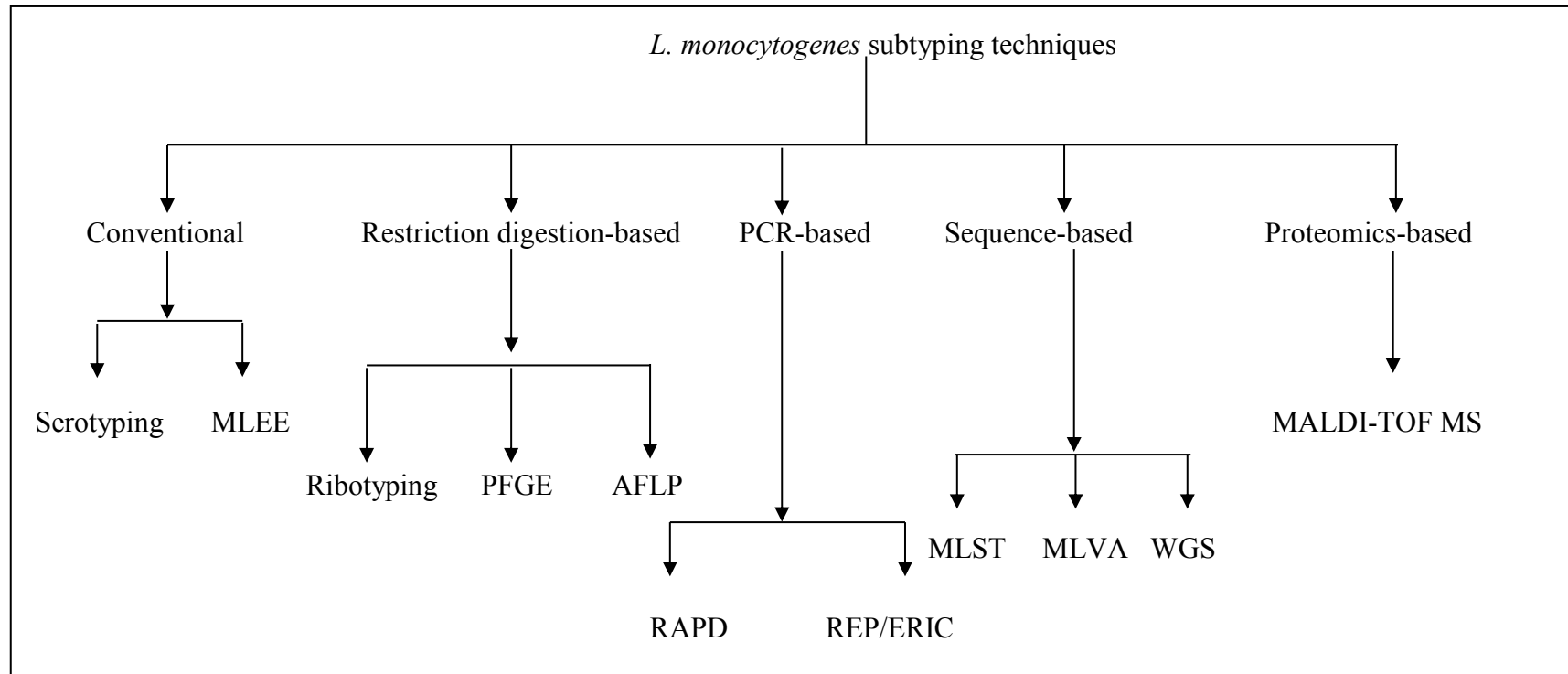
Figure 1.4 Flow chart representing techniques available for subtyping *L. monocytogenes*.

1.7   Conventional subtyping methods

1.7.1   Conventional serotyping

Conventional serotyping is performed by the slide agglutination method which is based on the agglutination of the somatic (O) and flagellar (H) surface antigens by various mono and polyvalent antisera. *L. monocytogenes* can be grouped into 13 serotypes, of which only four are responsible for human infections (1/2a, 1/2b, 1/2c and 4b) (Doumith *et al.,* 2004b).The most common serotype isolated from food processing environments is 1/2a, followed by the 4b, 1/2b, and 1/2c serotypes. Serotyping using monoclonal and polyclonal antibodies has the disadvantage of relying on high quality antisera prepared with standardised strains, is highly expensive, time consuming and shows low reproducibility (Palumbo *et al.,* 2003; Doumith *et al.,* 2004b). Antigens are also shared between *L. monocytogenes* strains, and closely-related strains diversified from a single ancestor by antigen switching mechanisms may lead to detection of a different serotype. Both *L. monocytogenes* and *L. seeligeri* have the serotypes 1/2a, 1/2b, 3b, 4a, 4b, 4c and 6b. Hence serotype detection in this case cannot be directly linked with the correct species (Liu, 2006). *L. monocytogenes* is categorised into three major phylogenetic lineages based on its serotype distribution. The two major lineages, lineage I and lineage II consist of serotypes 1/2b, 3b, 4d, 4e and 4b and serotypes 1/2a, 1/2c, 3c and 3a, respectively, while the minor lineage III consists of serotypes 4a, 4c and a few 4b (Ward *et al.,* 2008).

1.7.2   Serotyping using ELISA and multiplex PCR assays

Palumbo *et al.* (2003) developed an ELISA-based assay for serotyping. Although this method was in congruence with the slide agglutination technique, it was found to be time consuming and still dependent on high quality antisera. To overcome the issues with traditional serotyping, Borucki and Call, (2003) developed a PCR-based method for serotyping *L. monocytogenes* into five molecular serotype groups [1/2a (3a), 1/2b, 1/2c (3c), 4b (d, e) and 4 (a, c). Four primer sets used in the study serotyped the four major strains i.e. 1/2a, 1/2b, 1/2c and 4b having considerable congruency with slide agglutination. Further studies resulted in multiplex PCR-based serotyping primers based on the whole genome studies of Nelson *et al.* (2004). Microarray containing probes

specific for regions of genetic dissimilarity between three different *Listeria* spp. and a comparative genomics approach was used by Doumith *et al.* (2004a) to define three genetic lineages of *L. monocytogenes*, I, II and III, which were again subdivided into five serovar-specific subgroups: subgroup A (1/2a, 3a), subgroup B (1/2b, 3b, 7), subgroup C (1/2c, 3c), subgroup D (4b, 4d, 4e), and subgroup E (4a, 4c). However, this assay could not distinguish between 1/2a and 3a, 1/2c and 3c, 1/2b, 3b and 7, 4a and 4c and between 4b, 4d and 4e, but since 3a, 3c, 3b, 7, 4a, 4c, 4d and 4e are rarely involved in human listeriosis, this approach was considered to be suitable for rapid detection of serotypes. A similar multiplex PCR assay was proposed by Kérouanton *et al.* (2010) wherein all serotypes were found to belong to a molecular subgroup, except 4a, 4d and 7.

1.7.3 Multilocus Enzyme Electrophoresis (MLEE)

MLEE relies on the differences in electrophoretic mobility of different enzymes present in the bacterium due to differences in their amino acid sequences, which reflects differences in nucleic acid sequences (Shuckken *et al.,* 2003). Enzyme extracts are prepared using lysed bacteria and are separated by starch gel electrophoresis, followed by pulsed-field gel electrophoresis (PFGE) or native isoelectrophoretic focussing with specific enzyme stains. Each mobility variant of the enzyme is called as an electromorph and a group of electromorphs is called an electrophoretic type (ET). In a particular study by Graves *et al.* (1994), 305 *L. monocytogenes* isolates were differentiated into 78 electrophoretic types. MLEE has also been used for differentiating *L. monocytogenes* strains based on the serotype differentiation. This technique divides the species into two sub-groups: division I (serotypes 1/2b, 4a and 4b) and division II (serotypes 1/2a and 1/2c) (Nadon *et al.,* 2001).

MLEE suffers from inter-laboratory reproducibility (Wiedmann, 2002), is laborious and is less discriminative than the molecular typing methods (Shuckken *et al.,* 2003). On the other hand, it is economical, easy to use or interpret and gives 100 % typeability (Graves *et al.,* 2007).

1.8   Subtyping methods based on use of restrictions enzymes

1.8.1   Ribotyping

Ribotyping is a restriction fragment length polymorphism technique based on the polymorphism observed in the rRNA gene. In this technique, genomic DNA is initially digested with frequently cutting restriction enzymes such as *Eco*RI, *Pvu*II and *Xho*I. As many as 500 small fragments are generated and a Southern blot is performed with probes specific for the genes coding for ribosomal RNA.

An automated system of ribotyping is now available (RiboPrinter® System by Qualicon Inc.) wherein the ribopattern of the DNA digest is compared with an already existing ribotype library (Shuckken *et al.,* 2003). In a study by Kabuki *et al.,* (2004), three processing environments producing cheeses from pasteurised milk were investigated in order to source track the *L. monocytogenes* contamination using automated ribotyping. Analysis of the isolates obtained after sampling the plants revealed a peculiar ribotype (DUP-1044A), which had previously been associated with a 1998 multi-state outbreak in the Unites States due to contaminated hot dogs and deli meats. Since this isolate was isolated from two of the three plants and had a high prevalence, it was concluded that isolates belonging to this ribotype had the ability to persist in food plants (Kabuki *et al.,* 2004). The use of *Eco*RI and *Pvu*II for ribotyping has been observed to result in good strain discrimination in *L. monocytogenes* strains (Wiedmann, 2002). This dual enzyme strategy was used by de Cesare *et al.* (2007) to study the prevalence of *L. monocytogenes* in fermented and fresh sausages from Italy. Isolates with the same *Eco*RI ribogroup were categorised into five different *Pvu*II ribogroups, highlighting the higher discrimination obtained by using this enzyme.

Ribotyping is known to have a similar discriminatory power to MLEE and has been used frequently to type *Listeria* species (Nadon *et al.,* 2001). Although it has the advantage of being reproducible, it cannot efficiently differentiate between strains belonging to the 1/2b and 4b serotypes (Graves *et al.,* 2007).

1.8.2   Pulsed-field gel electrophoresis (PFGE)

Unlike ribotyping, PFGE uses restriction enzymes that cut the genomic DNA less frequently resulting in 8 to 25 larger fragments ranging between 40 to 600 kb. In this technique the bacteria are lysed and the genomic DNA is digested (generally using *Asc*I and *Apa*I enzymes) in agarose plugs (Weidman, 2002). The DNA banding patterns obtained after restriction digestion are compared to distinguish the isolates into different pulsotypes. PFGE is considered the gold standard method for epidemiological studies in foodborne outbreaks (Jiang *et al.,* 2008).

The Centre for Disease Control and Prevention (CDC) of the United States has developed PulseNet (www.cdc.gov/pulsenet/), a network of health and food regulatory laboratories that use PFGE for subtyping foodborne pathogens, including those involved in listeriosis outbreaks (Lorber, 2007). PFGE can be standardised easily and is a sensitive method that detects genetic changes such as point mutations, insertions, deletions and transpositions. It has been particularly successful in typing 4b serotypes which are responsible for most of the outbreaks and PFGE has been used widely for subtyping *L. monocytogenes* isolates in epidemiological studies for outbreaks (Whittam and Bergholz, 2007). In addition, PFGE is known to show complete correlation with serotyping and lineage distribution in case of *L. monocytogenes* (Brosch *et al.,* 1994). Although PFGE is an efficient discriminatory technique, it requires skilled labour, specialised equipment, expensive restriction endonucleases, and is laborious and time consuming (Murphy *et al.,* 2007). The results are difficult to compare between laboratories as they vary with minor changes in the experimental conditions and the electrophoresis apparatus used and are highly subjective due to the band marking involved. Thus, inter-laboratory reproducibility is an issue often encountered with PFGE.

PFGE is routinely used as a source-tracking tool for *L. monocytogenes* isolates obtained from food processing environments. Fox *et al.* (2011b) used PFGE to subtype 250 isolates obtained from thirteen cheese making environments in Ireland. Among the 52 pulsotypes obtained, no pulsotype was shared between the different facilities and most of the contamination (75 %) was attributed to sporadic contaminations. Some of the pulsotypes from external sources (raw material and farm environment) were also found

in product and processing environments, suggesting that incoming materials from the farms may also be an important source of contamination. Such PFGE analysis can help develop effective cleaning and monitoring regimes. In a more recent study by Barancelli *et al.* (2014), the prevalence of *L. monocytogenes* in three dairy plants in Brazil was investigated using serotyping and PFGE. Only *L. seeligeri* was isolated from one of the dairy plants having more rigorous cleaning regimes, while the other two plants, that had insufficient cleaning regimes and did not follow the appropriate good manufacturing processes, were facing a persistent type of *L. monocytogenes* contamination with a predominance of serotype 4b (responsible for most human infections).

1.8.3   Amplified fragment length polymorphism (AFLP)

In AFLP, the genomic DNA is digested with two restriction enzymes, and double-stranded adaptors are ligated to the fragments (50-100) generated on denaturing polyacrylamide gels. These fragments are amplified using PCR and subjected to capillary gel electrophoresis with an automatic sequencer (Graves *et al.,* 2007; Parisi *et al.,* 2010). In a recent study conducted by Lomanaco *et al.* (2011), 103 *L. monocytogenes* strains obtained from food and environmental sources were typed using AFLP with *Bam*HI and *Eco*RI (AFLPI) and with *Hind*III and *Hha*I (AFLPII). The typeability was then compared with PFGE. All the strains were typeable using PFGE and AFLPII, however only 82 were typeable using the AFLPI and all except one of the untypeable strains were dairy isolates. This change in experimental design may be required for subtyping certain untypeable strains. Since both AFLP and PFGE were found to be discriminative, the study concluded that their combination would be highly discriminative. AFLP is a sensitive and reproducible technique providing species and strain level discrimination for *L. monocytogenes* (Graves *et al.,* 2007) with the advantage of possible automation. Since DNA sequence information is obtained in this technique, it can also be used for determining the evolutionary history of the organism. However, it is time consuming and expensive due to the added step of adding adaptors to the digested DNA. Nonetheless, AFLP has been used in source-tracking of food processing environment contamination along with other subtyping methods.

Other techniques used for subtyping *L. monocytogenes* which involve restriction digestion include restriction endonuclease analysis and PCR-Restriction fragment

length polymorphism (RFLP) (Wiedmann *et al.,* 1997; Waak *et al.,* 2002; Kabuki *et al.,* 2004).

1.9   PCR-based subtyping techniques

1.9.1   Random amplified polymorphic DNA (RAPD)

In RAPD, arbitrary sequences are used as single primers (generally 10 bp in length) that target an unspecified genomic sequence in order to generate a genetic profile. Amplification is conducted at low annealing temperature (~37 °C) allowing mismatches as the primer binds non-specifically and specifically to the DNA template. Differences observed between gel profiles of related strains are due to loss of restriction sites by mutations, deletions or insertions of genetic elements (Tyler *et al.,* 1997). RAPD analysis is not considered suitable for determining evolutionary relationships between organisms or for epidemiological investigations (Tyler *et al.,* 1997). Although less discriminative than other subtyping techniques, this method is simple, inexpensive, rapid and useful for typing of a low number of strains (Gravesen *et al.,* 2000). The main drawback of this technique is that it has a low reproducibility since very low annealing temperatures are used, which may give rise to different results in different labs. However, in a study by Gravesen *et al.* (2000), considerable inter- and intra-laboratory reproducibility in the RAPD profiles was observed for *L. monocytogenes* isolates with some minor differences in the band patterns which did not affect the level of typing. RAPD has been used widely for *L. monocytogenes* to type dairy, poultry, seafood and pork isolates. In a study conducted by Aurora *et al.* (2009), RAPD was able to differentiate between isolates belonging to the same serovar and isolates obtained from the same sampling areas for 18 *L. monocytogenes* strains isolated from milk and RTE milk products. Similar profiles for some strains belonging to 4b, 4d and 4e serovars were observed from different sampling regions. As 4b is a major disease causing serotype, its discrimination may be considered essential in case of an outbreak. In this study, it was found that different strains have the potential to contaminate the same food item or processing plant.

1.9.2 Repetitive extragenic palindromic (REP)-PCR and enterobacterial repetitive intergenic consensus sequence based (ERIC)-PCR

The REP and ERIC repeats were first discovered in Gram-negative bacteria (Gilson *et al.,* 1984) and their distribution in a wide range of bacteria was first studied by Versalovic *et al.* (1991). They are imperfect palindromes which can form stem and loop structures (Treangen *et al.,* 2009). REP palindromic sequences (generally 21-65 bp long) are found in the extragenic regions of the genome in direct or reverse orientation (Roth, 2005). ERIC sequences, originally characterised in *Enterobacteriaceae*, are generally 69-127 bp long and consist of a central highly conserved inverted repeat (Treangen *et al.,* 2009) Unlike REP elements, these sequences have sometimes been found in the intergenic transcribed regions (Wilson and Sharp, 2006). The repetitive elements which serve as primer binding sites for the REP and ERIC are primers often located a few kilobases apart. As there are numerous repetitive sequences in the genome, the size of the fragments generated varies between strains (Harvey, 2004). While using REP/ERIC-PCR for species in which these elements have not been characterised, one must confirm that the amplification is taking place between genuine repetitive elements. In the absence of genuine repeat elements it may be possible that REP-PCR or ERIC-PCR is functioning only like RAPD-PCR for such species if very low annealing temperatures (40-50 °C) are used (Tyler *et al.,* 1997).

Jersek *et al.* (1999) used REP and ERIC primers for typing *L. monocytogenes* isolates from humans, animals and food sources and differentiated these into four major clusters depending upon their source of isolation. Moreover, a clear differentiation between serotypes 1/2a, 1/2b and 4b was observed. The use of an automated REP typing system (Agilent 2100, Bioanalyser) to group serotypes and establish relationships between their regional associations was evaluated by Van Kessel *et al.* (2005). The 'DiversiLab *Listeria* kit' was used to type *L. monocytogenes* strains obtained from raw bulk tank milk. The technique was found to be useful for source-tracking *L. monocytogenes* 1/2a serotypes and differentiating between the clonal groups of this serovar, but it could not efficiently differentiate 1/2b and 4b isolates which are important for listeriosis outbreaks. An advantage of REP-PCR over arbitrary RAPD-PCR is that a single primer set can be used for Gram-positive and Gram-negative bacteria, whereas RAPD requires

the design of species-specific arbitrary primers (Harvey, 2004). Overall, REP-PCR has been found to be more economical, less tedious and less time consuming compared to other techniques and should be considered as an important tool for subtyping *L. monocytogenes*. However, this technique faces the problem of inter-laboratory reproducibility and needs standardisation.

1.10   Sequencing-based subtyping techniques

1.10.1 Multilocus sequence typing (MLST)

In MLST, seven different loci from the housekeeping genes (which are known to diversify gradually) of the bacterial genome are selected, and analysed (Parisi *et al.*, 2010) using PCR followed by sequencing. Multi-virulence locus sequence typing (MVLST) types isolates based on differences in sequences of virulence and virulence-associated genes (Whittam and Bergholz, 2007). Conventionally MLST is performed by selecting gene loci that have gradually differentiated from each other by accumulation of neutral changes (e.g. certain housekeeping genes). However, Cai *et al.* (2002) selected seven different loci which included two housekeeping genes, a stress response gene, two virulence genes, and two large intergenic regions from the virulence cluster of *L. monocytogenes* as markers for MLST typing. Selection of only virulence genes can make the typing scheme too discriminatory for determining evolutionary relationships between strains as these genes are constantly under selection pressure and hence rapidly accumulate changes. Thus a combination of both housekeeping and virulence gene targets were selected in this study to obtain a higher and more reliable discrimination. As also indicated by Revazishvili *et al.* (2004), it is important that multiple genes be sequenced, as a single gene may be too highly conserved to provide discrimination or too variable for reliable differentiation.

L. monocytogenes strains involved in geographically distinct outbreaks are sometimes found to be genetically related with the possibility of a common ancestor. Such strains are grouped together as epidemic clones that represent closely-related strains with a high potential for causing disease. Subtyping of isolates from various outbreaks suggests that most of the outbreaks are caused by group of four epidemic clones: ECI, ECII, ECIII and ECIa. MLST was unable to differentiate ECII isolates from two

different outbreaks in US, which were then differentiated by PFGE in a study conducted by Chen and Knabel, (2007). However, MLST has the added advantage of allowing phylogenetic analysis, which is not possible with typing methods based on DNA sizing (Graves *et al.,* 2007). MLST appears to be one of the most accurate and simplest procedures for inter-laboratory comparison as it is a sequencing-based approach (Moorman *et al.,* 2010). In a more recent study, Martín *et al.* (2014) used MLST to investigate the diversity in *L. monocytogenes* isolates obtained from Spanish meat processing plants. In some plants, similar strain types were recovered from food-contact surfaces and products, supporting the requirement for thorough cleaning and disinfection procedures. Dominant sequence types ST9 and ST121 were shared between different meat plants suggesting that these strains were better adapted to these environments.

1.10.2 Multilocus variable number of tandem repeat analysis (MLVA)

MLVA works on the principle of detection of variation in the number of tandem repeats at a particular locus in the genomic DNA (Murphy *et al.,* 2007). Using programmes such as Tandem Repeat Finder, loci which give the highest allelic variation are selected and PCR primers are designed from the flanking regions of the repeat sequences (Miya *et al.,* 2008). The PCR products for each locus can then be separated in agarose gels or sequencing to detect differences in copy number. MLVA has proved to be highly efficient in typing other microbes including *Bacillus anthracis, E. coli* and *Francisella tularensis* (Sperry *et al.,* 2008).

Forty five food isolates from Ireland were typed using MLVA for the first time by Murphy *et al.* (2007) by using six primer sets for six tandem repeat loci present in the *L. monocytogenes* genome. Subsequently, Sperry *et al.* (2008) developed an MLVA assay by targeting eight loci containing tandem repeats (6-15 bp) in this genome and amplifying them into two multiplex PCRs to produce 54 MLVA patterns from 193 isolates. The eight loci were identified by analysing two fully sequenced *L. monocytogenes* strains, EGD-e and F2365. MLVA was also used for source-tracking of *L. monocytogenes* contamination in a cold smoked salmon plant where in it was observed that strains found at each processing site clustered together (Dass*,* 2010). This study also found MLVA to be as efficient as PFGE typing. In another study, Chen *et al.*

(2011) used MLVA to subtype *L. monocytogenes* isolates directly from spiked and enriched food samples using capillary gel electrophoresis with labelled primers.

1.10.3 Whole genome sequencing (WGS)

Whole genome sequencing of bacteria provides an opportunity to perform interspecies or intraspecies comparison. *L. monocytogenes* EGD-e (1/2a serotype) and the non-pathogenic *L. innocua* CLIP 11262 (serotype 6a) were the first two strains to be completely sequenced (Glaser *et al.,* 2001). The comparison of these two revealed many important strain-specific proteins. These sequences were then compared to three other whole genome sequences of *L. monocytogenes* strains previously linked to foodborne illnesses in U.S.A (Nelson *et al.,* 2004). With the advent of next generation sequencing, the entire genome of the bacteria can be sequenced in a cost-effective and time saving manner (Datta *et al.,* 2012). Data obtained from WGS can also be used to find information relevant to other subtyping techniques. For instance, in a study conducted by Larsen *et al.* (2012) a freely available web-based application has been developed for finding existing MLST sequence types from whole genome sequence data of pathogens. Recently, Schmid *et al.* (2014) used WGS to subtype outbreak strains of *L. monocytogenes* from Austria and Germany and found this to be more discriminatory than PFGE and fluorescent AFLP for serotype 1/2b isolates. Other *L. monocytogenes* strains are currently being sequenced by the Broad Institute and four strains (*L. monocytogenes* FSL R2-561, *L. monocytogenes* Finland 1988, *L. monocytogenes* J0161 and *L. monocytogenes* 10403S) have been completely sequenced to date, (Broad Institute, 2010;

http://www.broadinstitute.org/annotation/genome/*Listeria*_group/MultiHome.htmL).

These efforts will allow a better understanding of the virulence determinants in the pathogenic *Listeria* strains, which in turn will offer the possible development of more specific detection and typing strategies. Rudi *et al.* (2006) and Honjoh *et al.* (2008) have also investigated the use of single nucleotide polymorphism (SNP) typing, which relies on identifying SNPs in certain loci which vary in different strains (Datta *et al.,* 2012). Selection of a higher number of loci and polymorphisms can increase the discriminatory power of this technique. These genomic studies will also help construct libraries of SNPs found in certain regions of the genome, which may be more diverse in different

species of *Listeria* or the different strains of *L. monocytogenes;* thus, enabling specific detection and typing strategies (Nightingale, 2010).

1.11   MALDI-TOF MS-based subtyping

Several studies have validated the diagnostic capabilities of MALDI-TOF MS. A few studies have also focussed on its ability to perform strain level discrimination including for *Streptococcus pneumoniae* (Williamson *et al.,* 2008), *L. monocytogenes* (Barbuddhe *et al.,* 2008; Hsueh *et al.,* 2014), *Streptococcus agalactia* (Lartigue *et al.,* 2009), *S. aureus* (Wolters, 2011; Bőhme *et al.,* 2012; Wang *et al.,* 2013), *Enterococcus* (Griffin *et al.,* 2012; Quintela-Baluja *et al.,* 2013); *Neisseria gonorrhoeae* (Schmid *et al.,* 2005); *Yersinia enterocolitica* (Stephan *et al.,* 2011); *Corynebacterium striatum* (Verroken *et al.,* 2014) and *Cronobacter sakazakii* (Lu *et al.,* 2014). The use of a single platform to detect and subtype multiple pathogens makes MALDI-TOF MS an attractive alternative method to other subtyping techniques. Simple sample preparation and the availability of commercial databases have encouraged the adoption of this technology in high-throughput laboratories. In the case of *L. monocytogenes*, Barbuddhe *et al.* (2008) and Hsueh *et al.* (2014) found MALDI-TOF MS to be a simple, rapid and efficient strain level discrimination tool that was also in congruence with PFGE. However, unlike microbial identification to the species level using MALDI-TOF MS, strain level subtyping requires more detailed investigations as changes in culture conditions, sample preparation methods, quality of the spectra and the type of data analysis tools used can influence its outcomes (Sandrin *et al.,* 2013).

In summary it is clear that *L. monocytogenes* does prevail in a range of food processing facilities and various subtyping approaches are available for tracing the sources of contamination. Table 1.3 summarises the advantages and disadvantages associated with the different subtyping methods. Adoption of good manufacturing practises (GMP), HACCP plans and appropriate work flows can help in reducing the incidence of this pathogen in the processing facility. However, the foremost priority of manufacturers should be to prevent *L. monocytogenes* from residing in their facilities. This requires efficient disinfection and sanitation approaches that can eradicate both planktonic and sessile communities of *L. monocytogenes*.

Table 1.3 Advantages and disadvantages of subtyping techniques used for *L. monocytogenes* strains

Subtyping Technique and basis of discrimination	Advantages	Drawbacks	References
Conventional serotyping: Slide agglutination of somatic (O) and flagellar (H) antigens	• First approach of subtyping used to identify outbreak associated serotypes	• Expensive and good quality antisera required • Is laborious and time consuming process • Cross-reactivity between related strains	Palumbo *et al.,* (2003); Doumith *et al.* (2004b)
Molecular Serotyping: Regions of genomic differences between strains of different serotypes	• Rapid and inexpensive • Highly reliable	• Cannot distinguish between all serotypes • Used in combination with other subtyping techniques	Doumith *et al.,* (2004a); Kerouanton *et al.,* (2010)
MLEE: Differential electrophoretic mobility of bacterial enzymes due to differences in their amino acid composition	• All strains are typeable using this technique	• Time consuming and laborious • Inter-laboratory variation	Wiedmann *et al.,* (2002); Shuckken *et al.,* (2003
Ribotyping: Comparison of restriction digestion profiles obtained after digestion of chromosomal DNA by frequently cutting restriction enzyme	• Highly reproducible and discriminative • Automated version available	• Inter-laboratory comparisons difficult • Inefficient differentiation between 1/2b and 4b serotypes in some studies	Kabuki *et al.,* (2004); de Cesare *et al.,* (2007); Graves *et al.,* (2007)
PFGE: Comparison of restriction digestion pattern obtained after digestion of chromosomal DNA by rarely cutting restriction enzyme	• Highly discriminative • Efficiently subtypes isolates belonging to 4b serotype	• Time consuming and laborious process requiring a special equipment setup • Inter-laboratory variations • Skilled labour required	Whittam and Bergholz, (2007); Murphy *et al.,* (2007); Jiang *et al.,* (2008);
AFLP: Polymorphism in DNA band sizes	• Highly	• Time consuming and expensive	Graves *et al.,*

Subtyping Technique and basis of discrimination	Advantages	Drawbacks	References
obtained post PCR amplification of the products of restriction digestion of genomic DNA	discriminative, and reproducible • Automation available	• Incomplete digestion of chromosomal DNA can lead to false results	(2007); Parisi *et al.,* (2010); Lomanaco *et al.,* (2011)
RAPD: Polymorphism in PCR amplification of genomic DNA by random primers	• Rapid, simple and inexpensive	• Non-specific annealing of primers can lead to inter-laboratory variations • Less discriminative and less reproducible	Graveson *et al.,* (2000); Jeyaletchumi *et al.,* (2010)
REP and ERIC PCR: Polymorphism in band pattern produced by amplification between REP and ERIC elements in genomic DNA	• Rapid, inexpensive and simple technique • Less laborious • Species-specific primers don't have to be designed	• Amplification between two REP/ERIC elements may not be genuine with use of low annealing temperatures • Inter-laboratory variation	Harvey, (2004); Wojciech *et al.,* (2004)
MLST: Polymorphism in genomic sequences of housekeeping or virulence genes	• Inter-laboratory comparisons feasible • Highly discriminative • Provides insights in the evolutionary history of the organism	• Highly expensive and time consuming • Less discriminatory for 4b serotype • Targeting only virulence genes is discriminatory but less reliable due to their fast evolution • Selection of gene loci influences the discriminatory index	Cai *et al.,* (2002); Revazishvilli *et al.,* (2004); Chen and Knabel, (2007); Moorman *et al.,* (2010)
MLVA: Variance in copy number of targeted tandem repeats in genomic DNA	• Simple, rapid and inexpensive • Can be performed using agarose gel	• Multiplexing of primers requires standardisation and optimisation • Tandem repeats giving variation in copy numbers have to be targeted and	Sperry *et al.,* (2008); Dass *et al.,* (2011)

Subtyping Technique and basis of discrimination	Advantages	Drawbacks	References
	electrophoresis or capillary gel electrophoresis • Highly discriminative • Inter-laboratory comparisons feasible	specific primers have to be designed for them	

1.12   Control of *L. monocytogenes* in food processing environments

In the food industry, different control strategies are applied to different foods and food processing environments. For example, addition of antimicrobials such as potassium lactate or sodium di-acetate during product manufacturing, thermal treatment of the final product with steam, application of high hydrostatic pressure or irradiation or reduction in the product shelf life would be appropriate control measures for RTE meat products (Mataragas *et al.,* 2010). However, alternative strategies would be required for some other foods depending on their physical and chemical properties. Another issue is the use of specific 'in-house' microbiota that are often used to give sensory characteristics such as taste, odour or other organoleptic properties to foods. Thus, it is important that the cleaning and disinfection routines remove only the spoilage or pathogenic bacteria without affecting the beneficial microbes (Lebert *et al.,* 2007).

This part of the literature review is mainly focussed on control of *L. monocytogenes* in the processing environment. Dealing with a routinely encountered pathogen such as *Listeria* requires a practical approach to achieve the food safety objectives (FSO) in any production environment. It is important to devise and implement various risk management strategies to reduce the incidence of listeriosis (Walls and Buchanan, 2005; Pérez-Rodríguez *et al.,* 2006). The numerous control strategies include GMP, sanitation, standard operating procedures (SOP), HACCP programs, intensive environmental sampling, time and temperature controls throughout the entire processing and storage period, and post-packaging treatments to destroy *L. monocytogenes* on products.

1.13   Biofilms

While developing control strategies for *Listeria*, it is crucial to consider its biofilm forming ability. Biofilms are highly organised cellular arrangements formed through irreversible attachment of cells to various surfaces such as water pipes, industrial equipment, processing facilities, food handling surfaces, conveyer belts and stainless steel equipment (Gandhi and Chikindas, 2007; Takahashi *et al.,* 2010; Xu *et al.,* 2011). The ability of *L. monocytogenes* to persist has been attributed to its ability to adhere to niches and form biofilms that are harder to eradicate and to survive extreme environmental conditions such as low temperatures (Carpentier and Cerf, 2011).

Biofilm formation takes place in multiple stages. In the initial stage, free floating planktonic cells adhere to a solid surface, generally by intermolecular forces and hydrophobic interactions. Once adhered, the cells proliferate and start generating extracellular polymeric substances (EPS), which play a role in providing the mechanical stability to the biofilm and blocking the entry of disinfectants or cleaning agents. Following this, the biofilm transforms into a more complex arrangement or a mature biofilm that contains very small channels for the flow of nutrients. In the final stage, cells start detaching from the mature biofilms and can keep contaminating product/environment, making it a persistent biofilm (da Silva and de Martinis, 2013).

In particular, cells in these sessile communities are difficult to manage as they are known to develop an increased resistance to disinfectants (Chorianopoulos *et al.,* 2008; Sandasi *et al.,* 2008). Thus, the presence of resident biofilms involving a mixture of pathogenic microbes, can lead to a persistent contamination. In a study conducted by Pan *et al.* (2006), a five strain mixture of *L. monocytogenes* was exposed to a sanitation regime which would be typically experienced by the bacteria in a food processing environment to evaluate the resistance of biofilms formed on stainless steel and Teflon coupons towards various sanitizers such as a hydrogen peroxide-based reagent, chlorine and quaternary ammonium compounds. The biofilms were exposed to conditions of dehydration, starvation and disinfection treatments, conditions they would experience in food processing environments. In the first week, the biofilm cell number was reduced under all treatments; however the biofilms subsequently developed resistance towards all sanitizers (Pan *et al.,* 2006). Thus, there should be an emphasis on using cleaning regimes that reduce the probability of biofilm formation. The resistance to disinfectants has been attributed to many factors: a) the inability of disinfectants to penetrate the EPS surrounding the biofilm matrix, b) unlike planktonic cells, cells in a sessile community have a slow growth rate (due to lack of nutrients) and thus are less susceptible to antimicrobials, c) differential protein expression under stress conditions, compared to that in their planktonic counterparts. The three-dimensional arrangements of biofilms under static and flow conditions also play important roles. Generally, under static conditions, a monolayer of cells is observed while under flow conditions, a more complex dense network of cells is observed which hinders the penetration of antimicrobials (Oliveira *et al.,* 2012b).

1.14   Control strategies used for *L. monocytogenes*

A number of studies aimed at finding effective strategies to eliminate biofilms from food processing environments have been published. Disinfection with commonly used chemical disinfectants such as chlorine, acetic acid, quaternary ammonium compounds, hydrogen peroxide and sodium hypochlorite have been the most popular strategies to control *L. monocytogenes* (da Silva and Martinis, 2013). However, cells in biofilms can sometimes acquire resistance to chemical disinfectants. This was evaluated in a study conducted by Norwood and Gilmour, (2000). In this investigation, exposure to 1000 ppm free chlorine for 20 min was suggested to reduce biofilms whereas a 10 ppm dose was found to be sufficient for planktonic cells. In another investigation, the effects of aqueous sodium hypochlorite, aqueous chlorine dioxide and gaseous chlorine dioxide on a five-strain biofilm (of *L. monocytogenes* strains) formed on stainless steel surface were reported. In comparison to aqueous sodium hypochlorite, low levels of chlorine dioxide gas and aqueous chlorine dioxide were found to be required for the inactivation of listerial biofilms. The authors suggested that, unlike chlorine, chlorine dioxide gas produces less amounts of toxic disinfection by-products (which can sometimes be carcinogenic) and also effective over a wide pH range (Vaid *et al.*, 2010). Romanova *et al.* (2007) showed that, for eradication of listerial biofilms, concentrations of greater than 10 mg/mL of benzalkonium chloride (a major component of commonly used industrial sanitizers) had to be used for at least 30 min. However, the same concentration was insufficient for removal of biofilms formed on plastic surfaces. One of the problems associated with the use of commercial disinfectants containing hypochlorites, quaternary ammonium compounds and iodophors is their reduced efficacy at low temperatures. Food processing environments generally operate at refrigeration temperatures. As a solution Amalaradjou *et al.* (2009) reported the effect of octenidine hydrochloride (a positively charged bispyridinamine) on free-floating and preformed biofilms of *L. monocytogenes* at a temperature range of 4 °C to 37 °C. The non-toxic, non-mutagenic and non-carcinogenic octenidine hydrochloride exhibited significant anti-listerial activity on planktonic and biofilms cells. However, acquired resistance can often create delay in the elimination of mature biofilms. In such cases, peracetic acid has been suggested as an efficient disinfectant as its high oxidizing

capacity and low molecular weight facilitates penetration into the interior pockets of mature biofilms (Ibusquiza *et al.,* 2011).

An alternative biological control approach that has been the used is the application of lactic acid bacteria (LAB) or bacteriocins (Guerrieri *et al.,* 2009). Polymers containing such natural antimicrobial agents that block or interfere with the initial adhesion and eventual biofilm formation may prove to be an efficient strategy for controlling biofilms in the food industry, especially for food packaging systems (Romanova *et al.,* 2007; Nguyen et *al.,* 2008). Winkelströter *et al.* (2011) concluded that bacteriocins produced by a *Lactobacillus sakei* (used in preservation of fermented meat and fish products) strain were able to inhibit biofilms formation by *L. monocytogenes* on a stainless steel surface.

Since bacteria generally exist as biofilms in multispecies communities, the use of non-spoilage or non-pathogenic bacteria to counter pathogen biofilms is another approach. Zhang *et al.* (2007) confirmed the inhibition of *L. monocytogenes* cells in biofilms by metabolites produced by *Lactococcus lactis* and *Enterococcus durans* strains isolated from floor drains of food processing environments. Bacteriophages that are safe for consumption have also been used as antimicrobial agents in foods; e.g. Soni and Nannapaneni, (2010) used phage P100 (safe for consumption) to inhibit *L. monocytogenes* biofilms on stainless steel surfaces. However, their host specificity raises a possibility of certain host strains acquiring resistance to phages due to genetic mutations. Also, since biofilms generally exist as multispecies communities, a cocktail of phages would be required to eradicate more than one pathogen. Detergents containing nucleases has also been proposed as an alternative strategy. Since EPS is known to contain extracellular DNA and DNA from dead cells in biofilms, addition of nucleases can lower the stability of biofilm formation. However, as EPS composition can vary with the pathogen involved, a mix of enzymes would be required, which can make it an expensive solution. It is also important to check their stability at temperatures found in the food processing environments (Di Ciccio *et al.,* 2012).

Consumers today are more inclined towards products derived from natural sources instead of synthetic chemicals; this tendency has been termed 'green consumerism' (Sandasi *et al.,* 2008; Al-Reza *et al.,* 2010; Oliveira *et al.* 2012b). The emergence of

highly resistant strains and the reluctance of consumers towards chemically treated goods have created the need for the use of natural agents for controlling *L. monocytogenes* in food processing environments.

1.15   Essential oils (EOs) as anti-listerial agents

Also called ethereal oils, EOs are volatile secondary plant metabolites with characteristic odours that can be obtained from buds, flowers, seeds, leaves, fruits, bark and roots (Oliveira *et al.,* 2012a; Nazzaro *et al.,* 2013). EOs and their constituents have been explored extensively for their antioxidant, antidiabetic, anticancerous, antiviral, antithrombosis and antimicrobial effects (Djabou *et al.,* 2013). Although many techniques are available for obtaining EOs, steam distillation is the most widely accepted method (Burt, 2004). EOs obtained from plants belonging to similar species often possesses varying chemical compositions, influenced by geographical locations, physiological conditions of cultivation/growth, and time of harvest, the plant organ used for extraction and its developmental stage and the technique used for oil extraction (Jordán *et al.,* 2013).

The antimicrobial action of EOs is determined by their composition, which typically includes terpenes, terpenoids, sesquiterpenes and phenylpropanoids (Figure 1.5) (Carson and Hammer, 2011; Oliveira *et al.,* 2012a). Gram-positive bacteria are generally known to be more susceptible to EOs in comparison with Gram-negative bacteria. Being lipophilic, EOs disrupt the bacterial cell membrane and arrangement of the polysaccharide, fatty acid and phospholipid layers in the cytoplasmic membrane, thus altering the membrane permeability. Certain essential enzymes in the cell then become inactive and coagulation of the cytoplasm is observed, along with a significant reduction in the proton motive force (Oliveira *et al.,* 2012b). Some EOs has also been associated with inhibition of virulence factors, quorum sensing, toxin synthesis and flagellin production in certain bacteria (Carson and Hammer, 2011). The resistance of bacteria to antimicrobial EOs is less likely, as they generally have multiple bioactive components that may have multiple modes of action (Delaquis and Stanich, 2011). Apolónio *et al.* (2014) investigated this hypothesis by subjecting *L. monocytogenes* and *S. aureus* (including methicillin-resistant *S. aureus* [MRSA] strains) to a continuous exposure of two EO components (at varying concentrations), eugenol and citral, after

which the strains did not develop resistance against the EO components or the tested antibiotics. Additionally, exposure to sub-inhibitory concentrations of the EO components impaired the ability of the strains to adhere to polystyrene surfaces.

Several EOs have been reported to exhibit anti-listerial effects. EOs of clove (*Syzygium aromaticum*), bay (*Laurus nobilis*), cinnamon (*Cinnamomum zeylanicum*), thyme (*Thymus vulgaris*) and pimento (*Pimenta Dioica*) (Owen and Palombo; 2007); rosemary *(Rosmarinus officinalis)* and oregano (*Origanum vulgare*) (Dimitrijević *et al.,* 2007; de Azerado *et al.,* 2012); lemongrass (*Cymbopogan citratus*) (Oliveira *et al.,* 2010b) and cilantro (*Coriandrum sativum*) (Delaquis and Stanich, 2011) have exhibited significant anti-listerial properties. Many of the EO constituents exhibit synergistic antimicrobial effects when used as mixtures (Dimitrijević *et al.,* 2007). Gutierrez *et al.* (2008) observed a synergistic anti-listerial effect when a mixture of marjoram (*Majorana hortensis*) and thyme EOs were used in combination with other EOs. Interestingly, the synergy was not observed when a mixture of two strong antimicrobial oils, oregano and thyme, were used in combination with other EOs. Since some EOs have similar chemical composition, it is possible that their mixtures exhibit additive rather than synergistic effects. Hence, using EOs in combination with compounds which have a different chemical structure may produce desirable synergistic behaviour.

The bioactive components in some of these EOs have been elucidated. For instance, the antimicrobial effects of thyme and oregano EOs have been attributed to the presence of phenolic constituents such as carvacrol and thymol that target the bacterial cell membrane altering its structure and function (Dimitrijević *et al.,* 2007). Certain EO constituents such as carvacrol, thymol, p-cymene and γ-terpinene have been issued generally-recognised-as-safe (GRAS) status by the USFDA (USFDA, 2013) and have also been registered as safe flavouring agents by the European authorities (EFSA, 2013).

A) Terpenes
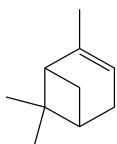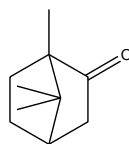
α-pinene   Camphor

B) Terpenoids
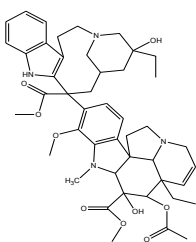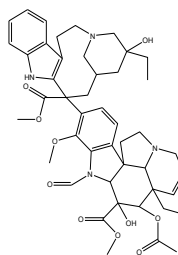
Vinblastine   Vincristine

C) Sesquiterpenes
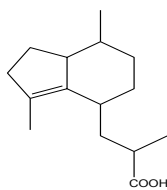
R = cinnamoyl
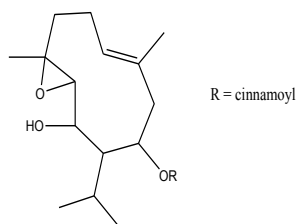
Valerenic acid   Cinnamoyl echinadiol

D) Phenylpropanoids
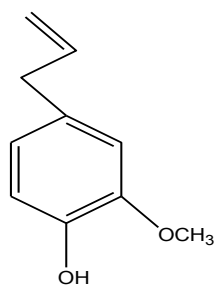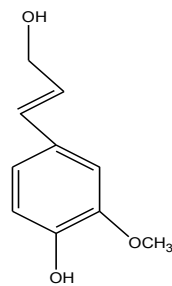
Eugenol   Coniferyl alcohol

Figure 1.5 Chemical constituents of EOs.

1.16   Biofilm inhibitory effects of essential oils

Although many studies have focussed on the anti-listerial activity of EOs, very few have focused on their biofilm inhibitory effects. Chorianopoulos *et al.* (2008) investigated the disinfecting ability of *Satureja thymbra* EO and its hydrosol fraction against monoculture and mixed culture biofilms and also compared the effect to three chemical sanitizers (hydrochloric acid, lactic acid and sodium hydroxide). The authors observed that chemical sanitizers were not as efficient as the EO and its hydrosol fractions in disinfecting listerial biofilms. An acquired adaptation of the cells to maintain pH fluctuations and the inability of the molecules to diffuse into the biofilms were the possible explanations for reduced efficacy of the chemical disinfectants. Oliveira *et al.* (2010b) found disinfectant solutions of *Cymbopogan citratus* (D.C.) Stapf. and *Cymbopogan nardus* (L.) Rendle EOs (used alone or in combination) to be effective against *L. monocytogenes* adhered to stainless steel after a treatment time of 60 min. EOs of *Syzygium aromaticum, Mentha spicata, Lippia rehmannii* and *Cymbopogon citratus* and their major constituents were studied against listerial biofilms (Leonard *et al.,* 2010). EOs of *Syzygium aromaticum* and *Mentha spicata* and the pure compounds nerol and citral produced strong biofilm inhibitory effects. However, the major chemical constituents of *S. aromaticum* and *M. spicata*, i.e. eugenol and carvone, respectively, when tested individually, caused biofilm enhancement rather than inhibition. Thus, it is important to study the actual mechanism of the bioactive components in EOs and also their synergistic or antagonistic behaviours with other EOs or EO components. On the contrary, *L. rehmanni* and *C. citratus* EOs failed to inhibit biofilm formation but their chemical constituent citral showed the same anti-listerial acitivity as the positive control (ciprofloxacin). Although these may seem as conflicting to the study by Oliveira *et al.* (2010b), the differences in the concentration of EOs of *C. citratus* used in both studies could account for the discrepancy.

L. monocytogenes intra-strain diversity in biofilm formation has been the focus of many studies (Nilsson *et al.*, 2011; Kostaki *et al.,* 2012; Kadam *et al.*, 2013). Desai *et al.* (2012) tested nine different EOs against 21 different strains of *L. monocytogenes* representing all 13 serotypes. Although minor differences in sensitivities of different strains were observed, all were sensitive towards thyme, oregano and carvacrol EOs.

The EOs successfully inactivated early and mature phase biofilms formed on stainless steel and polystyrene surfaces. The strong biofilm inhibitory effect of carvacrol has been previously reported by Pérez-Conesa *et al.* (2011).

Since biofilms generally exist as multispecies communities in food processing environments (Chorianopoulos *et al.*, 2008), Oliveira *et al.* (2012a) evaluated the effects of EOs of *Cinnamonum cassia* against single and multispecies sessile communities of enteropathogenic *E. coli* (EPEC) and *L. monocytogenes* formed on stainless steel coupons, and compared this effect to that of commercial chemical sanitizers. In comparison to EPEC, *L. monocytogenes* cells were more resistant to the EO treatment when present as single-species biofilms. However, in mixed-species biofilms, both bacteria showed increased sensitivity to the EO treatment. This emphasizes the importance of studying the effects of EOs against mixed-species biofilms as they represent the true natural environment. Interestingly, *C. cassia* EOs performed as efficiently as chemical sanitizers (hydrogen peroxide and Diprol containing a quaternary ammonium compound) in killing all the viable bacterial cells from the stainless steel surfaces, thus highlighting the potency of EOs as sanitizers. The same group investigated the anti-listerial and biofilm inhibitory activity of EOs obtained from the bark of *Cinnamonum cassia*, and leaves of *Melaleuca alternifolia* and *Cymbopogan flexuosus*, and found *C. Cassia* EO to be the most effective anti-listerial agent, but no synergistic effects were observed when combinations of the three EOs were used (Oliveira *et al.*, 2012b).

1.17   Limitations of essential oils

Numerous studies have been performed to assess the effects of disinfectants used in industrial environments, including quaternary ammonium compounds, peracetic acids and peroxides. Industrially-used antimicrobials are often hazardous to health and some can even be carcinogenic (Rahnama *et al.*, 2012). In contrast, few studies have explored the use of EOs as surface disinfectants (Chorianopoulos *et al.*, 2008; Ibusquiza *et al.*, 2011). Most studies have focussed on the ability of EOs to be used as food preservatives since some have been given GRAS status (Dimitrijević *et al.*, 2007; Al-Reza, *et al.*, 2010). However, their use as preservatives has also been limited since EOs in foods can possibly alter the organoleptic, textural, odour and other sensory food characteristics

(Gutierrez *et al.,* 2008; Ehsani and Mahmoudi, 2013). It is also possible that some EOs may be better suited as surface disinfectants rather than food additives in spite of being safe for consumption. For instance, cilantro EO was found to be very strongly anti-listerial (MIC ≤ 0.01 % v/v) in a study conducted by Delaquis and Stanich, (2011). The authors suggested its use as an effective sanitizer or disinfectant as its volatile and oxidative properties may alter the sensory characteristics of the foods if used as a food preservative. In case of EOs that are not considered safe for consumption, it is important to investigate the cytotoxicity of the oils.

Another problem associated with EOs is the variation in chemical composition of the source plant with changes in climate, geographical conditions and/or technique of isolation. Hence, using the bioactive constituents in formulating sanitizers and disinfectants can provide a more standardised approach (Oliveira *et al.,* 2012a). Jordán *et al.* (2013) performed an interesting study in which the antimicrobial activity of different chemotypes (the major chemical constituents defining the oil) of rosemary EOs obtained from multiple climatic locations were compared against different foodborne pathogens including *L. monocytogenes,* and the activity was found to be similar for all chemotypes in the case of *L. monocytogenes* but not for all pathogens.

Using EOs in sanitizing or disinfecting formulations would require considerable standardisation in regards to the optimum concentration, contact time and temperature needed to maximise the effect of the oil and the removal of any residual odour. Along with this, toxicity of the oils should also be assessed for possible associated allergen or irritant properties if they are to be applied in industrial environments. Another important factor would be the cost involved in manufacturing EOs, especially in case of EOs that need to be imported. Using EOs only occasionally or in combination with conventional chemical sanitizers can also prove to be an alternative solution to make them cost-effective (Oliveira *et al.,* 2012a). However, more investigation is required to understand the efficacy of EOs as antibacterial agents in the presence of other synthetic disinfectants or sanitisers.

Assessing the efficacy of any disinfectant, including EOs, should be performed under conditions which attempt to replicate those found in real-world industrial scenarios. It is also important not to limit antimicrobial studies to planktonic cells as sessile cells may

exhibit different behaviours. Studies highlighting the antibacterial properties of EOs are often carried out at temperatures suitable for microbial growth. However, in food processing plants, the operating temperatures are often around refrigeration; hence it is important to test the efficacy of the oils under conditions mimicking specific food processing environments.

Another approach in applying the biofilm inhibitory effects of EOs is their incorporation in the production of materials used in food industries. Nostro *et al.* (2012) investigated the biofilm inhibitory effects of some EO components with GRAS status, and incorporated these in polyethylene-co-vinylacetate (EVA) films. The EVA films with carvacrol and cinnamaldehyde showed biofilm inhibitory effects against *L. monocytogenes*. Although the authors advocated the use of such films in food packaging, the inclusion of EOs and their constituents into materials used in food processing equipment seems an attractive approach control foodborne pathogens.

To summarise, incorporation of EOs in disinfectants can create a broad spectrum effect against foodborne pathogens (Nazzaro *et al.,* 2013). Currently the use of EOs as disinfectants is still limited due to reduced solubility in the aqueous phase, loss in activity due to reaction with food components and alteration of sensory qualities of the food (Delaquis and Stanich, 2011). Further research into understanding the mechanisms and behaviour of EOs in real-world scenarios will provide the knowledge and evidence needed to assist in the development of disinfectant/sanitiser formulations based on these complex products.

1.18   Project Rationale

Foodborne diseases such as listeriosis have a severe impact on public health and economic stability of all nations. In Australia, approximately 5.4 million cases of foodborne illnesses are reported annually, with listeriosis being an important contributor (Popovic *et al.,* 2014). Anually, approximately 60-80 cases of invasive listeriosis are reported in Australia (Australian Department of Health, 2015). Assuming underreporting or underdiagnosis of cases by a factor of 2, this infers about 150 cases per year (Mead *et al.,* 1999). Although the overall incidence of listeriosis in Australia is low as compared to some European countries, the fatality rates in patients is relatively

high (20-30 %) with aged individuals being the most susceptible (Popovic *et al.,* 2014). One of the major reasons for the low incidence of this disease is the efficient monitoring and control of the pathogen in food products and processing environments. However, as consumers today are more inclined towards consumption of RTE foods (meat, poultry or dairy products), it is extremely important that there is an adequate surveillance of this pathogen.

The dairy industry is the third largest rural industry in Australia ($13 billion farm, manufacturing and export industry value) and exports its products (mainly milk, cheese and butter) to Japan, China, Singapore, Indonesia and Malaysia (Dairy Australia, 2013). *L. monocytogenes* is known to exist in a range of dairy products and processing environments in Australia. The presence of *Listeria* in products has caused product recalls, disposal of products, shutting down of plants and disassembling of product lines for cleaning (Australian Dairy Authorities Standards Committee, 2000). Such expensive and goodwill affecting recalls are thus an area of concern for the dairy industry.

After reviewing the above literature it is clearly evident that extensive research has been performed on developing highly sensitive and sophisticated detection (e.g. molecular techniques) and subtyping techniques (e.g. PFGE) for *L. monocytogenes*. However, many of these techniques are more suited for epidemiological investigations or surveillance studies and are expensive, time consuming and labour-intensive. Rigorous sampling is performed routinely in high-risk food industries, such as the dairy industry. This requires simple, rapid cost-effective and reliable solutions to monitor the presence of *L. monocytogenes* and other pathogens. Additionally, since *L. monocytogenes* can form biofilms which can develop resistance to chemical disinfectants or sanitisers, it is important to investigate if alternative anti-listerial agents such as essential oils can be used to eliminate the sources of contamination in the plant environment.

1.19   Research Aims and Objectives

The major aims of this project were (i) to develop rapid and reliable techniques for the detection and source-tracking of *L. monocytogenes* isolates obtained from food processing environments in Australia, and (ii) to investigate if plant essential oils can be used as efficient control measures for planktonic and biofilm communities of *Listeria.*

To achieve these aims, a series of tasks were undertaken, which were designed to:

a) Investigate the ability of MALDI-TOF MS as a rapid and reliable detection tool for *L. monocytogenes* isolates grown under different culture conditions;

b) Develop a novel MALDI-TOF MS-based detection scheme to facilitate rapid detection of *L. monocytogenes* directly from selective enrichment broth containing spiked foods (without reliance on solid culture media);

c) Explore the utility of MALDI-TOF MS as a source-tracking tool for *L. monocytogenes* isolates obtained from dairy processing environments within Australia and compare its discriminatory ability with the gold standard PFGE technique; and

d) Investigate the antimicrobial effects of various essential oils and study the biofilm inhibitory effects of essential oils of *Achillea millefolium* (having strong anti-listerial effect) against *Listeria* isolates obtained from food processing environments.

1.20   Thesis outline

The thesis is comprised of six chapters. Chapter 1 provides a comprehensive and critical review of the currently existing detection and subtyping techniques for *L. monocytogenes*, highlighting their advantages and limitations. It also provides an insight into the biofilm forming abilities of *Listeria* and some of the control strategies available. Chapter 2 describes some of the general materials and methods adopted in this research, while chapter-specific methodologies will be discussed in the subsequent chapters. Chapter 3 investigates the ability of MALDI-TOF MS to detect a collection of *L. monocytogenes* isolates (obtained from food processing environments) from different solid culture media and selective enrichment broths containing spiked foods. Chapter 4 investigates the ability of MALDI-TOF MS to source-track *L. monocytogenes* isolates obtained mainly from dairy processing environments, in comparison to the gold standard PFGE technique. In Chapter 5, preliminary screening of various essential oils for anti-listerial properties was undertaken and the essential oils of yarrow (*Achillea millefolium*) were investigated for their biofilm inhibitory effects against two biofilm

forming isolates of *Listeria.* Chapter 6 summarises the major findings of this research and suggests certain future directions.

Chapter 2

Materials and Methods

2.1   Equipment

Table 2.1 Equipment used in the current study

Equipment	Manufacturer	Application
AXIMA Performance	Shimadzu Scientific, USA	Mass spectrometric analyses of samples
CHEF Mapper	Bio-Rad, Australia	Pulsed-filed gel electrophoresis
FastPrep bead beater	MP Biomedicals, Australia	Physical lysis of bacterial cells for DNA extraction
Finnpipette	Thermo Fisher Scientific, Australia	Dispensing liquids
Gel DOC XR system	Bio-Rad, Australia	Imaging of Agarose gels
GELAIRE Laminar flow cabinet	LAFtech, South America	Sterile work
Inverted Microscope	Olympus Imaging corporation, Australia	Observation of cell lines
Microtitire plate reader	Cary Eclipse Varian, Australia	Measuring absorbance of 96-well plates
Mini Spin Centrifuge	Eppendorf, Germany	Centrifugation of solutions
MyCycler	Bio-Rad, Australia	PCR
Orbital Shaker	Ratek, Australia	Incubation of cultures
PowerPac power supply	Bio-Rad, Australia	Agarose gel electrophoresis
Stomacher (Lab-blender 400)	FSE, Australia	Homogenisation of samples
Thermo Spectronic Spectrophotometer	Thermo Fisher Scientific, Australia	Measuring absorbance
Vitek Calorimeter	VITEK systems, bioMérieux, France	Standardizing McFarland standards
Vortex	Ratek, Australia	Mixing solutions
Water bath	Labec, Australia	Incubation of samples

2.2 Culture media

2.2.1 Microbiological media

Dehydrated microbiological media were purchased from Oxoid (Australia) and Micromedia (Australia). Selective enrichment media Oxoid Novel Enrichment (ONE) broth for *Listeria* was purchased from Oxoid (Australia). Media were prepared using de-ionised water (dH$_2$0) and sterilised by autoclaving at 121 °C for 16 min unless otherwise stated. 1.5 % (w/v) bacteriological agar (Micromedia) was used as a solidifying agent for preparing all culture media. Pre-made media plates (Baird Parker

Agar, Horse Blood Agar, Oxford Agar, PALCAM Agar, ALOA agar and MacConkey Agar) were purchased from Micromedia Australia.

Table 2.2 Microbiological media used in this study

Medium	Mass (g) per 1litre of water	pH
Brain Heart Infusion (BHI)	37.0	7.4 ± 0.2
Buffered Peptone water	20.0	7.2 ± 0.2
Tryptone Soy Broth (TSB)	30.0	7.3 ± 0.2
One Broth *Listeria* (OBL)[a]	22.0	7.4 ± 0.2

[a] Following sterilisation, media were cooled to 50 °C and then one vial of ONE Broth-*Listeria* Selective Supplement (Oxoid) was added.

2.2.2 Cell growth medium for mammalian cells

3T3-L1 mouse fibroblast cells were cultured in Dulbeco's Modifed Eagle's Medium (DMEM) purchased from Invitrogen, Australia. This medium was supplemented with 10 % (w/v) Foetal Bovine Serum (FBS, Invitrogen) and 1 % (w/v) antibiotic (penicillin and streptomycin, Invitrogen

2.3 Enzymes/Commercial Kits and chemicals

Table 2.3 Enzymes, commercial kits and chemicals used in this study

Enzymes/Kit/Materials	Manufacturer	Purpose
Biomix	Bioline, Australia	PCR
DNA ladder (1kb and 100 bp)	Promega, Australia	Molecular weight standards
Ethidium bromide	Sigma, Australia	Agarose gel electrophoresis
Lysozyme	Sigma Aldrich, Australia	Bacterial cell wall lysis
MyTaq DNA polymerase	Bioline, Australia	PCR
Penicillin G sodium	Invitrogen, Australia	Antimicrobial action
Proteinase K	Sigma Aldrich, Australia	Protein hydrolysis in bacterial cells
Restriction endonuclease, *Apa*I, *Asc*I, *Xba*I	New England BioLabs, USA	Restriction digestion of DNA plugs
Sarkosyl	Sigma, Australia	Surfactant used for cell lysis
SeaKem Gold Agarose	Lonza, Australia	Pulsed-field gel electrophoresis
Streptomycin	Invitrogen, Australia	Antimicrobial action
ZR genomic DNA extraction kit	Zymo Research, Australia	Genomic DNA purification

2.4 Preparation of Buffers

Chemicals used for preparing buffers were of analytical grade and purchased from Sigma Aldrich (Australia), unless specified otherwise. All solutions were sterilised by autoclaving or filtering through a 0.22 µM filter (Millipore, Madison, USA). All buffers were prepared using Milli-Q water. General buffers and solutions were prepared according to Sambrook and Russell (2001).

Table 2.4 Buffers used in this study

Buffers and solutions	Composition
0.5 X Tris Borate EDTA buffer	45 mM Tris, 45 mM borate, 1 mM EDTA
10 X TE buffer	0.1 M Tris-HCl and 10 mM EDTA, pH 7.5
Cell lysis buffer	50 mM Tris: 50 mM EDTA, 1% Sarcosyl + 0.1 mg/mL proteinase K, pH 8,
MTT solution	5 mg/mL in sterile PBS
Phosphate buffered saline (PBS, pH 7.2)	3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.2
Saline	0.85 g NaCl
TAE (tris-acetate EDTA buffer) 50 X	2 M Tris base, 0.05 M EDTA disodium salt, pH 8.0

2.5 Storage and maintenance of bacterial cultures

Agar slants were used for medium term storage of bacterial cultures. For preparing the slants, 1.5 % (w/v) bacteriological agar was added to BHI agar and digested by boiling. The dissolved solutions were dispensed in 10 mL tubes and were autoclaved (121 °C for 15 min). Overnight cultures of bacteria were streaked on the BHI agar slants and incubated at 37 °C for 24 hours prior to storage at 4 °C for up to six months. For long term storage of cultures, Protect Bead storage (Technical Service Consultants) was used which enables storage at -80 °C.

2.6 Agarose gel electrophoresis

PCR products were analysed using agarose gel electrophoresis. Samples were electrophoresed in 1 X TAE buffer in a Bio-Rad electrophoresis apparatus. The

concentration of agarose was adjusted according to the size of the DNA fragments. Gels were stained with ethidium bromide (final concentration of 0.5 µg/mL) to allow detection of DNA bands. Electrophoresis was performed at 100 V, 30-40 mA for 50 min. A molecular weight marker was included in each agarose gel to estimate the size of PCR products.

2.7 Genomic DNA purification

Prior to DNA extraction, bacteria were subcultured in BHI broth for 18-24 h. DNA was extracted using the ZR (Zymo Research, Australia) bacterial DNA extraction kit. Briefly 500 µL of bacterial culture was centrifuged at 10,000 x g (5 min) to harvest the cells. The pellet was resuspended in 200 µL of sterile water. This solution was added to the ZR bead bashing lysis tube. Cells were then lysed in a bead beater (FastPrep, MP Biomedicals) for 40 sec. Following this, the tubes were centrifuged at 10,000 x g for 60 sec to remove cellular debris. The DNA containing supernatant was then filtered in a Zymo-Spin IV filter (centrifugation at 10,000 x g for 60 sec) and DNA binding buffer (1,200 µL) was added to the filtrate. The DNA was purified further by filtration performed in the Zymo-Spin II filter. The DNA collected in the filter membrane was then treated with 200 µL of DNA pre-wash buffer followed by centrifugation (10,000 x g for 60 sec). The DNA was washed with 500 µL of wash buffer and re-centrifuged at 10,000 x g for 1 min. In the final step, a DNA elution buffer was added to elute the DNA in a 1.5 mL microcentrifuge tube.

2.8 PCR for confirmation of *L. monocytogenes* isolates

PCR was used to confirm the identification of *L. monocytogenes* isolates obtained from collaborating laboratories. The PCR assay was employed as previously described by Zhang *et al*. (2009), which amplifies the haemolysin gene fragment (*hly,* 596 bp) of *L. monocytogenes*. The primer sequences and amplification conditions used for this PCR are outlined in Table 2.5. PCR was performed in a total volume of 25 µL, using 50 ng template DNA, 1 µL (10 pmol) forward primer, 1 µL (10 pmol) reverse primer (primers were obtained from Sigma Aldrich, Australia) and 25 µL Biomix master mix (Bioline). Amplification was performed in a thermal cycler. The PCR products were resolved by 1.5 % agarose gel electrophoresis.

Table 2.5 PCR primers and PCR conditions used for *hly* PCR

Primer Sequence (5´-3´)	PCR conditions
Forward AGCACAACAAACTGAAGCAAAGGA Reverse ATTGTGATTCACTGTAAGCCATTTCGTCAT	1 cycle at 94 °C for 5 min, followed by 35 cycles consisting of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, and extension at 72 °C for 30 sec; followed by a final extension at 72 °C for 7 min.

2.9 Molecular serotyping of *L. monocytogenes* isolates using multiplex PCR

L. monocytogenes isolates used in this study were serotyped using the multiplex PCR assay by Doumith *et al.* (2004a). Five primer pairs (targeting the gene fragments *lmo*0737, *lmo*1118, ORF2819, ORF2110, *prs*) were used to differentiate the four major serotypes of *L. monocytogenes* responsible for disease in humans (1/2a, 1/2b, 1/2c and 4b). All primers were purchased from Sigma Aldrich (Australia). The primer sequences, amplicon sizes and proteins encoded by the target genes have been listed in Table 2.6. PCR was performed in a total volume of 25 μL using 50 ng template DNA. The five primer concentrations used were as follows; 1 μM for *lmo*0737, ORF2819 and ORF2110; 1.5 μM for *lmo*1118 and 0.2 μM for *prs*; 250 U of MyTaq DNA polymerase and 1 X MyTaq buffer (Bioline, Australia) were added to the reaction. The PCR conditions were as follows; initial denaturation cycle at 94 °C for 3 min, followed by 35 cycles consisting of denaturation at 94 °C for 40 sec, annealing at 53 °C for 75 sec, and extension at 72 °C for 75 sec followed by one final extension at 72 °C for 7 min. Amplification was performed in a thermal cycler. The PCR products were resolved by 2 % agarose gel electrophoresis.

Table 2.6 Primer sequences used for molecular serotyping (Doumith *et al.*, 2004a)

Gene target	Primer sequence (5'-3')	Amplicon size (bp)	Serotypes amplifying the gene target	Protein encoded by target gene
*lmo*0737	Forward: AGGGCTTCAAGGACTTACCC Reverse: ACGATTCTGCTTGCCATTC	691	*L. monocytogenes* serotypes 1/2a, 1/2c, 3a, 3C	Unknown
*lmo*1118	Forward: AGGGGTCTTAAATCCTGGAA Reverse: CGGCTTGTTCGGCATACTTA	906	*L. monocytogenes* serotypes 1/2c and 3c	Unknown
ORF2819	Forward: AGCAAAATGCCAAAACTCGT Reverse CATCACTAAAGCCTCCCATTG	471	*L. monocytogenes* serotypes 1/2b, 3b, 4b, 4d and 4e	Putative transcriptional regulator
ORF2110	Forward: AGTGGACAATTGATTGGTGAA Reverse CATCCATCCCTTACTTTGGAC	597	*L. monocytogenes* serotypes 4b, 4d, 4e	Putative secreted protein
Prs	Forward: GCTGAAGAGATTGCGAAAGAAG Reverse CAAAGAAACCTTGGATTTGCGG	370	All *Listeria* species	Putative phosphoribosyl pyrophosphate synthase

2.10 Direct smear method of spotting samples for MALDI-TOF MS

In the direct smear method, a small amount of bacterial colony was taken from the solid culture medium and spotted onto a disposable Flexi Mass-DS (Shimadzu-Biotech, Japan) target plate using sterile toothpicks. The spot was overlaid with 1 μL of matrix solution (10 mg/mL, α-cyano-4-hydroxy cinnamic acid [CHCA] solution, bioMérieux) and allowed to air dry. The target plates were loaded into the Axima Performance instrument, and the target spots were fired at using a 337 nm nitrogen laser (50 Hz

frequency). Mass spectra were generated in the positive linear mode in the mass range 2-20 kDa. Each spectrum was generated by accumulating 100 profiles for each sample using the Launchpad software (Version 2.9, Shimadzu), following which they were exported to the commercial database SARAMIS (version 4.10, bioMérieux, France) for identification.

2.11 Launchpad settings for MALDI-TOF MS analysis

Launchpad software was used to analyse the target spots and obtain peak lists which were exported to SARAMIS for identification. The parameters adjusted (as per the instructions of the manufacturer) for spectral acquisition and peak processing are indicated in Table 2.7.

Table 2.7 Parameters set up for MALDI-TOF MS analysis

Parameters	Setting
Spectral Acquisition	
Laser power	80-85 V
Profiles	100
Shots	5
Auto Quality	On
Ion gate	Off
Blank	1500 Da
Pulsed Extraction	8330 Da
Peak processing parameters	
Scenario	Advanced
Profile average	All profiles
Peak width	80
Smoothing method	Average
Smoothing filter width	50
Baseline subtraction	On
Baseline filter width	500
Peak detection method	Threshold-Apex
Peak picking	Off
Peak filtering	Off

2.12 Statistical methods

All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) for Windows Version 19.0. Statistical significance was defined at a p value of less than or equal to 0.05.

The generic materials and methods used in this study have been mentioned above. More specific methods are detailed in the subsequent results chapters.

Chapter 3

Detection of *Listeria monocytogenes* using MALDI-TOF MS

Abstract

Conventional methods used for primary detection of *L. monocytogenes* involve prolonged incubation and biochemical testing, which generally require four to five days to obtain a result. Public health concerns and stringent regulations associated with the presence of this pathogen in food and food processing environments underline the need for rapid and reliable detection techniques. In the current study, application of a simple and rapid proteomics-based MALDI-TOF MS approach was evaluated to detect *L. monocytogenes* from solid culture media and from selective enrichment broths. *L. monocytogenes* isolates obtained predominantly from dairy sources within Australia were cultured on different growth media and analysed using MALDI-TOF MS at two incubation times (24 and 48 h). While reliable genus-level identification was achieved from most media, identification at the species-level was found to be dependent on culture conditions. Successful speciation was highest for isolates cultured on the chromogenic Agar *Listeria* Ottaviani Agosti agar (ALOA, 91 % of isolates) and non-selective horse blood agar (HBA, 89 %) for 24 h. To evaluate the ability of MALDI-TOF MS to detect *L. monocytogenes* directly from selective enrichment broths, ultra-high-temperature (UHT) milk samples spiked with single species and multiple species cultures were incubated in a selective enrichment broth for 24 h, followed by an additional 6 h secondary enrichment. As few as 1 colony-forming unit (cfu) of *L. monocytogenes* per mL of initial selective broth culture could be detected within 30 h using this scheme. On applying the same approach to other solid foods previously implicated with listeriosis namely, chicken pâté, cantaloupe and camembert cheese, detection was achieved within the same time interval at inoculation levels of 10 cfu of *L. monocytogenes* per mL of enrichment broth. Unlike the routine application of MALDI-TOF MS for identification of bacteria from solid media, this study proposes a time saving detection scheme for direct identification of *L. monocytogenes* from broth cultures.

3.1 Introduction

Listeria monocytogenes, the causative agent of the foodborne disease 'listeriosis', is considered a serious public threat owing to its high case-fatality rate (20-30%) (Gasanov *et al.,* 2005; Fuchs *et al.,* 2012). Although listeriosis has a low incidence, it can result in severe invasive infections as discussed in Chapter 1 (section 1.3.1). Hence, most countries have stringent regulations requiring the absence of *Listeria* in certain high risk food samples. For some other RTE foods, levels exceeding 100 cfu/g during the shelf-life of the product are considered a health risk (EFSA 2012). A wide range of foods have been implicated in outbreaks of listeriosis including milk (contaminated post pasteurization) soft cheeses, smoked fish, ready-to eat (RTE) meat and pork products, pâté and fresh produce such as coleslaw and cantaloupes (Churchill *et al.,* 2006; Alessandria *et al.,* 2010; Laksanalamai *et al.,* 2012).

Existing methodologies of *Listeria* detection from foods include a combination of culture-based, immuno-based and molecular methods. Since *Listeria* are slow growing bacteria that can be outnumbered by other competitive microbes, culture-based methods rely on a series of enrichments (24-48 h) and are generally followed by lengthy biochemical analysis, taking four or five days to confirm a positive identification (Bruhn *et al.,* 2005; Moreno *et al.,* 2012). Hence, routine *Listeria* testing generally involves a period of incubation (24-48 h), depending on the relevant food standard in selective enrichment broth. Following this, a sample of the broth is plated onto *Listeria*-specific solid media such as Oxford/PALCAM (further 24/48 h incubation period) or the more recently developed chromogenic media such as *Listeria* Ottavani and Agosti (ALOA) for final confirmation using biochemical, immunological or molecular methods (Gasanov *et al.,* 2005). In comparison to culture-based methods, immunological or molecular approaches are more rapid and reliable, but are also more expensive and labour intensive (Anderson *et al.,* 2012). In addition to the food safety risk, the release and subsequent recall of contaminated foods in an outbreak situation is a major financial burden to the food industry and can damage brand image or consumer confidence. Hence, rapid and reliable pathogen detection systems are continually sought by the food industry.

The recent emergence of MALDI-TOF MS as a simple, rapid and economical technique for characterisation of bacteria, largely at the species level and occasionally at the strain level, has greatly revolutionised microbial diagnostics (Anderson *et al.,* 2012; Böhme *et al.,* 2010; Griffin *et al.,* 2012). The identification is based upon the analysis of the whole cell proteomes of microbes in the mass range of 2-20 kDa. Clinical applications of MALDI-TOF MS have been the focus of many studies (Ferreira *et al.,* 2010, 2011; Kok *et al.,* 2011; Schubert *et al.,* 2011). It is interesting to explore if this technology can also be used for rapid detection of foodborne pathogens. Thus the current research investigated the ability of MALDI-TOF MS to detect *L. monocytogenes* directly from solid culture media.

The profile obtained using this technique is known to comprise mainly of ribosomal proteins that are expected to be minimally affected by changes in culture conditions (Wieser *et al.,* 2012). However, some studies have reported that changes in culture conditions can affect the detection and strain level typing ability of MALDI-TOF MS (Anderson *et al.,* 2012; Sandrin *et al.,* 2013; Goldstein *et al.,* 2013; Šedo *et al.,* 2011). Thus, the current study also aimed to investigate the robustness of this technology by testing the detection ability of MALDI-TOF MS using *L. monocytogenes* isolates (obtained from different food sources in Australia) cultured on five different media (including non-selective, selective and chromogenic) for two incubation periods (24 and 48 h).

Although confirmation of *L. monocytogenes* directly from agar plates using MALDI-TOF MS would make the detection process rapid, direct identification and confirmation of the pathogen from the primary selective enrichment broth would make it even more rapid compared to routine procedures, probably saving an entire day. Hence, this research also explored the ability of MALDI-TOF MS to detect *L. monocytogenes* directly from a selective food enrichment broth (following a simple sample preparation) without culturing the bacteria on solid media. The initial experiments were carried out using UHT milk as a model food, following which the pathogen was detected from three different foods namely, a RTE meat product (chicken pâté), a fresh produce (cantaloupe) and a soft cheese (Camembert cheese). A recent study involved the detection of three common milk pathogens, *E. coli, S. aureus* and *Enterococcus*

faecalis, directly from spiked milk samples using MALDI-TOF MS (Barreiro *et al.,* 2012). However, the above study used pasteurized milk samples spiked with high bacterial inocula (10^7-10^8cfu/mL). In real-world scenarios, milk samples (pasteurized or raw milk) may contain numerous different contaminating bacteria at unknown concentrations. Thus, selective enrichment would be expected to be essential for accurate and reliable identification of the target microorganism.

3.2 Chapter Aims

The major aims of this chapter were to:

a. Assess the effect of changes in culture conditions (culture media and age of culture) on the ability of MALDI-TOF MS to detect *L. monocytogenes* isolates directly from solid culture media

b. Devise a simple and rapid detection scheme for *L. monocytogenes* directly from enrichment broth samples containing spiked food samples using MALDI-TOF MS

3.3 Materials and Methods

3.3.1 Bacterial strains and culture media used

For testing the detection ability of MALDI-TOF MS under different culture conditions, *L. monocytogenes* isolates were obtained from different dairy and non-dairy sources in Australia (Table 3.1). These were cultured on five different media, comprised of two non-selective media, i.e., brain heart infusion agar (BHIA) and horse blood agar (HBA); two *Listeria* selective media, i.e., Oxford agar (OA) and PALCAM agar (PA); and a chromogenic medium, i.e., Agar *Listeria* Ottaviani Agosti (ALOA). All media were purchased from Micromedia, Australia. The bacteria were incubated at 37 °C and analysed at two different time intervals, i.e., 24 and 48 h.

For investigating the direct detection of *L. monocytogenes* from enrichment broth, a standard *L. monocytogenes* strain (ACM 98; obtained from the Australian Collection of Microorganisms) was used. This strain, coded as LMC, was used for spiking different foods including 99.9% fat free UHT processed milk, Camembert cheese, chicken pâté and cantaloupe purchased from a local retail store. *S. aureus* (American Type Culture Collection [ATCC] 12600) and *E. coli* O157:H7 (ATCC 43895) were also used for the experiments involving spiking of UHT milk. All bacteria were initially cultured on brain heart infusion agar (BHIA) and 24 h cultures incubated at 37 °C were used for all experiments. The spiked foods were either enriched in non-selective BHI broth or the selective Oxoid Novel Enrichment (ONE) broth for *Listeria* (OBL, Oxoid, Australia). *E. coli* (ATCC 8739) cultured on BHIA was used as a calibrant for MALDI-TOF MS analyses.

Table 3.1 *L. monocytogenes* isolates used in this study

No.	Isolate number	Origin	Dairy Source
1	023	Dairy (Product)	1
2	226	Dairy (Product)	1
3	360	Dairy (Product)	1
4	489	Dairy (Product)	1
5	210	Dairy (Environmental swab)	1
6	212	Dairy (Environmental swab)	1
7	744	Dairy (Environmental swab)	1
8	745	Dairy (Environmental swab)	1
9	751	Dairy (Environmental swab)	1
10	758	Dairy (Environmental swab)	1
11	760	Dairy (Environmental swab)	1
12	761	Dairy (Environmental swab)	1
13	702	Dairy (Product)	2
14	850	Dairy (Product)	2
15	866	Dairy (Product)	2
16	160	Dairy (Product)	3
17	349	Dairy (Product)	3
18	522	Dairy (Product)	3
19	950	Dairy (Product)	3
20	N028	Dairy (Product)	4
21	N036	Dairy (Environmental swab)	4
22	N038	Dairy (Environmental swab)	4
23	N048	Dairy (Environmental swab)	4
24	N392	Non-Dairy (Fish)	UK[a]
25	N413	Dairy (Product)	UK
26	N726	Dairy (Product)	UK
27	N864	Non-Dairy (Turkey)	UK
28	N195	Non-Dairy (Vegetarian)	UK
29	N836	Non-Dairy (Fish)	UK
30	LMC	Non-Dairy[b]	Lab strain
31	OB	Non-Dairy (Clinical)[c]	Outbreak strain
32	295	Dairy (Product)	5
33	033	Dairy (Product)	UK
34	034	Non-Dairy (Pork)	UK
35	036	Non-Dairy (Turkey)	UK
36	041	Dairy (Environmental swab)	UK
37	046	Non-Dairy (Fish)	UK
38	049	Dairy (Environmental swab)	UK
39	058	Non-Dairy (Pork)	UK
40	060	Non-Dairy (Infused oil)	UK
41	067	Dairy (Environmental swab)	UK
42	118	Dairy (Product)	UK

No.	Isolate number	Origin	Dairy Source
43	124	Dairy (Environmental swab)	UK
44	125	Dairy (Environmental swab)	UK
45	158	Dairy (Product)	UK
46	167	Dairy (Product)	UK

[a] isolates with unknown manufacturing sources
[b] Non-dairy reference isolate ACM 98,(clinical)
[c] Strain from an Australian dairy outbreak

3.3.2 Confirmation by polymerase chain reaction (PCR)

Genomic DNA was extracted using the ZR (Zymo Research, Australia) bacterial DNA extraction kit (Chapter 2, section 2.7). All isolates obtained were confirmed as *L. monocytogenes* using a previously described PCR assay (Zhang *et al.,* 2009) that amplifies a 596 bp segment of the *L. monocytogenes*-specific haemolysin gene. The amplification products were electrophoresed in 1 % agarose gel stained with ethidium bromide (Chapter 2, section 2.6).

3.3.3 Detection of *L. monocytogenes* isolates from solid culture media

Identification was performed using the Axima Performance mass spectrometer (Shimadzu Scientific Instruments, USA) and the commercial database, Spectral Archiving and Microbial Identification System (SARAMIS) (version 4.10, bioMérieux). The direct smear technique (Chapter 2, section 2.10) was used for spoting the samples on the MALDI-TOF MS target plates as per the manufacturer's instructions. Each isolate was cultured on all five culture media, BHIA, HBA, OA, PA and ALOA (at 37 °C), and the cultures were analysed using MALDI-TOF MS after 24 and 48 h of incubation.

3.3.4 Detection of *L. monocytogenes* directly from enrichment broth

3.3.4.1 Spiking Procedure

For all spiking experiments a 24 h culture of LMC (cultured on BHIA) was adjusted to a 0.5 McFarland Standard (equivalent to 1.5×10^8 cfu/mL) in sterile saline using a Vitek calorimeter (Vitek Systems, bioMérieux, France). The standardised culture was utilised

to spike 25 mL or 25g of the test sample to the desired concentrations in 250 mL final broth volumes. The enrichment broths were incubated at 37 °C and aliquots (1 mL) at different sampling times.

3.3.4.2 Detection of *L. monocytogenes* directly from non-selective enrichment broth

To investigate the ability of MALDI-TOF MS to detect *L. monocytogenes* directly from enrichment broth, pilot experiments were performed with the non-selective BHI broth. A standardised 24 h culture of LMC (section 3.3.4.1) was added to BHI broth to achieve concentrations of 1 cfu/mL and 10 cfu/mL in 250 mL of final broth volumes. The broths were incubated at 37 °C without shaking. Two aliquots (1 mL each) were taken from the broth for MALDI-TOF MS analyses after 6 h, 18 h and 24 h of incubation.

After optimisation of the protocol for detection from broth culture, experiments were performed by spiking sterile fat-free UHT milk with *L. monocytogenes*. The methods described here were developed as a part of the work described in section 3.4.3.1. Milk samples were spiked with known concentrations of an adjusted LMC culture and enriched in 250 mL of BHI broth to a final concentration of 10 cfu/mL. Duplicate broth samples were incubated at 37 °C and sampled after 18 h (two aliquots of 1 mL each) for analyses using MALDI-TOF MS (section 3.3.5). At the time of sampling, a viable count was also performed to enumerate the bacterial load. Since milk contains a high amount of whey proteins that may interfere with the MALDI-TOF MS-based identification, several trials (Section 3.4.3.1.) were performed to reduce the load of milk proteins in the aliquoted samples. Since none of the methods were found to be effective, a simplified methodology was adopted for detecting LMC from spiked UHT milk samples.

Briefly, the spiked milk samples were inoculated in sterile BHI broth to achieve final concentrations of 1 cfu/mL (broth A) and 1000 cfu/mL (broth B) in 250 mL volumes. Broths A and B were incubated at 37 °C without agitation. Two aliquots (1 mL each) were taken from the BHI broths for MALDI-TOF MS analyses (section 3.3.5) after 6 h, 18 h and 24 h of incubation. A viable count was performed simultaneously to enumerate the cells. All experiments were performed in duplicate.

3.3.4.3 Detection of *L. monocytogenes* directly from selective enrichment broth

In order to investigate the suitability of Oxoid Novel Enrichment (ONE) broth for *Listeria* (OBL) medium for the selective enrichment broth experiments, the medium was inoculated with LMC to obtain a final cell number of 1000 cfu/mL. Duplicate broths were incubated at 37 °C for 24 h, and aliquots (1 mL each) were taken for MALDI-TOF MS analysis. A viable count was also performed. After achieving successful results with OBL broth, the same experiments were repeated with OBL containing spiked milk samples. Similar to the experiment with BHI broth, the UHT milk samples were spiked with LMC strain to concentrations of 1 cfu/mL (Broth A) and 1000 cfu/mL (Broth B) in the final broth volume of 250 mL. Two aliquots (1 mL each) were taken from both broths for MALDI-TOF MS analysis after 24 h incubation. Since the incubation period was not observed to be sufficient for reliable species-level identification (as discussed in section 3.4.3.2 below), 1mL aliquots from the enrichment broths A and B were transferred to tubes containing 9 mL sterile BHI broth for a secondary enrichment for a further 6 h period (at 37 °C). Following this, two aliquots (1 mL each) were taken for MALDI-TOF MS analyses (section 3.3.5). Viable counts were performed after the initial 24 h enrichment and the 6 h secondary enrichment period. All experiments were performed in duplicate.

3.3.4.4 Detection from selective enrichment broth containing microbial mixture

The proposed 30 h MALDI-TOF MS-based scheme for detecting *L. monocytogenes* from spiked milk samples was challenged further by spiking 25 mL UHT milk with a microbial mixture (ratio 1:1:1) of LMC, *S. aureus* (ATCC 12600) and *E. coli* O157:H7 (ATCC 43895) to achieve final concentrations of 1 cfu/mL each of all three bacteria in final broth volumes of OBL (250 mL). The broth containing the mixture of the three pathogens was incubated at 37 °C for 24 h following which 1 mL was transferred to 9 mL non-selective BHI broth and selective OBL broth for a 6 h secondary enrichment. The methodology described here is discussed in section 3.4.3.3. Prior to performing this experiment, 25 mL UHT milk samples were spiked with *S. aureus* and *E. coli* (spiking loads 1, 10 and 100 cfu/mL in final broth volumes of 250 mL) individually and enriched in OBL broth to confirm the selectivity of the broth. The enriched broths were sampled after 24 h for MALDI-TOF MS analysis and were also plated on MacConkey agar

(Micromedia, Australia) and Baird-Parker agar to assess the absence of *E. coli* and *S. aureus*, respectively.

3.3.4.5 Detection from selective enrichment broth containing spiked solid foods

After obtaining promising detections by using OBL for the 6 h secondary enrichment period (section 3.3.4.4), the same scheme was applied to three other foods; cantaloupe, chicken pâté and Camembert cheese. All foods were initially screened for the presence of *Listeria* by enriching the foods for 24 h in OBL, followed by isolation on *Listeria* selective Oxford agar (Micromedia, Australia). Only foods negative for *Listeria* were used for further experiments. 25 g portions of the foods were spiked with LMC and added to 225 mL OBL broth to achieve concentrations of 1 cfu/mL and 10 cfu/mL in final enrichment broth volumes of 250 mL. Post the 24 h incubation period at 37 °C, 1 mL aliquots of both broths were inoculated in 9 mL of sterile OBL broth for the secondary enrichment period of 6 h. Following this, two aliquots (1 mL each) from both broths were taken for MALDI-TOF MS analysis (section 3.3.5). All experiments were performed in duplicate.

3.3.5 Sample processing for MALDI-TOF MS

The aliquots taken for MALDI-TOF MS analyses were processed as per the reported protocol by Sparbier *et al.* (2012) with some modifications. Briefly, the aliquots (1 mL each) were centrifuged at high speed (13,000 x g) for 2 min to harvest the cells, and were washed with sterile distilled water and allowed to air dry for 15 min. This was followed by a treatment with formic acid and an equal volume of acetonitrile (volume proportional to the size of the cell pellet, approximately 30 µL). After a final centrifugation step (13,000 x g, 2 min), the supernatant was spotted onto disposable polymeric Flexi Mass-DS target slides (Shimadzu-Biotech, Japan) and the sample spots were allowed to air-dry. The spots were overlaid with 1µL of matrix solution (10 mg/mL α-cyano-4-hydroxy-cinnamic acid [CHCA], bioMérieux) and were allowed to dry again prior to subjecting it to MALD-TOF MS analysis.

3.3.6 Evaluation of MALDI-TOF MS profiles

Peak lists for the test samples were obtained in the mass range of 2 to 20 kDa using the Launchpad software (version 2.9, Shimadzu) and exported to the SARAMIS database for identification. This commercial database has a total of 18,831 reference spectra for prokaryotes and eukaryotes and a total of 3,178 SuperSpectra (6 SuperSpectra representing the genus *Listeria* and 12 representing the species *monocytogenes*). Reference spectra are a collection of individual MALDI-TOF MS spectra obtained for different isolates; whereas the SuperSpectra for a particular species have been generated from selected reference spectra already existing in the database that have passed quality criteria. The peaks present in the SuperSpectra of a particular species are already assigned a peak weight, such that higher peak weights are assigned to species-specific peaks and lower peak weights are assigned to higher taxonomic levels (genus or family). The peak list generated from the test sample is compared to the SuperSpectra in the database using a patented pattern-matching algorithm resulting in identification with a certain confidence score. For a reliable identification, it is not only important that the peaks from the test sample match the peaks in the SuperSpectrum but also that the sum of these peak weights adds up to a threshold score (Streit and Welker, 2010). In the current study, since well-characterised isolates were used, identifications exceeding the 75 % confidence value were considered as reliable identifications for all experiments.

3.4 Results and Discussion

Conventional culture-based methods and standard biochemical tests remain the core methods for large scale routine testing by food microbiology laboratories as they are cost-effective, simple to interpret and provide the sensitivity needed to meet standard regulations (Drancourt *et al.,* 2010; Murakami, 2012). The most widely used standard protocols for *Listeria* detection from food, namely the FDA BAM method and the ISO 11290 method, involve an initial enrichment of 25 g food sample in a selective enrichment broth, followed by plating on selective agar and analysis of presumptive listerial colonies (either by biochemical tests or API *Listeria* [bioMérieux] if speciation is intended). In addition, the presumptive colonies have to be selected and subcultured to obtain pure cultures if further testing is required. Confirmation of *L. monocytogenes* is generally performed using the BAX PCR system (Qualicon) (Gasanov *et al.,* 2005). Thus, confirmation of a positive sample is a time consuming process taking up to five days. The currently popular enzyme-linked fluorescent immunoassay assay, VIDAS, reduces the time for screening of *Listeria* presumptive samples to two days. However, it also requires confirmation by plating on specific media to eliminate false positives (Meyer *et al.,* 2011). This study explored the use of proteomics-based MALDI-TOF MS as a rapid tool to detect *L. monocytogenes* from solid culture media and food enrichment broths.

3.4.1 Species level confirmation using *hly* PCR

All isolates included in this study (Table 3.1) were initially screened for the presence of the *L. monocytogenes* specific haemolysin (*hly*) gene using a PCR previously described by Zhang *et al.* (2009). All isolates amplified the 576 bp fragment. Figure 3.1 shows confirmatory results obtained from selected *L. monocytogenes* isolates.
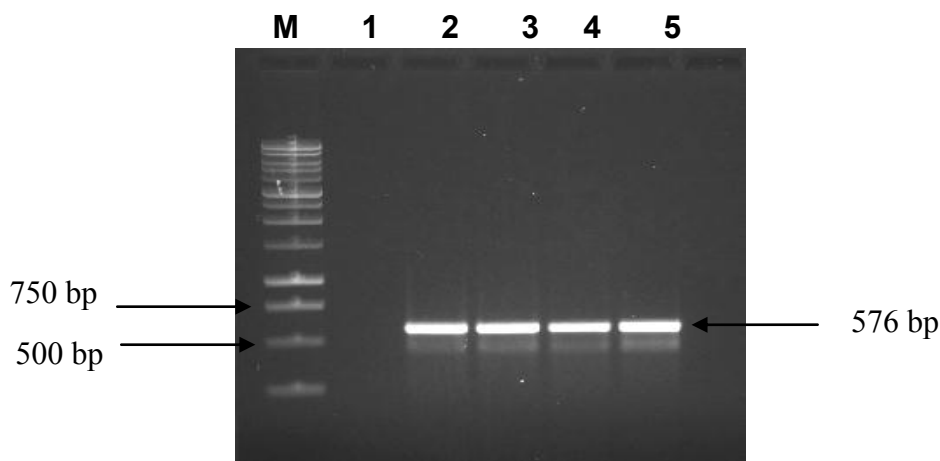
Figure 3.1 Agarose gel (1%) depicting amplification of *hly* gene fragment (576 bp). Lane M: molecular weight marker (1kb ladder Promega, Australia), Lane 1: negative control (DNA from a lab strain of *L. innocua*), Lane 2: positive control (*L. monocytogenes* reference strain 'LMC'), Lane 3 to 5: Isolates 023, 058 and 702, respectively.

3.4.2 Detection of *L. monocytogenes* isolates from solid culture media

Microbial identification using MALDI-TOF MS can be performed using two techniques: (a) direct spotting, where an individual colony is picked from a culture media plate using a toothpick, swab or disposable loop and placed on a MALDI target plate for analysis, or (b) extraction, which involves a simple formic acid-acetonitrile treatment. The latter is often used for microbes with a more complex cell wall wherein the formic acid is used for cell lysis and acetonitrile precipitates the protein in the sample (Anderson *et al.*, 2012). In the current study, the direct spotting technique was used, keeping in mind the usability and time constraints of high-throughput food laboratories and the recommendations of the manufacturer (bioMérieux).

Since well-characterised isolates were used in this study, any identification result above the 75 % confidence value in SARAMIS was considered as a reliable. Unlike previous studies by Barbuddhe *et al.* (2008) and Hseuh *et al.* (2014) that have demonstrated the ability of the commercial Biotyper (Bruker) database to detect *Listeria* isolates (mainly clinical isolates), this study explored the performance of the SARAMIS (bioMérieux) database to detect dairy isolates.

Five culture media routinely used for *Listeria* detection were selected for studying the influence of culture conditions on MALDI-TOF MS-based identifications. The two non-selective media, BHIA and HBA, are used as enriching media as they contain highly nutritious ingredients such as horse blood, bovine brain and heart tissue. The three selective media (ALOA, OA and PA) contain several antibiotics for selection of *Listeria*, including nalidixic acid, acriflavin hydrochloride, colistin sulphate, amphotericin B, ceftazidime, polymixin B and cyclohexamide (Leclercq, 2004). After 24 h of incubation, *L. monocytogenes* produces blue colonies surrounded by opaque halos on ALOA agar, pinpoint green colonies, sometimes with a black centre and a black halo on PALCAM agar, and pinpoint black colonies surrounded by black halos on Oxford agar. Due to the presence of selective agents, the growth of listerial colonies was observed to be slower in comparison to BHIA or HBA.

Table 3.2 depicts the overall identification results at genus and species levels for the different media. After 24 h of incubation, more than 90 % of the isolates cultured on all five media were identified to the genus level. Particularly in the case of ALOA and HBA, all isolates were identified to the genus level after 24 h of incubation. Since ALOA is a chromogenic medium that is used to identify presumptive *L. monocytogenes* colonies, a combination of this medium and MALDI-TOF MS-based identification can serve as a robust confirmatory test. In contrast, species-level identification was observed to vary with the culture media. It was evident that some media, such as ALOA, HBA and BHIA, performed better (more than 80 % isolates identified to species level) when compared with other media such as OA or PA. Although ALOA, OA and PA are all selective media, only OA and PA contain esculin and ferric ammonium citrate salt (Leclercq, 2004). Whether these compounds interfere with MALDI-TOF MS-based spectral acquisition needs to be investigated. After 48 h of incubation, genus and species level identifications were considerably lower (less than 75 % isolates identified to genus level and 60 % isolates identified to species level) for isolates cultured on all media except for PA where identification improved after 48 h of incubation. Overall the percentage of 'no detections' also increased after 48 h, suggesting the loss of biomarker peaks that are significant for identification.

Table 3.2 Detection of *L. monocytogenes* using MALDI-TOF MS

Medium	Time of incubation	Genus level ID* (%)	Species level ID (%)	No detection (%)
ALOA	24 h	100	91	-
	48 h	94	37	7
BHIA	24 h	96	80	4
	48 h	87	59	13
HBA	24 h	100	89	-
	48 h	87	30	13
OA	24 h	94	78	7
	48 h	78	37	22
PA	24 h	91	50	9
	48 h	98	70	2

* ID denotes identification

Some of the reasons suggested to influence the performance of MALDI-TOF MS include the high salt content in media and pigments or dyes (such as crystal violet) that interfere with the ionisation of the sample in MALDI-TOF MS (Anderson *et al.,* 2012; Croxatto *et al.,* 2012). High salt content can lead to an ion suppression effect, wherein the presence of ions (other than those of the analyte) can affect the ionisation process (Anderson *et al.,* 2012). Another factor to be considered for the direct smear method is the ease of spotting. In the current study, direct spotting from the two selective media, OA and PA, was observed to be less convenient compared to the other three media due to the nature of the bacterial growth. These media contain esculin, and *L. monocytogenes* produces slow growing (due to selective agents) and small pinpoint colonies with black precipitation (Leclercq, 2004). In addition, picking the colonies without interference from the culture media was extremely difficult. This in turn, may have also contributed to the low genus and species level identification results. The development of in-house databases containing reference spectra acquired on these media may improve the identification rates. Alternatively, an extraction technique that allows the removal of certain media components could be investigated (Anderson *et al.,* 2012). Overall, these findings clearly reiterate the results from previous studies that media and culturing conditions influence MALDI-TOF MS profiles, which consequently influence identification rates (Walker *et al.,* 2002; Anderson *et al.,* 2012; Goldstein *et al.,* 2013; Šedo *et al.,* 2011; Balážová *et al.,* 2014).

A few studies have indicated that, although changes in culture conditions affect the mass spectra, there is minimal or no effect on identification rates for some bacteria (such as *E .coli* or *S. aureus*) using MALDI-TOF MS (Valentine *et al.,* 2005; Martiny *et al.,* 2012, Reich *et al.,* 2014). In contrast, Anderson *et al.* (2012) concluded that changes in culture media affect the overall confidence scores for MALDI-TOF MS. Balážová *et al.* (2014) also observed that, in the case of mycobacteria, identification to the species level was dependent on the culture media used. However, it is important to highlight that species more routinely encountered in clinical laboratories have greater representation (i.e., high number of reference spectra) in commercial databases compared to environmental or food pathogens. Since identification rates are driven by the quality and representation of mass spectra for a particular species in the database, the results for one species may not be comparable to another. Overall, although species-level identifications were affected by differences in culture media in the current study, it was noticeable that more than 90 % genus-level identifications were obtained across all media after 24 h of incubation. This is of particular significance in case of preliminary isolation studies for this pathogen from food or clinical samples.

3.4.3 Detection of *L. monocytogenes* directly from enrichment broth using MALDI-TOF MS

From the results presented above (section 3.4.2), it is evident that, although differences in culture conditions have an impact on the identification for *L. monocytogenes*, MALDI-TOF MS nonetheless provides a reliable and rapid alternative for other tests that may require further confirmation. However, direct detection from a selective enrichment broth would make the process even more rapid and also help in eliminating any samples negative for *Listeria.*

3.4.3.1 Detection of *L. monocytogenes* from non-selective BHI broth

Pilot experiments were carried out in non-selective media by inoculating BHI broth with LMC to a final concentration of 1 cfu/mL and 10 cfu/mL. No detection was obtained after 6 h of sampling. A positive *L. monocytogenes* identification was obtained for both broths after 24 and 18 h of incubation (37 °C), respectively. However at this

point, the broth did not contain any food matrix that could possibly interfere with the identification process.

UHT milk was selected as a model food for the initial spiking experiments since products made from raw milk and pasteurised milk have been associated with listeriosis outbreaks (Garrido *et al.,* 2010). Milk samples were spiked and enriched in non-selective BHI broth to a final concentration of 10 cfu/mL in 250 mL and sampled after 18 h of incubation (two aliquots of 1 mL each) for MALDI-TOF MS analysis. Milk is a highly proteinaceous food and caesin (~ 80 %) is the major constituent of its total protein content, followed by whey proteins. Most of the casein in milk exits in the form of micelles along with other components such as calcium, phosphate, citrate, some ions and enzymes. Low levels of calcium and pH alteration (to pH 4.6) have been associated with casein precipitation (Goff and Hill, 1993). Since proteins originating from the food matrix can be ionised and interfere with MALDI-TOF MS-based identification, different trials were performed to lower the amount of milk proteins in the sample aliquots taken for MALDI-TOF MS analysis (Table 3.3).

EDTA was used to chelate the calcium in milk, thereby destabilising the micellar structure of casein and leading to its precipitation. The casein micellar structure is responsible for the white colouration of milk. Thus, after addition of EDTA, it was observed that the solution cleared instantly. Following this, different strategies were adopted such as centrifugation (Trials 1 and 3) and filtration (Trial 2) to eliminate the precipitated casein. Precipitation of casein using acetic acid was also attempted in Trial 4 since casein is known to precipitate at pH 4.6.

The spectra obtained after these treatments were exported to SARAMIS for identification. Unlike the earlier experiments with BHI broth (without spiked milk) where species level detection was obtained after 18 h of incubation for the same bacterial load, the current trails did not provide a successful species level outcome (Table 3.3.). Only genus level detections were obtained by performing Trials 1 and 3. Figure 3.2 depicts the spectral profiles obtained after performing the trials. Firstly, on comparison of profiles obtained from enrichment broth containing spiked UHT milk (D) to BHI broth without UHT milk (E), an increase in the number of peaks was observed, presumably arising from ionisation of the UHT milk or its breakdown products. Trials 1

and 3 produced profiles that were quite similar to profile D, indicating that the EDTA treatment was not successful in reducing the background peaks. One reason for this could be that the treatment eliminated casein peaks in a mass range outside of the 2-20 kDa range. Acid precipitation (Trial 4) produced a spectral profile (A) that was missing peaks that are significant for identification of *L. monocytogenes*. Another factor that may have affected the detections is that the treatment with EDTA or acetic acid may have also contributed to some amount of cellular stress or loss of viability. Interestingly, the samples used for the two trials that did provide a genus level detection (Trial 1 and 3) had a viable count in the range of 10^7 cfu/mL while the other two trials that failed to provide any identification had comparatively lower viable counts (approximately 10^6 cfu/mL) suggesting that cell number/load may also play a role in MALDI-TOF MS-based detection.

Table 3.3 Trials performed to reduce the amount of milk proteins that may interfere with the MALDI-TOF MS-based detection of *L. monocytogenes*

Trial No.	Trial (initial spiking load 10 cfu/mL)	Theory behind the trial	Methodology	MALDI detection after 18 h incubation at 37 °C
1	Addition of Ethylenediamin etetraacetic acid (EDTA)	• EDTA chelates and disrupts the calcium in the milk casein micelle structure (Udabage *et al.*, 2000) • Caesin become more soluble in the aqueous phase and can be removed using centrifugation (Goff and Hill, 1993)	• 20 mM EDTA (Sigma Aldrich, Australia) was added to the 1 mL sampled aliquot of the enrichment broth • The mixture was centrifuged at 13,000 x g (Mini Spin Centrifuge, Eppendorf) for 2 min • Cell pellet was used for MALDI-TOF MS analysis	*Listeria* species
2	Addition of EDTA followed by filtration	• EDTA treatment disrupts miceller structure • Individual casein molecules will be filtered through a 0.45 µM filter, retaining the bacteria on the filter (Bouchoux *et al.*, 2009)	• 1 mL aliquot of the enrichment broth was filtered through a 0.45 µM filter (Saratorius, Australia) • The filter was transferred to a 10 mL sterile saline solution (0.85 % NaCl) and was vortexed vigorously for 30 sec • 1 mL aliquot from the saline solution was treated with EDTA as in Trial 1 before MALDI-TOF MS analysis	No detection
3	Addition of EDTA to cell pellet	• The enrichment broth would be centrifuged and EDTA added to the cell pellet would solubilise any milk casein present in the pellet (Udabage *et al.,* 2000)	• 1 mL aliquot of the enrichment broth was centrifuged at 13,000 x g for 2 min • 20 mM EDTA solution was added to the cell pellet (just enough to re-suspend the pellet) • The mixture was re-centrifuged and the pellet was washed with sterile water before MALDI-TOF MS analysis	*Listeria* species

Trial No.	Trial (initial spiking load 10 cfu/mL)	Theory behind the trial	Methodology	MALDI detection after 18 h incubation at 37 °C
4	Acid precipitation	• Caesin can be precipitated at pH 4.6, and separated by low speed centrifugation retaining the bacteria in the supernatant (Goff and Hill, 1993; Recio and Olieman, 1996)	• The casein in the 1mL aliquot of the enrichment broth was precipitated using 10 % acetic acid and 1 M sodium acetate solution (Sigma Aldrich, Australia) • The mixture was centrifuged at 100 x g for 3 min • The resultant supernatant was analysed using MALDI-TOF MS	No detection
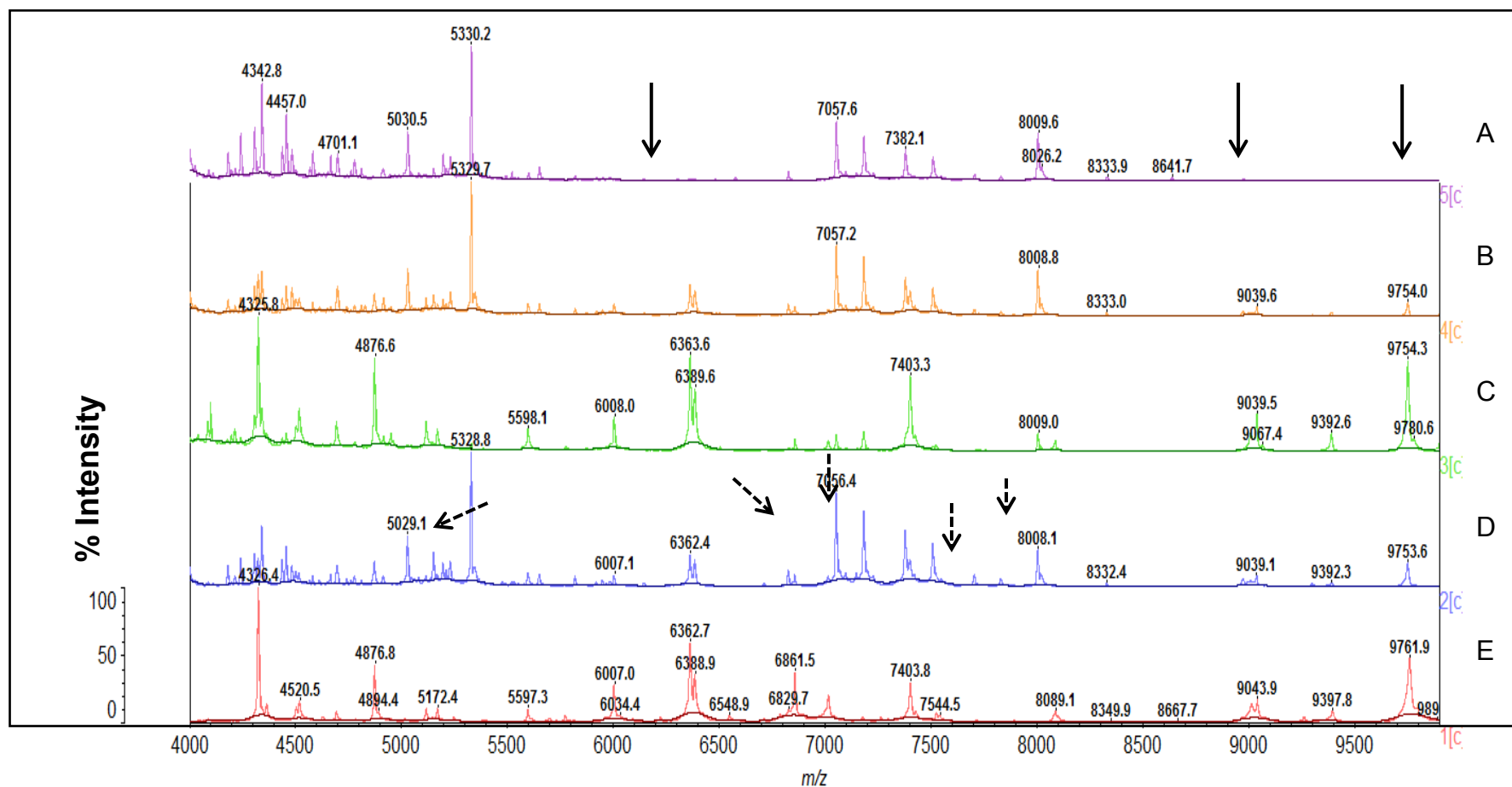
Figure 3.2 Comparison of MALDI-TOF-MS profiles of LMC strain spiked at 10 cfu/mL in 250 mL BHI and sampled after 18 h of incubation. (A) Trial 1: acid precipitation (B) Trial 2: addition of EDTA to cell pellet (C) Trial 3: addition of EDTA to enrichment broth (D) enrichment broth containing spiked UHT milk (E) enrichment broth without UHT milk. The downward arrows in profile (A) indicate the expected positions of missing peaks significant for identification of listerial samples in the acid precipitation trial. Profiles (B) and (C) appear to be more similar to profile (D) and were identified to the genus level using MALDI-TOF MS. Profile (D) contains numerous extra peaks (some indicated by dotted arrows) in comparison to profile (E), probably originating from UHT milk or its breakdown products.

Since the above trials did not yield a species level identification, a simple protocol suggested by Sparbier *et al.* (2012) was used to detect *L. monocytogenes* from spiked milk samples enriched in BHI broth. The final concentrations after spiking were 1 cfu/mL (broth A) and 1000 cfu/mL (broth B). Samples analysed at 6 h and 18 h post-inoculation failed to identify *L. monocytogenes* for broth A samples, while *L. monocytogenes* was identified only after 18 h incubation from broth B samples (Table 3.4). On observing the viable count after 18 h incubation for broth A (1.03×10^6 cfu/mL) and broth B (7.75×10^8 cfu/mL), the limit of detection for identification of *Listeria* from a sample was determined as approximately 10^8 cfu/mL. This supports the findings of others that MALDI-TOF MS detection is dependent on the number of cells present in the sample (Croxatto *et al.,* 2012).

Table 3.4 MALDI-TOF-MS detection of LMC from non-selective BHI broth

Broth	BHI (with spiked milk)					
Spiking concentration (strain LMC)	1 cfu/mL			1000 cfu/mL		
Time of sampling	6 h	18 h	24 h	6 h	18 h	24 h
MALDI-TOF MS identification	ND	ND	LM	ND	LM	LM

LM: detection as *L. monocytogenes;*
ND: no detection
Viable counts for all samples detected as LM were in the range 10^8-10^9cfu/mL

3.4.3.2 Detection of *L. monocytogenes* from selective enrichment broth (OBL) containing spiked milk

Once species-level identifications were obtained in non-selective broth after 24 h, the same procedure was repeated with the selective enrichment broth, OBL. This medium was selected because according to the manufacturer (Oxoid), it only requires a 24 h enrichment period to inhibit other contaminating bacteria in food samples as opposed to some other selective media which require a longer enrichment. In order to investigate the suitability of OBL for the selective enrichment broth experiments, the medium was inoculated with LMC strain at a concentration of 1000 cfu/mL (without milk). Successful *L. monocytogenes* detection was achieved after 18 h of incubation. Hence

further spiking experiments were carried out with OBL broth. In the case of OBL containing spiked milk (initial inocula of 1 cfu/mL and 1000 cfu/mL), SARAMIS was only able to identify the contaminant as "*Listeria* sp." after the 24 h enrichment period (broths A and B). However, successful identification to species level was achieved following a secondary enrichment of 6 h in BHI broth (Table 3.5).

Table 3.5 MALDI-TOF-MS detection of *L. monocytogenes* from spiked UHT milk samples enriched in selective OBL broth

Broth	OBL (primary enrichment medium)		BHI (secondary enrichment medium)	
Spiking concentration	Broth A (1 cfu/mL)	Broth B (1000 cfu/mL)	Broth A secondary enrichment	Broth B secondary enrichment
Time of sampling	24 h	24 h	6 h	6 h
MALDI ID	Lsp	Lsp	LM	LM

LM: detection as *L. monocytogenes;* Lsp: detection as *Listeria* species
Viable counts for all samples detected as LM were in the range 10^{8}-10^{9}cfu/mL and other samples detected as Lsp were in the range 10^{7}-10^{8} cfu/mL

Given the promising results with non-selective media (BHI) in pilot experiments with milk, the inability of the MALDI-TOF MS procedure to identify the same bacterium to the species level in OBL after 24 h was unexpected. However, this could be due to multiple factors. As reported by previous studies (Carbonnelle *et al.,* 2011; Anderson *et al.,* 2012) and the current study, alterations in MALDI-TOF MS spectra can result when culturing media are changed. Another important factor could be the presence of interfering spectral peaks due to media components or the ionisation of milk proteins. The spectrum for the OBL broth samples containing spiked milk sample (24 h) was observed to have numerous additional peaks compared to that obtained after the secondary enrichment period in BHI broth (Figure 3.3). Thus, the extra peaks may interfere with the algorithm used for speciation and this may explain the inability of SARAMIS to speciate *Listeria* after 24 h from OBL. A similar conclusion was reached by others (Sparbier *et al.,* 2012), where a lower identification rate for *Salmonella* sp. was observed due to the presence of interfering stool proteins in *Salmonella*-specific

enrichment broth. The reduction in the background milk peaks and the increase in cell numbers obtained after a secondary enrichment in BHI allowed the spectral profiles to reach the required threshold similarity score with the *Listeria monocytogenes* SuperSpectrum in SARAMIS to provide reliable identification. Table 3.6 shows the peaks detected in a sample of spiked milk that was first enriched in OBL broth (24 h primary enrichment) followed by a 6 h secondary enrichment in BHI. As explained in section 3.3.6, other than the presence of peaks matching the particular SuperSpectrum in the database, it is also important that the sum of the peak weights of these peaks (pre-assigned in the database) reaches the threshold score required for a positive identification. Thus, the score required to match the *L. monocytogenes* SuperSpectrum and achieve species-level identification was reached only after the secondary enrichment in BHI. It is important to note that interfering peaks were also observed in the case of BHI broth (24 h) containing spiked milk; however, it is possible that the bacteria reached the numbers required for detection more rapidly in BHI broth, generating profiles that could be identified to the species level using SARAMIS.
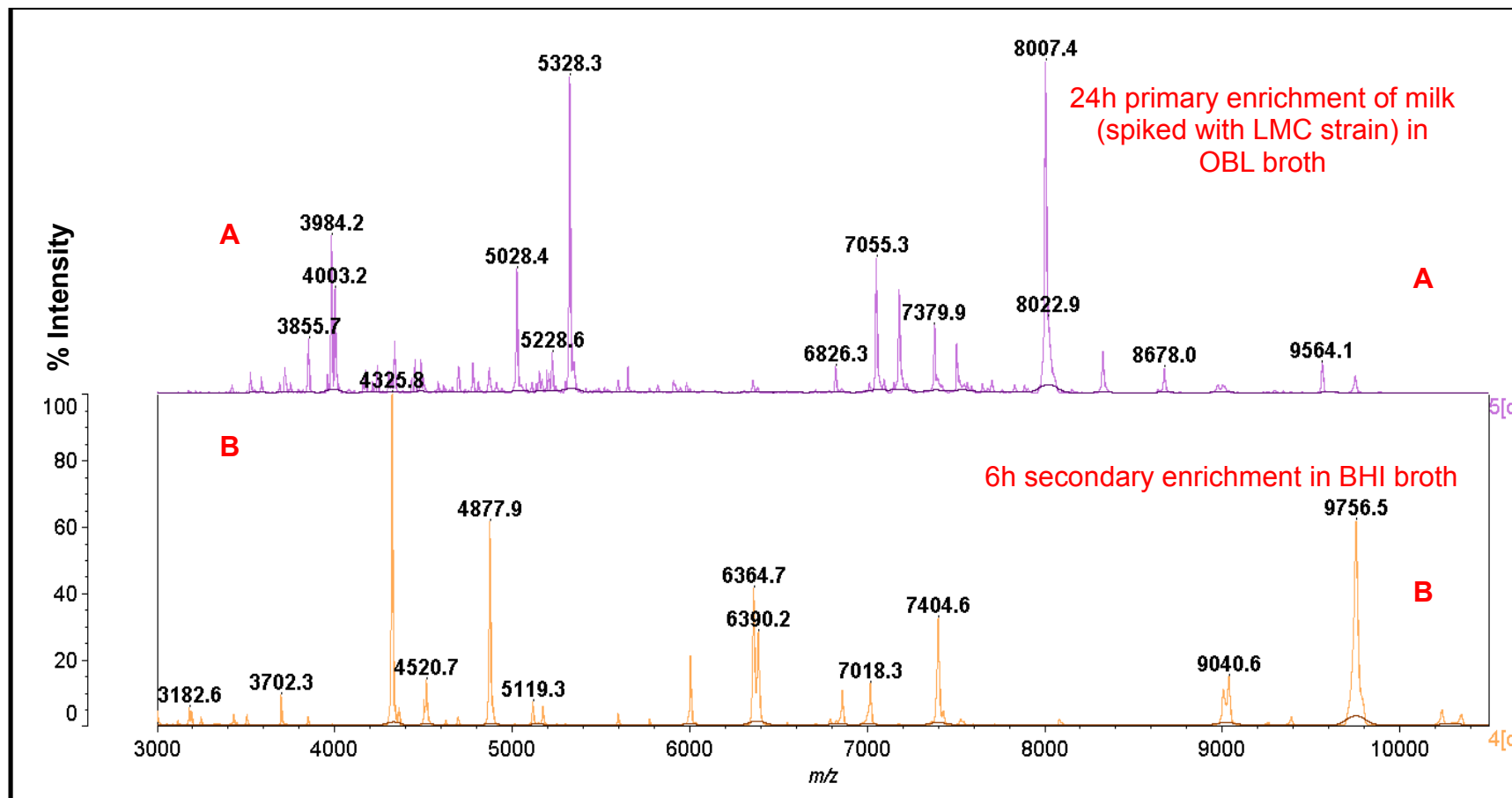
Figure 3.3 Comparison of MALDI-TOF-MS profiles of LMC in OBL broth in the presence of milk after 24 h enrichment (A), and after the 6 h secondary enrichment period in BHI (B). A was identified at genus level as *Listeria* species and B was unambiguously identified as *Listeria monocytogenes* after exporting to SARAMIS.

Table 3.6 Comparison of MALDI-TOF MS spectra obtained from OBL (after 24 h primary enrichment) and BHI (after 6 h secondary enrichment) broth to the *L. monocytogenes* SuperSpectrum in SARAMIS.

Peak No.	SARAMIS Superspectrum LM	Peak Weight	24 h primary enrichment in OBL	6 h secondary enrichment in BHI
1	3003.4	H	+	+
2	3181.2	H	+	+
3	3194.7	H	+	+
4	3223.5	L	-	-
5	3250.1	H	+	+
6	3357.9	H	+	+
7	3419.2	L	-	-
8	3430.2	H	+	+
9	3508.7	H	+	+
10	3526.9	L	+	+
11	3714.7	L	-	+
12	3795.1	H	+	+
13	3972.1	H	-	+
14	4361.9	H	-	+
15	4504.8	H	+	+
16	4519.2	H	+	+
17	4630.2	H	+	+
18	4696.1	H	+	+
19	4890.7	H	-	-
20	4944.3	L	+	-
21	5048.2	L	-	-
22	5118	H	+	+
23	5172.9	H	+	+
24	5253.7	H	+	+
25	5326.3	L	+	-
26	5398.6	H	-	-
27	5436	L	-	-
28	5460	H	+	-
29	5596.9	H	+	+
30	5701.6	H	-	+
31	5776.6	H	+	+
32	5968.1	H	+	+
33	6033.7	L	+	+
34	6170.5	H	-	-
35	6226.9	H	-	+
36	6525.2	L	-	-
37	6558.6	L	-	-

Peak No.	SARAMIS Superspectrum LM	Peak Weight	24 h primary enrichment in OBL	6 h secondary enrichment in BHI
38	7267	L	-	-
39	7299	H	-	-
40	7430.2	H	-	+
41	7541.1	H	-	-
42	7717.6	H	-	+
43	7943.3	H	-	+
44	9039.3	H	+	+
45	9260.3	H	+	+
46	10502.4	H	+	+
Number of high (and low) weightage peaks matching LM SuperSpectrum			22 (4)	28 (3)
Sum of peak weights			Lower than threshold score for positive identification	Higher than threshold score for positive identification
Identification by MALDI-TOF MS			Lsp.	LM

LM: detection as *L. monocytogenes;* Lsp: detection as *Listeria* species; H: denotes high weightage peaks (important for speciation) present in SARAMIS SuperSpectrum for *L. monocytogenes*; L: denotes low weightage peaks (less important for speciation compared to high weightage peaks) in SARAMIS SuperSpectrum for *L. monocytogenes.*

3.4.3.3 Detection of *L. monocytogenes* from selective enrichment broth containing milk spiked with a microbial mixture

Unlike UHT milk, other foods are more complex as they have their own microbiota derived from starter cultures (e.g. cheese or fermented meat products) or may be contaminated by more than one bacterium. In such samples, a single selective enrichment may not completely inhibit all background microbiota to allow *Listeria* to reach sufficiently high numbers (Nemattallah *et al.,* 2003). If these background microbiota undergo a secondary enrichment in non-selective broth (e.g. BHI), they may outgrow *Listeria* and lead to a non-reliable identification in the proposed scheme. Hence, a critical part of this study was to assess the detection scheme in the presence of other pathogenic bacteria in the model food (UHT milk) using OBL as the secondary enrichment medium. Preliminary experiments were performed by spiking the milk samples individually with a Gram positive (*S. aureus*) and Gram negative (*E. coli* O157:H7) pathogen at three different concentrations (1, 10 and 100 cfu/mL), as the presence of these pathogens is routinely tested in milk (Hill *et al.,* 2012). After the 24 h

enrichment in OBL, both bacteria were completely inhibited. MALDI-TOF MS analyses provided no identifications and plating on MacConkey agar and Baird Parker agar also failed to produce any colonies, suggesting that the bacteria were killed or inhibited to loads undetectable by culture-based methods. After successful inhibition of the bacteria, UHT milk was spiked with a mixture of LMC, *S. aureus* and *E. coli* O157:H7 strains. At this point, it was necessary to assess whether a secondary enrichment in a selective broth would be successful for achieving reliable species-level detection. To investigate the possibility of using selective broth for the 6 h secondary enrichment, an aliquot (1 mL) from the original 24 h enrichment broth was inoculated in 9 mL sterile BHI and OBL broth. After the 6 h secondary enrichment detection of *L. monocytogenes* to the species level was achieved using both OBL and BHI as the secondary enrichment media (Figure 3.4) implying that, along with reducing interfering peaks, the required cell numbers for reliable detection were also attained in OBL after the 6 h enrichment period (Table 3.8). Thus, OBL was used for all other foods to eliminate the possibility of any uninhibited microbiota outgrowing LMC during the secondary enrichment.
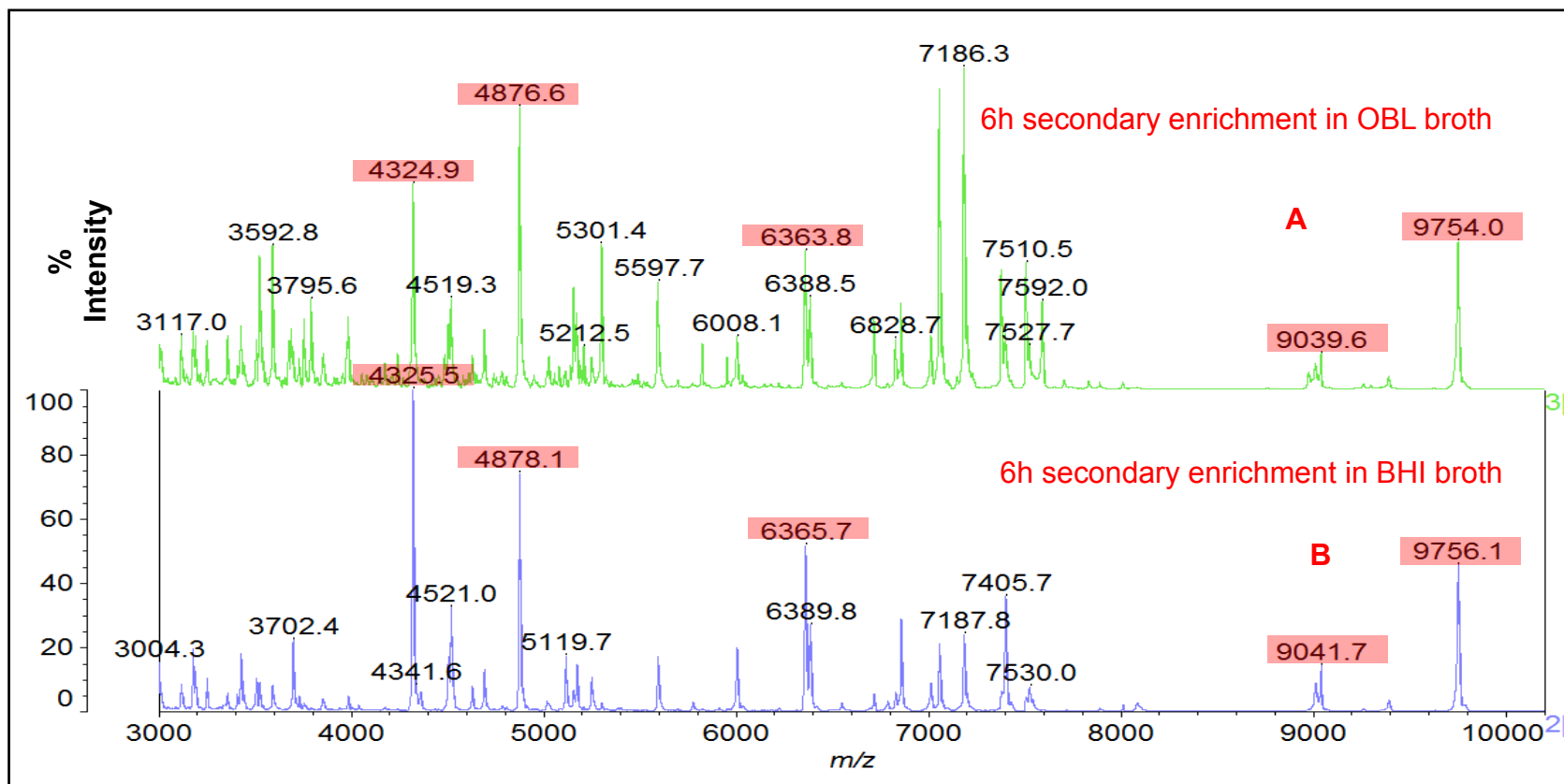
Figure 3.4 MALDI-TOF-MS profiles of LMC strain after 6 h enrichment in (A) OBL medium and (B) BHI medium. Both spectral profiles were reliably identified as *L. monocytogenes* after exporting to SARAMIS. Some of the diagnostic peaks required for identification (also present in the *L. monocytogenes* SuperSpectrum in SARAMIS) have been highlighted in the figure.

3.4.3.4 Detection of *L. monocytogenes* from spiked cantaloupe, chicken pâté and camembert cheese samples

Following the success of the detection scheme (24 h enrichment in OBL, followed by a 6 h secondary enrichment in OBL) in milk, its performance was assessed in three other solid foods. In the case of cantaloupe, detection was achieved at 1 and 10 cfu/mL levels of initial spiking, probably due to the minimal number of competing microbiota. For the foods with complex background microbiota (chicken pâté and Camembert cheese), species-level detections above the confidence level of 75 % were achieved only for initial loads of 10 cfu/mL (Table 3.7). The composition of Camembert cheese is particularly complex due to its high protein content and the use of multiple starter and secondary cultures generally including *Lactobacillus* species*, Streptococcus* species and moulds (Firmesse *et al.,* 2008). It is important to note here that although several species of *Lactobacillus* are known to produce bacteriocins (Loessner *et al.,* 2008) that can inhibit *Listeria*, the pathogen was successfully detected using MALDI-TOF MS in the current study. An interesting observation was made with one of the spiked Camembert cheese replicates where MALDI-TOF MS yielded no identification after the primary enrichment. To investigate this further, 1 mL aliquots of this broth were added to both BHI and OBL broths and a viable count was also performed. While the 6 h secondary enrichment in OBL provided a positive identification as *L. monocytogenes*, the secondary enrichment in BHI indicated mixed species identification as *Bacillus cereus and L. monocytogenes*. On observation of the BHIA plates used for performing the viable count, two types of colony morphologies were observed, small pinpoint white colonies typically seen for *L. monocytogenes* and large irregular shaped colonies generally observed for *Bacillus* species. The experiment was repeated with the remaining portion of the same cheese sample. The second replicate also provided the same results. Thus, the Camembert cheese sample may have been contaminated by a *Bacillus* species during the packaging stages or during the handling of the sample in the laboratory. These observations provide justification for the use of the selective secondary enrichment in the MALDI-TOF MS-based detection scheme as OBL broth was not able to completely inhibit the contaminant in the first 24 h enrichment. Nevertheless, MALDI-TOF MS was able to identify the presence of two microbes in the sample.

Table 3.7 Inoculum levels required for reliable identification of *L. monocytogenes* from spiked foods using MALDI-TOF MS after 6 h secondary enrichment in OBL medium

Food	Strain/s used for spiking	Spiking load (per mL of initial selective enrichment broth) required for MALDI-TOF MS detection
UHT milk	LMC + *S. aureus* (ATCC 12600) + *E. coli* O157:H7 (ATCC 43895)	1 cfu : 1 cfu : 1 cfu
Cantaloupe	LMC	1cfu; 10 cfu
Chicken pâté	LMC	10 cfu
Camembert cheese	LMC	10 cfu

3.5 Conclusions

The advent of MALDI-TOF MS has significantly transformed microbial identification in the clinical laboratory. However, its exploration in food microbiology laboratories remains restricted. The current chapter assessed the ability of MALDI-TOF MS to detect *L. monocytogenes,* a serious foodborne pathogen, from solid culture media and from selective enrichment broths containing spiked foods. This is the first study that has investigated the influence of culture conditions (culture media and age) on identification of *L. monocytogenes* and its detection directly from broth culture.

Overall, it was observed that species-level identifications seemed to be more affected by changes in culture conditions than genus-level identifications. Incubation time of 24 h was observed to be appropriate for obtaining better identification results for all media tested except PA. Barbuddhe *et al.* (2008) and Hsueh *et al.* (2014) used the extraction technique for detecting *L. monocytogenes* on the Bruker system and found 100 % and 90 % species-level typeability, respectively. In the current study, 80 % or more identifications to the species level were obtained (for ALOA, HBA and BHIA) using the bioMérieux system and a simple direct smear technique which can prove to be more time-effective in a high-throughput laboratory. Updating of the commercial databases with diverse spectra acquired under different culture conditions will increase the robustness of this technology.

In recent times, MALDI-TOF MS has been used extensively for detecting bloodborne pathogens in clinical settings (Kok *et al.,* 2011; Buchan *et al.,* 2012; Juiz *et al.,* 2012). Unlike blood cultures which usually contain single pathogens, detection of foodborne pathogens can be particularly challenging due to the complexity of food matrices and the likely presence of multiple bacteria in a single sample. Thus, selective enrichment is essential for pathogens like *Listeria* which are found in low numbers in food samples (Cornu *et al.,* 2002).

The current chapter provides proof-of-concept evidence that detection of *L. monocytogenes* from selective enrichment broth containing a variety of foods, including protein-rich food matrices (milk, cheese and meat), is possible with MALDI-TOF MS using the currently available SARAMIS database. Detection can be achieved more

rapidly than conventional methods with minimal sample processing. However, food samples can be very diverse and may contain components that can interfere with the MALDI-TOF MS analysis. Hence, more detailed investigations with different media and foods may be required to determine the actual limit of microbial detection in these foods. An optimised and more extensive database and algorithm for detecting *L. monocytogenes* in selective enrichment broth will help improve detection in naturally-contaminated food samples. Nonetheless, the current detection scheme from selective enrichment broths shows considerable promise for further exploration. Although the proposed methodology proved successful in a variety of foods, it should be highlighted that the robustness of the method will ultimately be determined by the ability of the selective enrichment media to completely inhibit the background microbiota so that *L. monocytogenes* can reach the required levels for detection.

The use of MALDI-TOF MS for bacterial identification from selective enrichment broth will reduce the overall costs involved in food testing as the same strategy could be used for other foodborne pathogens. Although the initial infrastructure investment for MALDI-TOF MS is high, the running and reagent costs are minimal (Drancourt *et al.,* 2010). Considering that fast-growing pathogens may require a shorter enrichment period compared to *Listeria*, this will enable even more rapid detection than reported in this chapter.

Chapter 4

Rapid source-tracking of *Listeria monocytogenes* using MALDI-TOF MS

Abstract

Stringent regulations associated with the presence of *L. monocytogenes* in foods result in most regulatory bodies mandating the continuous monitoring of this pathogen in certain food processing environments. The food industry requires simple and reliable subtyping techniques for tracing the sources of contamination in the processing environment in a timely manner. In this chapter, the application of MALDI-TOF MS as a source-tracking tool was explored using a collection of *L. monocytogenes* isolates obtained predominantly from dairy sources within Australia. The isolates were cultured on different growth media for 24 and 48 h prior to MALDI-TOF MS analysis. Chemometric statistical analysis of the MALDI-TOF MS data enabled source-tracking of *L. monocytogenes* isolates obtained from four different dairy sources. Comparable to the species level identification described in Chapter 3, strain level discrimination was also observed to be influenced by culture conditions. In addition, t-test/Analysis of Variance (ANOVA) was used to identify biomarker peaks that differentiated the isolates according to their source of isolation. Source-tracking using MALDI-TOF MS was compared to the gold standard pulsed-field gel electrophoresis (PFGE) technique. The discriminatory index and the congruence between both techniques were compared using the Simpsons Diversity Index, adjusted Rand and Wallace coefficients. Overall, MALDI-TOF MS-based source-tracking (using data obtained by culturing the isolates on horse blood agar) and PFGE demonstrated good congruence with a Wallace coefficient of 0.71 and comparable discriminatory indices of 0.89 and 0.86 respectively. MALDI-TOF MS thus represents a rapid and simple source-tracking technique for *L. monocytogenes*.

4.1 Introduction

Consumption of contaminated foods that include dairy, meat, seafood, poultry and fresh produce have been the major cause of almost all listeriosis outbreaks and sporadic incidents (Alessandria *et al.,* 2010). As discussed in Chapter 1 (refer section 1.7.1), based on the correlation of serotyping and ribotyping, *L. monocytogenes* can be classified into three lineages: lineage I comprising serotypes 1/2b, 3b, 4b, 4d and 4e; lineage II comprising serotypes 1/2a, 1/2c, 3a and 3c; and lineage III comprising serotypes 4a, 4b and 4c. The application of multilocus genotyping identified a distinct subset of divergent lineage III isolates, which have been classified as a separate lineage, lineage IV (Ward *et al.,* 2008). Many countries have a zero tolerance policy towards the presence of *L. monocytogenes* in certain high risk foods (Gasanov *et al.,* 2005) and mandate the requirement for hazard analysis and critical control point (HACCP) programs to periodically check the contamination levels of this bacterium in the processing facilities (Blatter *et al.,* 2010). Although pathogen detection is the preliminary stage of identifying problem areas in a food processing environment, strain level differentiation and subtyping is crucial in some situations. Real-time monitoring of foodborne pathogens requires rapid and reliable techniques to source-track the contamination occuring in processing plants. Techniques currently employed for source-tracking such as pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) or multilocus variable number tandem repeat analysis (MLVA) are highly discriminatory; however they are technically demanding, laborious, time consuming and expensive (Sandrin *et al*., 2012). Particularly in the case of *Listeria* detection in foods, industry requires timely information to avoid product recalls or delays in product release. In general, the ubiquitous nature of this pathogen and its ability to form biofilms makes the management of this pathogen a relentless challenge to the food industry globally, underscoring the need for rapid, reliable, robust and cost-effective source-tracking techniques for *L. monocytogenes.*

Considering the rapid turnaround time associated with MALDI-TOF MS analysis, it represents an attractive 'universal' detection and subtyping tool for multiple pathogens. Previous studies have explored the subtyping ability of MALDI-TOF MS for *Streptococcus pneumoniae* (Williamson *et al.,* 2008), *L. monocytogenes* (Barbuddhe *et*

al., 2008; Hsueh *et al.,* 2014), *Streptococcus agalactia* (Lartigue *et al.,* 2009), *S. aureus* (Wolters *et al.,* 2011; Bőhme *et al.,* 2012; Wang *et al.,* 2013)and *Cronobacter* species (Lu *et al.,* 2014).   Most of the above-mentioned studies have mainly focussed on MALDI-TOF MS as a tool for subtyping isolates during epidemiological or clinical investigations. In contrast, Siegrist *et al*. (2007) successfully used MALDI-TOF MS to classify environmental isolates of *E. coli* according to their origin.

Chapter 3 explored the ability of MALDI-TOF MS to detect *L. monocytogenes* isolates directly from selective enrichment broths and from different culture media. In the current study, the application of MALDI-TOF MS as a source-tracking tool for *L. monocytogenes* isolates obtained from different dairy and non-dairy sources within Australia was evaluated. Since changes in culture conditions are known to affect strain level typing ability of MALDI-TOF MS (Anderson *et al*., 2012; Sandrin *et al*., 2012; Goldstein *et al*., 2013; Šedo *et al*., 2011), the isolates were cultured on five different media including non-selective, selective and chromogenic and analysed after two incubation periods (24 and 48 h). To investigate the discriminatory power of MALDI-TOF MS, isolates were also subtyped using PFGE and repetitive element PCR and the correlations between the typing methods were explored.

4.2 Chapter Aims

The major aims of this chapter were to:

a. Assess the ability of MALDI-TOF MS to source-track a collection of *L. monocytogenes* isolates obtained from different dairy sources within Australia

b. Optimise the data analysis technique for finding strain level differences

c. Identify potential biomarker peaks that help distinguish between the strains

d. Compare the source-tracking ability of MALDI-TOF MS with the gold standard PFGE technique

4.3 Materials and Methods

4.3.1 Bacterial strains and culture media

The collection of *L. monocytogenes* isolates detailed in Chapter 3 was used in this study (Table 3.1). The collection constitutes dairy and non-dairy isolates obtained from different sources within Australia, and isolates from unspecified sources that were provided by collaborating laboratories. Since strain level differentiation using MALDI-TOF MS is hypothesised to vary with changes in culture conditions (Sandrin *et al.,* 2012), each isolate was cultured on five culture media, brain heart infusion agar (BHIA), horse blood agar (HBA), Oxford agar (OA), Palcam agar (PA) and Agar *Listeria* Ottaviani and Agosti (ALOA) at 37 °C, and the cultures were analysed after 24 and 48 h of incubation. All media were purchased from Micromedia, Australia. *E. coli* (ATCC) 8739 cultured on BHIA was used as a calibrant for MALDI-TOF MS analysis and *Salmonella* Braenderup strain (H9812) cultured on BHIA was used as a size standard for PFGE analysis (described in section 4.3.6).

4.3.2 Molecular serotyping of *L. monocytogenes* isolates

The isolates selected for the source-tracking study were serotyped using a multiplex PCR assay by Doumith *et al.* (2004). This assay can differentiate the four most common disease-causing serotypes of *L. monocytogenes* (i.e. 1/2a, 1/2b, 1/2c and 4b) into five distinctive subgroups: subgroup A (containing isolates belonging to serotypes 1/2a and 3a), subgroup B (containing isolates belonging to serotypes 1/2b, 3b and 7), subgroup C (containing isolates belonging to serotypes 1/2c and 3c), subgroup D (containing isolates belonging to serotype 4b, 4d and 4e) and subgroup E (containing isolates belonging to serotypes 4a and 4c) (Chapter 2, section 2.9).

4.3.3 Source-tracking of *L. monocytogenes* isolates using MALDI-TOF MS

Source-tracking using MALDI-TOF MS was performed using the Axima Performance Mass Spectrometer (Shimadzu Scientific Instruments, USA). Briefly, each isolate was spotted in quadruplicate using the direct smear technique (Chapter 2, section 2.10). Thus four peak lists (2-20 kDa) generated in SARAMIS were used for further analysis.

4.3.4 Data analysis using SPECLUST

For subtyping the isolates using MALDI-TOF MS, the four replicate peak lists obtained for each isolate were initially analysed using the freely available software SPECLUST (Alm *et al.,* 2006; http://bioinfo.thep.lu.se/speclust.html). SPECLUST calculates mass differences between two peaks from different peak lists to determine if the peaks match by considering a measurement uncertainty (σ) and a peak matching score (s). For every isolate, a consensus peak list containing peaks present in three out of four replicate spectra was generated using SPECLUST. Following this, the consensus peak lists for all isolates were clustered using the 'clustering' tool in SPECLUST. The average linkage method of clustering was used in the clustering tool to create a cluster for each peak list and then measuring the distance between different clusters.

4.3.5 Data analysis using SIMCA 13.0

Since the basis for differentiating strains using SPECLUST relies only on peak masses, different data analysis software, SIMCA (version 13.0, Umetrics) was adopted. SIMCA is a chemometric software package in which peak masses and their intensities for each isolate are statistically compared to find the discriminatory features that can separate the isolates into separate classes (Xia *et al.,* 2009). To perform this analysis, a consensus peak list containing peak masses and corresponding intensities present in three out of the four replicate spectra was generated for each isolate using SARAMIS. These consensus peak lists were further analysed using the multivariate data analysis tools of SIMCA (http://www.umetrics.com/products/simca).

Data analysis using the consensus peak lists for each isolate was initially performed with Principal Component Analysis (PCA), which is an unsupervised approach to find differences between datasets. PCA was followed by partial least square discriminant analysis (PLS-DA). Unlike PCA, PLS-DA is a supervised model that is a regression extension of PCA which relies on class information for better separation between groups and, hence, is not independent in finding the natural clusters between groups (Xia *et al.,* 2009). To determine the validity of the PLS-DA models, R^2X, R^2Y and Q^2 values were considered, where R^2X and R^2Y define the variation in X and Y variables in the sample set and Q^2 gives the predictability of the model (Azizan *et al.,* 2012).

Hierarchical cluster analysis (using Wards clustering algorithm) was performed with the PLS-DA data to generate dendograms displaying the clustering of isolates from the four dairy sources.

A separate PLS-DA analysis with the data obtained from isolates cultured on HBA (post 24 h incubation) was also performed to investigate whether MALDI-TOF MS spectra can be used for separating the isolates based on their serotypes. For identification of statistically significant biomarker peaks that help in discriminating isolates into separate clusters based on their source of isolation or serotype distribution, the data analysed using SIMCA were reanalysed by an independent metabolomics data analysis software package, MetaboAnalyst, version 2.0 (Xia *et al.,* 2012; http://www.metaboanalyst.ca/MetaboAnalyst/).

4.3.6 Source-tracking of *L. monocytogenes* isolates using PFGE

The ability of MALDI-TOF MS to source track the dairy isolates was compared with the gold standard PFGE technique. PFGE was performed using the standardised PulseNet protocol (PulseNet, 2013). All reagents/enzymes required were purchased from Sigma Aldrich (Australia) unless otherwise stated. Isolates were cultured on BHIA overnight at 37 °C. A standardised cell suspension of optical density 0.8-1.0 at 610 nm in sterile Tris-EDTA buffer was lysed with 20 mg/mL lysozyme and incubated at 55 °C for 40 min. After incubation, 20 mg/mL proteinase K was added to the 400 µL suspension and mixed gently with a pipette. To this solution, 400 µL of 1 % SeaKem Gold agarose was added and mixed gently. The suspension was dispensed into plug moulds and was allowed to solidify at room temperature for 15 min. The solid plugs were transferred from the plug moulds into 5 mL cell lysis buffer and were lysed for 2 h in an orbital shaker incubator at 54-55 °C. Subsequently, the plugs were washed with pre-warmed sterile water (twice) and TE buffer (four times). The genomic DNA in the plug was digested using two restriction enzymes, namely 25 U of *Asc*I, 37 °C for 2 h (New England Biolabs) and 25 U of *Apa*I, 25 °C overnight (New England Biolabs).

Salmonella serotype Braenderup H9812 strain was used as the size standard for the PFGE experiment (Hunter *et al.,* 2005). The *Salmonella* plugs were prepared and digested with 50 U of *Xba*I restriction enzyme (New England Biolabs, at 37 °C for 2 h),

according to the standard PulseNet procedure (PulseNet, 2013). The procedure used for plug preparation was very similar to that used for *L. monocytogenes* except that the standardised cell suspensions in TE buffer were only treated with proteinase K (20 mg/mL) without an extra treatment with lysozyme. The digested plugs for *L. monocytogenes* isolates and *Salmonella* ser. Braenderup H9812 strain were loaded into a 1 % SeaKem Gold Agarose gel in 0.5X Tris Borate EDTA buffer supplemented with 50 µM thiourea solutions to prevent DNA degradation during electrophoresis. The plugs were electrophoresed for 21 h in a CHEF Mapper instrument using initial switch time of 4.0 sec, final switch time of 40 sec, 6 V voltage at an included angle of 120 °. The gels were observed by staining with GelRed (30 µg/µL) for 30 min. Cluster analysis was performed with BioNumerics software (version 6.5, Applied Maths) by using the UPGMA (unweighted pair group) method and by calculating the Dice coefficient with optimisation and tolerance settings of 1 % (http://www.applied-maths.com/bionumerics).

4.3.7 Comparison of MALDI-TOF MS-based source-tracking and PFGE

To compare the discriminatory power of MALDI-TOF MS-based and PFGE-based source-tracking techniques, Simpson's Index of Discrimination (SID, with 95% confidence intervals) was calculated as per the method described by Hunter and Gaston (1988). The congruence between the subtyping techniques was determined by calculating the adjusted Rand coefficient which gives an estimation of the agreement between the techniques and the Wallace coefficient, which indicates if the clustering defined by one technique can be used to predict that defined by another technique (Waters *et al.,* 2012). All calculations were performed using the Comparing Partitions online tool (Carriço *et al.,* 2006; http://darwin.phyloviz.net/ComparingPartitions/).

The formulae for Simpson's index of diversity for a partition A (SID_A) and confidence interval ($CI_{95\%}$) calculation are:

$$SID_A = 1 - \frac{1}{N(N-1)} \sum_{i=1}^{S} n_i (n_i - 1) \qquad \text{... Equation 4.1}$$

$$CI_{95\%} = SID_A \pm 2 \times \sqrt{var(SID_A)} \qquad \text{... Equation 4.2}$$

where N denotes the total number of isolates, S is the total number of clusters formed and n_i is the number of isolates belonging to the individual clusters.

The Adjusted Rand co-efficeint is given by the equation,

$$Adjusted\ Rand = \frac{a+d-n_c}{a+b+c+d-n_c} \qquad \ldots \text{Equation 4.3}$$

Where a, b, c and d are individual entries in a mismatch matrix (created for comparing two typing methods) and n_c is calculated as,

$$n_c = \frac{N(N^2+1)-(N+1)\sum n_i^2-(N+1)\sum n_j^2+\sum\sum\frac{n_{ij}^2}{N}}{2(N-1)} \qquad \ldots \text{Equation 4.4}$$

Where N denotes the total number of isolates, n_i is the number of isolates belonging to cluster i and n_j is the number of isolates belonging to cluster j.

The Wallace co-efficeint is given by the equation

$$W_{A \to B} = \frac{a}{a+b} \qquad W_{B \to A} = \frac{a}{a+c} \qquad\qquad \ldots \text{Equation 4.5}$$

Where a, b, c, d are the entries in the mismatch matrix.

4.4 Results and Discussion

Several studies have found MALDI-TOF MS to be a reliable and rapid tool for the identification of bacteria to the species level; however, few studies have explored its ability to subtype bacteria at the strain level (Sandrin *et al.,* 2012). Whilst some studies focus on using MALDI-TOF MS to find biomarkers that differentiate or identify individual strains, the current study focussed on discriminating the isolates based on their source of isolation. The source-tracking ability of MALDI-TOF MS was compared to the routinely used PFGE technique and the ERIC PCR technique.

4.4.1 Molecular serotyping

L. monocytogenes isolates were serotyped using the molecular serotyping assay by Doumith *et al.* (2004). The isolates were classified into four subgroups based on their genetic differences; subgroup A (containing isolates belonging to serotypes 1/2a or 3a), subgroup B (containing isolates belonging to serotypes 1/2b, 3b or 7), subgroup C (containing isolates belonging to serotype 1/2c and 3c) or subgroup D (containing isolates belonging to serotype 4b, 4d and 4e) (Table 4.1). No subgroup E isolates were found in this sample set. All *L. monocytogenes* isolates amplified the *prs* gene fragment (370 bp), since this was a genus specific marker. The *lmo0737* gene fragment (691 bp) was amplified from subgroup A isolates (e.g. isolate 745), the *ORF2819* gene fragment (471 bp) was amplified from subgroup B isolates (e.g. isolate 702), both *lmo0737* and *lmo1118* (906 bp) gene fragments were amplified from subgroup C isolates (e.g. isolate 058), and both *ORF2819* and *ORF2110* (597 bp) gene fragments were amplified from subgroup D isolates (e.g. isolate LMC). Figure 4.1 shows the serotyping results obtained for selected *L. monocytogenes* isolates from the collection.
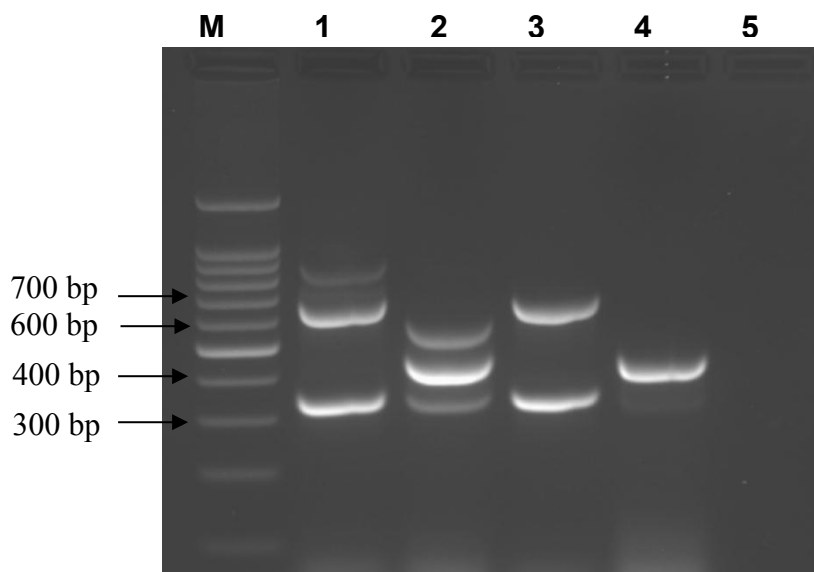
Figure 4.1 Agarose gel (1%) depicting molecular serotyping of *L. monocytogenes* isolates representing different subgroups. Lane M: molecular weight marker (100 bp ladder Promega, Australia), Lane 1: Isolate 058 (amplification of *prs, lmo0737* and *lmo118* gene fragments) Lane 2: Isolate LMC, Reference isolate LMC from Australian culture collection of microbes (amplification of *prs, ORF2819* and *ORF2110* gene fragment); Lane 3: Isolate 745 (amplification of *prs* and *lmo0737* gene fragment); Lane 4: Isolate 702 (amplification of *prs* and *ORF2819* gene fragment), Lane 5: negative control (DNA from a lab strain of *E. coli*).

4.4.2 MALDI-TOF MS-based source-tracking

MALDI-TOF MS can be performed using two techniques – direct spotting (where an individual colony is picked from a culture media plate using a toothpick, swab or disposable loop and placed on a MALDI target slide) or extraction (which involves a simple formic acid-acetonitrile treatment for cell wall lysis and protein precipitation). The latter is often used for microbes with a more complex cell wall (Anderson *et al.,* 2012). In the current study, the direct spotting technique was used, keeping in mind the usability and time constraints of high-throughput food laboratories and the recommendations of the manufacturer of the system used in this investigation (bioMérieux).

Contrary to species level identifications, strain level discrimination using MALDI-TOF MS is expected to be influenced by minor changes in the mass spectra (Kern *et al.,*

2014). Thus it is important to investigate the effect of culture conditions when considering the adoption of this technology for such applications. Since some variation between replicates is common in MALDI-TOF MS analysis, each isolate was spotted in quadruplicate. Unlike PFGE, that differentiates strains based on the differences in banding patterns following gel electrophoresis of enzymatically restricted genomic DNA (10-20 bands in general), the data obtained from MALDI-TOF MS consist of extensive peak lists that contain numerous peaks with relative intensities. Hence, the data analysis tool employed will greatly influence the quality of typing information obtained. Thus, the application of two data analysis tools to source-track the isolates was investigated.

Analysis using SPECLUST

The data obtained for the *L. monocytogenes* isolates using MALDI-TOF MS were first analysed using SPECLUST. Overall, it was observed that culture media had a profound effect on the subtyping of *L. monocytogenes,* using since the dendograms generated using different media and culture conditions clustered the isolates differently. Figures 4.2 A and 4.2 B depict the dendograms derived from the data generated by culturing the isolates on ALOA agar for 24 and 48 h. None of the data generated from any single media at either time of incubation was able to show consistent grouping of isolates obtained from different sources. Dendograms derived from data obtained by culturing the isolates on other media are shown in the Appendix (Figures A 4.1 to A 4.4). Peak lists obtained from some media (e.g. HBA) were more extensive than those obtained from other media. Since SPECLUST considers only on the presence of differentiating peaks in the peak lists, the presence of many peaks in the spectra may be the reason for the analysis being too discriminatory. A probable solution to this would be to filter the peak lists based on intensity. Here an attempt was made to reduce the peak lists by applying a 2 % intensity filter. However, the consensus peak lists were reduced to only about 20-30 peaks on average. Since the study focuses on strain level typing of a pathogen that is known to have high clonal complexes, it is important that the data analysis does not reduce the peak lists to a point where strain level differences are lost. Thus, this approach was not adopted for further analysis.

SPECLUST has been used in different studies to study the inter-relatedness between isolates belonging to the same and/or different species (Böhme *et al.,* 2010; Böhme *et al.,* 2012; Qunitela-Baluja *et al.,* 2013). In most of these studies, the authors compared the proteomic analysis with 16S rRNA gene based phylogentic analysis and found good congruence between the two analyses. However, it is important to consider that phylogenetic analysis may not always be sufficiently discriminatory due to high sequence similarity between certain species of bacteria.
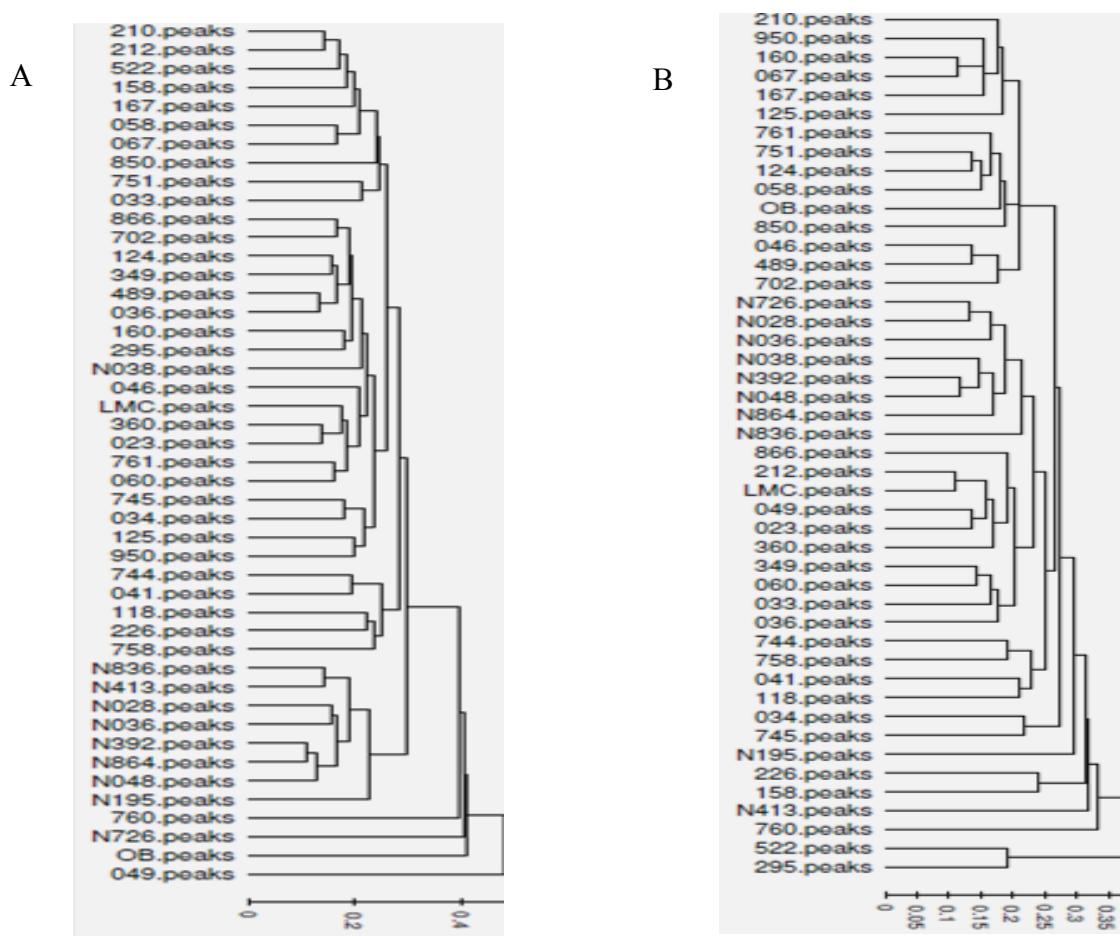
Figure 4.2 Cluster analyses of MALDI-TOF mass spectra of *Listeria monocytogenes* cultured on ALOA agar for (A) 24 hours and (B) 48 hours.

Analysis using SIMCA 13.0

Since SPECLUST relies solely on peak masses, full peak lists (2 to 20 kDa) were examined using SIMCA (version 13.1, Umetrics, Sweden). Initially, PCA was

performed for the data obtained from the five culture media at two different time intervals; however, the PCA models had low predictability values (Q^2 values less than 0.5) and could not discriminate between the isolates according to their source of isolation. Hence, PLS-DA analysis was performed, which required additional knowledge about the sample classes to differentiate between groups. Since strain typing studies deal with fully characterised bacteria, providing such knowledge is feasible. Hierarchical cluster analysis was performed after performing PLS-DA and dendograms were obtained. Similar to the PCA analysis, the PLS-DA models also had very low predictability values (Appendix Figures S 4.5 to S 5.4). Notably, culture media and the age of the culture were found to greatly affect strain level typing. The dendograms generated from different media or same media, albeit at different incubation times, did not cluster the isolates in a consistent manner. For almost all dendograms, Source 1 isolates were distributed throughout, suggesting that this environment was probably facing contamination by multiple strains of *L. monocytogenes*. Source 1 was a dairy site facing persistent *L. monocytogenes* contamination for two years. Overall, isolates from different sources were clustered together which highlighted the similarity between these isolates.

A major limitation of this study was that not all isolates in the dataset were well-defined. Many isolates were obtained from unspecified sources, including non-dairy sources. Thus, it was difficult to interpret any relationships between the isolates from defined and unspecified sources. Hence, a separate analysis was performed for only the dairy isolates obtained from four well-defined sources. Although this reduced the dataset to 23 isolates (Table 4.1), complete information about the strains was available which made it feasible to perform the multivariate data analysis. After performing the PCA and PLS-DA analysis for the new dataset, the dendograms were able to separate the isolates based on their sources with some minor discrepancies (Appendix Figures A 5.5 to A 6.3). Figure 4.3 illustrates the PCA plot and dendogram generated from PLS-DA analysis of the data obtained by culturing the isolates on HBA (24 h). As observed in the PCA plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The isolates from different sources were separated into different clusters in Figure 4.3 (B) which will be referred to as 'MALDI types' henceforth. Seven MALDI types (A-G) were identified in the dendogram, from which two MALDI

types (F and G) were shared between Sources 1 and 2. Similar to the PFGE analysis (section 4.4.2), Source 1 isolates were distributed throughout the dendogram, suggesting that this environment was probably facing contamination by multiple strains. In contrast, Source 3 and Source 4 isolates formed discrete clusters. Overall, all the PLS-DA models generated from the data obtained from other culture conditions had acceptable $R^2 X$, $R^2 Y$ and Q^2 values (values > 0.5 suggesting statistical significance) except for data from Palcam agar that had Q^2 values < 0.5 (Appendix Figures A 6.2 and A 6.3). However, consistent with the previous results, the clustering observed was not similar in all dendograms. Since the clustering varied with changes in culture conditions, standardisation of culture conditions was considered to be very important for effective subtyping using MALDI-TOF MS.

Table 4.1: *L. monocytogenes* isolates source-tracked using MALDI-TOF MS and PFGE

No.	Isolate number	Origin	Dairy Source	Clonal lineage according to molecular serotyping
1	023	Dairy (Product)	1	Lineage I
2	226	Dairy (Product)	1	Lineage I
3	360	Dairy (Product)	1	Lineage I
4	489	Dairy (Product)	1	Lineage II
5	210	Dairy (Environmental swab)	1	Lineage I
6	212	Dairy (Environmental swab)	1	Lineage I
7	744	Dairy (Environmental swab)	1	Lineage I
8	745	Dairy (Environmental swab)	1	Lineage II
9	751	Dairy (Environmental swab)	1	Lineage II
10	758	Dairy (Environmental swab)	1	Lineage I
11	760	Dairy (Environmental swab)	1	Lineage I
12	761	Dairy (Environmental swab)	1	Lineage II
13	702	Dairy (Product)	2	Lineage I
14	850	Dairy (Product)	2	Lineage I
15	866	Dairy (Product)	2	Lineage I
16	160	Dairy (Product)	3	Lineage I
17	349	Dairy (Product)	3	Lineage II
18	522	Dairy (Product)	3	Lineage II
19	950	Dairy (Product)	3	Lineage II
20	N028	Dairy (Product)	4	Lineage I
21	N036	Dairy (Environmental swab)	4	Lineage I
22	N038	Dairy (Environmental swab)	4	Lineage I
23	N048	Dairy (Environmental swab)	4	Lineage I
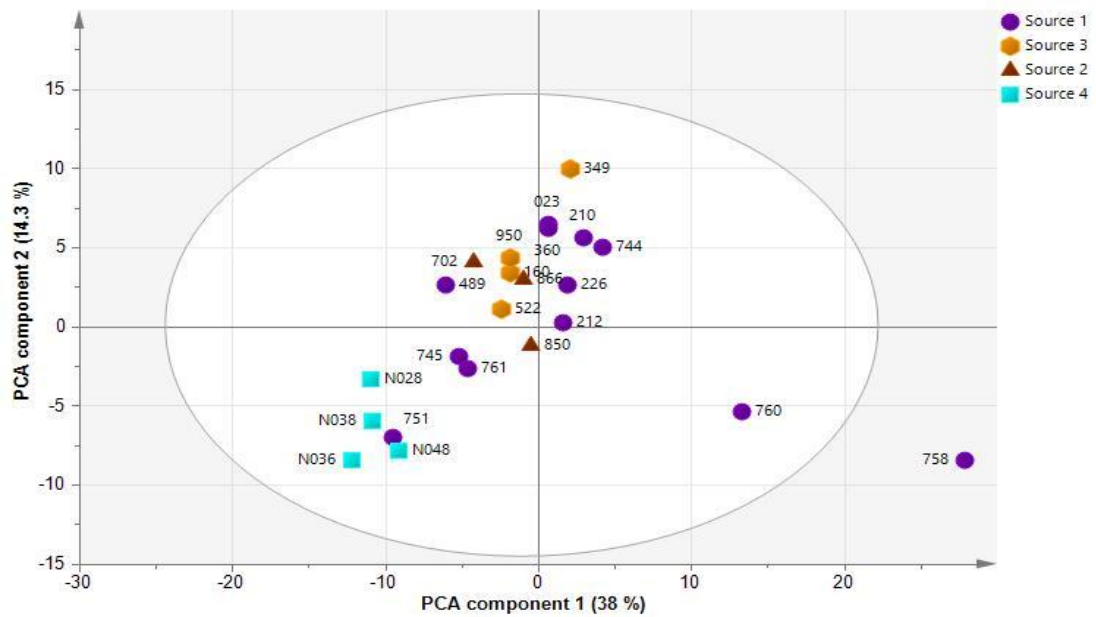
Figure 4.3 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on HBA agar, 24 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.52 and Q^2 (cumulative) = 0.20. The tolerance eclipse represents the 95% confidence interval limit and since observation 758 lies outside this eclipse, it is considered as an outlier.
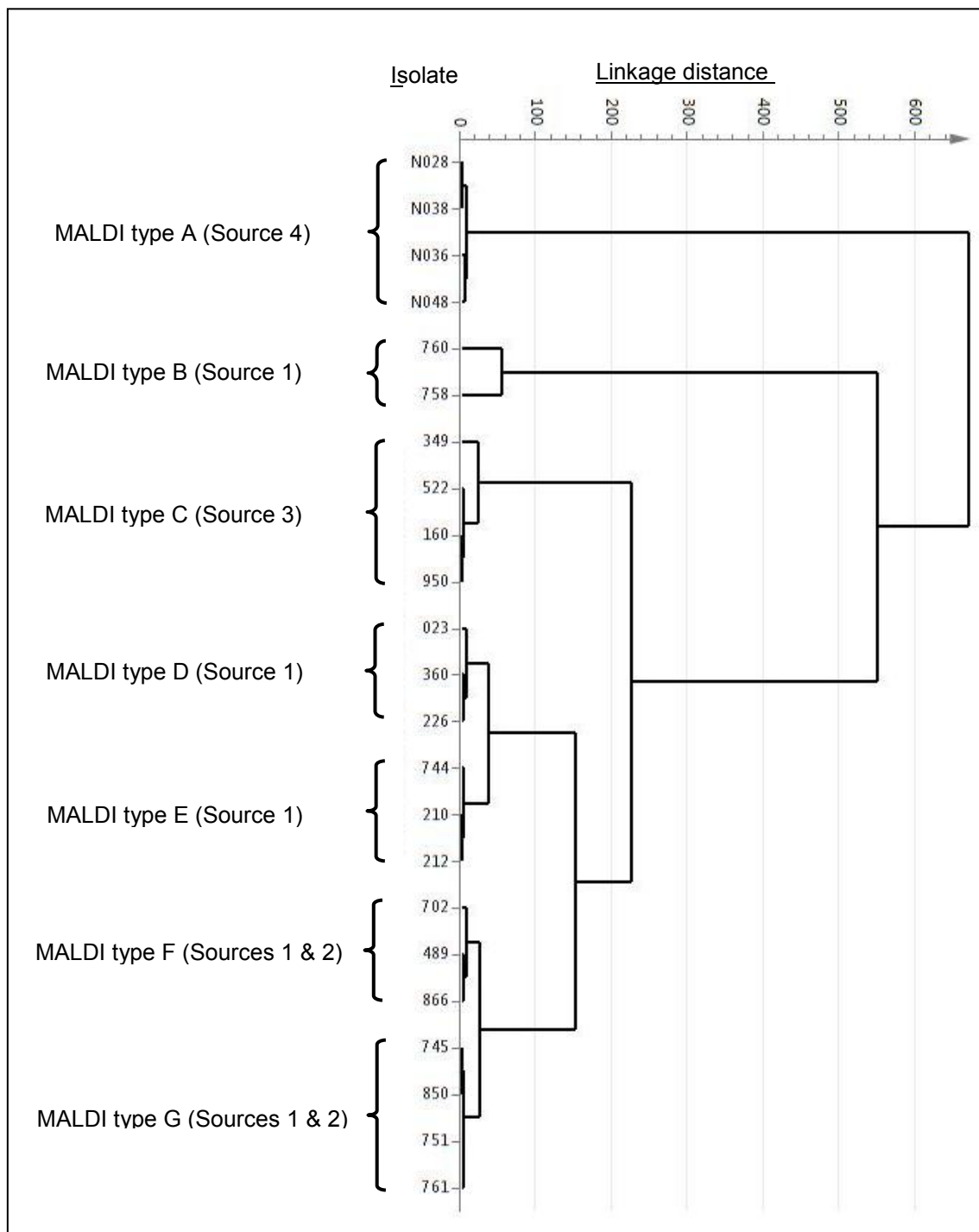
Figure 4.3 (B): The PLS-DA analysis plot generated from HBA (24 h) yielded R^2X, R^2Y and Q2 values of 0.76, 0.95 and 0.58, respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated from it.

4.4.2 PFGE based source-tracking

PFGE is a highly discriminatory 'gold standard' technique that has been used in several epidemiological and source-tracking investigations for *L. monocytogenes* (Fox *et al.,* 2011a). In the current study, the 23 isolates that were subtyped using MALDI-TOF MS (Table 4.2) were also subtyped using PFGE. Two restriction enzymes, *Asc*I and *Apa*I, were used since a combination of two enzymes is known to provide improved discrimination, and identify closely-related subtypes (Fox *et al.,* 2011b). Isolates from all four dairy sources were divided among six pulsotypes of *L. monocytogenes* (Figure 4.4). Based on the PFGE patterns and molecular serotyping (Table 4.2), two lineages were observed in Figure 4.4: lineage I (containing 1/2b or 3b serotype isolates) and lineage II (containing 1/2a or 3a serotype isolates). The isolates obtained from Source 1 (dairy site experiencing persistent contamination) included three different pulsotypes (1, 4 and 6). This indicated that, although the site was experiencing persistent contamination, more than one strain was involved. Interestingly, isolates 023, 226 and 360 (pulsotype 4) were product detections and clustered separately from the other isolates of Source 1, suggesting that the product and the processing environment may have been contaminated with different strains. In contrast, isolate 489, also obtained from product at a different time of sampling, clustered with the environmental isolates, indicating a possible transmission route from product to environment, or vice versa. Isolate 160 from Source 3 clustered with isolates from Source 1 (pulsotype 6). Unlike the other three isolates from Source 3, isolate 160 belonged to lineage I (serotype 1/2b or 3b), and clustered away from the remaining isolates of Source 3, which belonged to lineage II. This grouping of pulsotype 6 isolates was the only instance of a pulsotype identified at multiple dairy processing sites, suggesting these sites may be colonised by the same strain (or a closely-related clone) of *L. monocytogenes.* Overall, although PFGE proved to be sufficiently discriminatory, it proved to be a technically demanding and laborious process.
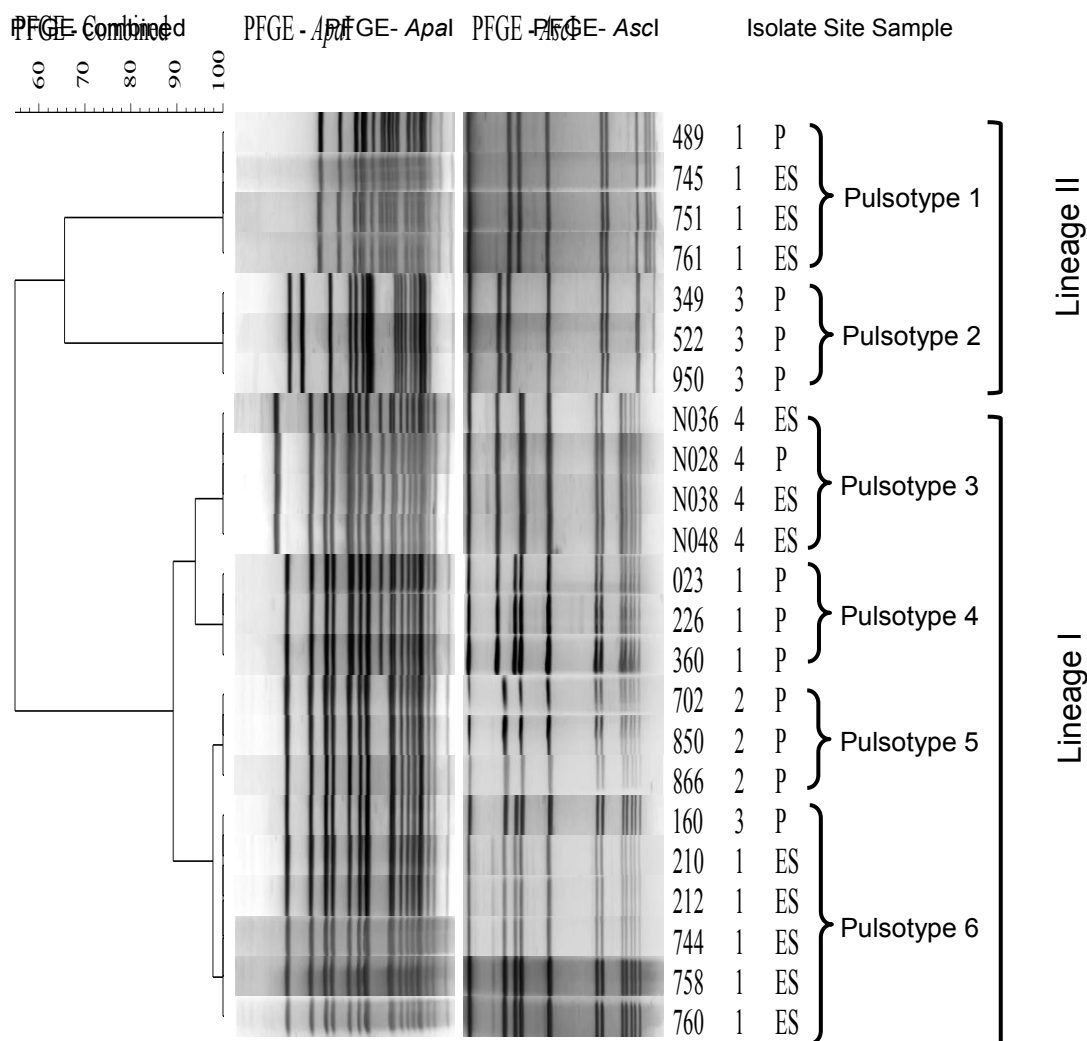
Figure 4.4 Dendogram showing PFGE analysis of the isolates used in this study obtained by using two restriction enzymes (*Asc*I and *Apa*I).

4.4.3 Congruence between MALDI-TOF MS-based subtyping and PFGE

Since MALDI based source-tracking was found to be affected by culture conditions, congruence between the two source-tracking techniques was determined by calculating the adjusted Rand and Wallace coefficients (Tables 4.2 and 4.3).   While complete agreement between MALDI-TOF MS and PFGE was not found, both the adjusted Rand (0.57) and Wallace (0.71) coefficients suggested that 'MALDI typing' of isolates cultured on HBA (24 h of incubation) showed the best correlation with PFGE analysis.

Interestingly, data from both tables also suggested that MALDI-TOF MS-based clustering from the different culture media showed little congruence with each other. This clearly indicates the influence of culture conditions on MALDI-TOF MS data. Another important point to note is that, as per the results of Chapter 3, improved species level identifications were attained for *L. monocytogenes* isolates cultured on HBA after 24 h of incubation (Table 3.2). Thus, HBA, an in-expensive and readily available non-selective media, is a suitable choice of culture media for the simultaneous detection and subtyping of *L. monocytogenes* isolates using MALDI-TOF MS. In terms of discriminatory power (SID, Table 4.2), MALDI-TOF MS-based source-tracking from all culture conditions (except PA) were found to be more discriminatory than PFGE.

Table 4.2: Comparison of the MALDI-based source- tracking to PFGE by calculation of Adjusted Rand co-efficient

Source tracking technique	Number of clusters	SID (95 % confidence interval)	HBA 24h	HBA 48 h	ALOA 24h	ALOA 48h	BHIA 24h	BHIA 48h	OA 24h	OA 48h	PA 24h	PA 48h	PFGE
HBA (24 h)	8	0.89 (0.86-0.91)	1		-	-	-	-	-	-	-	-	-
HBA (48 h)	8	0.90 (0.85-0.95)	0.18	1	-	-	-	-	-	-	-	- `	-
ALOA (24 h)	7	0.90 (0.88-0.93)	0.23	0.21	1	-	-	-	-	-	-	-	-
ALOA (48 h)	7	0.87 (0.84-0.91)	0.43	0.15	0.16	1	-	-	-	-	-	-	-
BHIA (24 h)	8	0.86 (0.78-0.93)	0.19	0.1	0.14	0.05	1	-	-	-	-	-	-
BHIA (48 h)	7	0.89 (0.85-0.93)	0.32	0.18	0.14	0.21	0.22	1	-	-	-	-	-
OA (24 h)	9	0.90 (0.87-0.94)	0.15	0.08	0.09	0.21	0.19	0.06	1	-	-	-	-
OA (48 h)	8	0.89 (0.83-0.94)	0.23	0.05	0.05	0.16	0.11	0.15	0.15	1	-	-	-
PA (24 h)	10	0.89 (0.83-0.96)	0.20	0.19	0.15	0.18	0.08	0.04	0.00	0.20	1	-	-
PA (48 h)	8	0.85 (0.77-0.92)	0.34	0.12	0.05	0.17	0.15	0.03	0.17	0.47	0.10	1	-
PFGE	6	0.86 (0.81-0.90)	0.57	0.13	0.25	0.39	0.26	0.25	0.22	0.24	0.15	0.4	1

SID and Adjusted Rand co-efficient was calculated using the Comparing Partitions website (Refer section 4.3.7)

Table 4.3: Comparison of the MALDI-based source- tracking to PFGE by calculation of Wallace co-efficient

Source-tracking technique	HBA 24h	HBA 48 h	ALOA 24h	ALOA 48h	BHIA 24h	BHIA 48h	OA 24h	OA 48h	PA 24h	PA 48h	PFGE
HBA (24 h)	1	0.17	0.21	0.44	0.21	0.32	0.14	0.23	0.2	0.41	0.71
HBA (48 h)	0.19	1	0.21	0.18	0.12	0.19	0.08	0.05	0.19	0.15	0.16
ALOA (24 h)	0.25	0.21	1	0.19	0.18	0.16	0.08	0.06	0.16	0.07	0.32
ALOA (48 h)	0.40	0.13	0.14	1	0.06	0.19	0.18	0.15	0.16	0.19	0.42
BHIA (24 h)	0.17	0.08	0.12	0.05	1	0.20	0.15	0.10	0.07	0.16	0.27
BHIA (48 h)	0.32	0.17	0.13	0.22	0.25	1	0.06	0.15	0.04	0.03	0.29
OA (24 h)	0.17	0.08	0.09	0.25	0.24	0.07	1	0.17	0.00	0.23	0.29
OA (48 h)	0.23	0.04	0.05	0.17	0.12	0.15	0.13	1	0.19	0.56	0.28
PA (24 h)	0.21	0.18	0.14	0.19	0.10	0.04	0.00	0.20	1	0.13	0.18
PA (48 h)	0.29	0.09	0.04	0.16	0.15	0.02	0.13	0.41	0.09	1	0.39
PFGE	0.50	0.11	0.20	0.36	0.26	0.22	0.18	0.22	0.13	0.41	1

Wallace co-efficient was calculated using the Comparing Partitions website (Refer section 4.3.7)

Since MALDI-TOF MS analyses after culturing on HBA showed better congruence with PFGE, a separate PLS-DA analysis was performed with the data obtained from culturing on HBA (24 h) for isolates from Source 1. Source 1 was facing a recurrent *L. monocytogenes* contamination over a period of two years and, according to the PFGE data, more than one strain had contributed to this persistence. Hence, it was important to investigate if MALDI-TOF MS would verify this finding. On performing the analysis (Figure 4.5 A and 4.5 B), the clustering obtained using MALDI-TOF MS was found to be in close correlation, albeit more discriminatory, than PFGE. The pulsotype 1 isolates (all environmental samples) in Figure 4.5 A were distributed over two separate MALDI types (A and C) in Figure 4.5 B. It is worth highlighting that both techniques placed isolate 489 (originally isolated from product) in a cluster (pulsotype 3 or MALDI type D) containing environmental isolates. This clearly indicates a possible transmission route from product to environment or vice versa. Overall, both techniques were able to show strain level discrimination between isolates obtained from Source 1. On comparison of the discriminatory power of PFGE clustering (SID = 0.86) to that of the MALDI-TOF MS-based clustering (SID = 0.89, for isolates cultured on HBA for 24 h), the latter had a slightly higher discriminatory index. An important consideration when comparing MALDI-TOF MS clustering to other techniques is that the data obtained from MALDI-TOF MS represent ribosomal proteins while the data from many genomic subtyping techniques (other than 16S rRNA based subtyping) are derived from analysis of different regions of the bacterial genome (Böhme *et al.,* 2012). Hence, whether MALDI-TOF MS analysis will show complete correlation with other subtyping techniques needs to be further investigated for different species. Previous studies have also compared the subtyping ability of MALDI-TOF MS and PFGE for other microorganisms (Griffin *et al.,* 2012; Verroken *et al.,* 2014). Griffin *et al.* (2012) compared the subtyping ability of MALDI-TOF MS with PFGE for a set of vancomycin-resistant *Enterococcus* (VRE) isolates. Four VRE isolates considered to be identical by PFGE were found to be closely-related but in separate clusters according to the MALDI-TOF MS clustering, also suggesting that MALDI-TOF MS may be more discriminatory when compared with PFGE.

A

B
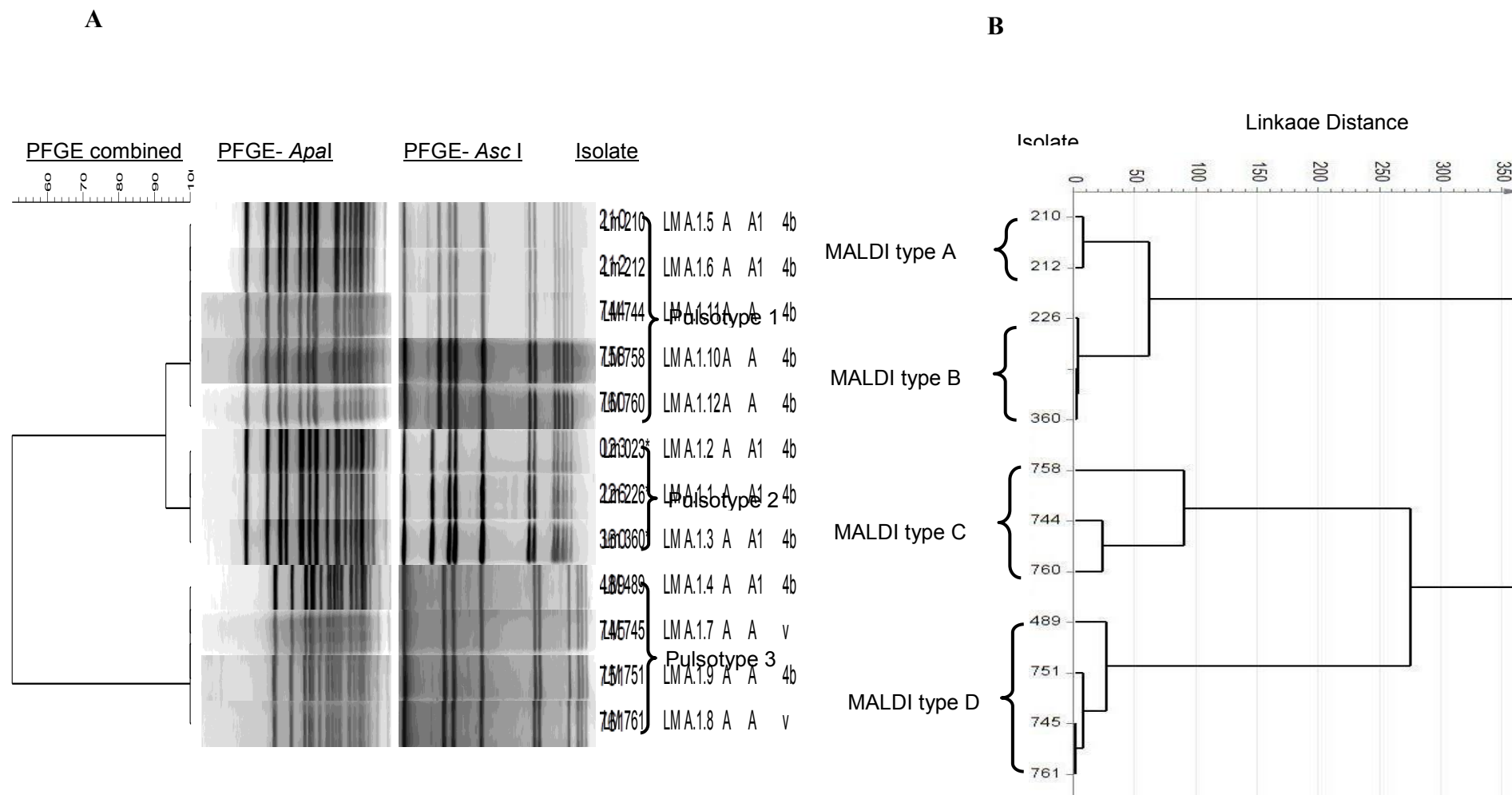
Figure 4.5 A: Dendogram obtained after PFGE analysis of Source I isolates; Figure 4.5 B: Dendogram obtained after PLS-DA analysis of MALDITOF MS data (for Source I isolates) obtained from HBA agar after 24h of analysis.

Since 'MALDI typing' from HBA after 24 h of incubation agreed the most with PFGE subtyping, these data were also analysed using MetaboAnalyst software to generate a heat map (Figure 4.6). Heat maps provide statistically significant features (identified by performing a t-test/ANOVA) that contribute to the hierarchical clustering of different samples. The biomarker peaks identified from the heat map are listed in Table 4.4. Interestingly, Source 1 isolates did not show a single signature peak that was present in all isolates from this source. This is consistent with the observation that, although this dairy site experienced persistent contamination, both PFGE and MALDI-TOF MS indicated that this was due to the presence of more than one type of strain.
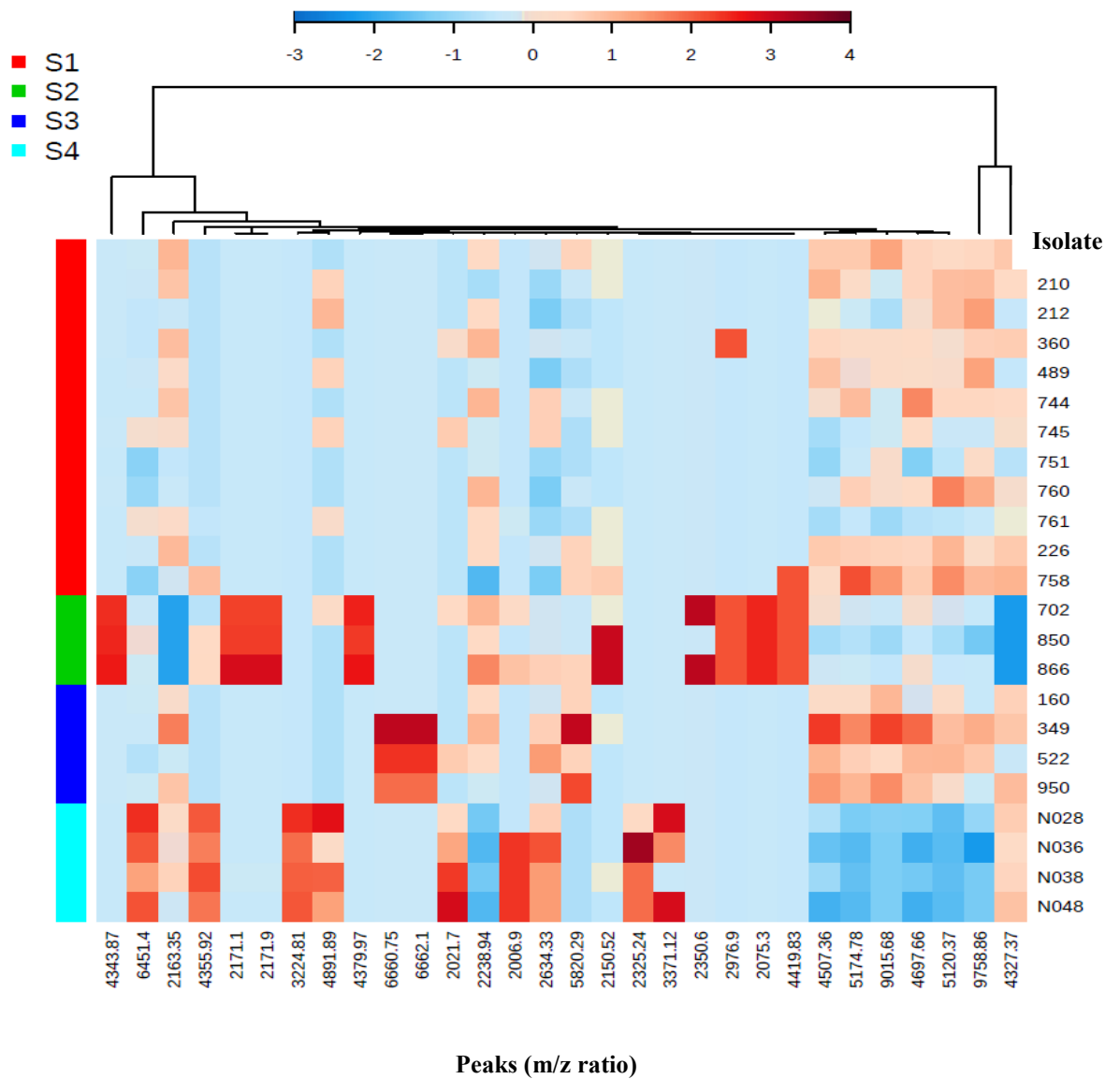
Figure 4.6 The heat map from HBA analysis (24h) was generated using Metaboanlayst indicating 30 statistically significant peaks (calculated using t-Test ANOVA) that discriminate the *L. monocytogenes* isolates, S1- Source 1; S2 - Source 2; S3 - Source 3; S4 - Source 4 (refer to Table 3.1 for detailed information about isolates).

Table 4.4: Potential biomarker peaks distinguishing isolates from different sources

Dairy Source	Isolates	Statistically significant biomarker peaks (m/z ratio)
Source-2	702,850,866	2075.3; 2171.1; 4343.87, 4379.97;
Source-3	349,522,950	5820; 6660.75; 6662.1
Source-4	N028, N036, N038, N048	2325.24; 3371.12; 3224.81; 4355.92; 4891.89; 6451.4;

Table 4.4 highlights the statistically significant biomarker peaks that distinguish isolates from Source 2, 3 and 4.

Barbuddhe *et al.* (2008) and Hseuh *et al.* (2014) also used MALDI-TOF MS to subtype *L. monocytogenes* isolates, the majority of these being of clinical origin and compared this technique to PFGE. Both studies found that MALDI-TOF MS was able to classify the strains based on their clonal lineages (determined by their serotype), as observed in PFGE. However, it is important to note that, unlike these studies, the current study consisted mainly of dairy isolates belonging to only two clonal lineages (lineage I and II), and this study aimed to evaluate the use of MALDI-TOF MS as a source-tracking tool. Hence, a separate PLS-DA analysis and t-test/ANOVA analysis was performed with the same data utilised in the source-tracking study to investigate if any biomarker peaks could differentiate between clonal lineages. The PLS-DA analysis clearly separated the 23 isolates included in the source-tracking study into two groups (R^2X, R^2Y and Q2 values of 0.75, 0.99, 0.81, respectively, Figure 4.6), with seven isolates belonging to lineage II (containing 1/2a or 3a serotype isolates) and 16 isolates belonging to lineage I (containing 1/2b or 3b serotype isolates). A heat map generated from this data identified biomarker peaks distinguishing the two lineages (Table 4.5). Thus, the current study is in agreement with the findings of Barbuddhe *et al.* (2008) and Hsueh *et al.* (2014) that MALDI-TOF MS data can be used to distinguish between the clonal lineages of *L. monocytogenes,* although further validation of this observation with a larger set of isolates should be performed.
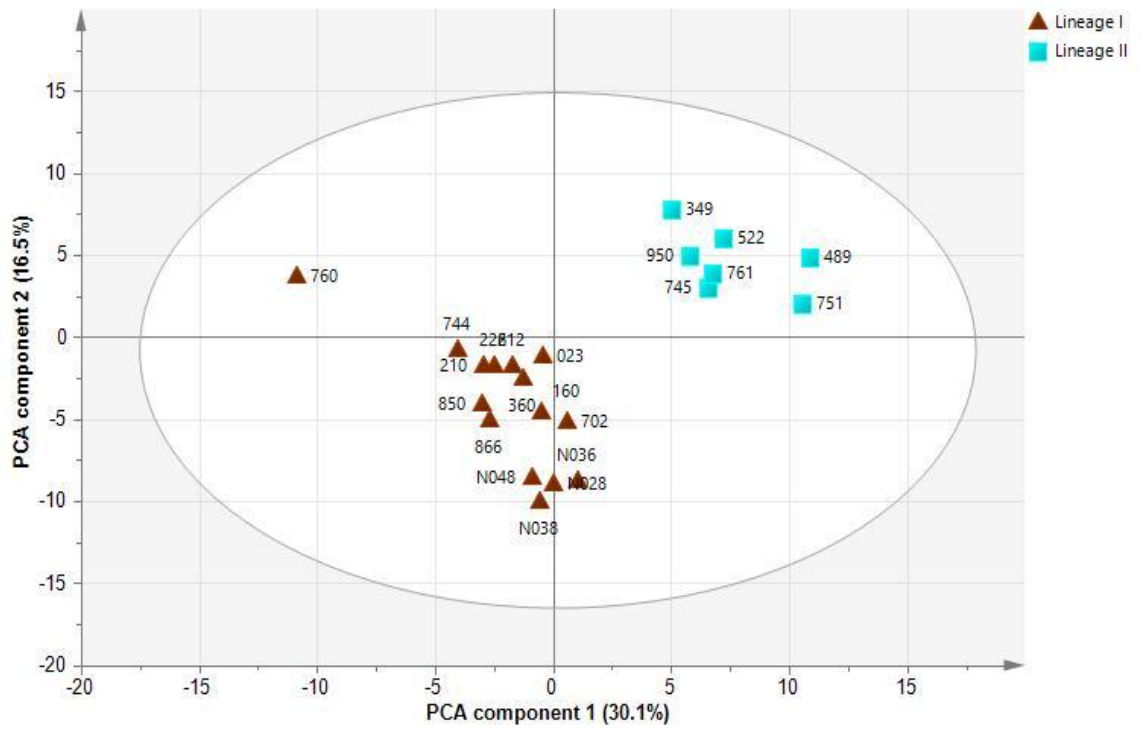
Figure 4.7 PLS-DA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on HBA agar, post 24 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.75, R^2Y =0.99 and Q^2 = 0.81.

Table 4.5: Biomarker peaks distinguishing isolates based on their serotypes

Clonal lineage	Molecular serotypes detected in this study	Statistically significant biomarker peaks (m/z ratio)
Lineage I	1/2b or 3b	2758.67; 6743.65;
Lineage II	1/2a or 3a	2141.31; 5591.67; 5792.16

4.5 Conclusions

The current study investigated the application of MALDI-TOF MS as a source-tracking tool for *L. monocytogenes* obtained mainly from dairy sources within Australia. The most important finding of this research was that changes in culture media and time of incubation had a profound effect on the reliability of source-tracking of *L. monocytogenes* using MALDI-TOF MS. Unlike microbial identification that considers a limited number of biomarkers, strain level typing using MALDI-TOF MS requires a more thorough exploration of the peak lists to define strain level differences. This in turn highlights the importance of the data analysis tool employed for finding such differences. In the current study, the application of two data analysis approaches, SPECLUST and SIMCA, for source-tracking the dairy isolates was assessed. While the application of SPECLUST may be more suited for studying species or strain level differences for other pathogens, multivariate data analysis using SIMCA was considered more suitable for the current research.

In this research, the mass spectrometry based source-tracking technique was also compared with the gold standard genetic technique, PFGE. Amongst all the culture conditions tested, data obtained by culturing the isolates on HBA (post 24 h of incubation) were found to be in good congruence with PFGE. Overall, MALDI-TOF MS-based source-tracking was found to be slightly more discriminatory than PFGE. Previous studies that compared the subtyping ability of MALDI-TOF MS and PFGE for other microorganisms also indicated that MALDI-TOF MS may be more discriminatory when compared with PFGE. However, a major limitation of most studies, including the current one, is the use of small sample sets. More extensive studies with larger sample sets will provide a deeper insight to the correlation between these techniques. In addition to being more discriminatory, the indisputable advantages of MALDI-TOF MS are its rapidity, simplicity and suitability for automation, making it more appropriate than PFGE for a high-throughput food laboratory. Thus, depending on the specific subtyping requirement (i.e. source attribution, source-tracking or outbreak investigation), the application of a single technique or a combination of techniques can be considered.

Overall, this study demonstrated that a standardised MALDI-TOF MS scheme can provide a rapid and simple solution for detecting and source-tracking *L. monocytogenes* isolates obtained from food processing environments. This technique, in combination with sophisticated data analyses such as PLS-DA and t-test/ANOVA, can be used to identify biomarkers that separate between isolates obtained from different sources. Larger epidemiological investigations involving this pathogen may still require very highly discriminatory techniques such as PFGE, MLST, MLVA or even whole genome sequencing. However, since these techniques can be technically and economically challenging, the proposed MALDI-TOF MS scheme can assist in immediate interventions required for outbreak and source-tracking applications. The same scheme may also be applied to other pathogens since MALDI-TOF MS is a 'universal' system for analysing numerous pathogens of relevance to the food industry.

Chapter 5

Control of *Listeria monocytogenes* in food processing environments using the essential oils of Yarrow

Abstract

L. monocytogenes is a major threat to the food industry since it can survive extreme physiological conditions encountered in food processing environments and can also attach to abiotic surfaces forming biofilms that can frequently contaminate food products and the production environment. Such biofilms tend to be more resistant to antimicrobial treatments. Considering the recent emergence of bacteria resistant to antimicrobials and the inclination of consumers towards products derived from natural sources, the current study investigated the anti-listerial effects of some plant essential oils and flavonoids. The essential oils obtained from yarrow (*Achillea millefolium*) were found to have strong bactericidal activity against *Listeria* planktonic cells which was found to be equivalent to that of tea tree essential oil (*Melaleuca alternifolia*). Inhibition of biofilm formation and growth of *Listeria* after incubation with different concentrations of yarrow essential oil (YEO) was also assessed by the crystal violet and *3*-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide reduction (MTT) assays. YEO significantly inhibited the initial cell attachment of the *Listeria* cells on polystyrene, stainless steel and high density polyethylene surfaces, but was less inhibitory towards biofilms preformed for 6 hours on polystyrene surface. MTT assay showed that metabolic activity of the biofilms decreased considerably after incubation with the oil. Gas chromatography coupled to mass spectrometry (GC-MS) was used to characterise the constituents of YEO. 1, 8-cineole and β-pinene were some of the major components of YEO. The cytotoxicity of the essential oil was determined against mouse fibroblast cells (3T3-L1) and found to be comparable to bleach (sodium hypochlorite). Thus, YEO, or its constituents, may be useful additives for the development of new surface disinfectant and sanitizer formulations for application in the food industry.

5.1 Introduction

L. monocytogenes is a ubiquitous pathogen that has been frequently isolated from numerous food processing environments including poultry, meat, trout and shrimp processing plants, and ice cream plants (Vogel *et al.,* 2001). It can be a serious threat to the food industry as it has the ability to adhere to various abiotic surfaces in the processing environments, forming biofilms that can continuously contaminate the food products (as discussed in Chapter 1, section 1.6). Biofilm formation generally takes place in multiple stages. The initial stage involves the reversible attachment of the cells to an inert surface, followed by irreversible adhesion of the cells (second stage). In the third stage, the bacteria grow on the surface to form microcolonies. In this maturation stage, a well-organised complex three dimensional biofilm is formed. Finally, the fourth stage involves detachment of cells from the mature biofilm (Rieu *et al.*, 2007; Xu *et al.,* 2010). Cells in a biofilm have been observed to be more resistant to heat, drying, acidic environments, salinity, antimicrobials and food preservatives, compared to their planktonic counterparts (Xu *et al.,* 2011). Hence, most jurisdictions mandate that high risk food processing environments have their own hazard analysis and critical control point (HACCP) program for controlling *Listeria* contamination in the food product as well as the entire facility (Blatter *et al.*, 2010). These programs require the industries to have systematic and periodic sampling regimes in their production facilities.

Recently, the emergence of antibiotic resistant bacteria and the reluctance of consumers towards consumption of chemically-treated goods have encouraged the development of natural antimicrobial agents. Substances obtained from plants are preferred over synthetic biocides as they may have been used in traditional medicine for a long time. Overall, they are generally considered to be safe by consumers and are not known to cause harm to the environment (Leonard *et al.,* 2010). In the current study, the anti-listerial effects of several essential oils and flavonoids were studied. This was followed by a more detailed investigation of the inhibitory effects of yarrow (*Achillea millefolium*) essential oil (YEO), which was found to be strongly anti-listerial on planktonic cells and biofilms formed by *L. monocytogenes* and *L. innocua* isolates obtained from food processing environments. *L. innocua* is a non-pathogenic species

which is often used as a surrogate to *L. monocytogenes*, as it is taxonomically closely-related (Oulahal *et al.,* 2008).

Native to Europe and western Asia, *Achillea millefolium* (family Asteraceae) can now be found in southern Australia, New Zealand and parts of North America (Rahimmalek *et al.*, 2009). YEO is conventionally obtained from different structural parts such as flowers, leaves and stems by steam distillation or hydrodistillation (Bocevska and Sovová, 2007). The annual worldwide production of the oil is 800 tonnes and it has been used traditionally as an anti-inflammatory medicine, to treat headaches and to control bleeding of wounds and haemorrhoids (Tajik *et al.*, 2008). Hydroalcoholic extracts of this herb have also exhibited anti-nociceptive activity (Lakshmi *et al.*, 2011). The antimicrobial activity of the alcoholic extract of this plant has been assessed against a range of other bacteria and the extract was found to be most active against *Staphylococcus aureus* (Tajik *et al.,* 2008).

The effect of various essential oils and their individual constituents on biofilms formed by *L. monocytogenes* has been investigated previously (Sandasi *et al.*, 2008; Leonard *et al.,* 2010; Oliviera *et al.,* 2010a; Sandasi *et al.*, 2010). However, to the knowledge of the author, this is the first report of the anti-listerial and anti-biofilm effects of YEO.

5.2 Chapter Aims

The major aims of this chapter were to:

a) Investigate the anti-listerial effects of various essential oils and study the biofilm inhibitory effects of the essential oils of yarrow (YEO) against *L. monocytogenes* and *L. innocua* isolates obtained from food processing environments, using the crystal violet (CV) and MTT assays

b) Characterise the chemical constituents of YEO using GC-MS

c) Study the cytotoxicity of YEO and compare it to that of a commercial sanitizer using the MTT assay.

5.3 Materials and methods

5.3.1 Preparation of bacterial cultures and selection of isolates

L. monocytogenes and *L. innocua* isolates used in this study were subcultured on Brain Heart Infusion (BHI, Oxoid) agar and incubated at 37 °C for 24 h. Prior to each experiment, a loopful of BHI culture was inoculated in Tryptone Soya broth (TSB, Oxoid) and incubated at 37 °C for 24 h. Following incubation, a hundred-fold dilution of the overnight culture matched to the 0.5 McFarland turbidity standard (approximately 10^8 cfu/mL) was used for experimental procedures.

For studying the anti-listerial and biofilm inhibitory effects of the essential oils and flavonoids, five *L. monocytogenes* isolates and three *L. innocua* isolates obtained from dairy processing environments within Australia (Table 5.1) were initially screened for their ability to form biofilms using a CV assay (Djordjevic *et al.,* 2002). Initially, 100 μL of bacterial cultures (prepared as above) were added to a sterile 96-well flat bottom microtitre plate in quadriplicates. Sterile TSB was also added as an additional control to confirm the sterility of the medium. The plates were sealed and incubated for 24 h at 37 °C to allow cell attachment, following which the culture medium from each well was gently removed. The plates were washed five times with sterile distilled water to remove loosely attached bacterial cells. The plates were air-dried and further oven-dried at 60 °C for 45 min. The attached cells were then stained with 1 % crystal violet solution (100 μL) and incubated at room temperature for 15 min. The wells were then washed with sterile distilled water to remove the excess stain. One hundred and twenty five μL of 95 % ethanol were then added to the wells to destain the wells and 100 μL of the destaining solution from each well were transferred to a new plate. The absorbance of the second plate was measured at 595 nm using a microplate reader (Cary Eclipse-Varian, Australia). One isolate each representing both species (*L. monocytogenes* and *L. innocua*) showing highest biomass attachment according to the CV assay were selected for further experiments.

Table 5.1 Isolates screened for biofilm formation

Number	Isolate number	*Listeria* Species	Dairy Source
1	023	*monocytogenes*	1
2	226	*monocytogenes*	1
3	360	*monocytogenes*	1
4	210	*monocytogenes*	1
5	125	*monocytogenes*	Unknown
6	500	*innocua*	1
7	713	*innocua*	1
8	260	*innocua*	1

5.3.2 Essential oils and Flavonoids

Various essential oils were screened for potential anti-listerial activity. These included Cold-Pressed Lemon (*Citrus limon*), Bergamot (*Citrus bergamia*), Pine Scotch (*Pinus sylvestris*), Peppemint Premium (*Mentha piperita*) and Yarrow essential oils obtained from New Directions (Sydney, Australia); essential oils of Traditional Aniseed (*Pimipinella anisum*), Anise Myrtle (*Syzgium anisata*), Kunzea (*Kunzea ambigua*), Fragonia (*Agonis fragrans*) and Sandalwood (*Santalum spicatum*) obtained from Essentially Australia (Byron Bay, New South Wales) and essential oil of *Melaleuca alternifolia,* commonly known as tea tree oil (TTO, 100 % pure) obtained from Thursday Plantations (Queensland, Australia). Eight flavonoids, naringin, hesperidin, quercitin, neohesperidin, naringenin, hesperitin and gallic acid (obtained from Sigma-Aldrich Co. Ltd. Australia), were also screened for anti-listerial properties.

5.3.3 Agar disk diffusion assay

The anti-listerial activities of the essential oils and flavonoids were evaluated using the agar disk diffusion assay, carried out as per Fisher and Phillips, (2006), with some modifications. Initially, 100 µL of 24 h bacterial cultures (in TSB) adjusted to a 0.5 McFarland standard were uniformly spread on the surface of BHI agar plates. Three sterile discs impregnated individually with 20 µL of the essential oils and flavonoids (1 mg/mL) mentioned in section 5.3.2 were then placed on the surface of the plates. This experiment was carried out on two independent occasions. The solvent used for subsequent preparation of dilutions of the essential oils and flavonoids (methanol) was

used as negative control while chloramphenicol discs (30 µg) were used as positive control. The plates were incubated at 37 °C for 24 h before measuring the zone of inhibition (diameter in mm).

5.3.4 Determination of Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of YEO and tea tree oil (TTO)

The MIC of YEO was determined in BHI broth using a broth microdilution method in the 96-well round-bottomed polystyrene microtitre plates (Corning, NY), as described by Mihajilov-Krstev *et al.* (2010), with some modifications. One hundred microlitres of YEO (50 % v/v) were added to an equal volume of BHI broth and a series of two-fold dilutions was performed with BHI broth in the microtitre plate to achieve a concentration gradient from 25 % v/v to 0.078 % v/v. Inhibition of bacterial growth in the wells containing YEO was assessed by comparison with growth in blank control wells and the inhibitory activity was compared to that of TTO by simultaneous determination of the MIC of TTO against the *Listeria* isolates. Bacterial cultures in TSB were adjusted to 0.5 McFarland turbidity equivalents. Ten microlitres of the inoculum were added to each well and incubated for 24 h at 37 °C. Positive growth controls (inoculum + BHI) and vehicle growth controls (inoculum + BHI + methanol) were also included in the plate. The MIC values were defined as the lowest concentrations of the oil which inhibited visible growth of the bacteria. For determination of MBC of YEO and TTO, 50 µL of solution from all wells having no turbidity were spread onto the surface of BHI agar plates and incubated for 24 h at 37 °C. The sample with the lowest concentration of the oil that showed no growth on BHI agar was recorded as the MBC.

5.3.5 Determination of biofilm inhibitory activity of YEO

5.3.5.1 Inhibition of initial cell attachment

The effect of YEO on biofilm formation was evaluated as described by Sandasi *et al.* (2010). Solutions of YEO (equivalent to 0.5xMIC, 1xMIC and 2xMIC) were prepared. One hundred microlitres of each solution were added to individual wells of a sterile flat-bottomed 96-well polystyrene microtitre plates (Corning, NY). Equal volumes of methanol or water were added as negative controls, while tetracycline (0.00125 mg/mL)

was added as a positive control. One hundred microlitres of the bacterial cultures (prepared as described above in section 5.3.1) were then added to the wells to yield a final volume of 200 µL in each well. The cultures were added in quadruplicate and sterile TSB was also added as an additional control to confirm the sterility of the medium. The plates were sealed and incubated for 24 h at 37 °C to allow cell attachment. Biofilm formation was assessed using the modified crystal violet assay (section 5.3.6) and the metabolic activity of the cells incubated with YEO was investigated using the MTT assay (section 5.3.7).

5.3.5.2 Inhibition of preformed biofilm

The effect of YEO on biofilm growth and development was evaluated as described by Sandasi *et al.* (2010), with some modifications. Biofilms were allowed to form for 6 h prior to addition of YEO. Biofilm formation was achieved by transferring 100 µL of bacterial culture (prepared as described in section 5.3.1) into the wells of sterile flat-bottomed 96-well polystyrene microtitre plates in quadruplicates. The plates were covered and incubated for 6 h at 37 °C to allow cell attachment and biofilm formation. Following incubation, 100 µL of each stock solution of the oil were added to each well to yield a final volume of 200 µL. Equal volumes of methanol or water were added as negative controls while tetracycline (0.00125 mg/mL) was added as a positive control. After the treatment of preformed biofilms with YEO, the plates were incubated for 1 h, 5 h, and 20 h. Following incubation, the biofilms were assessed for biomass attachment using the crystal violet assay and MTT assays described below.

5.3.6 Biofilm biomass assay (modified crystal violet assay)

Indirect assessment of cell attachment for *Listeria* isolates was evaluated using the modified crystal violet (CV) assay described by Djordjevic *et al.* (2002). Following the 24 h incubation (section 5.3.5.1) and the 1 h, 5 h and 20 h incubations (section 5.3.5.2), plates were processed as described in section 5.3.1. One hundred microlitres of the destaining solution from each well was then transferred to a new plate, and the absorbance was measured at 595 nm using a microplate reader (Cary Eclipse-Varian, Australia). The mean absorbance (OD_{595nm}) was used for determining the percentage

inhibition of biomass formation for each concentration of the oil according to the following equation:

Percentage inhibition = 100 − [{OD$_{595nm}$ experimental well with YEO / OD$_{595nm}$ control well without YEO} x 100].

5.3.7 Biofilm metabolic activity assay

The metabolic activity of the biofilms formed by the bacteria was assessed using a modified *3*-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) reduction assay according to Schillaci *et al.* (2008). The MTT salt (Sigma-Aldrich, UK) was dissolved in phosphate buffered saline (PBS) to give a final concentration of 5 mg/mL.

For the plates containing cells incubated with YEO at 0.5xMIC, the culture medium was gently removed following the various incubation periods (1 h, 5 h and 20 h for preformed biofilm and 24 h for cell attachment) and the plates were air-dried. One hundred microlitres of PBS and 5 µL of MTT solution (5 mg/mL) were pipetted into each well and incubated for 3 h at 37 °C under sterile conditions. The insoluble purple formazan (obtained by enzymatic hydrolysis of MTT by the dehydrogenase enzyme found in living cells) was further dissolved in dimethyl sulphoxide (DMSO, Sigma-Aldrich, UK). The absorbance was then measured at 570 nm using the microplate reader (Cary Eclipse-Varian, Australia).

5.3.8 Inhibition of initial cell attachment on stainless steel and high density polyethylene surfaces

The biofilm inhibitory effect of YEO on initial cell attachment of the listerial isolates was further investigated on two additional surfaces viz., stainless steel (SS) and high density polyethylene (HDPE). SS coupons (30 x 30 x 1mm) were initially cleaned and sterilised according to Valeriano *et al.* (2012) with some modifications. Briefly, the coupons were soaked in acetone overnight followed by washing with an alkaline (5 % potassium hydroxide) detergent. After a wash with distilled water, the coupons were dried at 60 ° C for 1 h and autoclaved at 121 °C for 15 min. In case of the HDPE coupons (20 x 20 x 1 mm), cleaning involved a first wash using a detergent followed by

a wash with distilled water. The coupon surfaces were surface sterilised using 70 % ethanol and air-dried for an hour before use.

Under sterile conditions, the coupons were placed in Petri plates (in triplicates) and 3.5 mL YEO solution were deposited on the coupons. Following this, an equal volume of the listerial culture (previously adjusted to a 0.5 McFarland standard in TSB) was added, to achieve the MIC of the oil in the final mixture. The plates were incubated at 37 °C for 24 h to allow cell attachment. After incubation, the coupons were washed thrice with sterile distilled water to remove any loosely attached cells. The washed coupons were immersed in a 10 mL PBS solution (pH 7.4) and were incubated on a mechanical shaker for 5 min at room temperature to remove the bound cells. Enumeration of the cells was performed using the viable cell count method. A similar treatment was given to the control plates without oil.

5.3.9 Identification of YEO components

The essential oil components were identified using a gas chromatograph coupled to a mass spectrometer as detector (GCMS-QP2010 Ultra, Shimadzu). An Rxi-5SIL-MS column was used for the separation of the oil components under the following operation conditions. The ion source temperature used was 200 °C and the initial column oven temperature was programmed at 50 °C for 2.5 min followed by an increase of 20 °C per min until it reached 270 °C. A 1 % solution of the oil in dichloromethane (1 µL) was injected in a split mode (1:20) using helium as a carrier gas (1.5 mL/min flow rate). The mass spectrometer scan range was 50-500 m/z and the scan rate was 1666 amu/s. The compounds were identified by comparison with the NIST and Wiley 8.0 MS library. Confirmation was achieved by comparative analysis of pure standards for two constituents of the oil (1, 8-cineole and linalool).

5.3.10 Cytotoxicity of YEO

The cytotoxicity of YEO was determined using the MTT assay and was compared to a commercial sanitizer bleach (sodium hypochlorite concentration, 4.2 % v/v). The cytotoxicity was also compared to TTO since it is used commercially in some disinfectant formulations.

5.3.10.1 Media and chemicals

Thiazolyl blue tetrazolium bromide and DMSO used in the cytotoxicity assay were purchased from Sigma-Aldrich (Australia) and Merck (Australia), respectively. Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Invitrogen Corp.), foetal bovine serum (FBS), penicillin G sodium (10,000 units/mL) and streptomycin (10,000 μg/mL) were obtained from Invitrogen Technologies (Australia). Dulbecco's phosphate buffered solution (PBS) was provided by Sigma-Aldrich (Australia). Ultra-purified water was obtained from a MilliQ® Plus purification system (Millipore, Germany) and all other chemicals and reagents were commercially available and of analytical grade.

5.3.10.2 Maintenance of cell lines

The mouse embryonic fibroblast 3T3-L1 cell lines were maintained at confluence in complete DMEM supplemented with 10 % FBS and 1 % antibiotic in a humidified incubator at 37 °C with a 5 % CO_2 atmosphere.

5.3.10.3 Exposure of cells to essential oils

The 3T3-L1 cells were seeded into 96-well plates at a density of 1 x 10^4 cells per well in 200 μL complete DMEM and allowed to attach for 24 h after which the media were aspirated. Essential oils and bleach diluted in DMEM (two-fold dilutions, ranging from 0.5-0.0156 % v/v) and another 100 μL of complete growth medium were then added to each well and incubated for 24 h at 37 °C / 5 % CO_2. Triton-X 100 (1 % solution in water) was used as a positive control.

5.3.10.4 MTT assay

The viability of cells following exposure to essential oils or bleach was evaluated by measuring the metabolic activity of cells using the MTT assay as described by Rajasekaran *et al.* (2013) with minor modifications. Following 24 h incubation, the medium with the essential oils or bleach was removed by aspiration and 170 μL MTT solution (5 mg/mL MTT in sterile PBS, pH 7.4) was added to the DMEM (final concentration of 0.32 mg/mL) in the well and incubated for 4 h at 37 °C. The medium with MTT solution was discarded, 150 μL of DMSO was added to the wells and the

plates were incubated for 15 min in the dark at room temperature. Absorbance, which is directly proportional to cellular metabolism, was measured at 570 nm using a Cary Eclipse Varian microplate reader (Australia). Cell viability was calculated as follows:

Cell viability (%) = [Absorbance of treated cells / Absorbance of control cells] x 100

According to ISO 10993-5, a reduction of cell viability by more than 30 % is considered a cytotoxic effect (ISO, 2009). For this study, cell viability values > 75 % were considered as non-toxic. Cell viability values between 25 % and 75 % were considered to have reduced viability, and values below 25 % indicated complete loss of viability.

5.3.11 Statistical analysis

The ANOVA test was used to examine any significant differences in the crystal violet, MTT and inhibition of cell attachment (SS and HDPE surfaces) assays. Differences with *p* values < 0.05 were considered statistically significant.

5.4 Results and Discussion

Listeria species have often been observed to form biofilms on abiotic surfaces found in food processing environments (Djordjevic *et al.,* 2002). Such biofilms have been observed to be more resistant to antimicrobials compared to planktonic cells. Their ability to resist antimicrobials has been attributed to many factors, such as (i) the presence of anionic, extracellular polysaccharide (also called glycocalyx) which serves as the physical barrier for the diffusion of antimicrobials into the cells embedded in biofilms; (ii) differential gene expression of sessile cells as opposed to planktonic cells; (iii) the inactivation of antimicrobial peptides; (iv) the presence of efflux pumps which enable the removal of these compounds from the bacterial cell (Sandasi *et al.,* 2008; Purkrtová *et al.,* 2010; Sandasi *et al.,* 2010). Considering the resistance of pathogens to antimicrobial treatments and the growing interest of consumers in foods with minimal synthetic chemical treatment, it is important to explore new antimicrobials that already exist in nature.

Since *Listeria* can readily survive extreme physiological environments found in food processing environments by forming biofilms, the use of natural anti-listerial agents such as flavonoids and essential oils were investigated in the current study.

5.4.1 Biofilm formation by *Listeria* isolates

Preliminary characterisation of the biofilm forming abilities of eight isolates of *Listeria* was performed, comprising of five *L. monocytogenes* isolates and three *L. innocua* isolates (Table 5.1). It should be noted that the selection of isolates was made from a limited collection of isolates available for the study. According to the CV assay adsorption of crystal violet by attached cells is a direct indication of biomass attachment, which is estimated by measuring the absorbance of the destaining solution at 595 nm. After comparing the absorbance values obtained for the eight isolates (ranging from 1.1 to 2.1 AU at 595 nm) *L. monocytogenes* isolate 125 and *L. innocua* isolate 713 (isolated from environmental swabs of two independent sources) were selected for further experiments since they showed the highest absorbance readings (2.0 and 2.1 AU respectively), indicating greater biomass attachment.

5.4.2 Anti-listerial effect of essential oils and flavonoids

Essential oils and their individual components have been previously explored for their antimicrobial properties (Saad *et al.,* 2013). Essential oils of clove (*Syzygium aromaticum*), cinnamon (*Cinnamomum zeylanicum*), thyme (*Thymus vulgaris*) (Owen and Palombo; 2007); rosemary *(Rosmarinus officinalis)* (Dimitrijević *et al.,* 2007) and lemongrass (*Cymbopogan citratus*) (Oliveira *et al.,* 2010b) have exhibited significant anti-listerial properties. However, comparatively few studies have explored their abilities to inhibit biofilms. Essential oils being moderately water soluble can diffuse through water and enter bacterial cell membranes; disrupting and destabilising them which can eventually result in osmotic shock (Taweechaisupapong *et al.*, 2012). Flavonoids, which are phytochemicals obtained from plants, have also been studied for their antimicrobial, anti-allergic, anti-inflammatory, antioxidant and anti-viral properties (Kuponiyi and Ibibia, 2013).

The anti-listerial effects of plant essential oils and flavonoids were assessed using the disk diffusion assay with methanol and chloramphenicol as negative and positive controls, respectively. Table 5.2 and 5.3 summarise the results of the disk diffusion assay after an overnight incubation period. Disk diffusion assays have been used extensively by researchers to study the antimicrobial activity of essential oils. Since essential oils are generally poorly water soluble, they may have limited diffusion through the agar (Griffin *et al.,* 2000). Here the author would like to emphasize that in the current study this assay was only used to confirm the presence or absence of anti-listerial effects. Essential oils of yarrow, cold pressed lemon, bergamot, pine scotch and peppermint were observed to show considerable anti-listerial activity. Some of the essential oils that did not show such activity (essential oils 7-10 in Table 5.2) were obtained from Australian native plants that have not been explored completely for their antimicrobial properties. Thus, more research needs to be conducted to investigate their effect against other microbes. With the exception of quercitin, all flavonoids tested showed some anti-listerial effects (Table 5.3). However, since the effects were not as strong as the essential oils, flavonoids were not studied further. The anti-listerial activities of lemon, bergamot, pine and peppermint essential oils have been studied previously (Fisher and Phillips, 2006; Gómez-Estaca *et al.,* 2010; Bonyadin and

Moshtaghi, 2008). However, YEO has not been studied previously for its anti-listerial effects. Thus, this oil was selected for the further experiments.

Since the agar disk diffusion assay only serves as a preliminary screening assay for selection of antimicrobial compounds, MIC and MBC of YEO were determined to estimate its anti-listerial effect. The estimated MIC and MBC were compared to that of TTO as it is well-known for its strong antimicrobial properties (Palombo, 2011), its anti-listerial activity has been investigated by Lis-Balchin *et al.* (2000) and is also used in commercial formulations of sanitizers. The MIC of both YEO and TTO was determined to be equivalent for both the listerial isolates (3.13 % v/v). The MBC of YEO and TTO for *L. monocytogenes* and *L. innocua* were determined as 6.25 % v/v and 12.5 % v/v, respectively.

Table 5.2 Screening of essential oils for anti-listerial activity

Number	Essential oils	Plant of origin	Zone of inhibition (mm)	
			L. monocytogenes isolate 125	*L. innocua* isolate 713
1	Yarrow	*Achillea millefolium*	16	23
2	Cold-pressed Lemon	*Citrus limon*	14	16
3	Bergamot	*Citrus bergamia*	12	14
4	Pine Scotch	*Pinus sylvestris*	14	12
5	Peppermint premium	*Mentha piperita*	14	14
6	Traditional Aniseed	*Pimpinella anisum*	< 8[a]	< 8
7	Anise Myrtle	*Syzygium anisata*	< 8	< 8
8	Kunzea	*Kunzea ambigua*	< 8	< 8
9	Fragonia	*Agonis fragrans*	< 8	< 8
10	Sandalwood	*Santalum spicatum*	< 8	< 8

[a] For this study, essential oils giving a zone of inhibition <8 mm were not considered to be anti-listerial. Chloramphenicol (30 µg) was used as positive control and it gave a zone of inhibition of 24 mm

Table 5.3 Screening of flavonoids for anti-listerial activity

Number	Flavonoids	Zone of inhibition (mm)	
		L. monocytogenes isolate 125	*L. innocua* isolate 713
1	Naringin	11	11
2	Hesperidin	11	10
3	Quercetin	< 8[a]	< 8
4	Neohesperidin	10	11
5	Naringenin	12	12
6	Hesperetin	10	10
7	Gallic acid	10	10

[a] For this study, essential oils giving a zone of inhibition <8 mm were not considered to be anti-listerial.

Chloramphenicol (30 µg) was used as positive control and it gave a zone of inhibition of 24 mm

5.4.3 Biofilm inhibitory effects of YEO on polystyrene surface assessed by the CV assay

In order to understand the anti-biofilm action of YEO, its effect was tested on both the initial cell attachment by planktonic cells as well as on preformed (6 h) biofilms. The crystal violet assay indicated that the effect of YEO on initial cell attachment was dosage-dependent ($p < 0.05$), although, even at 0.5xMIC, biomass attachment was reduced by 79.4 % for *L. innocua* (Figure 5.2 A) and 77.7 % for *L. monocytogenes* (Figure 5.2 B). However, complete inhibition of cell attachment was not achieved despite using 2xMIC of YEO. Nonetheless, the use of natural compounds to condition surfaces and make them less suitable for attachment seems a useful approach to deal with microbial adhesion in food processing environments (Sandasi *et al.,* 2010).

When the same oil was tested against a preformed biofilm, its inhibitory effect was reduced greatly (Figures 5.1 A and 5.1 B). As assessed by the CV assay, after an hour of incubation of YEO with the preformed biofilm, only 28.7 % and 52.2 % inhibition occurred at MIC levels for the *L. innocua and L. monocytogenes* strains, respectively. Thus, inhibition of biofilm formation was highest at 2xMIC of YEO. Overall all three concentrations of YEO inhibited biofilm formation ($p < 0.05$), however the effect of YEO on preformed biofilms was reduced compared to the effect on initial cell attachment. Biofilm formation involves an initial reversible (weak) attachment phase followed by an irreversible (strong) attachment phase (Oliveira *et al.*, 2010a). Hence, it

appears that a higher concentration of YEO is required to disrupt established biofilms which may be in the irreversible attachment phase. This again emphasises the greater resistance of biofilm-forming sessile cells compared to planktonic cells (Chaieb *et al.,* 2011).

The effect of YEO reduced significantly with time (Figures 5.1 A and 5.1 B) with the oil being most effective after one hour of incubation. This observed resistance of a preformed biofilm may be associated with the negatively charged extracellular polysaccharide layer which may repel the entry of antimicrobials, or to the compact three dimensional arrangement of a mature biofilm that may hinder the entry of these compounds into the biofilm (Sandasi *et al.,* 2008). Another factor which may contribute to this increased resistance is that most antimicrobial compounds are more effective against actively growing cells. The cells in a biofilm have a poor growth rate due to lack of nutrients and oxygen, which may reduce the antimicrobial effects of compounds against them (Sandasi *et al.,* 2010).

(A)
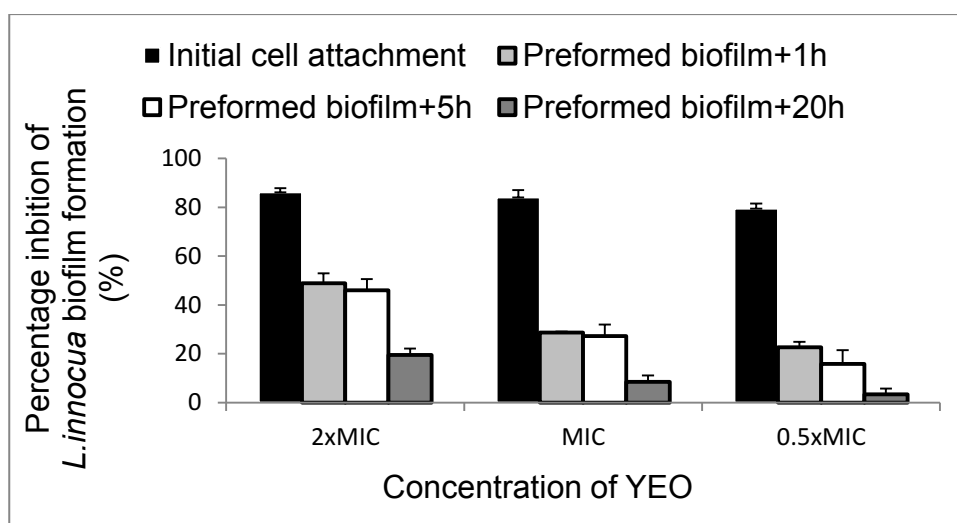
(B)
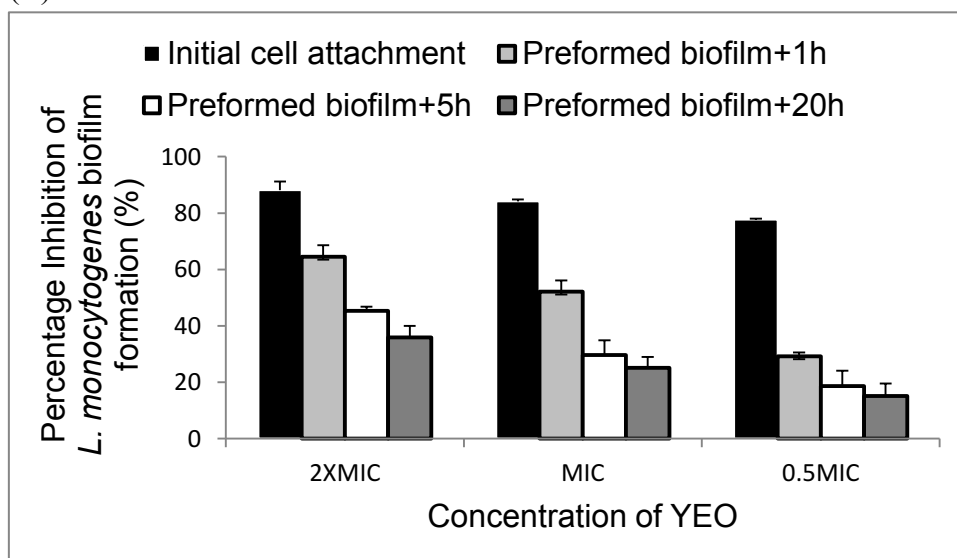
Figure 5.1 Effects of different concentrations of YEO (expressed as percentage inhibition of biofilm formation) on initial cell attachment and on 6 h preformed biofilms of *L. innocua* (A) and *L. monocytogenes* (B) incubated with the oil for 1 h, 5 h and 20 h, as determined by the crystal violet assay.

5.4.4 Biofilm inhibitory effects of YEO on polystyrene surface assessed by the MTT assay

Although the CV assay serves as an indicator of attached biomass, it does not reveal the metabolic status of the cells. MTT is a tetrazolium salt which, in the presence of metabolically active cells, is reduced into a product that can be measured calorimetrically, thus serving as a respiratory indicator of live cells (Krom *et al.,* 2007).

In biofilms, the MTT assay is used as an indicator of attached viable cells, while CV stains both viable and non-viable cells that may be attached (Kouidhi *et al.,* 2010). The results of the MTT assay confirmed that the essential oil significantly inhibited metabolic activity of the biofilms formed by both the *Listeria* species ($p < 0.05$) at 0.5x MIC. The addition of YEO to prevent initial cell attachment not only reduced the biomass (as indicated by CV assay) but also the metabolic activity of the cells, which resulted in 65.3 % inhibition of *L. monocytogenes* and 63.7 % inhibition of *L. innocua.* However, in the case of preformed biofilms, the metabolic activity suppression was found to increase with increased time of exposure, with activity being lowest after a 20 h exposure (Figure 5.2). This can be explained by the fact that most essential oils are hydrophobic and remain associated with the lipid-rich cells walls, thus leading to increased cell permeability and eventually cell death. Thus, as the time of exposure is increased, the loss of constituents from the bacterial cell will also increase (Oliveira *et al.*, 2010a).
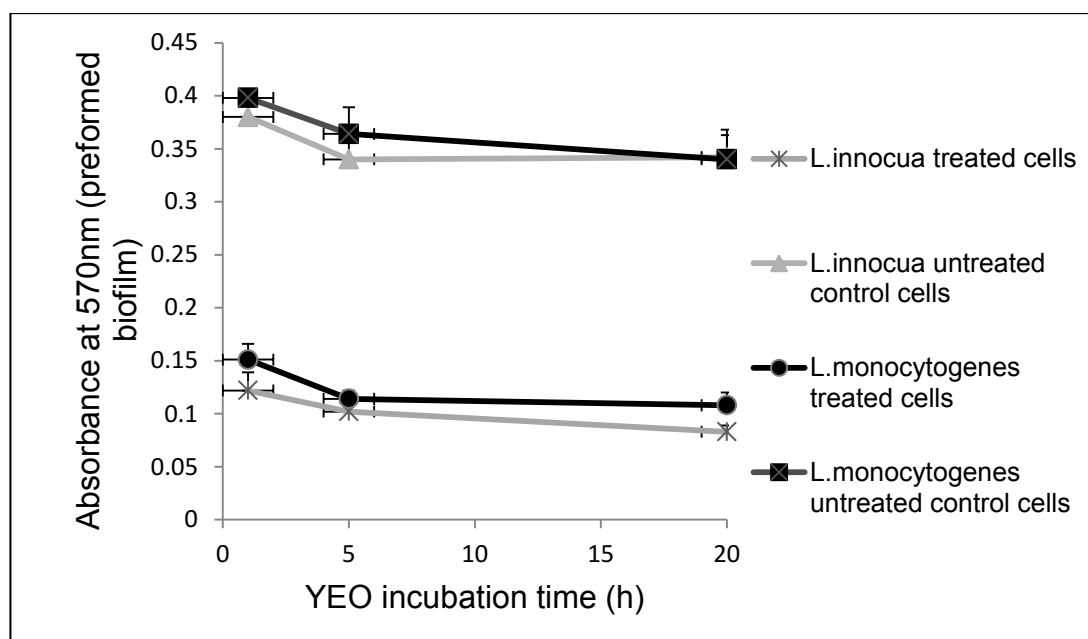
Figure 5.2 Effect of YEO on the metabolic activity of preformed biofilm cells of *L. innocua* and *L. monocytogenes* incubated with the oil for 1 h, 5 h and 20 h, as determined by the MTT assay.

5.4.5 Biofilm inhibitory effects of YEO on SS and HDPE surfaces

Since treatment with YEO resulted in significant inhibition of initial cell attachment on polystyrene surface for both the listerial isolates, a further study was undertaken to investigate its effect on two additional abiotic surfaces commonly encountered in food processing environments (Dourou *et al.,* 2011). Similar to previous findings (Sinde and Carballo, 2000; Di Bonaventura *et al.,* 2008; Oliveira *et al.*, 2010b), the current study highlighted the ability of *L. monocytogenes* to adhere to abiotic surfaces found in food processing environments. After using an initial inoculum of 8 log cfu/mL and a 24 h incubation period, it was observed that an average of 5.71 log cfu/cm^2 (*L. monocytogenes*) and 6.18 log cfu/cm^2 (*L. innocua*) cells adhered to the SS surface. In case of the HDPE surface, an average of 5.72 log cfu/cm^2 (*L. monocytogenes*) and 5.66 log cfu/cm^2 (*L. innocua*) cells were found adhered. The inhibition of attachment at MIC concentration of YEO was exhibited as a logarithmic reduction ($p < 0.05$) in the initial number of cells attached to the SS and HDPE surfaces and the final number of attached cells after treatment with the oil. The results from Table 5.4 indicate that the logarithmic reduction on each surface was almost similar between the two isolates. However, the effect of YEO was more prominent on the SS surface as it led to a greater logarithmic reduction (3.34 ± 0.015 for *L. monocytogenes* and 4.56 ± 0.11 for *L. innocua*) in the attached cell number compared to the HDPE surface (2.24 ± 0.07 for *L. monocytogenes* and 2.50 ± 0.09 for *L. innocua*).

Table 5.4: Inhibition of cell attachment to stainless steel and high density polyethylene surfaces expressed in terms of log reduction (p < 0.05)

Abiotic surfaces	Organism	log reduction[a]
SS	*L. monocytogenes*	3.34 ± 0.15
	L. innocua	4.56 ± 0.11
HDPE	*L. monocytogenes*	2.24 ± 0.07
	L. innocua	2.50 ± 0.09

[a] expressed as mean value ± standard deviation

5.4.6 GC-MS analysis of YEO

In accordance with previous studies (Rohloff *et al.*, 2000; Smelcerovic *et al.,* 2010; Nadim *et al.,* 2011), GC-MS analysis identified several monoterpenes such as β-pinene,

1, 8-cineole, terpinene-4-ol and caryophyllene as major components of YEO (Table 5.5). Two of the major components detected in the current study, viz. 1, 8-cineole and β-pinene, have been previously identified in conifer essential oil and also reported to exhibit significant anti-*L. monocytogenes* activity (Mourey and Canillac, 2002). Monoterpenes have been found to interfere with the cell membrane functions in bacteria. These components cross the bacterial cell membrane, penetrate into the interior and interact with the intracellular sites, which eventually cause cell death (Oliveira *et al.,* 2010b). Some studies have reported that essential oil constituents can show synergistic antimicrobial effects when used in certain combinations (Dimitrijevic *et al.,* 2007). Thus, it is important to study the actual mechanism of action of the bioactive components in essential oils and also their synergistic or antagonistic behaviours with other essential oils or components.

Table 5.5: Chemical composition of YEO

Compound	Percentage	Compound	Percentage
Caryophyllene	15.9	Gamma-terpinene	4.8
1,8-cineole[a]	12.0	Cymene	4.6
Bornyl acetate	9.9	Alpha caryophyllene	4.1
1-terpinen-4-ol	8.2	Germacrene D	3.9
β-pinene	8.1	Carvacrol	2.4
Camphor	6.6	Lavandulol aceate	2.3
Linalool[b]	5.5	Piperitenone oxide	2.3
Sabinene	5.0	Beta-thujone	2.2
		Thymol	2.1

[a,b] Verified using authentic samples

5.4.7 Cytotoxicity of YEO

In recent years, there has been increased interest in plant based antimicrobials such as essential oils. However it is very important that these compounds are also tested for toxicity if they are to be applied in situations where contact with humans is possible. This is because in the case of most essential oils, minimal information exists about the actual bioactive components that are responsible for their antimicrobial activity. In addition, essential oils are made up of a range of constituents which may affect many cell types differently (Russo *et al.,* 2013).

In the current study, the potential application of YEO as a surface sanitizer has been proposed and, in a typical food processing plant, it will be mandatory to be familiar with its cytotoxic effects. Thus, to determine the non-toxic concentration of essential oils, the viability of cells was determined on proliferating mouse 3T3-L1 cells using the MTT assay and the cytotoxicity was compared to bleach. Figures 5.6 depicts the percentage cell viability of 3T3-L1 cells when treated with various concentrations (ranging from 0.5-0.016 % v/v) of YEO, TTO and bleach. The viability of cells decreased with increasing concentrations of essential oils and both essential oils had similar cytotoxic effects. At lower concentrations (< 0.25 %), none of the compounds tested induced any cytotoxicity whereas at concentration of 0.5 % and higher, YEO and TTO showed a slightly increased reduction in cell viability when compared to bleach. Ramage *et al.* (2013) investigated the cytotoxicity of TTO against mammalian fibroblast (PRF cell line) and epithelial cells (OKF6-TERT2 cell line) and found it to be cytotoxic at concentrations of 0.5 % (v/v) and higher after a 2 min exposure period. Since different studies have used different cell lines and exposure periods, it is difficult to generalise or interpret the cytotoxic effects. In the current study the cytotoxic effects of the oils were found to be comparable to bleach which further supports the application of oils as surface sanitizers. It is important to note that while bleach is composed mainly of a single antimicrobial ingredient, sodium hypochlorite, essential oils contain many constituents that can contribute to their cytotoxicity. Since the cytotoxicity of all components of YEO was not evaluated individually, it is not possible to identify the actual component/s responsible for this effect. This effect could also be due to the synergistic effects of different terpenes in the oil. This again highlights the importance of deciphering the actual mechanism and components involved in the antibacterial action of essential oils.
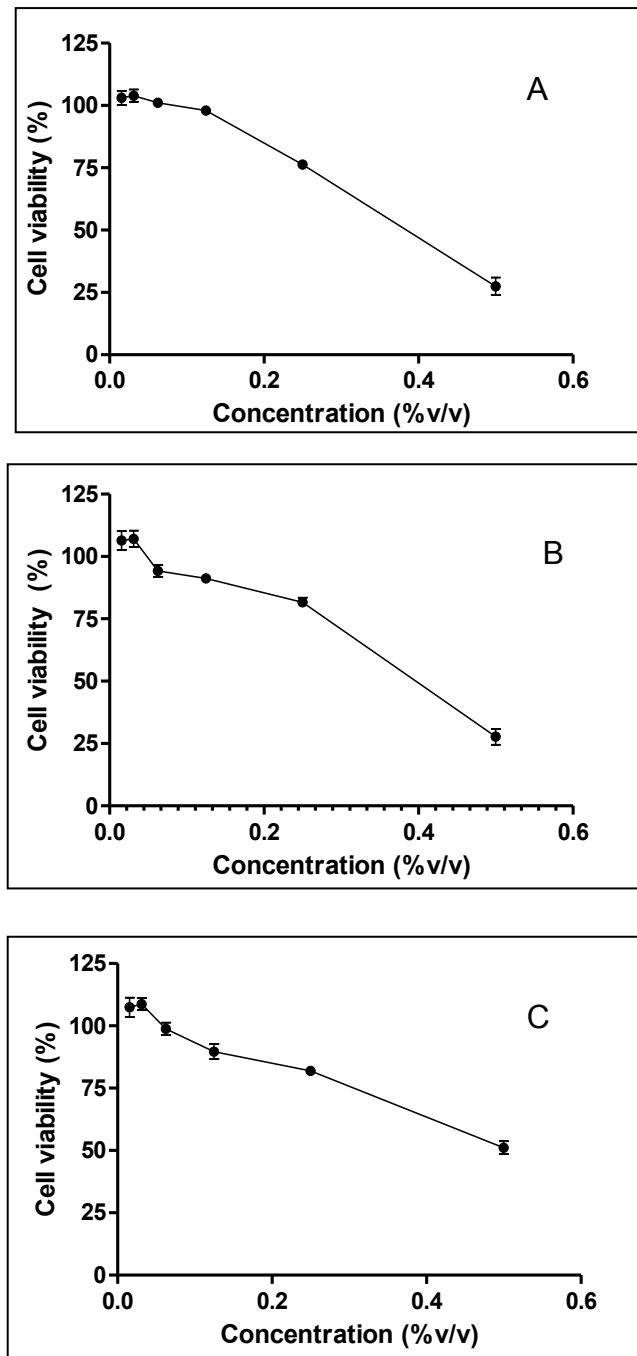
Figure 5.3 Cell viability of 3T3-L1 cells at different concentrations of (A) yarrow essential oil, (B) tea tree oil and (C) bleach.

5.5 Conclusions

Eradication of the foodborne pathogen *L. monocytogenes* from food processing environments is a major challenge for the food industry. The primary reasons for this are the severity of listeriosis, its ubiquitous nature and the ability to form biofilms on abiotic surfaces which tend to be more resistant to certain antimicrobial agents compared to their planktonic counterparts. The common sites of listerial contamination include conveyor belts, drains, equipment surfaces, cold rooms and transportation areas (Soni and Nannapaneni, 2010).

Recently, plant based antimicrobial agents, such as essential oils, have attracted considerable interest amongst consumers as they are considered safer than synthetic sanitizing agents. The current study investigated the anti-listerial and biofilm inhibitory effects of YEO against *Listeria* species and demonstrated that YEO is an effective antimicrobial agent against sessile and planktonic cells of *Listeria*. It was particularly effective in inhibiting initial cell attachment to polystyrene, SS and HDPE surfaces compared to preformed biofilms. The MTT assay also suggested that YEO was effective at significantly reducing the metabolic activity of the biofilms. This indicates that it can be used to condition the abiotic surfaces in food processing plants by incorporation into sanitizer formulations, in order to inhibit the adherence of listerial cells. Alternatively, it may also be used during the manufacture of such surfaces making them resistant to bacterial adhesion. Essential oils are known to show additive, antagonistic or synergistic effects with other essential oils and essential oil components (Nestor Bassole and Juliani, 2012; Yap *et al.,* 2014).Since preformed biofilms were found to be more resistant to YEO treatment, a combination of YEO with other oils or sanitizers that produce synergistic effects may be considered. It is important to highlight that the current study also evaluated the cytotoxicty of YEO and compared it to bleach. The cytotoxic effects of YEO and bleach were found to be similar, indicating that it is important to regulate the final concentrations of YEO in sanitzer formulations to be used in food processing environments.

Chemical intervention using disinfectants or sanitizers, such as quaternary ammonium compounds, sodium hypochlorite, hydrogen peroxide or benzalkonium chloride, is the most commonly used strategy for controlling this pathogen (Pan *et al.,* 2006). However,

continual exposure to some of these chemicals can lead to development of resistance in pathogens. In contrast, some plant-based essential oils have multiple bioactive components, thus making it difficult for the pathogens to acquire such resistance (Soni *et al.,* 2013). Hence, the use of EOs in sanitizing or disinfecting formulations is a plausible strategy for controlling *Listeria* in food processing environments.

Chapter 6

Conclusions and future directions

Foodborne diseases are a major concern for public health globally. In Australia, about 5.4 million cases of foodborne illnesses occur annually, costing the community approximately $1.2 billion dollars (OzFoodNet, 2012). In the period from 2000-2010, *Listeria monocytogenes* was one of the leading foodborne pathogens responsible for deaths in Australia (Kirk *et al.,* 2014). Since ingestion of raw and uncooked foods such as dairy, meat and RTE products are the primary source for *Listeria* infections, it is essential that this bacterium is monitored and controlled by the industries involved in manufacturing such foods. Thus, the food industry requires rapid, reliable and robust methods to continually detect, subtype and control this pathogen. Keeping this in mind, the main aims of the current research project were to investigate a) the suitability of MALDI-TOF MS as a rapid and reliable detection tool for *L. monocytogenes* grown under different culture conditions and food enrichment broths; b) the application of MALDI-TOF MS as a source-tracking tool for *L. monocytogenes* isolates obtained from food processing environments; and c) the antimicrobial and biofilm inhibitory effects of essential oils against *Listeria* obtained from food processing environments.

This chapter provides an overview of the major findings of this research and identifies the scope for further research.

6.1 Summary of Conclusions

6.1.1 MALDI-TOF MS as a detection tool for *L. monocytogenes*

Chapter 3 investigated MALDI-TOF MS as a detection tool for *L. monocytogenes* isolates obtained from food processing environments. Some studies have previously reported rapid species level identifications of *Listeria* using this technology (Barbuddhe *et al.,* 2008; Hseuh *et al.,* 2014); however, the current study also explored the influence of culture conditions on the overall success of identification obtained using this technique. The type of culture media and the age of the culture significantly impacted on species level identifications, while genus level identifications were less affected. An incubation period of 24 h was determined to be ideal for achieving higher species level identifications using the direct smear technique. Indeed, incubation for a further 24 h did not improve identification and, in some cases, led to poorer identification rates. Culturing on specific media, namely ALOA (chromogenic media), HBA (non-selective

media) and BHIA (non-selective media) yielded identification rates of 80 % or more to the species level. In general, spotting from selective media (OA and PA) was found to be more cumbersome compared to other media. The results clearly shows that although this proteomics-based approach is rapid, simple and has high through-put capacity, it requires sufficient standardisation of culture conditions to obtain reliable results.

The second focus of Chapter 3 was to investigate the detection of *L. monocytogenes* directly from food enrichment broths. To the knowledge of the author, this is the first study reporting direct detection of *L. monocytogenes* from different food matrices using MALDI-TOF MS. MALDI-TOF MS has mostly been used to detect microbes from solid culture media and from blood and urine samples (Ferreira *et al.,* 2010, 2011, Juiz *et al.,* 2012). A simple sample preparation method was proposed to analyse the broth samples. Similar to the results from Chapter 3, culture media influenced the time for detection in the case of spiked UHT milk samples. In the case of the non-selective BHI broth, low levels of contamination (as few as 1 colony-forming unit (cfu) of *L. monocytogenes* per mL of initial broth culture) could be detected within 24 h of incubation at 37 °C, while a 30 h incubation period (24 h primary enrichment followed by 6 h secondary enrichment) was required for the selective OBL broth. The number of bacterial cells in the final broth sample and the presence of extraneous peaks from the food matrix influenced the accuracy of MALDI-TOF MS detection, suggesting that sample clean-up procedures to reduce background proteins may help to achieve faster detection. The limit of detection for identification of *Listeria* from a broth sample was approximately 10^8 cfu/mL.

The reliability of the 30 h detection scheme from OBL broth was challenged by introducing a mix of microbial pathogens (1 cfu/mL each of *L. monocytogenes, S. aureus* and *E. coli* O157:H7). Even in the presence of competing microbes, successful detection was obtained for the pathogen of interest. This was an important finding since many foods have their natural microbiota which can potentially interfere with MALDI-TOF MS identification. Subsequently, the detections scheme successfully detected the pathogen in three other solid foods i.e. cantaloupe, chicken pâté and Camembert cheese for initial spiking loads of 10 cfu/mL. Overall, successful detection was found to be dependent on complete inhibition of competing bacteria. The results provide proof-of-

principle evidence that direct detection of foodborne pathogens from enrichment broths using MALDI-TOF MS is plausible. The time and cost savings associated with this technology should prove highly beneficial for the food industry.

6.1.2 MALDI-TOF MS as a source-tracking tool for *L. monocytogenes*

Chapter 4 addressed the second aim of this research. Most studies have focussed on the diagnostic capabilities of MALDI-TOF MS. However, this study explored its ability to find differences at the subspecies level for *L. monocytogenes* isolates obtained from different dairy sources within Australia. Since the type of data analysis tool used can influence the final outcome of subtyping using MALDI-TOF MS, application of two data analysis tools, SPECLUST and multivariate data analysis (SIMCA), was investigated in the current study. Both approaches suggested that culture conditions influenced MALDI-TOF MS-based subtyping which reinforced the findings of Chapter 3. The clustering of isolates differed with changes in media and time of incubation. With SPECLUST, the clustering was random and no relatedness between isolates could be established. Previously, SPECLUST has been used to investigate species-level differences in microbes (Böhme *et al.,* 2010*,* 2012), hence it is possible that a more refined approach is required to reveal strain-level relatedness in *L. monocytogenes* (Chen *et al*., 2007) very long line and the relevance of clonal complexes is not quite clear to strain-level ID.

The second approach adopted was multivariate data analysis (using SIMCA). PCA and PLS-DA modelling followed by hierarchical clustering of data obtained from almost all media was able to discriminate between dairy isolates obtained from four sources within Australia with few discrepancies. Clustering obtained using HBA (24 h incubation) showed maximum congruence with the gold standard PFGE technique and was also more discriminatory than PFGE (Simpsons Index of Diversity for HBA = 0.89, versus 0.86 for PFGE). Both PFGE and MALDI-TOF MS-based source-tracking indicated that Source 1, which was facing a persistent *L. monocytogenes* problem for two years, was contaminated by diverse *L. monocytogenes* strains. Biomarker peaks discriminating the isolates based on their sources were identified using t-test/ANOVA.

Similar to the conclusions of other studies (Barbudhhe *et al.,* 2008 and Hseuh *et al.,* 2014), MALDI-TOF MS-based source-tracking was also able to discriminate the dairy isolates based on their clonal lineages. Lineage I (1/2b or 3b serotypes) and II (1/2a or 3a serotypes) isolates were discriminated using PLS-DA modelling followed by hierarchical clustering. Characteristic peaks specific to lineage I (2758.67, 6743.64 m/z ratio) and II (2141.31, 5591.67 and 5792.16 m/z ratio) were identified. Thus, the findings of the current study demonstrated that MALDI-TOF MS-based source-tracking correlated well with PFGE and serotyping. However, in comparison to PFGE, MALDI-TOF MS-based source-tracking was found to be more simple and rapid and less labour intensive.

6.1.3 Control of *L. monocytogenes* in food processing environments

While monitoring the presence of a foodborne pathogen like *L. monocytogenes* is a routine matter, controlling its prevalence in a processing environment is the ultimate goal of the food industry. Recurrent contamination with the pathogen spanning over a decade have been reported in the past (Swaminathan and Garner-Smidt, 2007). Such a persistent type of contamination can be attributed to the organism's ability to survive extreme physiological environments by forming biofilms. Chapter 5 investigated the use of plant based essential oils as an effective control strategy. These compounds were selected because of the need to discover new antimicrobials (to overcome the problem of resistance in microbes), especially ones that are derived from natural sources such as plants (Leonard *et al.,* 2010).

Essential oils of yarrow, cold pressed lemon, bergamot, pine scotch and peppermint showed anti-listerial effects. Yarrow essential oil also exhibited biofilm inhibitory effects against *L. monocytogenes* and *L. innocua* isolates obtained from independent dairy sources. The essential oil was particularly effective in inhibiting the initial attachment of cells to polystyrene, stainless steel and high density polyethylene surfaces. Results of the MTT assay indicated that the essential oil not only reduced biomass attachment but was also reducing the metabolic activity of the cells in the biofilm at sub MIC levels. In the case of preformed biofilms, the effect of the oil reduced significantly. The oil displayed dosage dependent effects and was most effective after one hour of incubation. Unlike most studies that propose the use of

essential oils as food additives, the current study proposed the application of yarrow essential oil as a surface sanitizer. Hence, the cytotoxicity of the oil was compared to bleach, which is commonly used as a sanitizer in the dairy industry. The toxicity of the oil against 3T3-L1 cell line was found to be comparable to both bleach and tea tree oil.

Some studies have reported *L. monocytogenes* strains resistant to one or more antimicrobials or antimicrobial strategies used by the food industry to control microbes in the processing environments (Carpentier and Cerf 2011, Luz *et al.,* 2012). However, since essential oils are generally comprised of multiple bioactive constituents, it will be more challenging for the bacterium to develop resistance against all of them. Hence, essential oils represent an efficient strategy to control the prevelance of foodborne pathogens on abiotic surfaces found in food processing environments.

6.2 Future Directions

6.2.1 MALDI-TOF MS-based detection and subtyping

In the current research, alterations in culture conditions were found to influence both species-level detections and subtyping using MALDI-TOF MS. This clearly highlights limitations in the currently available, commercial databases. Such limitations have also been reported previously (Seng *et al.,* 2009; Vithanage *et al.,* 2014). Hence, in-house databases including reference spectra of bacteria collected from diverse geographical locations and cultivating under different culture conditions should be developed. This should greatly improve the success rate of microbial identification using MALDI-TOF MS.

The current study provided proof-of-concept evidence that MALDI-TOF MS can be used to detect *L. monocytogenes* from food enrichment broths using a 30 h detection scheme. However, only a limited number of spiked foods were tested. The robustness of this detection should be investigated by testing a larger variety of foods since food matrices can differ considerably in their protein constitution and inherent microbiota. A major hurdle in the current experiments was the background food matrix peaks, thus emphasizing the importance of sample clean up prior to analysis. Use of chemical or physical separation techniques, such as protein precipitation or filtration, should be

explored. Alternatively, a separate algorithm may be introduced into the databases that can recognise the specific peaks arising from the food. The proposed 30 h detection scheme can also be tested for other foodborne pathogens such as *E. coli* O157:H7, *Salmonella* or *S. aureus,* to assess if faster detection can be achieved for these pathogens which have faster growth rates than *Listeria*.

MALDI-TOF MS-based source-tracking was compared to PFGE in this research. However, some studies, including the current one, concluded that MALDI-TOF MS-based subtyping was not found to be in complete agreement with PFGE (Griffin *et al.,* 2012; Verroken *et al.,* 2014). Hence, more studies comparing MALDI-TOF MS-based subtyping with other techniques, such as SNP based subtyping, MLST or MLVA, should be performed. This can eventually help to select a single or combinatorial approach for subtyping foodborne pathogens to obtain maximum discrimination in the most time saving manner.

6.2.2 Essential oils as control agents

Essential oils have been studied extensively for their antimicrobial properties. However, their mechanism of action has not been deciphered completely. In the current study, essential oils of yarrow were studied for its anti-listerial and biofilm inhibitory effects. Identification of the bioactive components of this oil and the mechanism of action can assist in determining whether the effects are the result of single bioactive ingredients or the combined effect of all components. Since interactions between essential oils and their components can produce additive, antagonistic or synergistic effects, more studies investigating such interactions can assist in developing strong antimicrobial combinations that can inhibit multiple pathogens simultaneously.

Growing interest in the application of essential oils as antimicrobial agents has led to the hypothesis that they may also play a role in quorum sensing. Biofilm formation in Gram-positive and Gram-negative bacteria is regulated by quorum sensing which involves the secretion of certain signalling molecules in bacteria. Essential oils of oregano, marjoram, and the essential oil component eugenol have been found to produce anti-quorum sensing activities which may eventually be responsible for biofilm inhibition (Kerekes *et al.,* 2013, Burt *et al.,* 2014; Kim *et al.,* 2015). It will be

interesting to investigate if essential oils of yarrow may be involved in such a mechanism.

Lastly, since this research proposed the application of yarrow essential oil as a surface sanitiser, a more extensive study should be undertaken to compare its anti-listerial effects in combination with commercially used sanitisers. In addition the biofilm inhibitory effects of the oil and sanitizers should be studied in condition mimicking food processing environments. Simulating extreme physiological conditions found in these environments which include less water and nutrient availability, refrigeration temperatures, conditioned abiotic surfaces and periodic cleaning cycles will be able to provide a realistic assessment of the response of the pathogen on exposure to these sanitisers.

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Zhang, D., Zhang, H., Yang, L., Guo, J., Li, X., Feng, Y. (2009). Simultaneous detection of *Listeria monocytogenes, Staphylococcus aureus, Salmonella enterica and Escherichia coli* O157:H7 in food samples using multiplex PCR method, *Journal of Food Safety, 29*, 348-363.

Zhang, Y., Yeh, E., Hall, G., Cripe, J., Bhagwat, A. A., Meng, J. (2007). Characterization of *Listeria monocytogenes* isolated from retail foods. *International Journal of Food Microbiology, 113,* 47-53.

Appendices

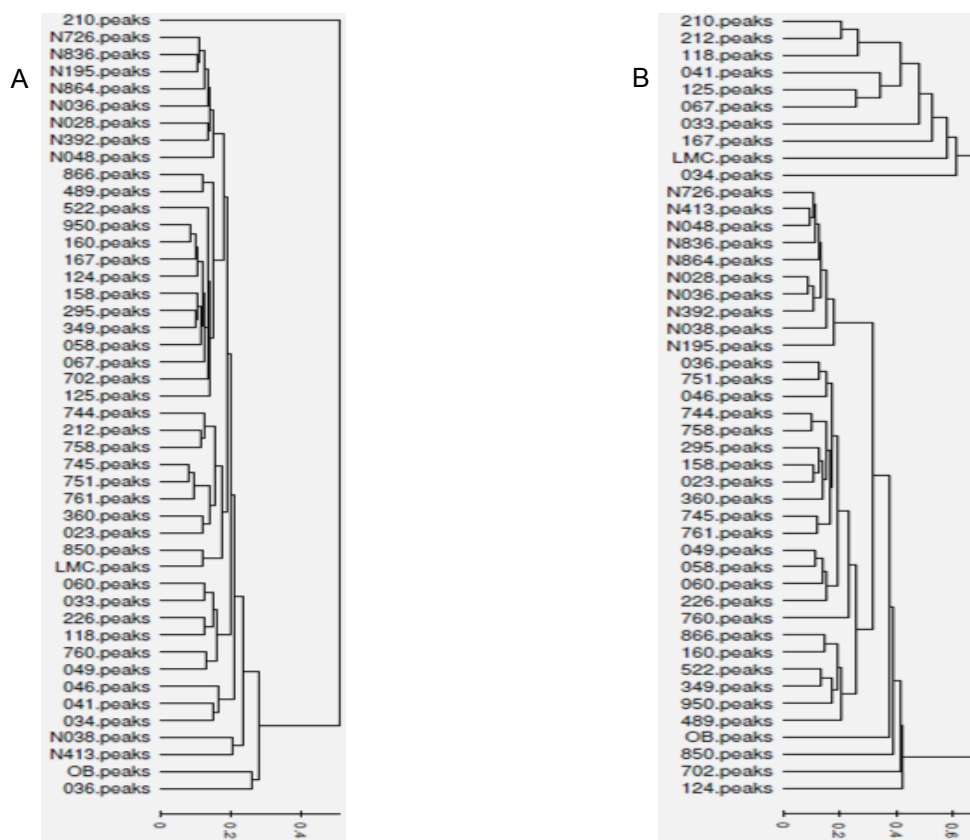**Figure A 4.1.** Cluster analyses of MALDI-TOF mass spectra of *Listeria*

Figure A 4.2. Cluster analyses of MALDI-TOF mass spectra of *Listeria monocytogenes* cultured on HBA agar for (A) 24 hours and (B) 48 hours.
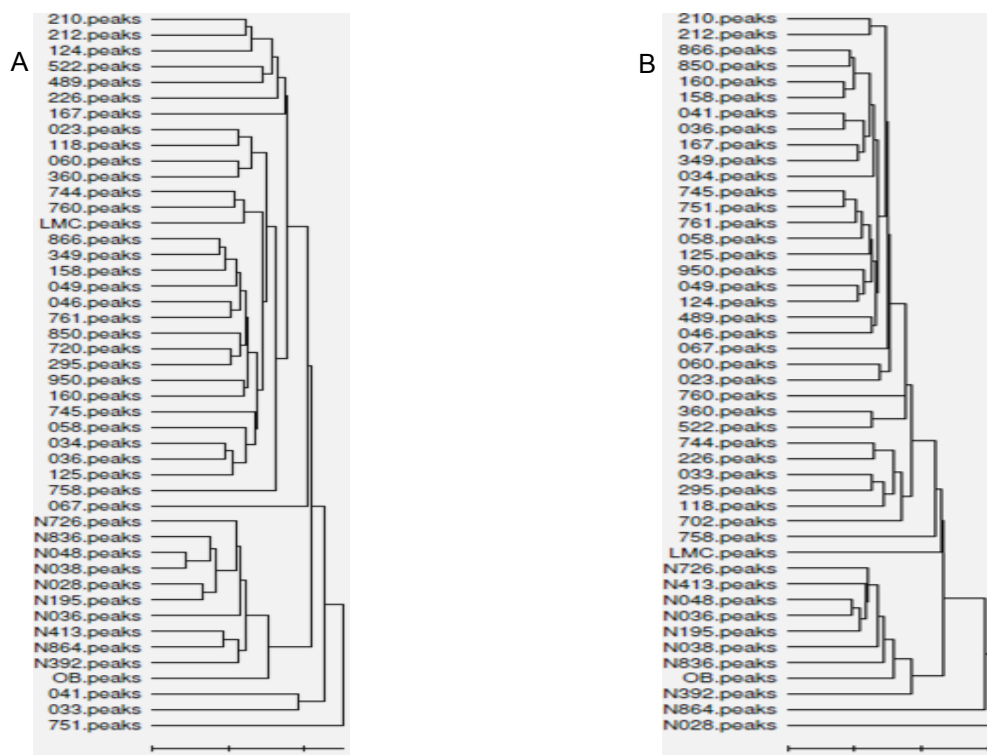
Figure A 4.3. Cluster analyses of MALDI-TOF mass spectra of *Listeria monocytogenes* cultured on Oxford agar for (A) 24 hours and (B) 48 hours.

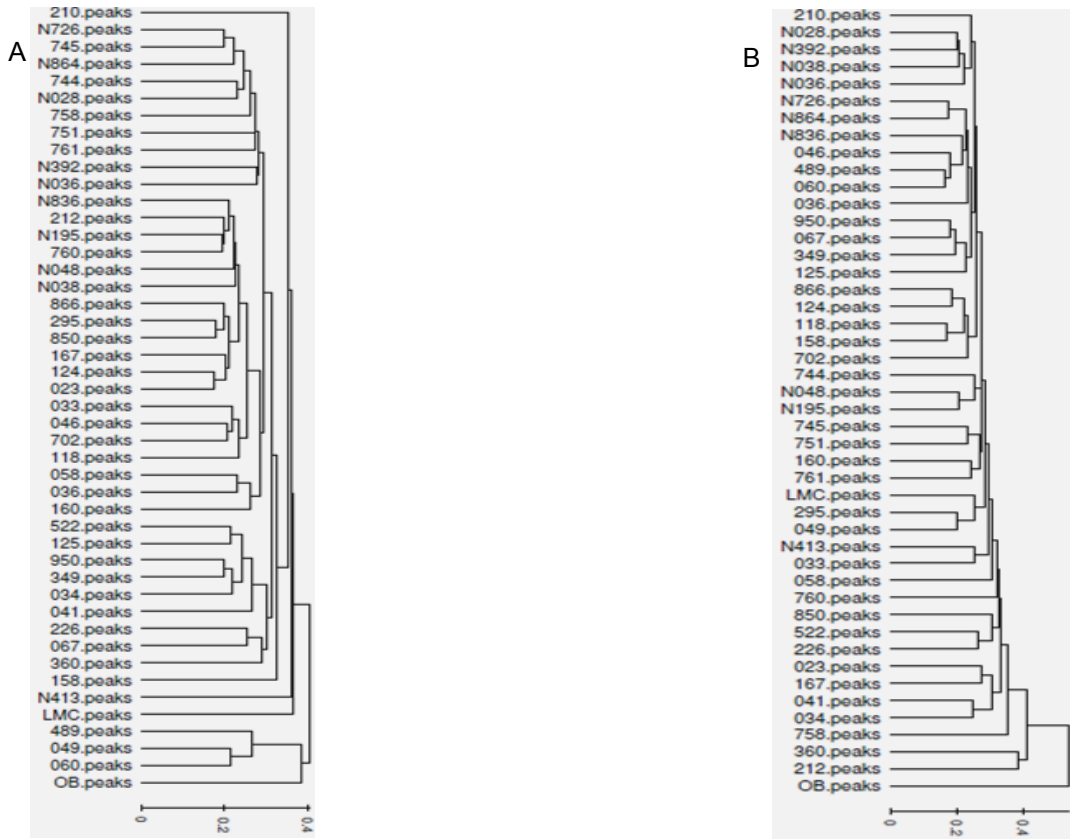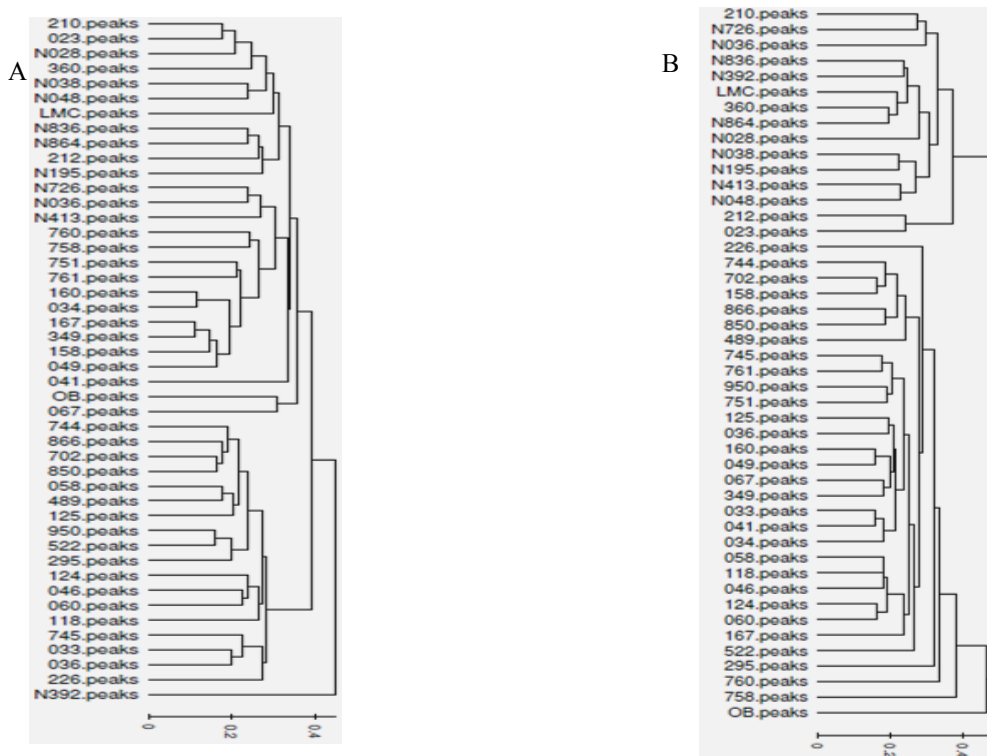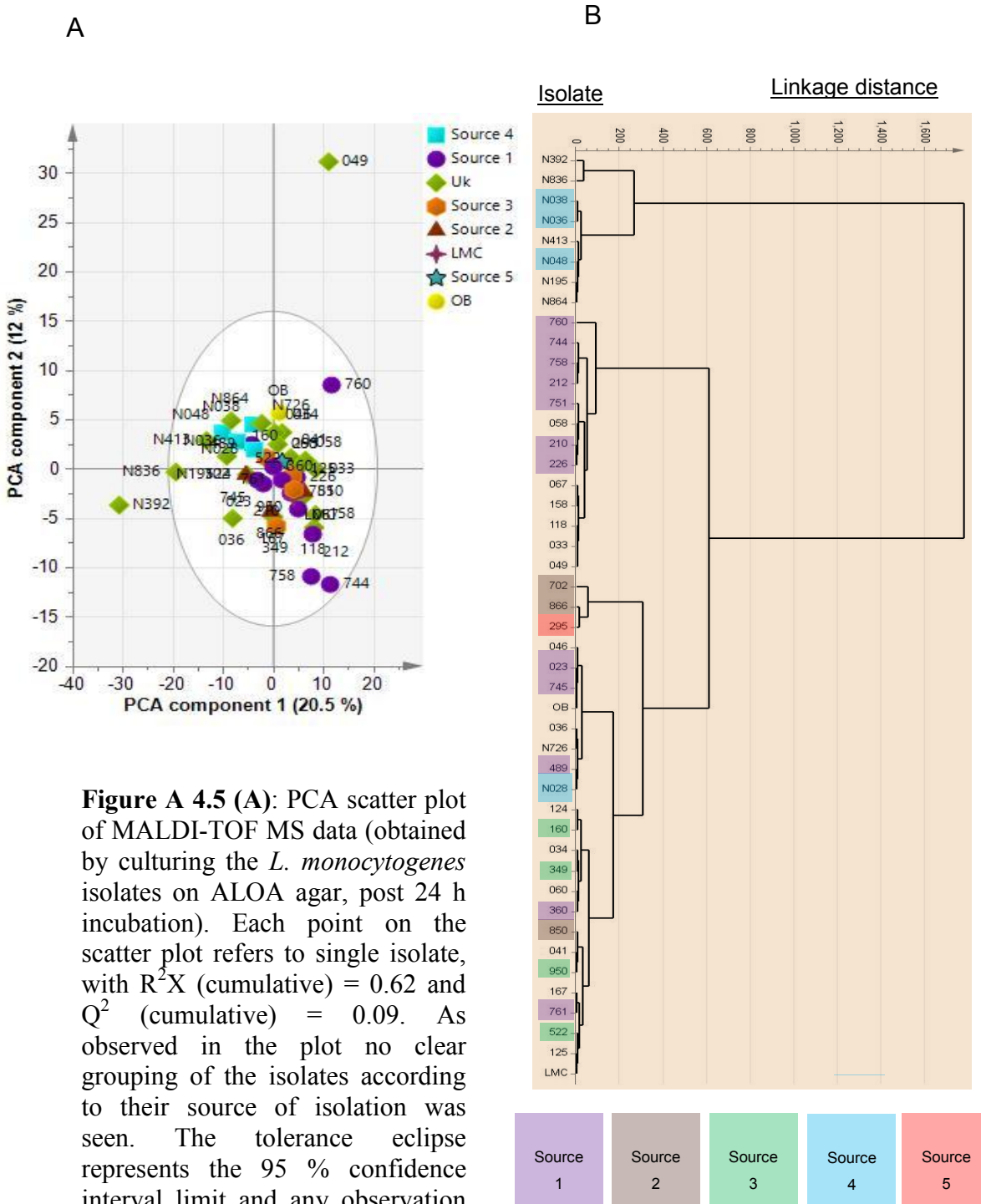**Figure A 4.4.** Cluster analyses of MALDI-TOF mass spectra of *Listeria monocytogenes* cultured on PALCAM agar for (A) 24 hours and (B) 48 hours.

A

Figure A 4.5 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on ALOA agar, post 24 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.62 and Q^2 (cumulative) = 0.09. As observed in the plot no clear grouping of the isolates according to their source of isolation was seen. The tolerance eclipse represents the 95 % confidence interval limit and any observation this eclipse, is considered as an outlier.

B

Figure A 4.5 (B): The PLS-DA analysis plot generated from ALOA (24 h) yielded R^2X, R^2Y and Q2 values of 0.48, 0.44, 0.13 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.

A

B
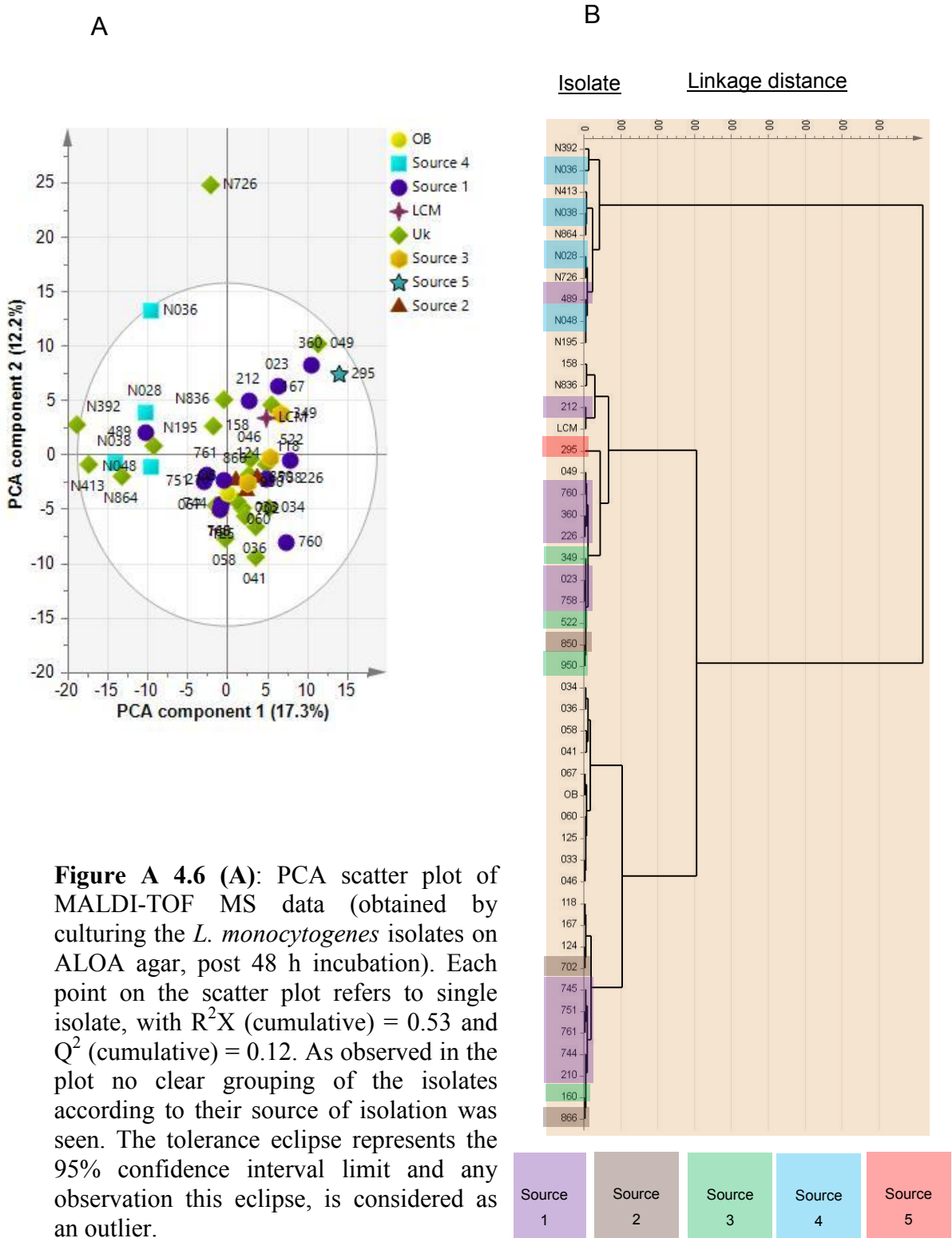
Figure A 4.6 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on ALOA agar, post 48 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.53 and Q^2 (cumulative) = 0.12. As observed in the plot no clear grouping of the isolates according to their source of isolation was seen. The tolerance eclipse represents the 95% confidence interval limit and any observation this eclipse, is considered as an outlier.

Figure A 4.6 (B): The PLS-DA analysis plot generated from ALOA (48 h) yielded R^2X, R^2Y and Q2 values of 0.52, 0.6, 0.12 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.

A

B

Figure A 4.7 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on HBA agar, post 24 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.44 and Q^2 (cumulative) = 0.14. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation outside it is considered as an outlier.

Figure A 4.7 (B): The PLS-DA analysis plot generated from HBA (24 h) yielded R^2X, R^2Y and Q2 values of 0.47, 0.6, 0.13 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.

A

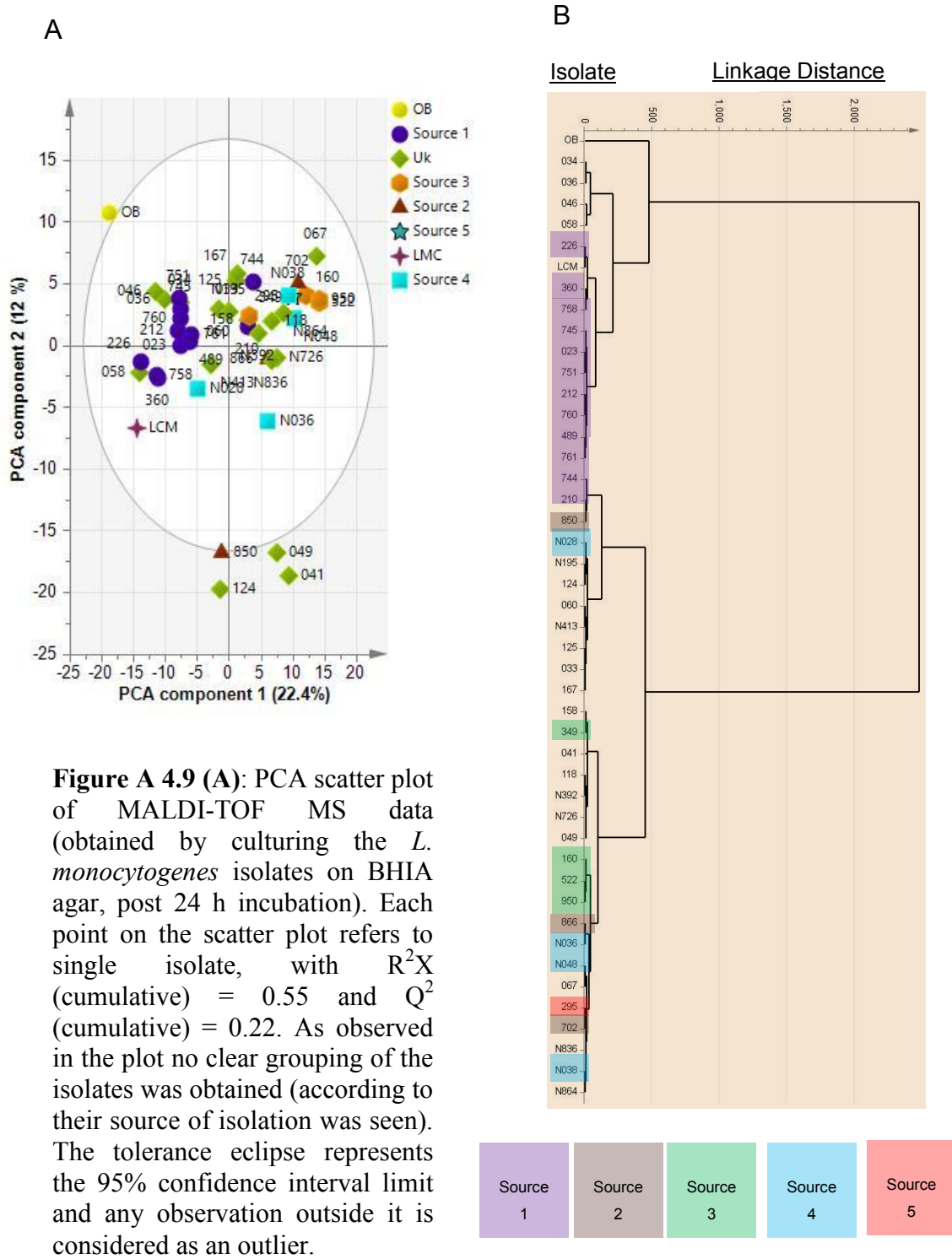
Figure A 4.8 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on HBA agar, post 48 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.49 and Q^2 (cumulative) = 0.02. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation outside it is considered as an outlier.

B

Figure A 4.8 (B): The PLS-DA analysis plot generated from HBA (48 h) yielded R^2X, R^2Y and Q2 values of 0.51, 0.6, 0.15 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.
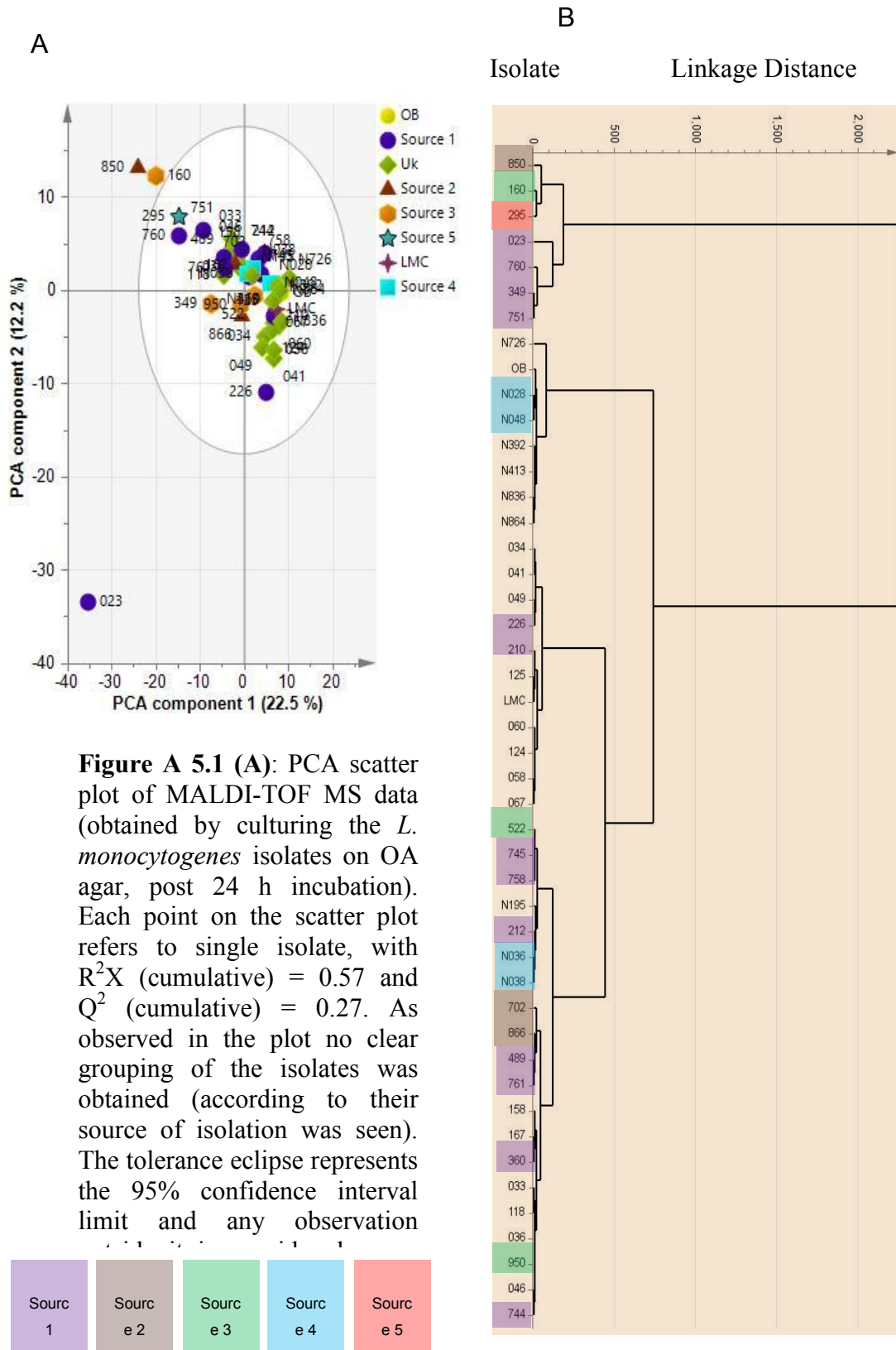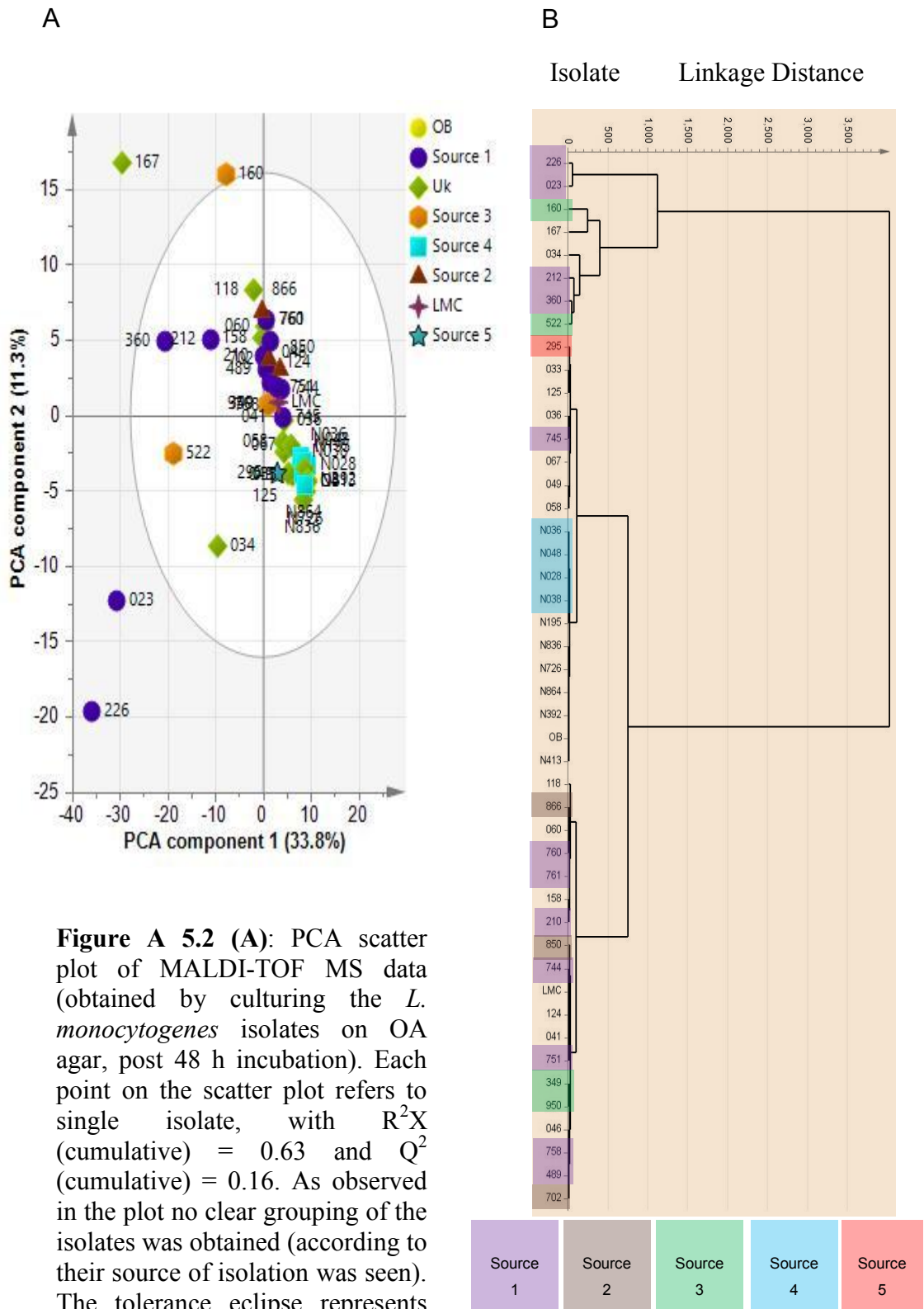
A

Figure A 4.9 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on BHIA agar, post 24 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.55 and Q^2 (cumulative) = 0.22. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation outside it is considered as an outlier.

B

Figure A 4.9 (B): The PLS-DA analysis plot generated from BHIA (24 h) yielded R^2X, R^2Y and Q2 values of 0.57, 0.75, 0.31 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.
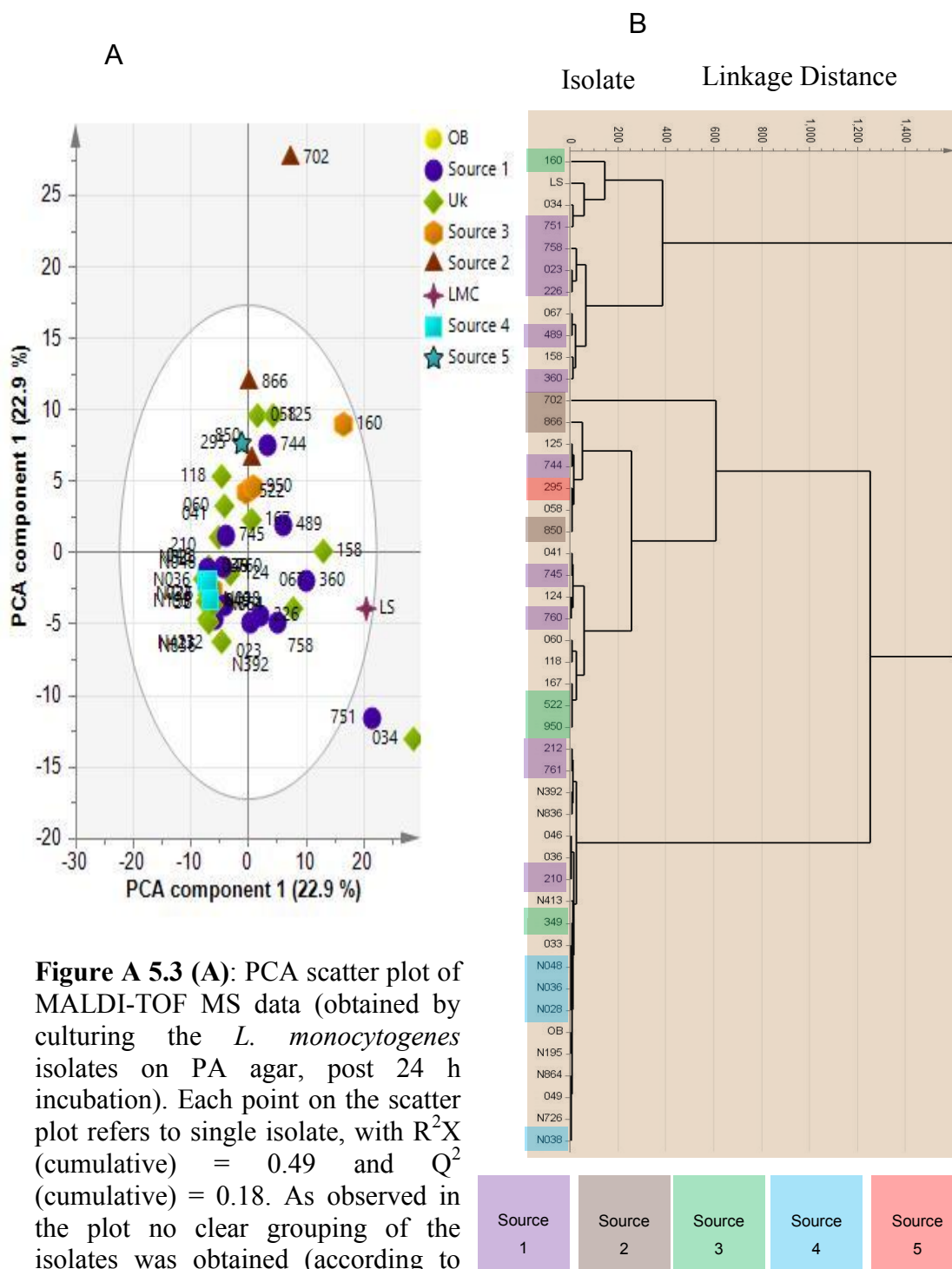
A

B

Figure A 5.0 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on BHIA agar, post 48 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.57 and Q^2 (cumulative) = 0.27. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation outside it is considered as an outlier.
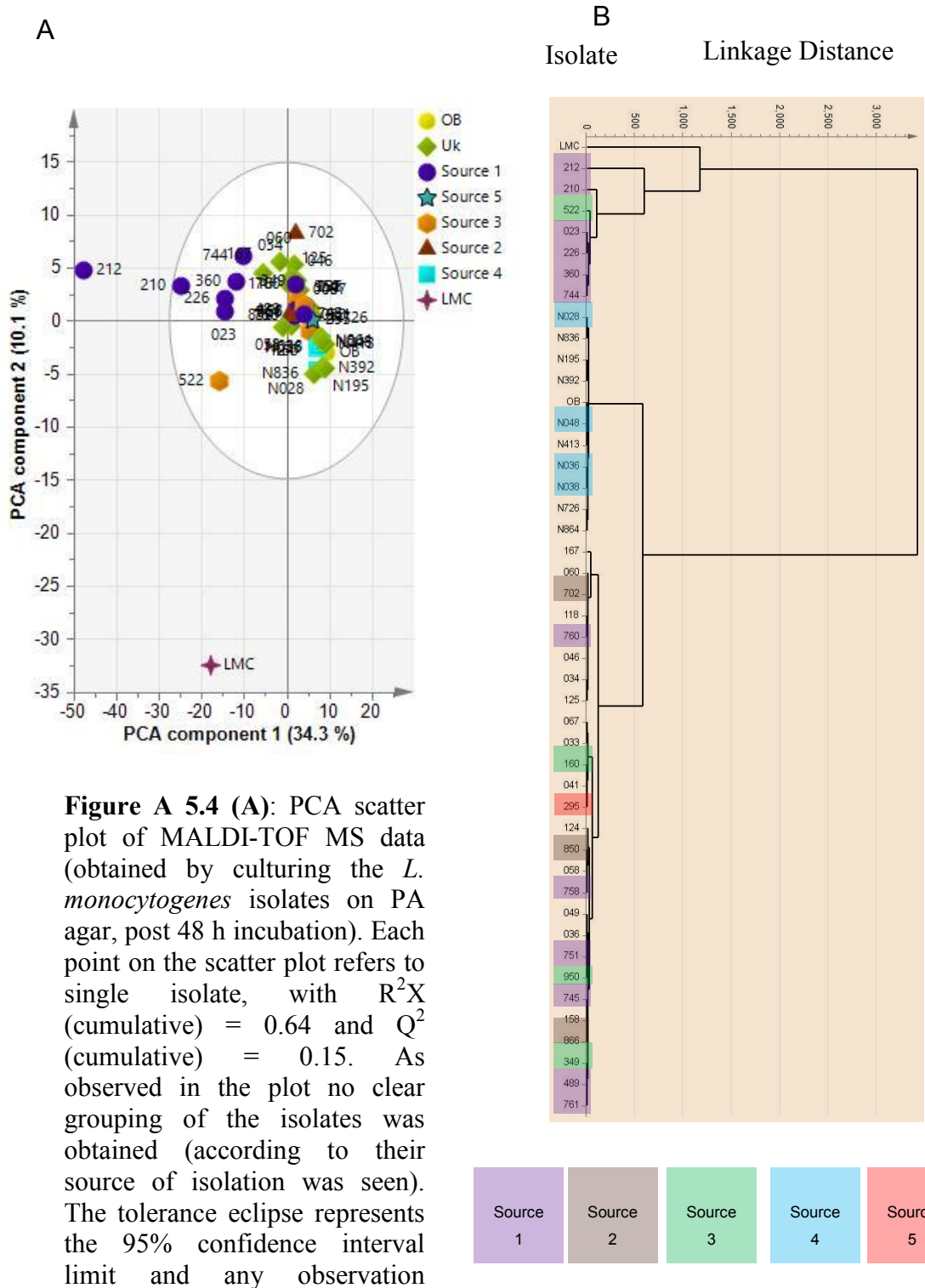
Figure A 5.0 (B): The PLS-DA analysis plot generated from BHIA (48 h) yielded R^2X, R^2Y and Q2 values of 0.59, 0.75, 0.12 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.

A

Figure A 5.1 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on OA agar, post 24 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.57 and Q^2 (cumulative) = 0.27. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation

Sourc 1	Sourc e 2	Sourc e 3	Sourc e 4	Sourc e 5

B

Isolate   Linkage Distance

Figure A 5.1 (B): The PLS-DA analysis plot generated from OA (24 h) yielded R^2X, R^2Y and Q2 values of 0.50, 0.52, 0.14 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.

A

B

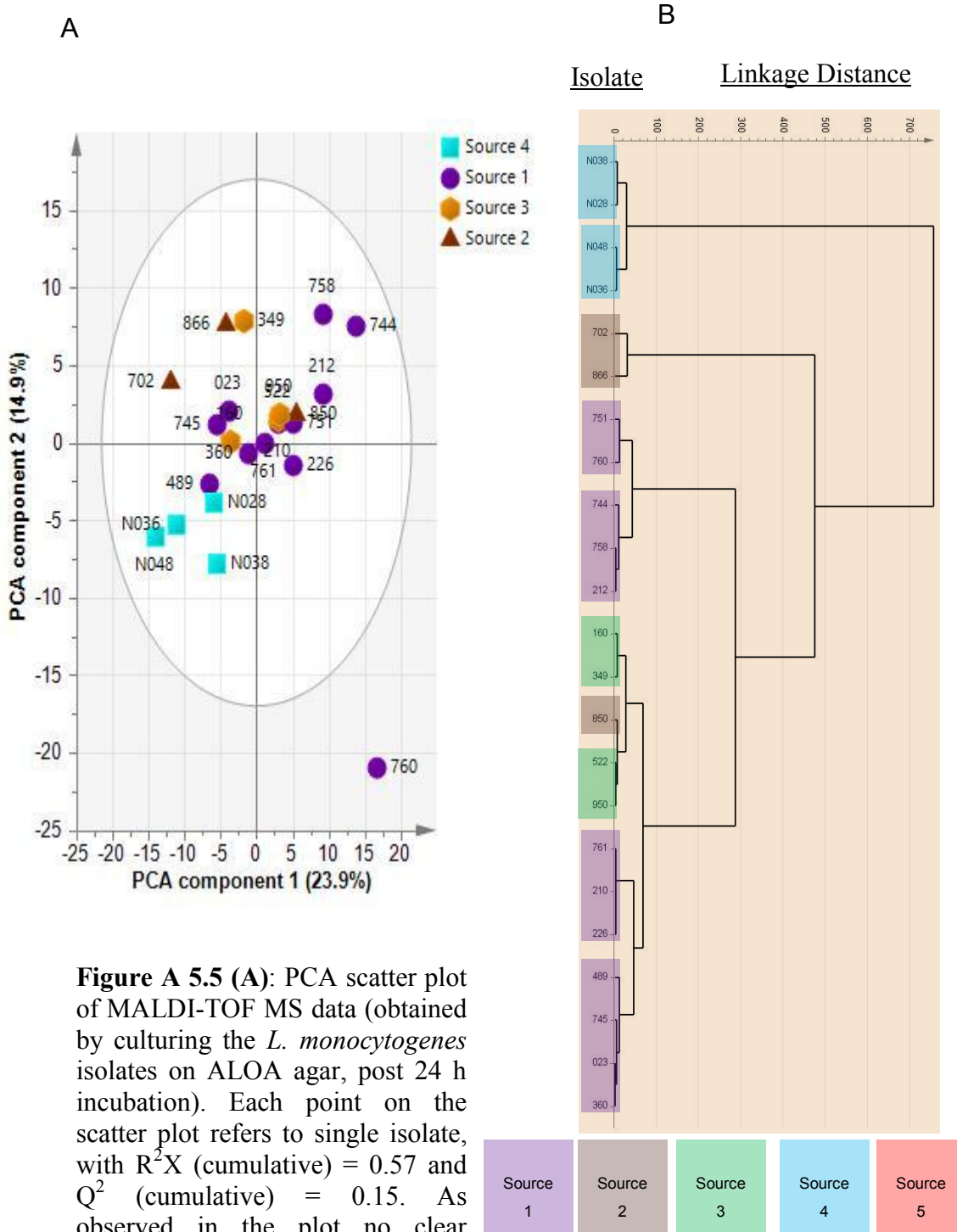Isolate   Linkage Distance

Figure A 5.2 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on OA agar, post 48 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.63 and Q^2 (cumulative) = 0.16. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation outside it is considered as an outlier.

Figure A 5.2 (B): The PLS-DA analysis plot generated from OA (48 h) yielded R^2X, R^2Y and Q2 values of 0.57, 0.37, 0.05 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.
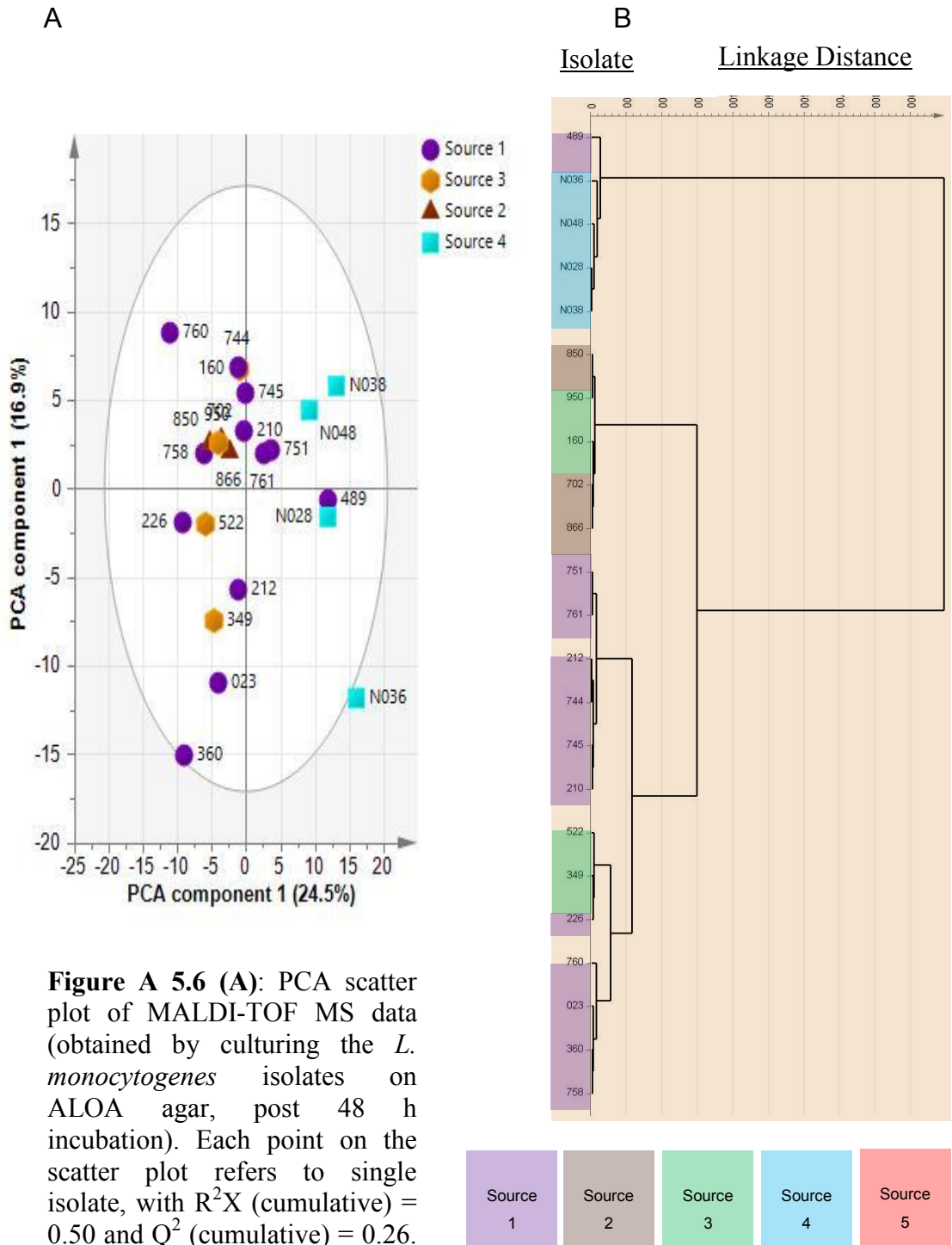
B

A

Isolate   Linkage Distance

Figure A 5.3 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on PA agar, post 24 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.49 and Q^2 (cumulative) = 0.18. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation outside it is considered as an outlier.

Figure A 5.3 (B): The PLS-DA analysis plot generated from PA (48 h) yielded R^2X, R^2Y and Q2 values of 0.53, 0.25, 0.03 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.
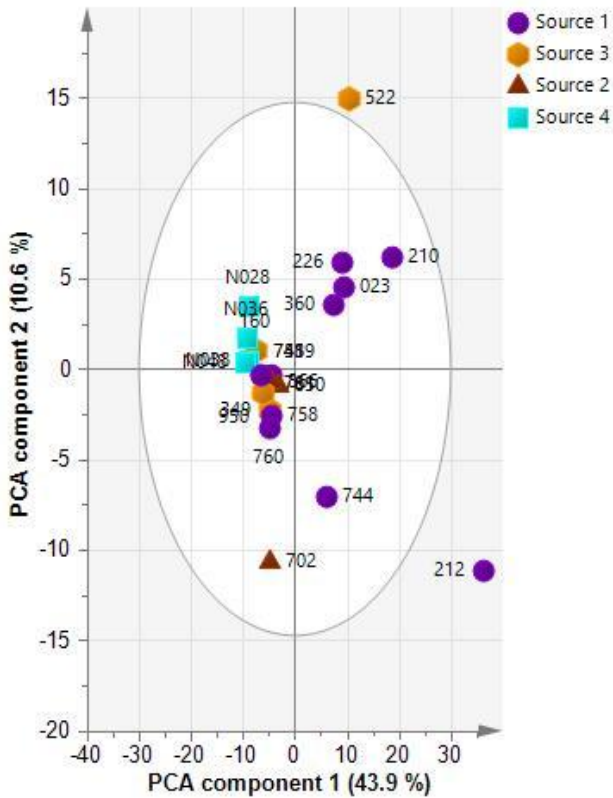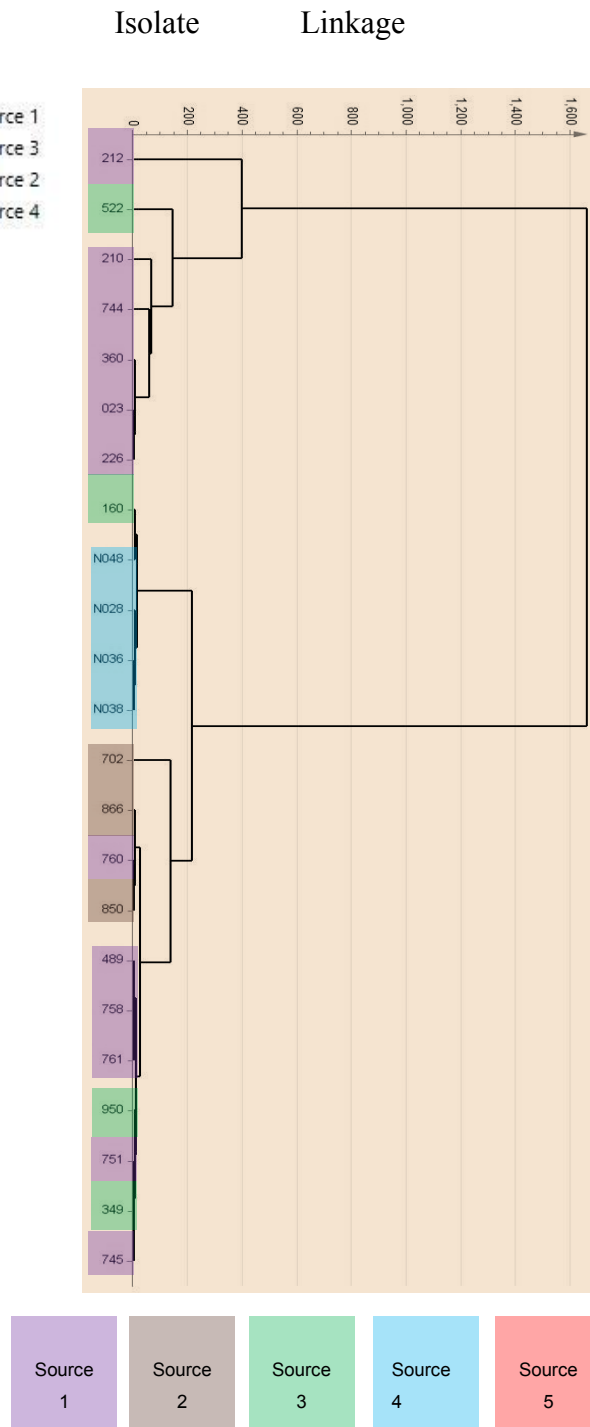
A

Figure A 5.4 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on PA agar, post 48 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.64 and Q^2 (cumulative) = 0.15. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation

B

Isolate   Linkage Distance

Figure A 5.4 (B): The PLS-DA analysis plot generated from PA (48 h) yielded R^2X, R^2Y and Q2 values of 0.69, 0.69, 0.2 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.
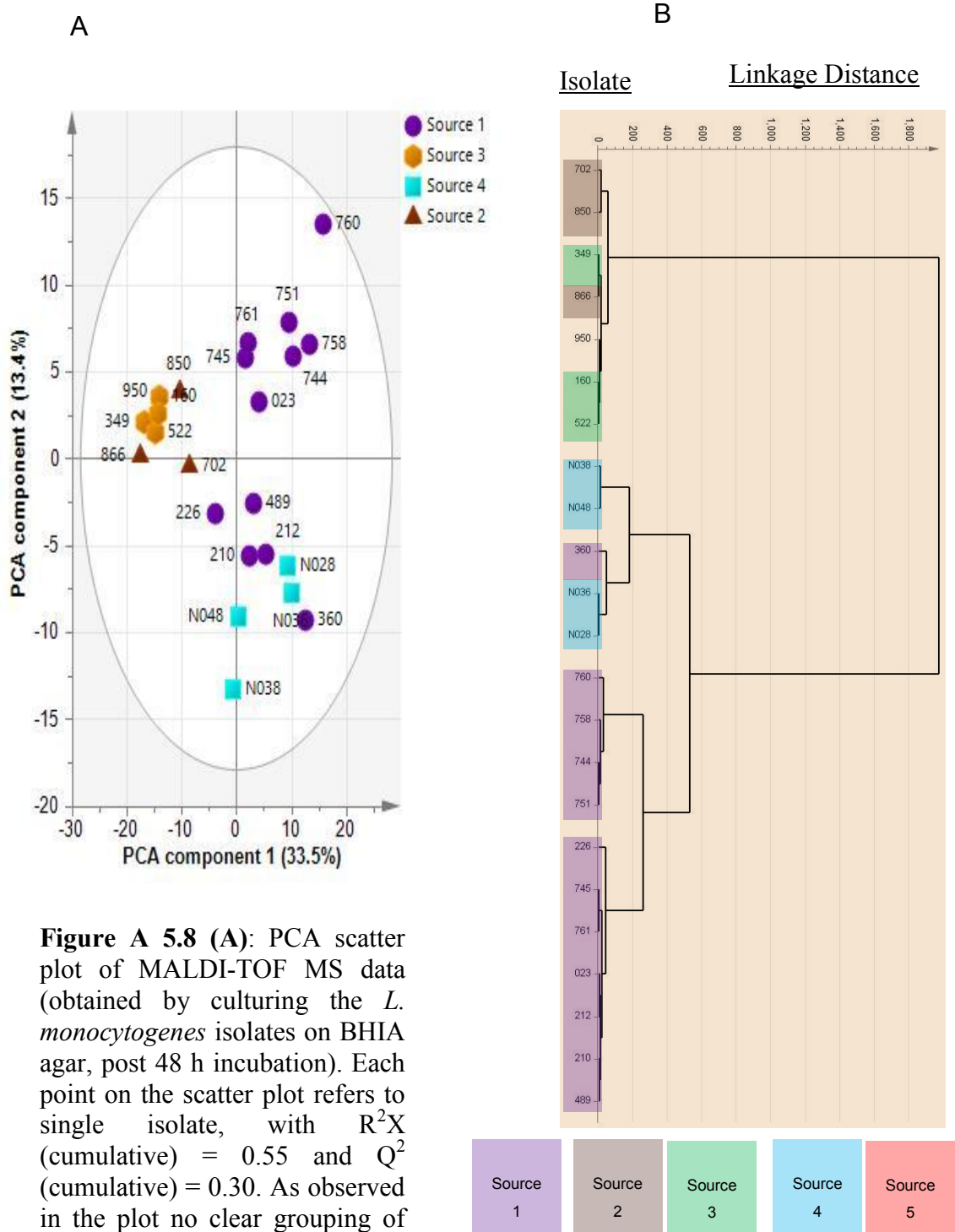
A

B

Figure A 5.5 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on ALOA agar, post 24 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.57 and Q^2 (cumulative) = 0.15. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation outside it is considered as an outlier.

Isolate   Linkage Distance

| Source 1 | Source 2 | Source 3 | Source 4 | Source 5 |

Figure A 5.5 (B): The PLS-DA analysis plot generated from ALOA (24 h) yielded R^2X, R^2Y and Q2 values of 0.65, 0.92, 0.58 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.

A

B

Figure A 5.6 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on ALOA agar, post 48 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.50 and Q^2 (cumulative) = 0.26. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation outside it is considered as an outlier.

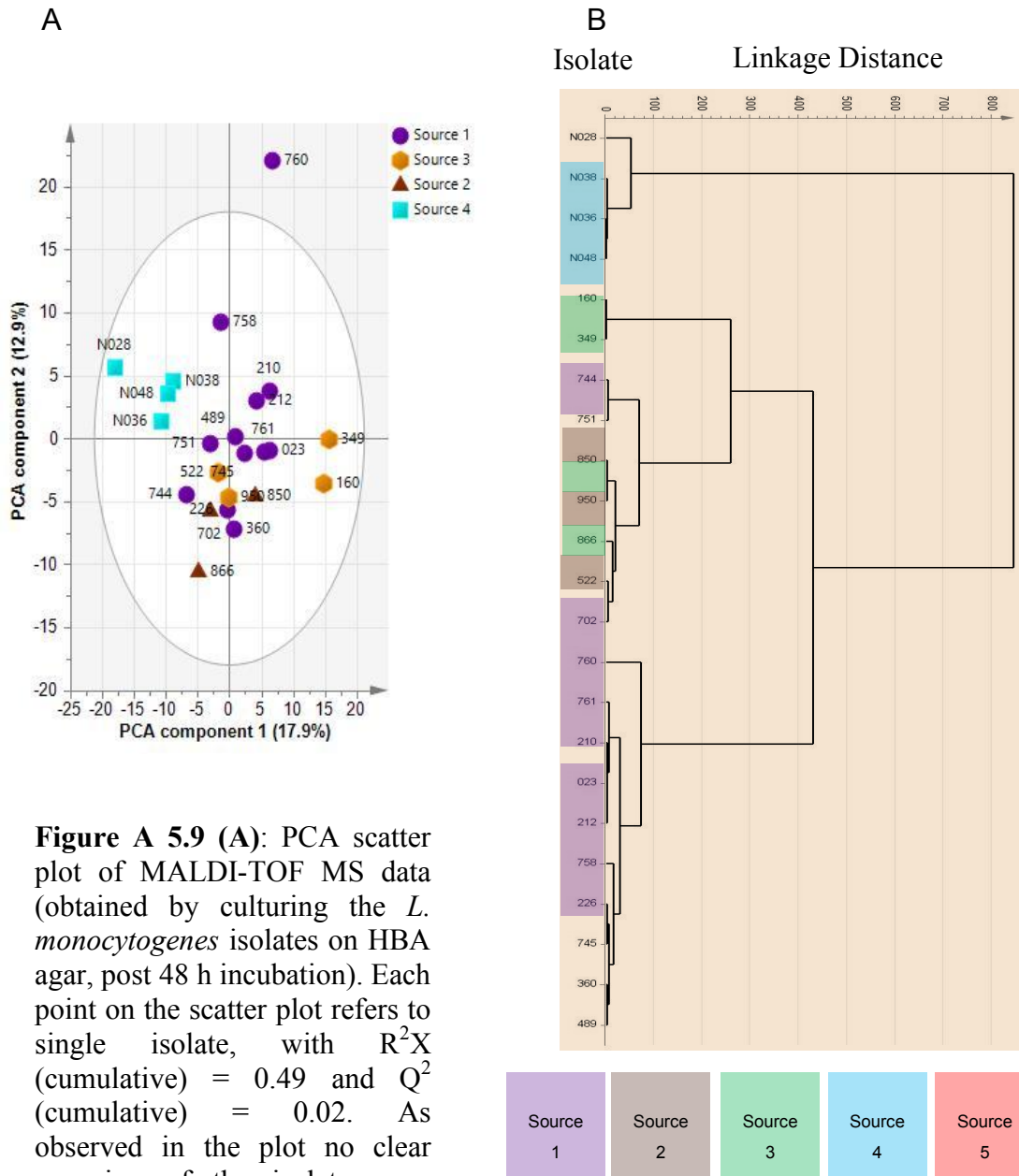**Figure A 5.6 (B):** The PLS-DA analysis plot generated from ALOA (48 h) yielded R^2X, R^2Y and Q2 values of 0.71, 0.98, 0.74 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.
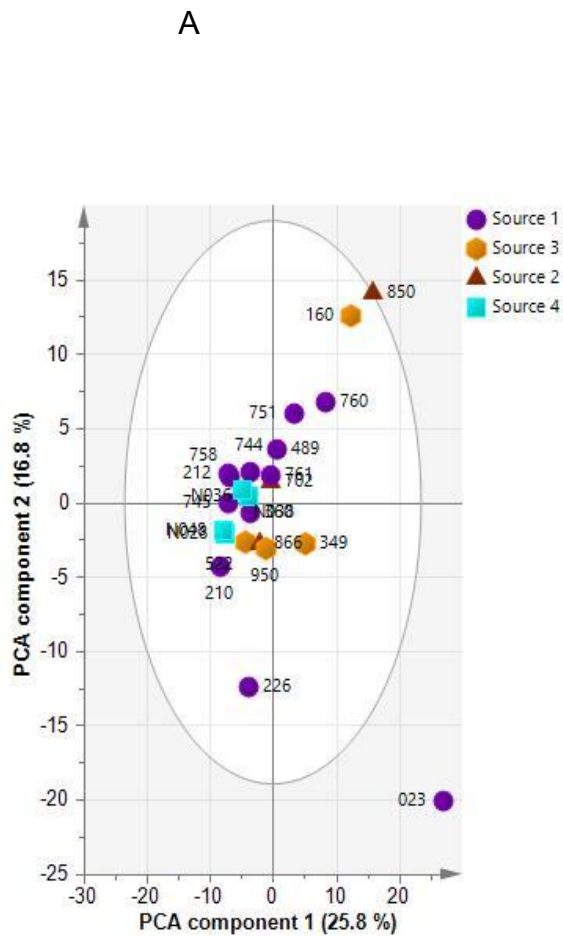
A

B

Figure A 5.7 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on BHIA agar, post 24 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.69 and Q^2 (cumulative) = 0.21. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation outside it is considered as an outlier.

Figure A 5.7 (B): The PLS-DA analysis plot generated from BHIA (24 h) yielded R^2X, R^2Y and Q2 values of 0.83, 0.94, 0.53 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.

A

B

Isolate   Linkage Distance

Figure A 5.8 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on BHIA agar, post 48 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.55 and Q^2 (cumulative) = 0.30. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation outside it is considered as an outlier.
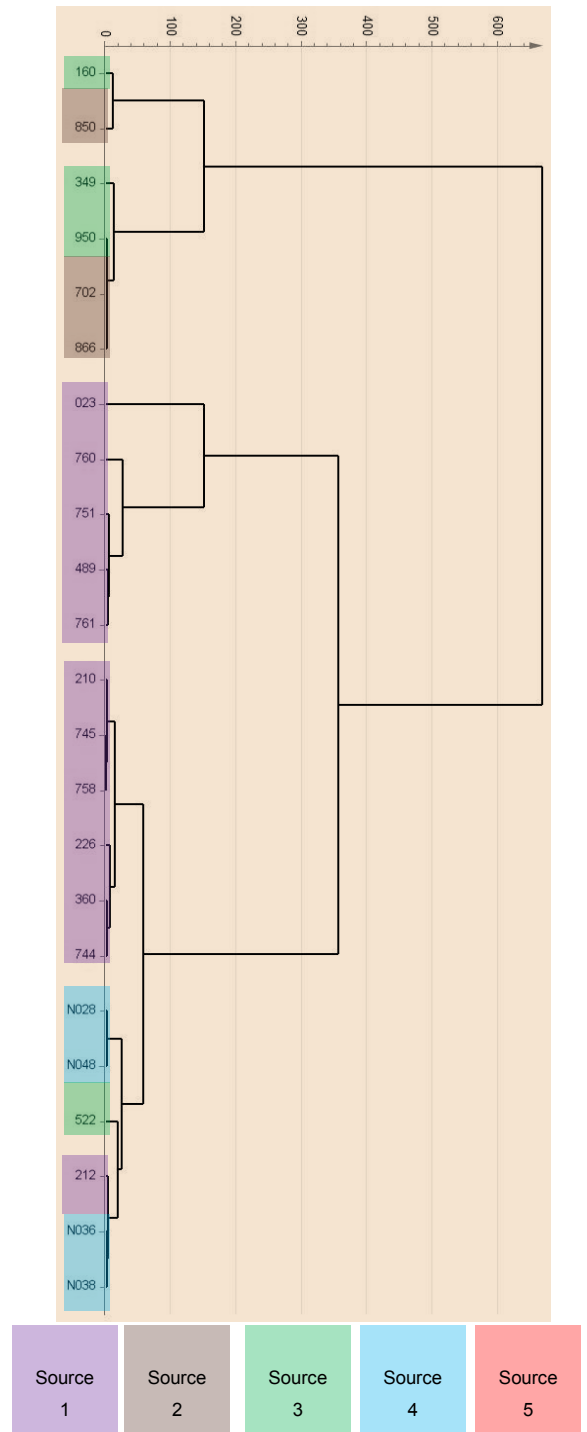
Figure A 5.8 (B): The PLS-DA analysis plot generated from BHIA (48 h) yielded R^2X, R^2Y and Q2 values of 0.73, 0.99, 0.73 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.
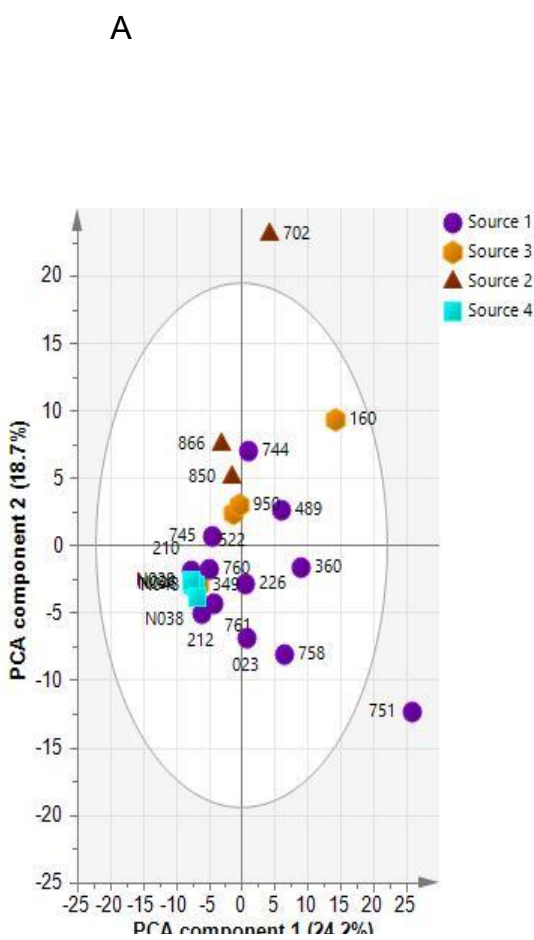
A

B

Isolate   Linkage Distance

Figure A 5.9 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on HBA agar, post 48 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.49 and Q^2 (cumulative) = 0.02. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation outside it is considered as an outlier.
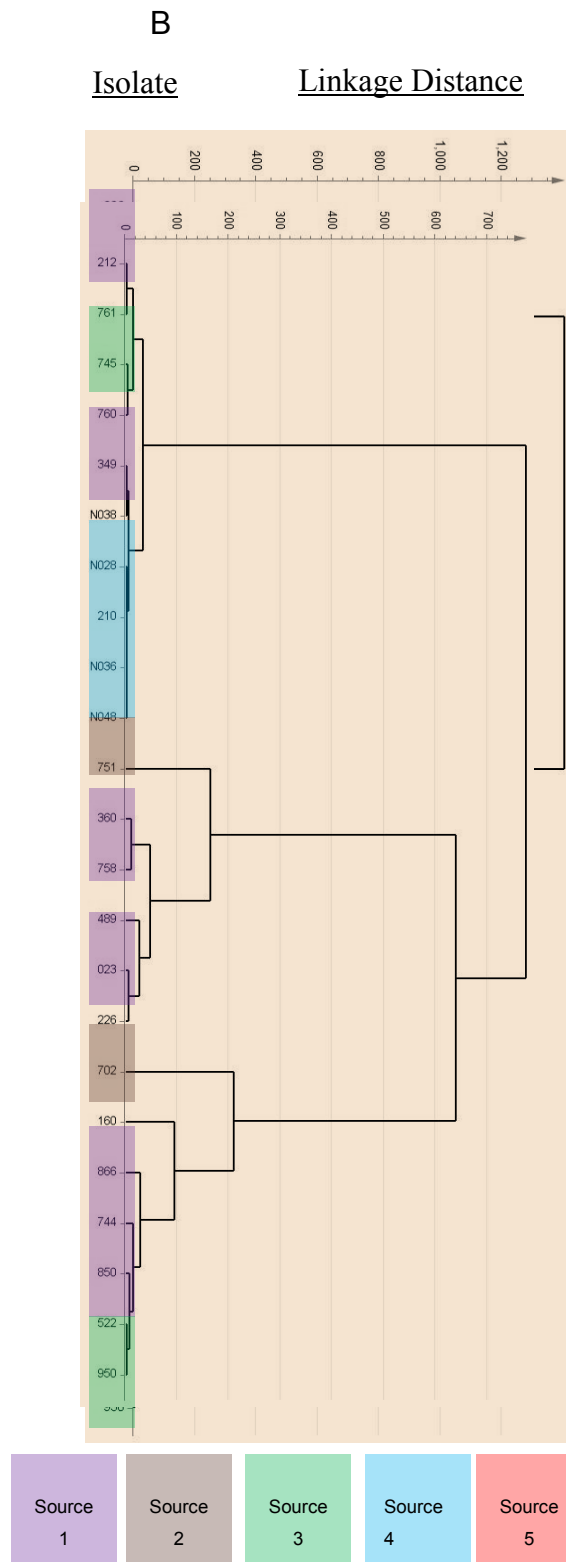
Figure A 5.9 (B): The PLS-DA analysis plot generated from HBA (48 h) yielded R^2X, R^2Y and Q2 values of 0.64, 0.98, 0.77 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.

A

B

Isolate   Linkage Distance

Figure A 6.0 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on OA agar, post 24 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.52 and Q^2 (cumulative) = 0.09. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation outside it is considered as an outlier.
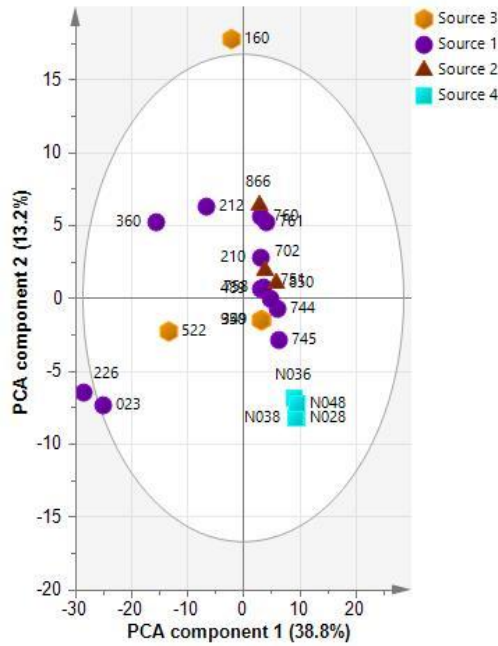
Figure A 6.0 (B): The PLS-DA analysis plot generated from OA (24 h) yielded R^2X, R^2Y and Q2 values of 0.71, 0.98, 0.69 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.
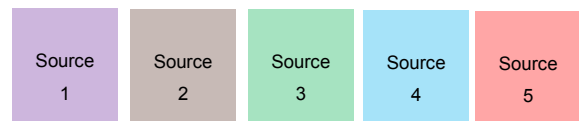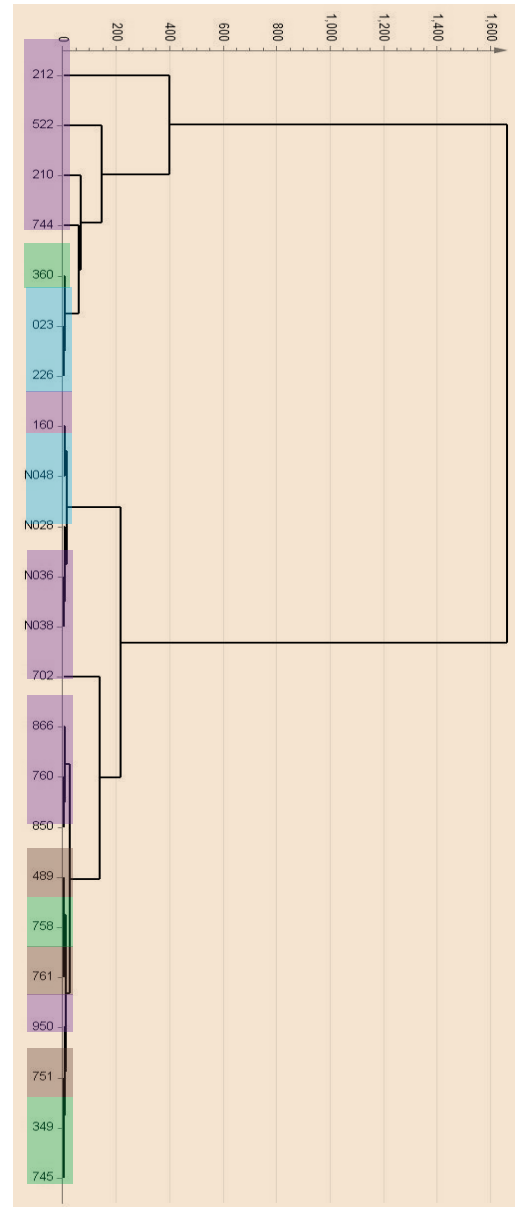
A

Figure A 6.1 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on OA agar, post 48 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.62 and Q^2 (cumulative) = 0.23. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation outside it is considered as an outlier.

B

Figure A 6.1 (B): The PLS-DA analysis plot generated from OA (48 h) yielded R^2X, R^2Y and Q2 values of 0.82, 0.97, 0.63 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.
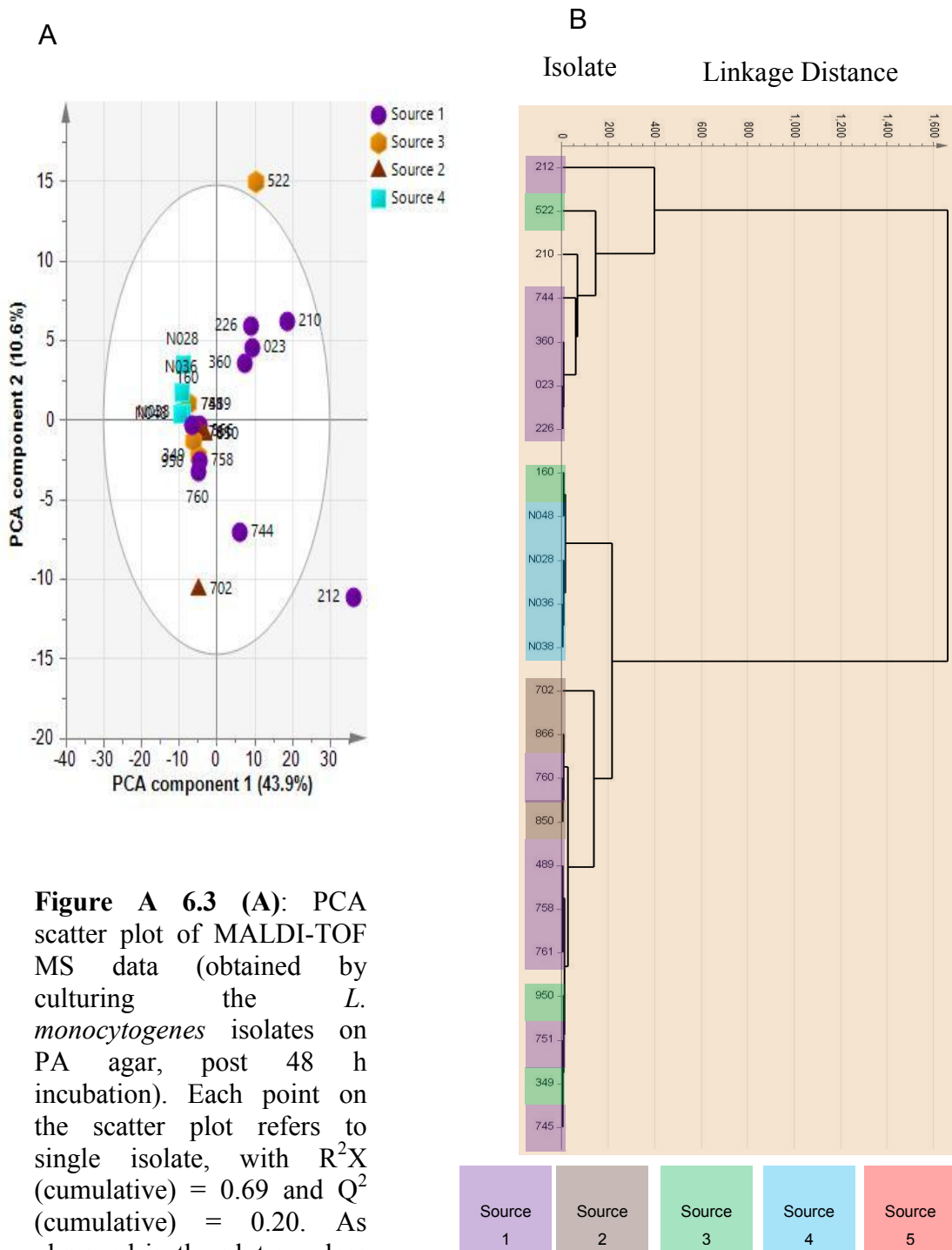
Figure A 6.2 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on PA agar, post 24 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.69 and Q^2 (cumulative) = 0.33. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation outside it is considered as an outlier.

Figure A 6.2 (B): The PLS-DA analysis plot generated from PA (24 h) yielded R^2X, R^2Y and Q2 values of 0.55, 0.50, 0.13 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.

A

Figure A 6.3 (A): PCA scatter plot of MALDI-TOF MS data (obtained by culturing the *L. monocytogenes* isolates on PA agar, post 48 h incubation). Each point on the scatter plot refers to single isolate, with R^2X (cumulative) = 0.69 and Q^2 (cumulative) = 0.20. As observed in the plot no clear grouping of the isolates was obtained (according to their source of isolation was seen). The tolerance eclipse represents the 95% confidence interval limit and any observation outside it is

B

Isolate   Linkage Distance

Figure A 6.3 (B): The PLS-DA analysis plot generated from PA (48 h) yielded R^2X, R^2Y and Q2 values of 0.76, 0.89, 0.49 respectively. Hierarchical cluster analysis was performed following PLS-DA and the above dendogram was generated.