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Comparative Proteomic Analysis of *Listeria monocytogenes* Isolates from New Zealand

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A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Master of Applied Science

at
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by
GE HUANG

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Master Applied Science.

Comparative Proteomics Analysis of *Listeria monocytogenes* Isolates from New Zealand

by

GE HUANG

Listeria monocytogenes is one of the top ranking foodborne pathogens implicated in a number of foodborne outbreaks that have caused human illness and death. About 90% of the cases of Listeriosis in New Zealand are associated with the consumption of food contaminated with *L. monocytogenes*. Recently, many researchers have used proteomic approaches to analyse foodborne pathogens, especially *L. monocytogenes*. Proteomics is a protein-based technology that is the large-scale study of the expressed protein components of an organism's genome. However, in New Zealand, there have been few studies carried out using proteomic techniques to analyse the protein profile of foodborne pathogens, such as *L. monocytogenes*, *Salmonella* and *Campylobacter*. Comparative proteomic analysis was conducted in this research using 1D SDS-PAGE, 2DE analyses, MALDI-TOF/TOF mass spectrometry and Ion Trap mass spectrometry to identify key differences in protein expression of three strains of *L. moncytogenes* (V7, SB92/844 and SB92/870) that have different ecological origins.

The protein banding patterns generated by 1D SDS PAGE of the three strains of *L. monocytogenes* showed no significant differences. Visual observations from 2DE gels revealed that certain spots appeared to be expressed differentially/differently?. Key differences in protein expression of the three strains of *L. monocytogenes* were found using PDQest ™ 2D analysis software. One hundred and eighty nine spots were detected using SB92/844 as the reference gel. There were 99 spots in the reference gel that matched the spots in *L. monocytogenes* strain V7 and 102 spots were detected that were similar in *L. monocytogenes* strain SB92/870. Seventy out of 189 spots detected (37% of detected spots) were present in all three strains studied. By estimating the relative differences in protein expression, 10 protein spots had decreased expression in SB92/844 relative to V7 and five

protein spots were significantly over-expressed. Besides this, 14 protein spots showed no significant protein expression differences in SB92/844 relative to V7. As for the relative protein expression pattern in *L. monocytogenes* strain SB92/870 relative to V7, five protein spots were significantly over-expressed and six protein spots were down-regulated. Additionally, 11 protein spots showed no differential expression in SB92/870 relative to V7. A comparison of the protein expression of SB92/844 vs SB92/870 showed that 22 protein spots had no significant differences. Only three protein spots were over-expressed and the expression of eight protein spots decreased in SB92/844 relative to SB92/870.

The identity of selected protein spots was achieved using MALDI-TOF mass spectrometry and Ion Trap mass spectrometry. It was found that certain proteins (i.e. a major cold shock protein and superoxide dismutase) were expressed differently between the two local strains from New Zealand. The key findings of this study indicated that differentially?? expressed proteins may be related to virulence and pathogenicity. This work also represented the first proteomic study on *L. monocytogenes* isolates from New Zealand.

Keywords: *Listeria monocytogenes*, foodborne pathogenes, proteomics

List of Publications

1. Huang, G. and Hussain, M.A. (2012). Advances in proteomics-based detection techniques of *Listeria monocytogenes*: a potential risk in New Zealand. Internet Journal of Food Safety. 14, 70-74.

2. Huang, G., Mason, S., Hudson, A., Clerens, S., Plowman, J.E. and Hussain, M.A. (2012) Microbiological assessment of four probiotic feed supplements in New Zealand. The NZMS Conference 2012 held on 27-29 November 2012 at Dunedin, New Zealand.

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Symbols and Abbreviations

2DE	Two-dimensional gel electrophoresis
2D-DIGE	Two-dimensional fluorescence difference gel electrophoresis
2DnLC-MS/MS	Two-dimensional nanoliquid chromatography coupled to ion-trap mass spectrometry
AcN	Acetonitrile
APS	Ammonium persulphate solution
BHI	Brain heart infusion
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESR	Institute of Environmental Science and Research Ltd, New Zealand
GalE	UDPglucose- 4-epimerase
HCl	Hydrochloric acid
IEF	Isoelectric focusing
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDA	Kilodaltons
LC	Liquid chromatography
LC/LC	Two-dimensional liquid chromatography
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
LLO	Listeriolysin O

MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MALDI-TOF MS	Matrix-assisted laser desorption ionization-time of flight mass spectrometry
MudPIT	Multi-dimensional Protein Identification Technology
OD	Optical density
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
Pfk	6-phosphofructokinase
pI	Isoelectric point
PLcA	Phospholipases A
PLcB	Phospholipases B
rmp	Revolutions per minute
RNA	Ribonucleic acid
RTE	Ready-to-eat food
RP	Reversed phase
SCX	Strong cation exchange
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	N,N,N’-tetramethylethylenediamine
TFA	Trifluoroacetic acid
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
USA	The Unites States of America

Chapter 1
Introduction and Review of Literature

1.1 Introduction

1.1.1 Background

The genus *Listeria* belongs to family *Listeriaceae* (previously known as *Corynebacteriaceae*) with microbiological features as non-spore formers, catalase-positive, and oxidase-negative (Collins et al., 1991). This genus contains six species: *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. innocua* and the recently found species, *L. grayi* (Collins et al., 1991; Graves et al., 2009). *L. monocytogenes* is the main cause of listeriosis in both humans and animals. It is one of the most serious food-borne illnesses worldwide. This bacterium can express a beta haemolysin that causes the destruction of red blood cells. One of the unique features of this bacterium is its tumbling motility when viewed with light microscopy. This bacterium is actively motile by means of peritrichous flagella at room temperature (20−25°C) but the synthesis of the flagella does not occur at body temperature (37°C). When present, the flagella cause the bacterial cells to move within eukaryotic cells by explosive polymerisation of their actin filaments (Hain et al., 2006; de Velde et al., 2009). The actin polymerisation plays a key role in the infectious cycle of the bacterium.

Twenty to thirty percent of clinical infections of listeriosis result in death (Mead et al., 1999; Orsi et al. 2011). This fatality rate from listeriosis is even greater than for *Salmonella* and *Clostridium botulinum*. *L. monocytogenes* is distributed widely in the environment. Raw, cooked and processed foods can be a mode for the transmission of the infection. *L.moncytogenes* is widely present in nature and usually infects humans through contaminated vegetables or unpasteurised dairy products. *L. monocytogenes* has the ability to survive very well between -20°C and 50°C and also in other adverse growth conditions, e.g. high salt concentrations, a wide range of pH and temperatures, and low water availability, by forming biofilms. It is known that refrigeration is able to reduce or prevent the growth of most food-poisoning bacteria; however, this is not true for *L. monocytogenes*, which may continue to grow slowly at low temperatures (Bryan 2004). Moreover, *L. monocytogenes* has shown an ability to resist a number of sanitisers, such as ethanol, sodium hypochlorite and sodium hypochlorite, while methanol and quaternary ammonium compounds are ineffective in killing *L. monocytogenes.*

1.1.2 Importance of this study

In New Zealand, around 90% of listeriosis cases are due to the consumption of food contaminated with *L. monocytogenes* (MAF 2011). *L. monocytogenes* is able to be found in soil and water. Hence, this bacterium can be found in a variety of raw, cooked and processed foods, such as fruit and vegetables. Animals could be carriers without showing symptoms and they may contaminate foods of animal origin, such as uncooked meats and unpasteurised milk. Moreover, ready-to-eat food, such as soft cheese, processed meat and poultry, and smoked seafood, can also be contaminated with *L. monocytogenes* (Gilbert et al. 2009; MAF 2011; Crerar et al. 2011). The majority of listeriosis victims are very young, elderly, people with lowered immunity (usually due to chronic diseases) and pregnant women. Due to the growth of an ageing population, there is possibility that the incidences of listeriosis in New Zealand will surge in the future. In 2011, more than 30% of reported food-borne patients in New Zealand had listeriosis (Barnao 2011).

Therefore, it is very important to understand the spread of listeriosis and the physiological mechanisms of its transmission. Moreover, it is crucial to develop better control systems to prevent the spread of listeriosis.

1.1.3 Aims and Objectives

The aim of this research is to understand the proteomics of three strains of *L. monocytogenes:* serotype 1/2a strains SB92/844 and SB92/870 (New Zealand isolates) and V7 (an isolate from the USA). There is very limited research about the proteomic analysis of *L. monocytogenes* that has been undertaken in New Zealand. This research could be the first proteomic study that aims to understand the differences in *L. monocytogenes* isolates from New Zealand and with different ecological origins.

There were two objectives of this research:

(1) To study the proteomic profiles of three strains of *L. monocytogenes* using 1D SDS-PAGE, 2DE analyses, MALD-TOF mass spectrometry and Ion Trap mass spectrometry.

(2) Comparative proteomic analysis to identify key differences in protein expression of the three strains from different ecological origins.

1.2 Literature review

1.2.1 The Genus *Listeria*

The genus *Listeria* consists of a group of gram-positive bacteria that is classified in the family *Listeriaceae* (previously known as *Corynebacteriaceae*) with microbiological features as non-spore formers, and facultative anaerobe. *Listeria spp.* have no capsules and are motile at 10-25°C (Collins, et al., 1991). This genus can be found in a variety of sources, such as soil, water, plants, faeces, decaying vegetables, meat, seafood and, dairy products. This genus currently contains six species: *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii,* and the recently found species, *L. grayi* (Collins et al., 1991; Graves et al., 2009). *L. moncytogenes* and *L. innocua* are the only pathogens in the genus *Listeria*. *L. monocytogenes* is a food-borne pathogen, which is able to cause severe illness in both humans and animals. *L.innocua* is an animal pathogen.

The genus of *Listeria* comprises bacteria with a low G+C content of between 36.4% and 41.5%, which are closely related to *Bacillus*, *Clostridium*, *Enterococcus*, *Streptococcus*, and *Staphylococcus* (Hain et al., 2007; Sallen et al., 1996*).* All *Listeria* chromosomes are circular with lengths ranging from 2.7 to 3.0 Mb. Although both strain- and serotype-specific genes have been identified previously, all *Listeria* genomes are highly similar in gene organisation and content (Sallen et al., 1996). The genome sequence encodes approximately 2800 putative protein coding genes with about 65% of the genes having been identified.

1.2.2 L. monocytogenes and its virulence gene cluster

L. monocytogenes is a gram-positive food borne pathogen of humans and animals. It is a facultative anaerobic rod, which is catalase positive and oxidase negative. *L. monocytogenes* also expresses a β-haemolysin that is able to form clear zones when grown on blood agar. One of the unique features of this bacterium is the formation of pertrichous flagella, which give the bacterium the capability of tumbling motility when viewed under light microscopy. The forming of peritrichous flagella usually occurs at room temperature (20−25°C) but at body temperature (37°C) the formation of flagella usually reduced. Flagella help the bacterial cells move within eukaryotic cells by explosive polymerisation of actin filaments (Hain et al. 2006; de Velde et al. 2009).

The virulence gene cluster of *L. monocytogenes* is encoded on a cluster of six genes (*prfA*, *hly*, *plcA*, *plcB*, *mpl*, and *actA*) 9 Kb in length (Cossart et al., 1989; Vázquez-Boland et al., 2001). The virulence gene cluster encodes the proteins that play an important role in the major

virulence functions of *L. monocytogenes* (Kreft & Vázquez-Boland, 2001*)*. The product of *hly* is Hly (usually called listeriolysin O in *L. monocytogenes*), which is a sulfhydryl-activated pore-forming listeriolysin. This protein is required for the disruption of the phagocytic vacuole necessary for the bacteria to escape from the phagosomes of the host cells into the host cytosol. Therefore, Hly is an essential virulence factor and its presence will lead to avirulence. *actA* encodes for the surface protein, ActA, that has a function in actin-based motility and cell-to-cell spread (Kocks et al., 1992; Kreft & Vázquez-Boland, 2001). *plcB* encodes an inactive propeptide, phosphatidylcholine phospholipase C, which is extracellularly processed by the Mpl protease. Its function is to mediate the dissolution of the double-membrane, secondary phagosomes formed after cell-to-cell spread. Mpl is the product of the gene *mpl*. These two proteins are responsible for the lysis of host cell membranes. The gene *plcA* encodes a phosphatidylinositol-specific phospholipase C, which synergises with Hly and PlcB in the destabilisation of primary phagosomes.Gene *prfA* encodes the PrfA protein, which is a positive master-regulator of most of the gene cluster, including itself. PrfA is also the only virulence regulator identified, to date, in *Listeria* and is the main switch of a regulon comprising virulence-associated loci scattered throughout the listerial chromosome, including members of the internalin multigene family. The expression of the virulence regulon via PrfA can be modulated by a number of activation signals, such as high temperature, stress conditions, contact with a host cell, the eukaryotic cytoplasmic environment and the sequestration of extracellular growth medium components by activated charcoal.

1.2.3 Virulence factors of *L. monocytogenes*

Pathogenic *Listeria* species have a distinguishing feature; the destruction of the endothelial and epithetical barriers of the infected host, such as in the intestine and in the blood-brain and foeto-placental barriers. The infectious life cycle of *L. monocytogenes* starts with expression of two major proteins, internalins InlA and InlB, which can interact with E-cadherin and the hepatocyte growth factor receptor (Met), respectively (Dortet, Veiga-Chacon, & Cossart, 2009). These two proteins can induce bacteria into eukaryotic cells and incorporate them into a membrane-bound vacuole (Gaillard et al, 1991; lecuit et al., 1997). The pore-forming toxin, listeriolysin O (LLO) and two secreted, PLcA and PLcB, are released by these internalised bacteria and can lyse the membrane of the vacuole. The bacteria then move to the cytoplasm to multiply and polymerise. When present, the flagella cause the bacterial cells to move within eukaryotic cells by explosive polymerisation of actin filaments (Hain, et al., 2006;

Velde et al., 2009). The actin polymerisation plays key role in infectious cycle of the bacterium.

Internalin A (InlA) and Internalin (InlB) are a group of proteins that belong to the Internalin family and are characterised by the presence of leucine-rich repeats (LRRs) (Cabanes et al., 2002; Gaillard et al., 1991). InlA was first identified as a *Listeria* surface protein that is required for the penetration of *L. monocytogenes* into non-phagocytic cells, such as epithelial cells (Gaillard, et al., 1991). E-cadherin is receptor of InlA, which belongs to the cadherin superfamily of cell adhesion molecules (Mengaud et al., 1996). The interaction between InlA and E-cadherin stimulates the pathogenesis of *L. monocytogenes*. InlB is another membrane protein of the internalin family and is located in the same operon as InlA (Braun et al., 1998; Doyle, 2001). It is involved in the entry of *L. monocytogenes* into a broad range of cell lines such as hepatocytes and non-epithelial cells. It has been proven that InlB is crucial for the invasion of hepatocytes in the liver (Braun et al., 1998; Dramsi et al., 1995).

After invading host cells, phagocytic cells of *L. monocytogenes* are engulfed and enclosed within a vacuole and then tend to be killed by professional phagocytic cells. The survival of *L. monocytogenes* depends on escaping from the vacuole. Listeriolysin O (LLO) is a bacterial pore-forming toxin that is essential for lysing the vacuolar membrane to allow the bacteria to escape into the cytoplasm of the host cell (Beauregard, Lee, Collier, & Swanson, 1997). In addition to its pore-forming function, many cellular responses are involved in LLO stimulation, such as secretion of interleukin-1 in macrophages, activation of mitogen-activated protein kinase in HeLa cells, induction of apoptosis, expression of cell adhesion molecules on infected endothelial cells, secretion of various cytokines in spleen cells and cell-signalling in human embryonic kidney cells (Coconnier et al., 1998; Coconnier et al., 2000; Dramsi et al., 1995; Guzman et al., 1996; Kohda et al., 2002; Krull et al., 1997; Kuhn & Goebel, 1999; Nishibori et al., 1996; Schluter et al., 1998).

After invasion of the host cells, *L. monocytogenes* bacteria are enclosed in the vacuole and then the vacuole is lysed by LLO (Jacquet et al., 2002; Kreft & Vázquez-Boland, 2001). The bacteria need to move to other cells by polymerising the host cell actin through the activity of ActA (Kocks et al., 1992). ActA is a bacterial surface protein that can induce polymerisation of globular actin molecules to form a polarised actin filament. *L. monocytogenes* is able to move with the filaments to the target cell membrane and then cause portions of the membrane to form structures called listeriopods (Kocks et al., 1992). These listeriopods are engulfed by

adjacent cells, which can help *L. monocytogenes* disseminate and avoid antibodies or other immune active compounds. Therefore, ActA plays an important role for *L. monocytogenes* in the attachment and entry of target cells.

In order to lyse the intracellular vacuoles, *L. monocytogenes* can secrete two distinct phospholipases C, a phosphatidylinositol-specific phospholipase C (PI-PLC) and a broad-range of phosphatidycholine-specific phospholipase C (PC-PLC). These two phospholipases are crucial for *L. monocytogenes* to invade and spread into target cells. PI-PLC can assist bacteria to escape from the primary vacuole (Smith et al., 1995). While PC-PLC is active once the bacterial cells spread have into host cells, it can also damage vacuole membranes in epithelial cells and it also is involved in the cell-to cell spread of *L. monocytogenes* in the brain (Schluter et al., 1998; Smith et al., 1995).

L. monocytogenes also contains a Clp group of proteins that can act as either chaperones or proteases. These Clp proteins play an important role in pathogenesis. ClpC ATPase is a general stress protein that can cause the disruption of the vacuolar membrane and help the bacteria to survive intracellularly (Rouquette et al., 1998). ClpC ATPase can also modulate the expression of the ActA protein and the internalins at the transcriptional level (Nair et al., 2000; Rouquette et al., 1998). As for ClpE ATPase, it plays a role in the pathogenesis of *L. monocytogenes* (Nair et al., 2000). ClpP serine protease is required once the bacteria grow under stress conditions and has the ability to affect the activity of LLO (Gaillot et al 2000).

Other proteins can also act as virulence factors for *L. monocytogenes*. The recently identified surface protein, p104, plays a role in the adhesion of this bacteria to intestinal cells (Pandiripally et al., 1999). The metalloprotease secreted by *L. monocytogenes* is required to activate the phospholipase (Marquis et al., 1997). Another surface protein, called p60, can catalyse a reaction during the final stage of cell division in *L. monocytogenes* (Kuhn & Goebel, 1999). Hence, all of the virulence factors described above have their own way of assisting the infection process of *L. monocytogenes* and lead to the spread of the bacterial cells.

1.2.4 Lineages of *L. monocytogenes*

L. monocytogenes isolates can be classified into three evolutionary groups (Lineages I, II, III), which consist of at least 12 serotypes (i.e. 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and

7) based on previous subtyping studies (the serological reactions between *Listeria* Somatic (O)/flagellar (H) antigens and their corresponding antisera). Lineages I and II were first identified in 1989 using multilocus enzyme electrophoresis (Piffaretti et al., 1989). Lineage III was further identified based on analyses of partial DNA sequences for flaA, iap and hly, ribotyping, and PCR-RFLP analyses of *L. monocytogenes* virulence genes; and also comparative genomics and DNA sequencing studies (Gendel & Ulaszek, 2000; Rasmussen et al., 1995; Wiedmann et al., 1997). *L. monocytogenes* Lineage I consists of serotypes 1/2b, 3b, 3c and 4b, while Lineage II contains serotypes 1/2a, 1/2c and 3a (Nadon et al., 2001). Serotypes 4a, 4b and 4c belong to Lineage III. A number of studies have already indicated that Lineages I and II are widespread and comprise most *L. monocytogenes* food isolates (Nadon et al., 2001; Gray et al., 2004; Nightingale et al., 2005; Sauders et al., 2006). These two lineages are commonly associated with human clinical cases, including serotype 1/2a (Lineage II) and serotypes 1/2b and 4b (Lineage I). It is also known that most human listeriosis cases are usually associated with Lineage I while, as Lineage II strains are commonly isolated from natural and farm environments, they also have higher prevalence among animal listeriosis cases and sporadic human clinical cases (Gray et al., 2004; Nightingale et al., 2005; Sauders et al., 2006). Lineage III strains are rare and mainly associated with animals (Liu, et al., 2006). Recent studies suggest that isolates originally classified as Lineage III represent a polyphyletic group that includes at least three different lineages: Lineages IIIA, IIIB and IIIC. All strains from Lineage III contain serotypes 4a, 4c and atypical serotype 4b isolates (Roberts et al., 2006)

1.2.5 *L. monocytogenes* as a foodborne pathogen

Listeriosis is a serious bacterial infection, mainly caused by consumption of food that is contaminated with *L. monocytogenes* (Todar; WHO, 2004). Listeriosis is one of the most serious food-borne illnesses worldwide. It has been recognised as a foodborne pathogen since an listeriosis outbreak in the Maritime Provinces of Canada, in 1983 (Swaminathan & Gerner-Smidt, 2007). Twenty to thirty percent of clinical infections of listeriosis result in death (Mead et al., 1999; Orsi et al., 2011). *L. monocytogenes* can be found in many types of raw and processed foods. For example, it can be associated with milk and dairy products, meat products, vegetables, seafood and fish products. Soft cheese, hot dogs and seafood have also been reported as been involved in outbreaks of human listeriosis (Rocout & Bille, 1997; Rocourt & Cossart, 1997). *L. monocytogenes* can be killed by pasteurisation and cooking. However, some ready-to-eat foods, such as deli meats, salads and hot dogs may be

contaminated during the packaging processes, since the most dangerous feature of *L. monocytogenes* is that it can grow at low temperatures (Table 1.1). *L. monocytogenes* has the ability to grow at a wide range of temperatures (from 2 - 45 °C). The survival and growth of this bacterium are the main factors that contribute to the difficulty in controlling this pathogen (Rocourt and Cossart, 1997). It is known that refrigeration reduces, or prevents, the growth of most food-poisoning bacteria; however, this is not true in the case of *L. monocytogenes*, which may continue to grow slowly at low temperatures (Bryan, 2004). Some chemical agents are ineffective in reducing the numbers of *L. monocytogenes*. The ability of *L. monocytogenes* to withstand, adapt, survive and grow under stressful conditions, is another contributing factor towards the seriousness of listeriosis. *L. monocytogenes* can grow in a low pH environment by utilising a number of stress adaption mechanisms (Cotter & Hill, 2003). In addition, creating low water activity using salts is one of the methods in food preservation. Therefore, the ability of *L. monocytogenes* to adapt and survive in high concentrations of salts will make it very difficult to control the pathogen in various foods. Moreover, *L. monocytogenes* has shown a resistance phenotype to a number of sanitisers including ethanol, sodium hypochlorite, sodium hypochlorite along with methanol and quaternary ammonium compounds. In 2002, Romanova and his co-workers investigated the sensitivity of 19 strains of *L. monocytogenes* to the sanitisers widely used in the meat industry (Romanova et al., 2002). Some strains used in the study were isolated from listeriosis cases and the meat processing facilities where the authors found that five of these strains show a resistant phenotype to the sanitisers tested.

Table 1.1. Key characteristics of *L. monocytogenes* as a foodborne pathogen

• Widely present in nature • Can grow at low temperatures • Survives very well in unfavourable environments (freezing, high salt concentration, wide range of pH and temperature) • Ability to resist sanitation • Forms biofilms

1.2.6 Seriousness of Listeriosis

L. moncytogenes is the main cause of listeriosis in both humans and animals, while *L. ivanovii* often causes this disease in animals, especially sheep. *L.grayi* has also been reported in certain cases. Listeriosis is caused by the ingestion of contaminated food and displays a wide variety of symptoms that are similar in animals and humans. *Listeria* is ubiquitous in the environment and is primarily transmitted via the oral route after ingestion of contaminated food products, which may lead to digestive manifestations such as nausea, aqueous or bloody diarrhoea, abdominal pain and fever. The digestive manifestations are not only able to occur in healthy individuals but also in immunocompromised individuals. The disease may start as long as two months after the consumption of contaminated food. The bacteria are also able to penetrate the intestinal barrier and then spread from the mesenteric lymph nodes to the spleen and, even, the liver. If the bacteria cannot be controlled by the immune system at the liver and spleen level, they may reach the brain or the placenta and cause systemic infections, such as meningitis, brain abscesses or abortions. Pregnant women may experience a fever and other non-specific symptoms, such as fatigue and aches, followed by foetal loss or bacteraemia and meningitis in their newborns. *L. monocytogenes* has an ability to cross the materno-foetal barrier, which can lead to villitis and placental abscesses, chorionamnionitis and, ultimately, systemic infection of the foetus in pregnant women. In general, the disease usually affects people with a weakened immune system, older adults, newborns and pregnant women. The symptoms of listeriosis usually include fever, muscle aches and diarrhoea. Listeriosis primarily causes infections of the central nervous system (meningitis, meningoencephalitis, brain abscesses and cerebritis) in older adults and people with immune-compromising conditions.

The infective dose of *L. monocytogenes* may depend on the bacterial strain and the susceptibility of the victim. *L. monocytogenes* may invade the gastrointestinal epithelium. Once the bacterium enters the host's monocytes, macrophages, or polymorphonuclear leukocytes, it becomes blood-borne (septicaemic) and can grow. Its presence intracellularly in phagocytic cells also permits access to the brain and, probably, transplacental migration to the foetus in pregnant women. The pathogenesis of *L. monocytogenes* centres on its ability to survive and multiply in phagocytic host cells. It seems that *Listeria* originally evolved to invade membranes of the intestines, as an intracellular infection, and developed a chemical mechanism to do so. This involves the bacterial protein "internalin" which attaches to a protein, "cadherin", on the intestinal cell membrane. These adhesion molecules are also found

in two other unusually tough barriers in humans — the blood brain barrier and the foeto–placental barrier, and this may explain the apparent affinity that *L. monocytogenes* has for causing meningitis and affecting babies *in-utero*.

1.2.7 Outbreaks of Listeriosis in New Zealand

In New Zealand, 90% of cases of listeriosis are associated with *L. moncytogenes*. The incidence rates are usually higher than Australia. The majority of listeriosis victims are the very young, the elderly, people with lowered immunity (usually due to chronic diseases) and pregnant women. Incidences of listeriosis in New Zealand could surge in the future due to the ageing population (Barnao, 2011). The most recent outbreak of listeriosis was in 2012 and it induced two deaths caused by infection with *L.monocytogenes*. One of the victims was in his 80s, the other in his 60s. They were both older and had weaker immunity than younger people. The second contributor toward the increasing risk of listeriosis in New Zealand is the growing trend to use chilled ready-to-eat food (RTE) with extended shelf life (Gilbert et al., 2009). In the past, most of the outbreaks of listeriosis in New Zealand were associated with seafood, raw fish, shellfish, mussels and ready-to-eat cooked meats. Recently, contaminated foods are becoming much more diverse and include such things as non-dairy based flavoured dips, hummus and tahini, pate, pre-packaged salads, cooked and smoked chicken, chicken sandwiches, yoghurt, savoury dairy-based chilled dips, ice-cream and cheese, smoked fish, sliced deli meat and other small goods (Crerar et al., 2011; Gilbert, et al., 2009; MAF, 2011). More than 30% of the reported food-borne listeriosis patients in New Zealand in 2010 did not survive (Barnao 2011). This is alarming situation which demands strict monitoring of high risk foods and improvements in detection tools.

1.2.8 Research on *L. monocytogenes*

Research on *L. monocytogenes* has focused on its pathogenicity, epidemiology, functional genome studies and evolutionary aspects. Epidemiological studies focussed on the outbreaks of human listeriosis cases. Research on the population, genetics and evolutionary aspects helps in understanding how *L. monocytogenes* is transmitted from animals, or the environment, and through foods to humans. Research on pathogenicity initially focussed on the basic mechanisms of cellular pathogenesis of *L. monocytogenes*. A pioneering study in 1960s had demonstrated that *L. monocytogenes* is able to survive and multiply in macrophages (Macherky, 1997). Recent studies used the mouse as a model to understanding the cellular immune response (Shen, Tato, & Fan, 1998). Further studies on the cell-mediated

immunity of listeriosis were carried out with the murine model and gave rise to the basic theories about the inability of antibodies to protect against infections produced by intracellular pathogens, the importance of activated macrophages in the elimination of intracellular parasites and the T-cell as the macrophage-activating element required for cell-mediated immunity. (Mackaness, 1962, 1969). Recent technological developments have given the opportunity to better understand the infection pathway based on studies of whole genome sequences and virulence factors.

1.2.9 Proteomics of foodborne pathogens

Protein-based technologies (proteomics) are rapidly being developed for identifying and studying microorganisms. Proteomics is the large-scale study of the expressed protein components of the genome of an organism. Proteins are vital for living organisms as they are the main components of the physiological metabolic pathways of cells. Proteomics technologies are sensitive and can rapidly and easily detect pathogens by using different high-performance separation techniques, such as one-dimensional (1D), two-dimensional (2D) gel electrophoresis and multidimensional chromatography, combined with high-resolution mass spectrometry. Nowadays, proteomic techniques can be conveniently classified into six functional areas: expression proteomics, protein-protein interactions, functional proteomics, structural proteomics, proteome mining and post-translational modifications (Carbonaro, 2004).

An early technique widely used in the study of proteins is gel electrophoresis, which began with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Rabilloud, Chevallet, Luche, & Lelong, 2010). This technique can separate proteins in one dimension based on their molecular weight and electrophoretic mobility (Rabilloud et al., 2010). Two-dimensional gel electrophoresis (2DE) is another form of gel electrophoresis, where protein molecules are electrophoresed on the basis of pH in one direction and then by molecular weight in a second direction (Rabilloud, et al., 2010). 2D electrophoresis was mostly used in early studies in proteomics and it is still widely used due to its robustness, resolution and its ability to separate entire and intact proteins (Tannu & Hemby, 2006; Van den Bergh & Arckens, 2004). 2DE Fluorescence Difference Gel Electrophoresis (2D-DIGE) was developed to separate up to three different samples within the same 2DE gel (Tannu & Hemby, 2006; Van den Bergh & Arckens, 2004). This technique labels protein molecules first with

fluorescent dyes before running the 2DE. This can accurately reveal differences in protein abundance between samples (Tannu & Hemby, 2006; Van den Bergh & Arckens, 2004).

Recent methods for studying proteins can involve a number of combinations, such as gel electrophoresis or multidimensional nano-liquid chromatography (2DnLC-MS/MS) with the ultimate detection and identification of the proteins by high-resolution mass spectrometry (Calvo et al., 2005; Nägele et al., 2003). One of the most frequently used proteomic techniques is that of matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS). This technique is used increasingly to identify or classify microorganisms by the analysis of high copy proteins (Cabrita et al., 2010; Cacace et al., 2010; Calhoun et al., 2010; Dumas et al., 2008; Fagerquist et al., 2009; Fagerquist et al., 2007; Hefford et al., 2005; Mandrell et al., 2005 ; Stults, 1995). The sizes of these proteins range in mass from four to 20 kDa. The major advantages of MALDI-TOF MS are speed and simplicity. It usually requires only the time necessary to culture a sufficient number of bacterial cells. Currently, this technique is usually combined with 2DE for the quick and effective to identification of bacterial cells.

Multi-dimensional protein identification technology (MudPIT) is also widely used. This is a shotgun proteomics technique capable of identifying thousands of proteins in proteolytically-digested complex mixtures (Donaldson et al., 2011). Compared to traditional 2DE, MudPIT separates peptides according to two independent physicochemical properties but uses 2D liquid chromatography (LC/LC) alongside the ion source of a mass spectrometer. This separation relies on columns of strong cation exchange (SCX) and reversed phase (RP) material, back to back, inside fused silica capillaries.

1.2.10 Proteomics of *L. monocytogenes*

Recently, several studies attempted to use different gel-based and non-gel-based proteomic techniques to detect food-borne pathogens, especially *L. monocytogenes*. 2DE, combined with mass spectrophotometry, are the most commonly used techniques to study protein changes in *L. monocytogenes* in response to stresses such as the presence of antimicrobial chemicals, low pH, high salinity or cold shock (Cabrita et al., 2010; Cacace et al., 2010; Duché et al., 2002; Folio et al., 2004; Gardan et al., 2003; Ramnath et al., 2003; Trost et al., 2005; Wemekamp-Kamphuis et al., 2004). Most of the recent studies primarily focused on the identification of *L. monocytogenes,* the analysis of cell wall and secretory proteins, the development of a partial

proteome reference map and bacterial virulence or bacterial protein expression under strict growth conditions (Figure 1.1).

2DE was used to analyse the response of *L. monocytogenes* under acidic conditions or following the application of osmotic shock conditions (Duché et al., 2002; Phan-Thanh & Mahouin, 1999). These two early studies found that stress conditions may either induce a number of proteins or overexpress certain proteins. Following those studies, proteins Ctc and GbuA were identified from the 59 proteins that were identified under the different salinities of the growth media (Gardan et al., 2003; Wemekamp-Kamphuis et al., 2004). Both these proteins are σ^B-dependent and act as osmotic stress tolerances for the bacterium (Cetin et al., 2004; Kazmierczak et al., 2003). The role of σ^B were further investigated by comparing the proteome expression profile between *L. monocytognes* EGD-e and its isogenic deletion mutant, ΔsigB, under acidic conditions (Wemekamp-Kamphuis et al., 2004). It was found that nine proteins were assigned as putative σ^B-regulated proteins involved in acidic adaptation. These proteins are 6-phosphofructokinase (Pfk), UDPglucose- 4-epimerase (GalE), ClpP protease and an unknown protein, Lmo1580.

Biofilms play an important role in bacterial survival and they cause serious problems in food industries, especially with *L. monocytogenes*. Thus, a number of comparative proteomic studies of the global protein expression from biofilm and planktonic-grown cells of *L. monocytogenes* have been carried out (Hefford et al., 2005; Helloin et al., 2003; Trémoulet et al., 2002). Hefford and colleagues' work identified 19 proteins that were up-regulated in *Listeria* biofilms (Hefford et al., 2005). These proteins are involved in the overall stress response, envelope and protein synthesis, biosynthesis, energy generation, and regulatory functions.

A number of studies have been carried out to compare protein expression between normal and stress conditions for *L. monocytogenes (*Ramnath et al., 2003; Folio et al., 2004). In early 2003, Ramnath and colleagues used a reproducible total protein extraction and 2-DE method to develop a partial annotated proteome reference map of the food-borne pathogen, *L. monocytogenes* (Ramnath et al., 2003). Although this study was not fully able to detect all the major proteins from food strains, it has provided a valuable starting point for the analysis of the bacteria food.

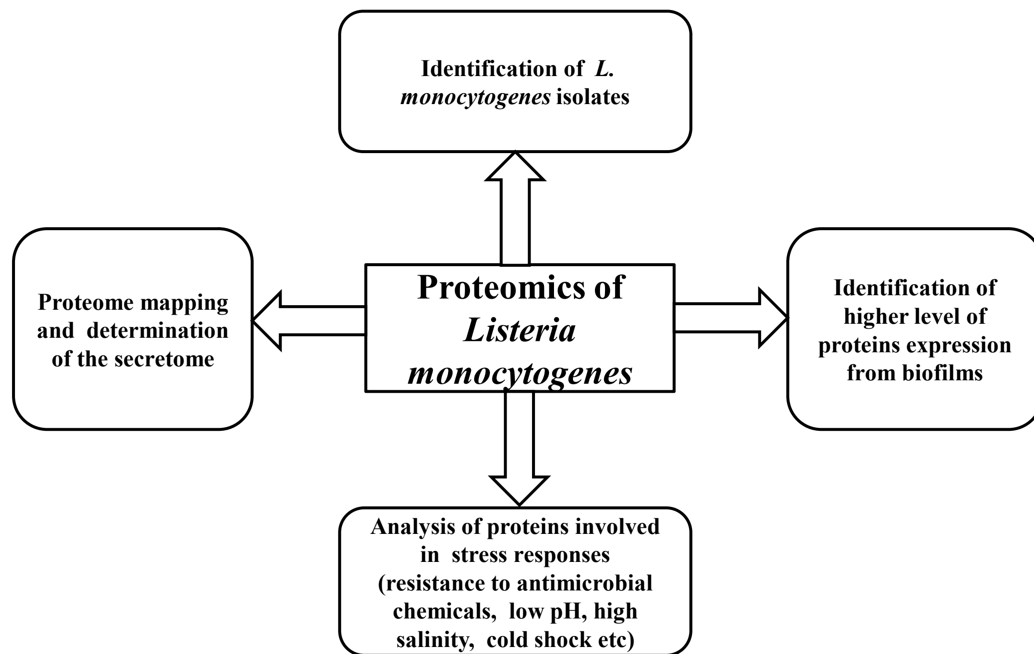

Figure 1.1. Different applications of proteomics analysis to characterise *L. monocytogenes* (Huang & Hussain, 2012)

Biofilms play an important role in bacterial survival, causing a serious problem in food industries, especially for *L. monocytogenes*. Hefford and colleagues' work identified 19 proteins that were up-regulated in *Listeria* biofilms (Hefford et al., 2005). These proteins are involved in the overall stress response, envelope and protein synthesis, biosynthesis, energy generation, and regulatory functions.

A number of studies have been carried out to compare protein expression in L. monocytogenes under normal and stress conditions. A 2DE reference map for *L. monocytogenes* EGDe serotype 1/2a strain was generated by compariing the proteomes between *L. monocytogenes* EGD-e cells in the exponential or stationary phase of growth at 37°C. (Folio et al., 2004). This reference map covered 28.8% of the potential gene products from the soluble subproteome. This study also identified 38 regulated proteins, which were involved in translation, cellular metabolism and stress adaptation.

The development of quantitative proteomics, high-throughput gel- and non-gel-based protein fractionation techniques, such as liquid chromatography (LC) are now widely coupled with protein identification by high-throughput tandem mass spectrometry (MS/MS)-based on automated software algorithms. This has been used to analyse protein expression in *L. monocytogenes*. Development of intracellular and extracellular proteome maps of *L. monocytogenes* has been attempted using the multiplexing fluorescent two-dimensional fluorescence difference gel electrophoresis (2DE-DIGE) and MALDI-TOF MS (Dumas et al., 2008; Velde et al., 2009).

The 2DE-DIGE technique uses different fluorescent dyes, such as Cy2, Cy3 or Cy5, to label the protein, allowing protein mixtures of different origins to be analysed within the same gel run (Velde, et al., 2009). In 2007, Folsom and Frank found 19 proteins that were differentially expressed between planktonic and biofilm cells of a hypochlorous acid-tolerant variant of *L. monocytogenes* ScottA (4b) strain using 2DE-DIGE (Folsom & Frank, 2007). Six of these 19 proteins were later identified by peptide-mass mapping, of which three were the ribosomal proteins (L7, L10 and L12), along with the peroxide resistance protein (Dpr/Flp/Fri), the sugar-binding protein (Lmo0181) and a putative protein Lmo1888 of a yet unknown function.

More recent investigations used advanced proteome analysis techniques (2DnLC- coupled to an ion-trap mass spectrometer and MuDPIT) to study *L. monocytogenes* protein expression

(Calvo et al., 2005; Donaldson et al., 2009; Pucciarelli et al., 2005). A cell-free supernatant of *L. moncytogenes* EDGe was analysed using LC-MS/MS and 105 proteins were identified using LC-MS/MS compared to 54 detected by traditional 2DE techniques (Trost et al., 2005). A total of 301 membrane-associated proteins from *L. monocytogenes* EDGe were successfully identified by using SDS-PAGE combined with LC-MS/MS (Wehmhöner et al., 2005). 2DnLC-MS/MS was applied to identify cell-wall proteins from *L. monocytogenes* and *L. innocua.* (Calvo et al., 2005). They identified 30 proteins, 19 of which were from *L. monocytogenes* and 11 proteins from *L. innocua*, most of them belonging to the class of LPXTG motif-harbouring proteins. Recently, a combination of LC-LC-MS/MS, essentially, a MudPIT approach, was used to study differences of protein expression between *L. monocytogenes* strains EDGe and F2365 (Donaldson et al., 2009). They were able to identify 1754 proteins from strain EDGe and 1427 proteins from strain F2365, accounting for a total *L. monocytogenes* proteome coverage of 50-60%. They found that a total of 1077 proteins were common to both strains and 423 proteins were expressed significantly differently. In another example, Hefford et al. (2005) identified higher levels of protein expression in biofilms of *L. monocytogenes* using 2DE analysis combined with MALDI-TOF-MS and MS/MS. Most of these studies focused primarily on the identification of *L. monocytogenes* cell wall and secretory proteins, the development of a partial proteome reference map and bacterial virulence or bacterial protein expression under certain growth conditions (Table 1.2). Current research on proteomics of *L. monocytogenes* provides a promising future for the development of detection technologies based on specific protein markers.

In comparison with traditional methods and nucleic acid-based techniques, proteomic techniques offer a high quality, sensitive and specific analysis for the classification and identification of bacteria (Sauer & Kliem, 2010). Therefore, there is potential for using proteomic techniques as tools for bacterial detection and identification.

Table 1.2. Important publications on *L. monocytogenes* using proteomics analysis

Year	Authors	Title	Technique(s) used
2003	Ramnath et al.	Development of a *Listeria monocytogenes* EDGe partial proteome reference map and comparision with the protein profiles of food isolates	2DE
2005	Hefford et al.	Proteomic and microscopic analysis of biofilms formed by *Listeria monocytogenes* 568	2DE Matrix assisted laser desorption/ionization time of flight (MALDI-TOF) and mass spectrometry/mass spectrometry (MS/MS) analysis
2005	Calvo et al.	Analysis of the *Listeria* cell wall proteome by two-dimensional nano-liquid chromatography coupled to mass spectrometry	Two-dimensional nano-liquid chromatography coupled to ion-trap mass spectrometry (2DnLC-MS/MS) analysis
2005	Trost et al.	Comparative proteome analysis of secretory proteins from pathogenic and non-pathogenic *Listeria* species	2DE MALDI-TOF/MS analysis High performance liquid chromatography(HPLC)/electrospray ionization-mass spectrometry (EI-MS)
2005	Pucciarelli et al.	Identification of substrates of the *Listeria monocytogenes* sortases A and B by a non-gel proteomic analysis	2DnlC-MS/MS analysis
2008	Dumas et al.	Comparative analysis of extracellular and intracellular proteomes of *Listeria monocytogenes* strains reveals a correlation between protein expression and serovar	2DE MALDI-TOF/MS analysis
2009	Donaldson et al.	Comparative proteomic analysis of *Listeria monocytogenes* strains F2365 and EGD	Multidimensional protein identification technology(MuDPIT)
2009	de Velde et al.	Isolation and 2-D-DiGE proteomic analysis of intracellular and extracellular forms of *Listeria monocytogenes*	2-D-DIGE MALDI-TOF analysis
2010	Carbrita et al.	A secretome-based methodology may provide a better characterization of the virulence of *Listeria monocytogenes*: preliminary results	SDS-PAGE MALDI-TOF-MS analysis
2010	Cacace et al.	Proteomics for the elucidation of cold adaptation mechanisms in *Listeria monocytogenes*	2DE MALDI-TOF analysis

Chapter 2
Methods and Materials

2.1 Sources of bacterial strains, chemicals, reagents and microbiological media

2.1.1 Bacterial Strains

The bacterial strains used in this research are listed in Table 2.1. The bacterial strains were purchased from the Institute of Environmental Science and Research Ltd (ESR), New Zealand.

2.1.2 Chemicals and Reagents

All chemicals were obtained from BDH Chemicals Company (Corporate Avenue, Rowville, Australia), Sigma-Aldrich Chemical Company (Auckland, New Zealand), Merck Pty Ltd (Victoria, Australia), Bio-Rad Laboratories (Albany, Auckland, New Zealand), Invitrogen (Penrose, Auckland, New Zealand), and Thermo Fisher Scientific (Albany, North Shore City, New Zealand). Ultra-pure water was prepared using either MODULAB™ Water Purification System or deionised water using the Milli-Q reagent water system (Millipore) throughput.

2.1.3 Microbiological media

Unless otherwise stated, all media were supplied by Oxoid (Hampshire, England) and sterilised by autoclaving at 121°C for 15 minutes. The pH of each broth was checked and, if necessary, adjusted immediately before autoclaving.

2.2 General methods

All media and stock solution were prepared using ultra-pure distilled water after weighing on an OHAUS E12140 analytical balance (for small quantities of chemicals) and an A& D Company FX2000 laboratory weighing balance (for large quantities of chemicals). The pH was measured using a Desktop-pH-meter SevenEasy pH meter. Solutions were stored at room temperature unless otherwise specified. Optical densities were measured using a UV-1610 spectrophotometer (Shimadzu). Bacterial cultures and protein extracts were centrifuged in a Labofuge 400R refrigerated centrifuge (large volumes of up to 250 ml) or an Eppendorf

Table 2.1. List of *L. monocytogenes* strains used in this study

Strain	NZRM No.	Source
Listeria monocytogenes (Murray et al.) Pirie Strain V7. Serotype 1a1.	3371	FDA, Bothell, Washington. Milk. (Medium 1, 37C)
Listeria monocytogenes (Murray et al.) Pirie NZ isolate SB92/870. (FH L6652a, CLIP 36389)	3349	Referred from Auckland Public Health Laboratory. Serotype 1/2a, phage type 1967, 881. Smoked mussels. (Medium 1, 37C))
Listeria monocytogenes NZ isolate SB92/844. (FH L6653b, CLIP 39392, 36387	3350	Serotype 1/2a, phage type 1967, 881. Blood, perinatal case, associated with consumption of smoked mussels. (Medium 1, 37C)

centrifuge (for small volumes of up to 1.5 ml). Stock solutions and media additives were sterilised by autoclaving at 121°C for 15 minutes, unless otherwise specified.

2.3 Stock solutions, buffers, reagents and media

2.3.1 Stock solutions

All solutions and media were prepared in accordance with Chambers (1993) and Sambrook (1989) using distilled (dH_2O) sterilised by autoclaving at 121°C for 15 min and then kept at room temperature unless otherwise specified.

Rehydration solution

7 M Urea, 2M Thiourea, 4% CHAPS, 0.25% Pharmalyte 4-6.5, 0.25% Pharmalyte 5-8, 0.4% dithiothreitol and 0.1% bromophenol blue were added to ultra-pure water.

Gel buffer stock solution

1.875 M Tris was adjusted to pH 8.8 with hydrochloric acid.

15% resolving gel solution

16 ml of gel buffer stock solution (1.875 M Tris), 30 ml of acrylamide stock solution, 0.8 ml of 10% SDS and 33.2 ml of ultrapure water were mixed together.

4% T?? stacking acrylamide solution (10.25 ml volume)

2 ml of gel buffer stock solution (1.875 M Tris), 1 ml of acrylamide, 0.1 ml of 10% SDS and 7.15 ml of ultrapure water were mixed together.

10% Ammonium persulphate solution (APS)

0.1 g of ammonium persulphate was dissolved in 1 ml of ultrapure water.

Equilibration stock solution

0.05 M Tris, 6 M Urea, 20% glycerol and 2% SDS were dissolved in ultrapure water. The solution was titrated to approximately pH 8.8 with HCl and stored at 4°C.

Fixative solution (150 ml for one gel)

75 ml of ethanol, 15 ml of concentrated acetic acid and 60 ml of ultra-pure water were mixed together.

Stain solution for 2DE gel (150 ml for one gel)

30 ml of methanol, 15 g of ammonium sulphate, 15 ml of 85% phosphoric acid, 0.18 g of Coomassie Brilliant Blue-G250 and 105 ml of ultrapure water were mixed together.

Spot destaining solution

0.158 g of NH_4HCO_3 were dissolved in 10 ml of a solution containing 50% AcN (acetonitrile)

Reduction and Alkylation solution

(a) 10 0?? mM NH_4HCO_3 (0.079 g of NH_4HCO_3 dissolved in 10 ml of ultrapure water)

(b) 50 mM Tris(2-carboxyethyl)phosphine (TCEP) (0.1433 g of TCEP dissolved in 10 ml of 100 mM NH_4HCO_3)

(c) 150 mM IAM (0.2775 g of IAM dissolved in 10 ml of 100 mM NH_4HCO_3

Working trypsin solution

Diluted 2-fold trypsin solution by adding 50 mM NH_4HCO_3 to create a working trypsin solution.??

50%AcN/0.5TFA solution

5 ml of AcN and 0.5 ml of TFA were dissolved in 4.5 ml of ultrapure water.

2.3.2 Buffers

Buffers were prepared according to the methods described in Biochemistry LabFax (Chambers, 1993).

Tris buffer pH 8.0

A tris(hydroxymethyl)aminomethane buffer solution was prepared from the appropriate concentrations of Tris base. The pH was adjusted to 8.0 using HCl.

4X SDS buffer

40 mM of Tris, 40% of glycerol, 40 mM EDTA, 2.5% SDS and ~ 0.2 mg/ml bromophenol blue were dissolved in distilled water.

***Listeria* protein solubilisation buffer**

7 M Urea, 20 mM Tris-Cl (pH8.0), 5 mM EDTA, 5 mM $MgCl_2$, 4% CHAPS and 1 mM phenymethanesulfonyl fluoride were dissolved in dH_2O.

***Listeria* lysis buffer**

2% Triton X-100, 2.6 mg/ml sodium azide, 0.1 M Tris (pH 8.0) and 8 mM phenylmethanesulfonyl fluoride were dissolved in dH_2O.

2.3.3 Media

Brain Heart Infusion (BHI) Broth

Dehydrated brain heart infusion broth (37.0 g) was dissolved in one litre of dH_2O and, if needed, the pH was adjusted with HCl before sterilisation by autoclaving.

BHI Agar

For BHI agar plates, 1.5% bacteriological agar was added to the BHI broth. Before autoclaving, the BHI components were dissolved completely by leaving the bottles in a hot water bath at 51°C until all the components dissolved.

2.4 Microbial growth and conditions

Bacteria were streaked on to Brain Heart Infusion agar and incubated for 24 h at 37°C. Colonies were then picked and inoculated into Brain Heart Infusion broth and grown for 24 h at 37°C (Moorhead, *et al*., 2003). A single bacterial colony was picked and inoculated into 5 ml of Brain Heart Infusion Broth and grown for 24 h at 37°C. This bacterial overnight culture was then transferred into 250 ml of BHI broth with an initial OD_{600} ~0.1, and grown at 37℃ with shacking at 130 rpm. The OD_{600} was measured every hour until bacterial growth reached the stationary phase (~13 h).

2.5 Protein analysis

SDS PAGE was used to determine the quality of the extracted protein and the banding patterns for these strains. Comparative proteomic analysis and identification of proteins was performed using 2DE analysis, MALDI-TOF-MS and Ion Trap MS.

2.5.1 Protein sample collection

One hundred µl of bacterial cells were placed in 5 ml of BHI broth and incubated at 37°C at 130 rpm for 24 h. Bacterial cultures were then transferred into 30 ml of BHI medium with

initial OD_{600} ~0 and grown at 37°C at 130 rpm to the exponential phase (optical density OD_{600} ~1.0). The bacterial cultures were pelleted by centrifugation at 5,600 x g for 10 min at 4°C and the supernatant discarded. The cell pellets were kept on ice.

Cell pellets were re-suspended in 4 ml of lysis solution. Bacteria were then incubated on ice for 1.5 hours. Samples were treated with 2 µl (10000U/ml) DNase I and 2 µl of 20 mg/ml RNase A for 30 min at 37°C, then 0.5 ml of the *Listeria* solubilisation buffer was added and the cell debris pelleted by centrifugation (18,000 x g for 5 min at 4°C). Supernatants containing the total protein were stored at -80°C until needed.

2.5.2 Protein sample quantification

The quantitation of the solubilised protein was undertaken using the bicinchoninic acid assay (BCA) kit and following the manufacturer's instructions.

The BCA reagent was mixed with Reagent B at a ratio of 50:1 (Reagent A: Reagent B=50:1) to make the working reagent. Twenty µg of the test protein sample was added to blank wells, and then 200 µl of working reagent was added and mixed using the pipette tip. The test plate was then covered with tinfoil and incubated at 37°C for 30 min. The plate was cooled to room temperature and the absorbance read at 562 nm.

2.5.3 One dimensional SDS PAGE

Dry protein samples were precipitated by adding 3x volumes of ice-cold acetone and incubated at -20°C overnight. The solutions were centrifuged at 4°C for 10 min. The acetone supernatants were discarded and the protein sample was freeze-dried and kept at -20°C until use.

Protein samples (20 µl of protein solubilisation buffer containing about 30 µg of dry protein) were mixed with 40 µl of 4x SDS sample buffer and then boiled for 10 min. Twenty-five µl were then loaded onto the gel, along with 5 µl of Precision Plus Protein Standards (Bio-Rad). All empty lanes were loaded with 10 µl of 4x SDS. After the protein samples were loaded, the gel was run at 200 V, 8 mA per gel and 15 W for approximately 1 hr. Gels were stained using colloidal Coomassie Brilliant Blue G250 (20% methanol, 10% ammonium sulphate, 10% phosphoric acid, 0.12% Coomassie Brilliant Blue G250) then de-stained with dH_2O.

2.5.4 2DE analysis

Two-dimensional polyacrylamide gel electrophoresis of protein samples isolated from *L. monocytogenes* isolates were performed according to the protocol described by Cacace, et al. (2010). Approximately ~850 µg of the freeze-dried protein samples were applied by in-gel rehydration in 18-cm IPG strips (pH 4-7 range strips).

The IPG strips were rehydrated with a solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.25% Pharmalyte 4-6.5, 0.25% Pharmalyte 5-8 and 0.4% DTT. Thirty-five µl of the solution above was used to dissolve ~850 µg of protein powder. This rehydration solution containing protein was pipetted into the rehydration tray. The IPG strips were placed over the top of the solution in the sealable rehydration tank and then flushed with nitrogen for 10 minutes. The IPG strips were then rehydrated for 24 h at room temperature.

After rehydration, the strips were rinsed with ultrapure water (18MΩ). The electrode wicks were dampened with 0.4% DTT solution, blotted dry and placed in the IPGphor electrofocussing system (GE Healthcare) over the electrodes and the rehydrated strips were then placed on top/above??. The strips were covered with degassed paraffin oil. The strips were then focussed overnight for ~80,000 V. After focusing, the strips were stored in a -85°C freezer until run in the second dimension.

A 15% T acrylamide (37.5:1 acrylamide:bis-acrylamide) resolving gel solution was prepared and degassed for 30 minutes. The Protean II gel casting cassettes were cleaned and assembled and the bottom of the cassette sealed with a solution of 0.5% agarose. Eighty ml of the acrylamide solution, 40 µl of tetramethylenediamine (TEMED) and 400 µl of 10% ammonium persulfate (APS) were added after pouring the gels into the casting cassette to begin polymerisation. After polymerisation, 10 ml of 4% stacking gel was poured into the cassette and polymerised with 5 µl of TEMED and 50 µl of APS. At each stage, the gel was overlain with 1 ml of water-saturated isobutanol, covered with polyethylene cling film and left to set (~90 mins).

The IPG strips in the second dimension were equilibrated, for 15 minutes in a strip rehydration tray on a platform rocket, in 2 ml of equilibration solution (6 M urea, 50 mM Tris, 20% glycerol and 2% sodium dodecylsulfate (SDS)) containing 1% dithiothreitol (DTT). The DTT equilibration solution was discarded and the strips equilibrated with the same solution containing 4% iodoacetamide (IAM) instead of DTT. The strips were removed from the equilibrating solution and cut to fit the gel, then allowed to settle onto the gel so as to ensure

that the strip was touching the surface of the gel. The water on the top of the gel was poured off and the top sealed with 10% agarose solution (containing 0.1% bromophenol blue dye). The gels were run at 200 V, 32 mA and 50 W per gel for 15 minutes, then at 275 V, 80 mA, 50 W, for approximately three hours.

The 2DE gels were fixed with 150 ml of fixative solution (50% ethanol and 10% acetic acid) with gentle rocking for 30 minutes. The gels were then stained with colloidal Coomassie Brilliant Blue G250 stain solution overnight. All the gels were washed with several changes of ultra-pure water, with the use of 1-2 Kimwipes per change to remove excess stain.

All samples were run in triplicate for a total of nine gel maps and three biological replicates for each bacteria strain were analysed.

Spots analyses were carried out on the gel images followed by the excision of spots, digestion in trypsin and then analysed by mass spectrometry. Proteins were identified using the bioinformatics resources of AgReseach Ltd (New Zealand).

2.5.5 Spot analysis

Spot detection, quantization, and analysis were performed using the PDQest ^{TM} 2-D Analysis software, Version 6.2 (Bio-Rad). After this, the spots were excised, digested with trypsin and analysed by mass spectrometry.

Images of the gels were captured with a Nikon D100 digital camera and the background subtracted with AnalySIS 5 (Olympus Imaging Systems). The images of the gels were aligned and the spots quantitated and matched by PDQuest v 6.2 2D gel analysis software (Bio-Rad Laboratories, Hercules, CA). The gels were initially processed using the automatic spot detection and matching routine, and then further aligned by manually defining key spots as landmarks. The spot intensities for all gels were then normalised using the Total Quantity in Gel Image routine. The results were reported as histograms of relative spot volume for each protein spot of interest.

2.5.6 Protein identification

Proteins were identified by MALDI-TOF-MS and Ion Trap MS in AgReseach Ltd (New Zealand).

2.5.6.1 Preparation of samples for Mass Spectrometry

Spots were excised from gels and destained by washing twice with a solution of 200 mM ammonium bicarbonate and 50% acetonitrile (AcN) for 1 hour at 37°C. The spots were reduced with a solution of 50 mM TCEP and 100 mM ammonium bicarbonate at 56°C for 45 minutes. Following this, they were shaken with 150 mM IAM in 100 mM ammonium bicarbonate for 30 minutes; they were then shaken with AcN for 10 minutes and dried on a Centrivap vacuum centrifugal concentrator (Labconco, Kansas City, MI, USA). After digestion with 2 μg TPCK-trypsin in 50 mM ammonium bicarbonate:AcN (8.625:1.375) for 18 hours at 37°C, peptides were extracted from the gel by vortexing for three hours in the presence of Empore™ disks that had been pre-wetted with AcN and methanol, following which the peptides were extracted with 75% AcN in 0.1 mM TFA. The extracts were dried on a Centrivap vacuum centrifugal concentrator and reconstituted in 0.1% TFA.

2.5.6.2 MALDI-TOF

Samples were prepared for MALDI-TOF by applying 1 ml of a α-cyanocinammic acid (CHCA)-saturated solution of 97:3 (acetone: 0.1%) TFA to an AnchorChip plate and then immediately removed. The sample was applied as 1 μL dissolved in 0.1% TFA, allowed to incubate for three minutes before removal, followed by the addition and immediate removal of 2 μL of 0.1% TFA. Finally, 1 μL of a solution of 0.1 mg/mL CHCA in 6:3:1 ethanol: acetone: 0/1% TFA was added and allowed to dry.

MS and MS/MS spectra were collected on a MALDI-TOF/TOF mass spectrometer (Ultraflex III, Bruker Daltonics). Spectra were calibrated externally using a Peptide Calibration Standard (Bruker) containing angiotensin II (m/z 1046.5418) and I (m/z 1296.6848), Substance P (m/z 1347.7354), bombesin (m/z 1619.8223), ACTH-clip (m/z 2093.0862), ACTH-clip (m/z 2465.1983) and somatostatin (m/z 3147.471) diluted six-fold with a matrix solution.

After the MS and MS/MS data acquisition, peak lists were extracted from the data using FlexAnalysis software (Bruker). These peak lists were searched against the NCBInr database using an in-house MASCOT server (Matrix Science, London, UK). The following search parameters were used: semitrypsin was chosen as the proteolytic enzyme; peptide and fragment ion tolerances were set at 0.2 Da; taxonomy was restricted to Firumicutes (gram-positive bacteria) and two missed cleavages were allowed.

2.5.6.3 Ion-trap Mass Spectrometry

LC-MS/MS was performed on a nanoAdvance UPLC coupled to an amaZon speed ETD mass spectrometer equipped with a CaptiveSpray source (Bruker Daltonik, Bremen, Germany). Two µl of protein sample was loaded on a C18AQ nano trap (Bruker, 75 µm x 2 cm, C18AQ, 3 µm particles, 200 Å pore size). The trap column was then switched in line with the analytical column (Bruker Magic C18AQ, 100 µm x 15 cm C18AQ, 3 µm particles, 200 Å pore size). The column oven temperature was 50°C. The elution was with a gradient from 0% to 40% B?? in 90 min at a flow rate of 800 nl/min. Solvent A was LCMS-grade water with 0.1% FA and 1% AcN; solvent B was LCMS-grade AcN with 0.1% FA and 1% water.

Automated information-dependent acquisition (IDA) was performed using Hystar PP 3.2.44.0 software, with a MS survey scan over the range m/z 50–2200 followed by three MS/MS spectra from 50–2200 m/z acquired during each cycle of 30 ms duration.

2.5.6.4 Data Analysis

After each LC-MS/MS run, peak lists were queried against Firumicutes (gram-positive bacteria) in the Uniprot database using the Mascot search engine (v2.2.03, Matrix Science) maintained on an in-house server. The Mascot search parameters included semitrypsin as the proteolytic enzyme with two repeat missed cleavages; standard modifications being carbamidomethylation (C), deamidation (NQ) and oxidation (M); the error tolerance was set to 0.15 Da for MS and 0.3 Da for MS/MS. Search results were compiled and analysed using ProteinScape 3.1.0 (Bruker) using the ProteinExtractor function and the automatic assessment of true and false positive identifications of protein and peptide matches. Acceptance thresholds for peptide and protein scores were set at 25 and 80, respectively. Results assessed as being true matches were used for further analysis. Error-tolerant searches were run on all spots in the Type I keratin region, all parameters being kept the same except the enzyme was set to trypsin and no modifications were searched for.

Chapter 3
Experimental Results

3.1 Growth Curve generation

In this research, three strains of *L. monocytogenes*, V 7, SB92/870 and SB92/844, were studied. Strain SB92/870 was isolated from a local New Zealand seafood product (mussels) and the other strain, SB92/844, was a clinical strain. V7 was isolated from milk products in the USA and has been extensively studied. The growth of the bacteria was first monitored using three biological replicates for each bacterial strain. All three strains showed identical growth patterns when growth conditions were kept similar. Under similar growth conditions, the exponential increase in OD_{600} for all three isolates occurred between 2-7 h of incubation at 37°C. Strains SB92/870 and V7 tended to grow a little slower than strain SB92/844 during the exponential phase. Interestingly isolates SB92/870 and V7 achieved similar OD_{600} values, 1.77(with SD 0.190 and 0.053 respectively), at around six hours of growth, which was slightly higher than for SB92/844 (1.53 with SD 0.046). Strain V7 first reached its highest OD (average OD_{600nm}=2.03 with SD 0.185) in 7 h, whereas the average OD of other two was about 1.9. After 7 h of growth, strain V7 reached the stationary phase, where the OD_{600} values fluctuated between 1.83~2.7. For isolates SB92/844 and SB92/870, they still had very slow growth after 8 h, and the highest OD_{600} values (2.09 with SD 0.078 and 2.04 with SD 0.042 respectively) were observed after 9 h (Figure 3.1). After nine hours growth, strains SB92/870 and SB92/844 tended to reach the stationary phase, and they both had average OD values ranging from 1.91 (SD 0.040)to 2.04(SD 0.042). In general, all three strains of bacteria had similar growth trends during the lag phase and exponential phases.

3.2 1D SDS PAGE analysis

For proteomics analysis purposes, bacterial cells were harvested at an OD_{600} of around 1.0 (mid exponential phase). The protein samples from three isolates of *L. monocytogenes* were first tested using SDS-PAGE (Figure 3.2). About 30 µg of protein sample from each strain was loaded on to a SDS gel. The gel was stained with Coomassie Blue G250 and imaged prior to examining the quality of the extracted protein and the banding patterns. There were no significant differences in the SDS PAGE gels, since the results showed that Lanes 2, 3, and 4

had similar banding patterns, they all migrated the same distance and had less abundant bands in the larger sizes (greater than

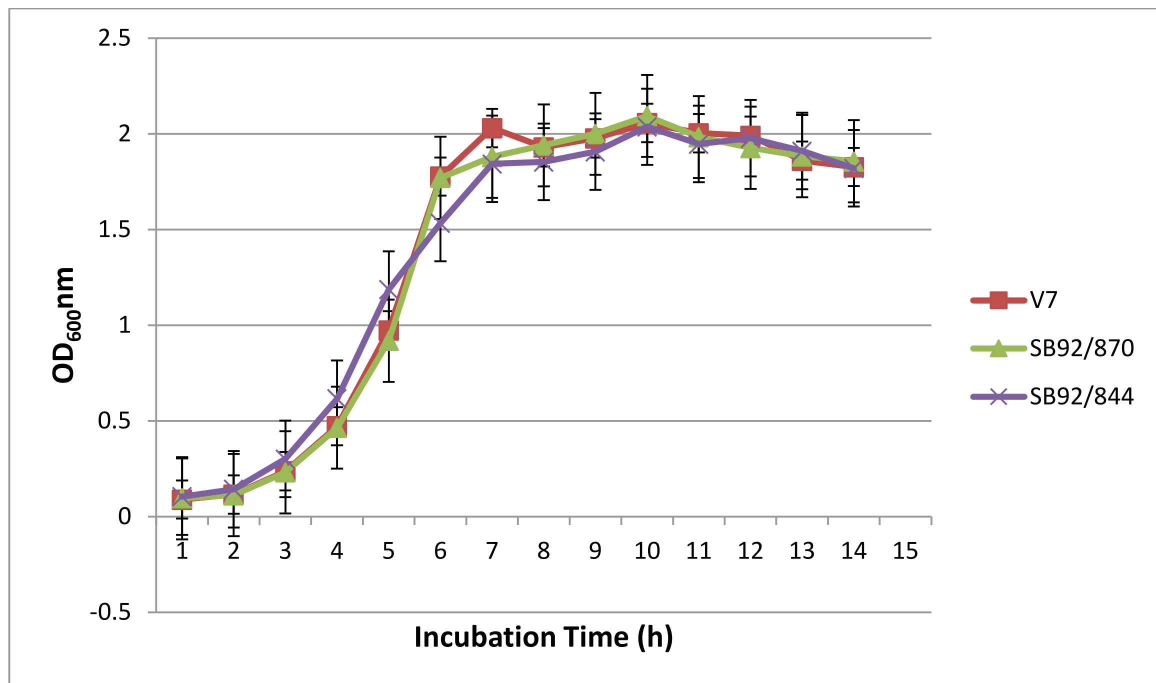

Figure 3.1. Growth of three isolates of *L. monocytogenes* (V7, SB92/870, SB92/844) in Brain Heart Infusion medium at 37°C until bacterial growth reached the stationary phase. The average OD_{600} values from three biological replicates of each bacterial isolate with the standard deviation is shown and the data are given in Appendix I.

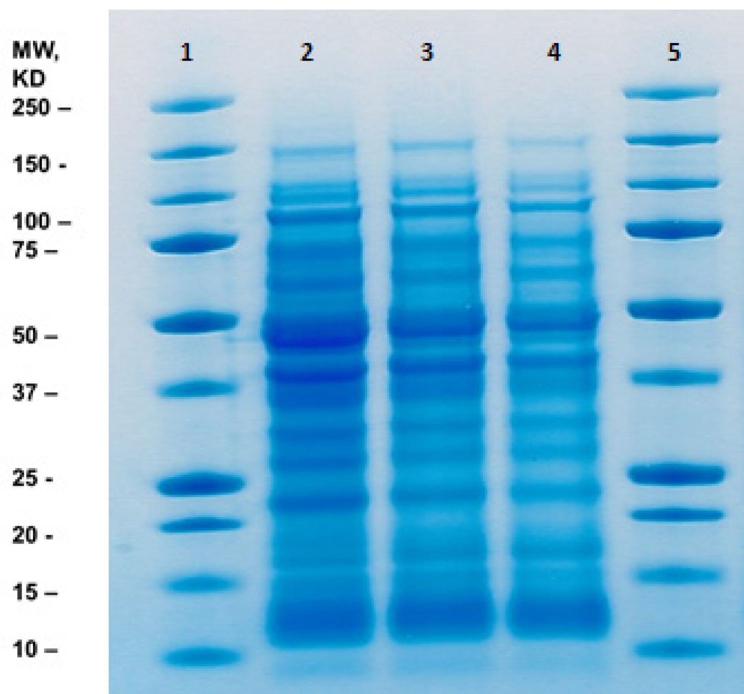

Figure. 3.2. SDS-PAGE gel showing the banding pattern of each strain. The protein samples were precipitated using a 3x volume of acetone to make 30 µg of dry protein samples. The dry protein samples were dissolved in 15 µlof protein solubilisation solution and 5 µl of 4x SDS buffer was added to make 20 µl of protein sample solution. For each lane, 20 µl of solution that contained ~ 30 µg of protein samples was loaded. Lanes 1 and 5 - Protein marker; Lane 2 – *L. monocytogenes* SB92/844; Lane 3 – *L. monocytogenes* SB92/870; Lane 4 – *L. monocytogenes* V7

50 kDA) and smaller sizes (between 50 kDA~15 kDA). For each strain, 20 bands could be observed by SDS PAGE. The sizes for all the protein bands migrated between 150 kDA and 10 kDA. The bands that formed well resulted in narrow bands with sharp edges (with sizes greater than 75 kDA), were soluble proteins. From the gels, it was shown that for the three strains of bacteria, protein bands with sizes between 100 kDA and 50 kDA had similar density and thickness and they all tended to migrate close together. For protein bands between sizes 10~15 kDA, all three strains have shown intrinsic protein patterns that form broader bands with less distinct edges. These protein bands might contain some proteins with lots of hydrophobic regions or non-protein substituents, such as multi-pass transmembrane proteins. Therefore, 2DE analysis was conducted to further study these protein samples from three isolates of *L. monocytogenes.*

3.3 2DE analysis

Two-dimensional electrophoresis (2DE) analysis of protein samples isolated from *L. monocytogenes* isolates was performed according to the protocol described by Cacace, et al. (2010). Detailed methods are given in Section 2.5.4. Protein samples were extracted in triplicate from each strain and grown in BHI broth to OD_{600} ~ 1.0. Protein samples from each culture were run on individual 2 DE gels. A total of nine gel maps from three strains were developed and analysed (images of the gels are shown in Appendices II, III and IV).

2DE was carried out on an equivalent number of proteins from the three different strains of *L. monocytogenes.* The gels were stained with Coomassie Blue G250 and imaged prior to performing spot analysis using PDQuest v7.0.0 software. The resulting 2DE maps showed the majority of protein spots for the three strains of bacteria were localised in a region between pH 4 and pH 6. Furthermore, most spots migrated to an isoelectric point (pI) of between pH 4 and 5.

Comparison of the gels from three strains of *L. monocytogenes* showed differences between these strains. Visual observation revealed that certain spots appeared to express differentially. Spot 1 was absent in *L. monocytogenes* V7 (Figure 3.3). Spot 2 underwent a positional shift in *L. monocytogenes* SB92/870. Spot 3 was absent in *L. monocytogenes* V7. Spot 4 underwent both a positional shift and intensity change between LM SB92/844 and *L. monocytogenes* SB92/870, and was absent in *L. monocytogenes* V7. Spot 5 underwent a positional shift in *L. monocytogenes* SB92/844. One gel was picked from *L. monocytogenes* SB92/844 as a reference gel for using the image analysis software

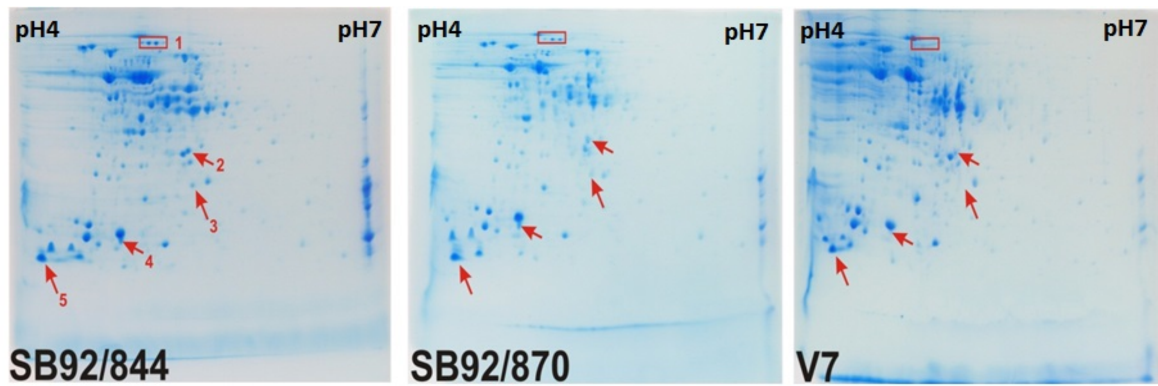

Figure 3.3. Typical images of whole cell protein 2DE gels of three *L. monocytogenes* strains grown in BHI broth at 37°C for 6 h. Some spots are marked which are assumed differentially??? expressed using visual observation.

PDQest v 6.2 to match the maximum number of spots and to visualise all the identified protein spots.

3.4 Comparison of differently expressed proteins of three different strains of *L. monocytogenes*

Spot detection, quantitation and analysis of the proteins in the three *L. monocytogenes* strains grown under similar conditions were performed using the PDQest^{TM} 2D analysis software (version 70.0). A total of 189 spots were detected with the software using SB92/844 as the reference map (Figure 3.4). Figure 3.4 represents the image of the reference gel that was created by the software, showing all possible spots that were marked on the reference image. From a comparison of *L. monocytogenes* strain V7 with strain SB92/844, there were 99 spots that matched SB92/844, which means 52.4% of detected spots in the reference gel were present in strain V7 (Table 3.1 and Appendix V). There were 102 detected spots (54.0% of detected spots) that able to be found in *L. monocytogenes* train SB92/870 (Table 3.1). Finally, 70 out of 189 detected spots (37% of detected spots) were present in all three strains studied. However, while some of the spots were neither in strain SB92/870 nor strain V7, it may be due to a real absence of protein spots, or it could be due to poor matching by the image analysis software. Therefore, in this study, we only took into account those spots that were able to be detected by the software.

For the purposes of this research, the relative difference in protein expression had to differ by more than 1.5-fold for it to be regarded as up-regulated or down-regulated. Spot differences of less than a 1.5-fold increase or decrease were regarded as not differentially/differently?? expressed. On this basis, the expression of five protein spots were found to increase in SB92/844 relative to V7, with two spots (Spot 6203 and Spot 6205) having a ratio of +2.3 indicating it was highly up-regulated (Table 3.2 a). Expression of 10 proteins spots were also shown to decrease in SB92/844 (Table 3.2 a), the ratio of the most down-regulated protein spot being -3.5 (Spot 9203). Furthermore, 14 spots showed no significant difference in protein expression in SB92/844 relative to V7. For the relative protein expression pattern in *L. monocytogenes* for SB92/870 relative to V7, five protein spots were significantly over expressed, and three of them had a ratio for the most up-regulated protein spot of +1.7 (Table 3.2b). Expression of six protein spots were significantly under expressed in SB92/870 relative to V7 with the most down-regulated having a ratio of -3.1. A further 11 protein spots showed no differential expression. By comparing the proteins expression between SB92/844 to

SB92/870, there were 22 protein spots that showed no significant differences (Table 3.2 c). Only three protein spots were over expressed,

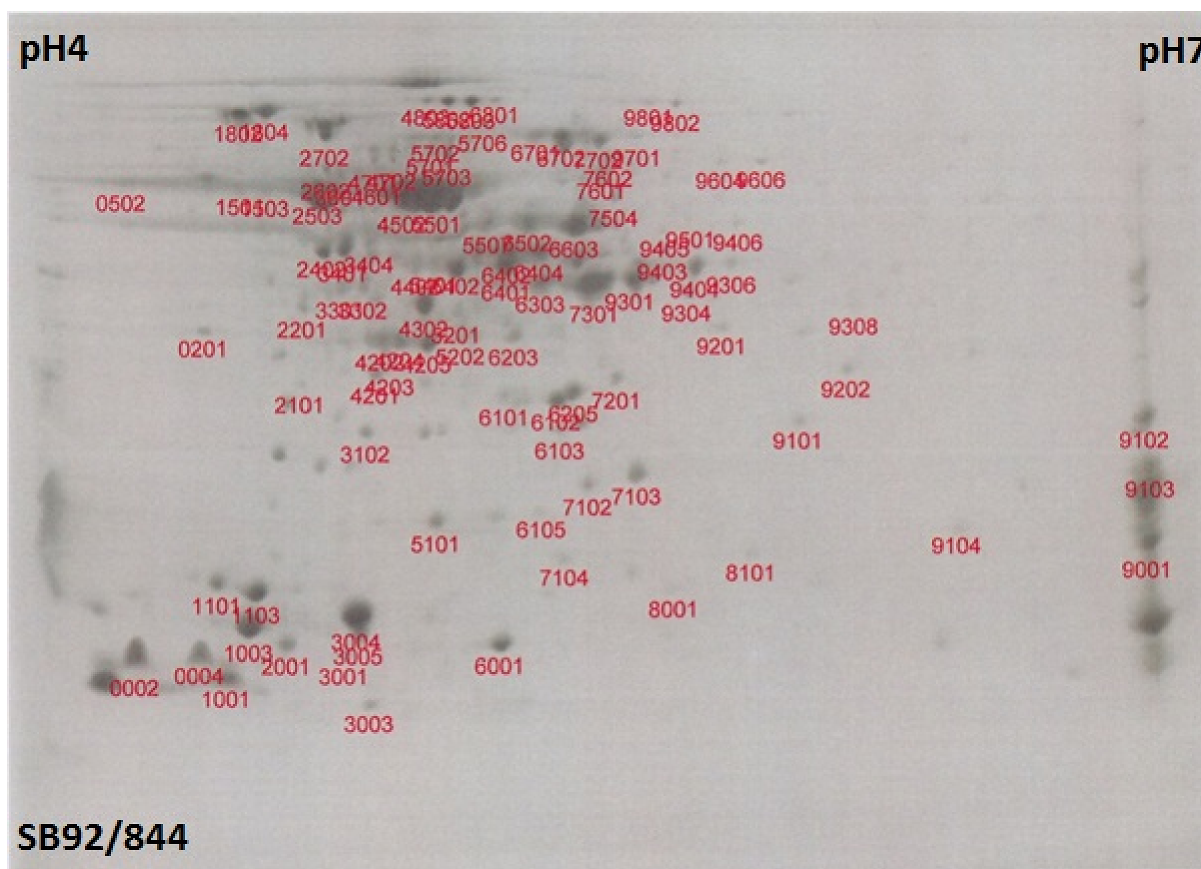

Figure 3.4. Image of the reference gel created by PDQest^{TM} 2D Analysis software to conduct comparative proteomic analysis

Table 3.1. Comparison of detected 2DE total protein spots of three strains of *L. monocytogenes* V7, SB92/844, and SB92/870

Total No. of spots detected in reference gel SB92/844	**No. of spots matched *L. monocytogenes* strain V7 (%)**	**No. of spots matched *L. monocytogenes* strain SB92/870 (%)**	**No. of spots matched three strains of *L. monocytogenes* (V7, SB92/844, SB92/870) (%)**
189	99 of 189 (52.4%)	102 of 189 (54.0%)	70 of 189 (37.0%)

Table 3.2a. Relative protein expression in *L. monocytogenes* SB92/844 to V7

Spot ID	Average Values in Reference Strain V7	Average Values in SB92/844	Relative protein expression SB92/844 vs V7
		Up-regulated spots	
0002	13359	23654	+1.8
1003	18975	37452	+2.0
6105	6713	3643	+1.8
6203	1775	4077	+2.3
6205	3272	7445	+2.3
		Down-regulated spots	
2001	8686	4599	-1.9
5101	7874	4724	-1.7
6102	15098	9703	-1.6
4203	4002	1307	-3.1
5101	7874	4724	-1.7
6102	15097	9702	-1.6
6105	6713	3643	-1.8
6502	27345	9246	-3.0
7201	4411	2345	-1.9
9203	4543	1296	-3.5
		Spots without differential expression	
0004	8697	10740	+1.2
0201	2184	1629	-1.3
1001	12485	11696	+1.1
1103	13271	13472	+1.0
2702	12899	12719	-1.0
3004	36430	42635	+1.2
3102	3104.9	2981	-1.0
6001	10338	9185	-1.1
6603	26225	26753	+1.0
3004	36429	37035	+1.0
7103	4056	3455	-1.2
7301	29527	40910	+1.4
7501	3315	3426	+1.0
9201	2047	1558	+1.3

Table 3.2b. Relative protein expressions in *L. monocytogenes* SB92/870 to V7

Spot ID	Average Values in Reference Strain V7	Average Values in SB92/870	Relative protein expression SB92/844 vs V7
	Up-regulated spots		
0002	13359	22338	+1.7
0004	8697	14774	+1.7
3004	36430	54226	+1.5
6205	3272	5671	+1.7
9203	4543	6947	+1.5
	Down-regulated spots		
6102	15098	7626	-2.0
2001	8686	4837	-1.8
6102	15098	7626	-2.0
6303	17509	11211	-1.6
6502	27345	8924	-3.1
6603	26225	10147	-2.6
	Spots without differential expression		
0201	2184	2197	+1.0
1001	12485	17385	+1.4
1003	18975	23053	+1.2
1103	13271	17391	+1.3
1104	5780	4593	-1.3
2702	12899	16714	+1.3
3102	3105	3007	-1.0
5101	7874	9164	+1.2
6001	10338	11174	+1.1
7103	4056	3620	-1.1
7301	29527	42203	+1.4

Table 3.2c. Relative protein expressions in *L. monocytogenes* SB92/844 to SB92/870

Spot ID	Average values in SB92/870	Average values in SB92/844	Relative protein expression SB92/844 vs SB92/870
		Up-regulated spots	
1003	23053	37452	+1.6
6603	10147	26753	+2.6
7104	1511	5519	+3.7
		Down-regulated spots	
1001	17385	11696	-1.5
1101	7428	5091	-1.5
2402	9609	5680	-1.7
2602	3381	2102	-1.6
3005	13312	7768	-1.7
3401	8453	3612	-2.3
5101	9164	4724	-1.9
5402	10595	7016	-1.5
		Spots without differential expression	
0002	22338	23654	+1.1
0004	14774	10740	-1.4
0201	2197	1629	-1.3
1103	17391	13472	-1.3
2001	4837	4599	-1.1
2201	1514	1689	+1.1
2702	16714	12719	-1.3
3003	3131	2323	-1.3
3004	54226	42635	-1.3
3102	3007	2981	-1.0
3301	2680	1940	-1.4
4302	14803	14603	-1.0
5802	4233	4017	-1.1
5803	3911	4422	+1.1
6001	11174	9184	+1.2
6102	7626	9702	+1.3
6205	5671	7445	+1.3
6303	11211	13625	+1.2
6502	8924	9246	+1.0
7103	3620	3455	-1.0
7301	42203	40910	+1.0
9802	1376	1373	+1.0

the ratio of the most up-regulated protein was +3.7 (Spot 7104); the expressions of eight protein spots decreased in SB92/844 in relation to SB92/870 and the ratio of the most down-regulated was -2.3 (Spot7104).

Comparison of the differentially expressed proteins in three strains of *L. monocytogenes*, revealed there were five spots that both showed up-regulation in SB92/844 relative to V7 and in SB92/870 relative to V7 (Table 3.3); whereas, there were only three over expressed spots in SB92/844 relative to SB92/870. For the down-regulated spots, nine spots were found to be down-regulated in SB92/844 relative to V7. There were eight spots showing down-regulation in SB92/844 relative to SB92/870 and only six spots showed decreased expression in SB92/870 relative to V7.

Comparative spot analysis was followed by the identification of the spots, marked on the reference gel (Figure 3.4), using MALD-TOF/TOF MS and Ion Trap MS. For identification of the protein, selected protein spots were excised, digested in trypsin, and analysed through mass spectrometry.

Table 3.3. Comparison of differential protein expressions in different strains of *L. monocytogenes*

	SB92/844 vs V7	SB92/870 vs V7	SB92/844 vs SB92/870
Up-regulated spots	5	5	3
Down-regulated spots	10	6	8

3.5 Identification of specific protein spots by MALDI-TOF MS and Ion Trap MS

Seventy-four spots were excised from triplicate gels of the reference strain SB92/844 and further analysed by MALD-TOF MS and Ion Trap MS. Only 19 were identified (Figure 3.5 and Table 3.4); the positions of the identified proteins are shown in reference gel SB92/844 (Figure 3.5). For those spots identified, four were cold shock proteins that play a role in detoxification and adaptation to atypical conditions (Spots 0001, 0002, 0004 and 1001) (Table 3.4). Three spots were found to contain co-chaperonin GroES (Spot 2001), phosphocarrier protein HPr (Spot 3005) and a CD4+ T-cell-stimulating antigen that functioned as transport/binding proteins (Spot 2402), lipoproteins and membrane bioenergetics, respectively. The five protein spots identified were found to be members of the carbohydrate metabolism group, including phosphoglycerate mutase, oligopeptide-binding protein and glyceraldehyde-3-phosphate dehydrogenase (Table 3.4 and Figure 3.5**.** Other spots were found to be of proteins involved in protein synthesis, protein folding, oxidative responses and steroid biosynthetic processes. In comparison, for the expression of proteins identified in *L. moncytogenes* strains SB92/844 and SB92/870 (Table 3.5), six of these identified proteins were not present in *L. monocytogenes* Strain V7, two were not present in *L. monocytogenes* SB92/870 (Spot 0001 and Spot 7201) and one of the identified proteins was missing from both V7 and SB92/870 (Spot 0001).

According to Figure 3.2, the marked spot group 1 contains two proteins that are both oligopeptides, ABC transporter and oligopeptide-binding protein, which are absent in *L. monocytogenes* V7 (Figure 3.5 and Table 3.5). Spot 2 is a ribosome recycling factor, which undergoes a positional shift in LM SB92/870. Spot 3 is absent in *L. monocytogenes* V7. Spot 4 is a protein that is involved in detoxification and adaptation to atypical conditions, which has undergone both a positional shift and intensity change between *L. monocytogenes* SB92/844 and LM SB92/870, and is absent in *L. monocytogenes* V7. Spot 5 is major cold-shock protein that has the functions to detoxify and adapt to atypical conditions. This protein has undergone a positional shift in *L. monocytogenes* SB92/844.

Table 3.4. Functional classification of the identified proteins from *Listeria monocytogenes* strain SB 92/844 by using MALD-TOF/TOF MS and Ion Trap MS

Spot No.	Ultraflex III (MALD-TOF MS)	amazon (Ion Trap MS)	Function
0001	Major cold shock protein, partial	Major cold shock protein	Detoxification and adaptation to atypical conditions
0002	Major cold shock protein	Major cold shock protein, partial	Detoxification and adaptation to atypical conditions
0004	Major cold shock protein, partial	Major cold shock protein, partial	Detoxification and adaptation to atypical conditions
1001		Major cold shock protein, partial	Detoxification and adaptation to atypical conditions
1003	50S ribosomal protein L7/L12		Protein synthesis
1103	Regulatory protein SpoVG		
2001	co-chaperonin GroES		Protein folding
2402	CD4+ T-cell-stimulating antigen		Transport/binding proteins, lipoproteins and membrane bioenergetics
3005		Phosphocarrier protein HPr	Transport/binding proteins, lipoproteins and membrane bioenergetics
3004		Phosphocarrier protein HPr	Transport/binding proteins, lipoproteins and membrane bioenergetics
5802	Oligopeptide ABC transporter, oligopeptide-binding protein	Glyceraldehyde-3-phosphate dehydrogenase	Metabolism of carbohydrates (Glycolysis/ gluconeogenesis)
5803		Glyceraldehyde-3-phosphate dehydrogenase	Metabolism of carbohydrates (Glycolysis/ gluconeogenesis)
6102		Superoxide dismutase	Oxidative stress
6205	Ribosome recycling factor	Ribosome recycling factor	Ribosomial protein
6502		Glyceraldehyde-3-phosphate dehydrogenase	Metabolism of carbohydrates (Glycolysis/ gluconeogenesis)
6603	Glyceraldehyde-3-phosphate dehydrogenase	Glyceraldehyde-3-phosphate dehydrogenase	Metabolism of carbohydrates(Glycolysis/gluconeogenesis)
7102		PTS system, glucose-specific, IIA component	Membrane transporter
7201	Phosphoglycerate mutase		Metabolism of carbohydrates (Glycolysis/ gluconeogenesis)
9203	NAD-dependent epimerase		steroid biosynthetic process

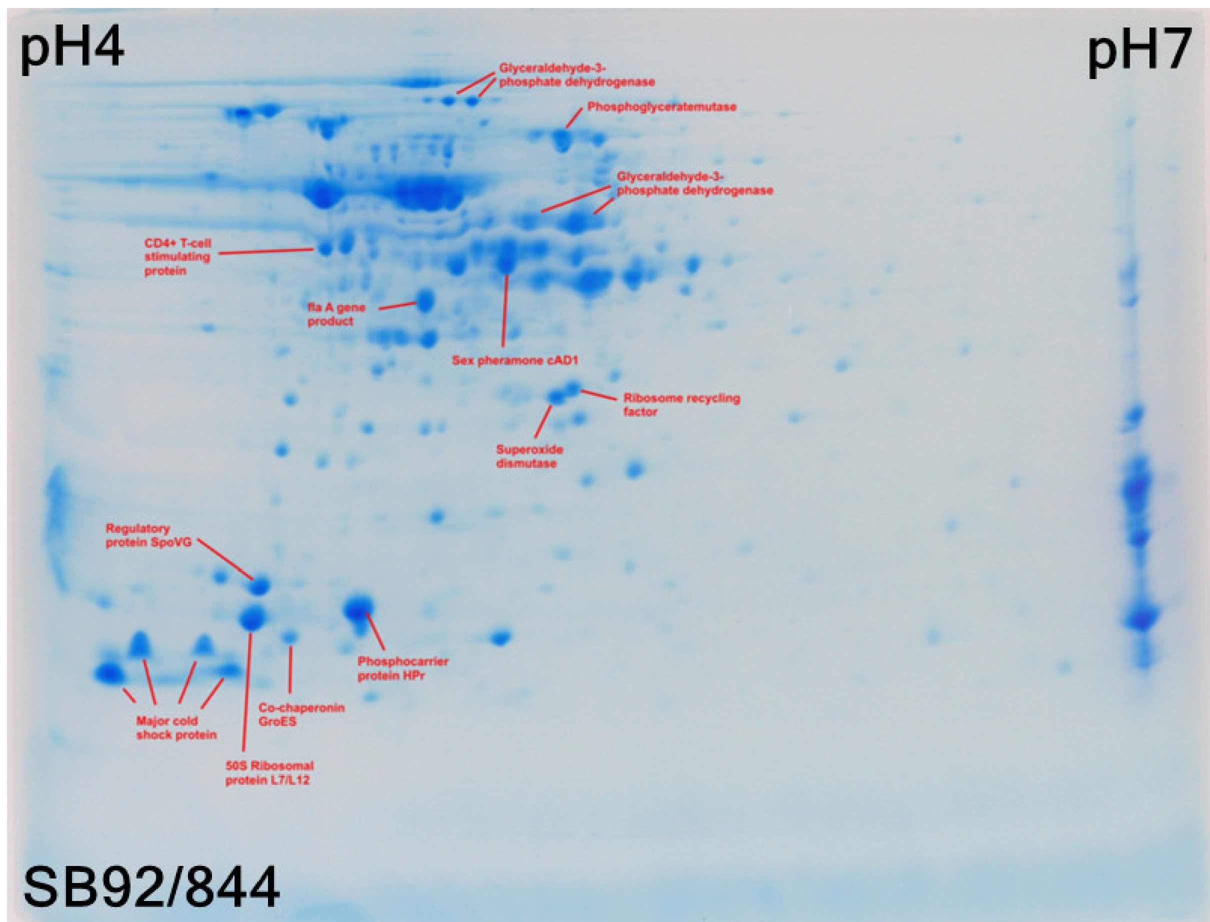

Figure 3.5. Spots identified through MALDI-MS/MS analysis are shown on the reference 2DE gel for *L. monocytogenes* SB92/844.

Chapter 4 Discussion

4.1 Comparison of growth *L. monocytogenes* strains in BHI broth

L. monocytogenes is an important foodborne pathogen. This research characterised the proteins expressed in three *L. monocytogenes* strains, SB92/844, SB92/870 and V7, during the exponential phase growth in BHI broth at 37°C. Strains SB92/844 and SB92/870 are New Zealand strains; one of the strains was isolated from a patient's blood sample and the other strain was isolated from a local seafood product. Strain V7 was imported from the USA. All three strains are representative of serotype 1/2a sporadic disease isolates from Lineage II and they all showed similar matching growth patterns when growth conditions were kept similar. The two local isolates showed very similar growth patterns under the same growth conditions. This indicated that those two local strains, SB92/844 and SB92/870, have almost identical growth profiles in comparison to the overseas strain under the same growth conditions.

4.2 1D SDS PAGE and 2DE profiles of three strains of *L. monocytogenes*

The initial investigation of the protein banding patterns of three strains of *L. monocytogenes* using 1D SDS PAGE showed no noticeable differences. All the protein bands from Lanes 2, 3 and 4 that migrated the same distance had less abundant bands of large and small sizes. Narrow bands with sharp edges were observed from three strains, which could be polypeptides from cytoplasmic proteins or membrane-associated proteins. The proteins bands with sizes around 100 kDA and 50 kDA, may be two polypeptides that shared the same amino acid sequence for the most part, or were closely related in structure, or, otherwise, were isoforms of the same protein. In nature, such polypeptides typically formed multiple-subunit proteins or were part of a "family" of isoenzymes that performed similar functions, but were each specialised for a specific tissue and/or purpose. For protein bands between 10~15 kDA for three strains have shown intrinsic protein patterns that were broader with less distinct edges. These protein bands may contain some hydrophobic and hydrophilic proteins, which may have polypeptides with different net charge depending on the amino acids sequence. For the proteins with many hydrophobic regions or non-protein substituents, they might be multi-

pass transmembrane proteins. Polypeptides that formed intrinsic proteins had sequences of hydrophobic side chains that may, or may not, denature completely. Moreover, these large bands may indicate either overloading of protein in a well or the presence of a predominant polypeptide in the three protein samples. In general, the band patterns were the same for each isolate, as they all migrated the same distance and the proteins bands were all distributed with sizes between 150 kDA to 10 kDA.

Further 2DE analyses followed by identification of individual spots by MALD-TOF/TOF mass spectrometry and Ion Trap mass spectrometry were carried to evaluate the protein expression in the strains of *L. monocytogenes*. Strain SB92/844 was used as a reference for comparative analysis of the protein patterns for the three strains of bacteria.

The 2DE technique is an important tool for proteomic study and is used to separate hundreds of proteins in a single gel (Trémoulet et al., 2002). In this study, the differential protein patterns of the three strains of *L. monocytogenes* were studied, initially, by using 2DE. From an examination of the 2DE gels for three strains of *L. monocytogenes*, cultivated under the same growth conditions, the majority of the protein spots were localised in a region between pH 4 and pH 6. As indicated by the protein patterns on the 2DE gels, most of the spots migrated to an isoelectric point (pI) of between pH 4 and pH 5. In this study, the same serotype and different strains of *L. monocytogenes* were compared using 2DE analysis first. From a comparison of the *L. monocytogenes* strain V7 to strain SB92/844, 52.4% of detected spots in the reference gel were present in strain V7. Meanwhile, 54.0% of detected spots were able to be found in *L. monocytogenes* Strain SB92/870. As early study, in 1995, demonstrated that 46.7% of the spots that were common among *L. monocytogenes* strains across serotypes and serotypes 1/2a and 1/2b??, were in two different major clusters (Gormon & Phan-Thanh, 1995). The results observed in this study were only from three strains of *L. monocytogenes*, and they were the same serotype. Therefore, a higher percentage of matched protein spots were observed then comparing the two strains of *L. monocytogenes*.It was also found that only 37% of spots detected were present in those three strains of bacteria. As Jungblut indicated, the greater variability observed at the proteomic level may be due to a single amino acid change in a protein (Jungblut, 2001). And these changes can result in a shift in pI, and then further resulting in a detectable modification in the 2D patter (Jungblut, 2001).

A comparison of the relative protein expression value between SB92/844 to V7 and SB92/870 to V7 showed that same numbers of up-regulated spots were observed, while slight differences were observed in down-regulated spots. This may indicate that the two local stains may have similar protein patterns, as shown in 2DE gel analysis, when compared with the overseas strain.

4.3 Comparative analysis of up- and down-regulated proteins that were identified in SB92/844

L. monocytogenes strains SB92/844 and SB92/879 are two local strains isolated in New Zealand. In this study, 19 proteins were identified, six of them were highly expressed in SB92/844 relative to V7 (Table 4.1). These were two major cold shock proteins (Spot 0002 and Spot 9203), 50S ribosomal protein L7/L12 (Spot 1103), co-chaperonin GroES (Spot 2001), phosphocarrier protein HPr (Spot 3004) and ribosome recycling factor (Spot 6205). Expression of the three proteins identified decreased in all 19 proteins identified, those being superoxide dismutase (Spot 6102), glyceraldehyde-3-phosphate dehydrogenase (Spot 6502), and phosphoglycerate mutase (Spot 7201) in SB92/844 (Table 4.1). There were four proteins that were not differentially?? expressed in SB92/844 relative to V7, i.e. two major cold shock proteins (Spot 0004 and Spot 1001), regulatory protein, SpoVG, and glyceraldehyde-3-phosphate dehydrogenase (Spot 6603). By comparing the identified proteins spots with *L. monocytogenes* strain SB92/870 relative to V7 (see Table 3.4), three out 19 proteins identified had lower expressions. They were two glyceraldehyde-3-phosphate dehydrogenases (Spot 6502 and Spot 6603) and one major cold shock protein (Spot 9203). Three major cold shock proteins showed up-regulation in SB92/870 (Spots 0002, 0004 and1001). Spot 0001 (major cold shock protein) looks/appears?? to be present in all three strains and was observed from the 2DE images, but it was not detected by PDQest^{TM} 2D Analysis software. Among the identified proteins, five of which were not present in V7 strain, but both were present in SB92/844 and SB92/870. These missing proteins are CD4^{+} T cell-stimulating antigen (Spot 2402), phosphocarrier protein HPr (Spot 3004, 3005), glyceraldenhyde-3-phosphate dehydrogenase (Spot 5803) z1q12cc and glucose-specific IIA component (Spot 7102). Moreover, despite the missing proteins in V7 and SB92/870, the rest of the identified proteins were highly expressed in SB92/870 relative to V7 when compared with SB92/844 relative to V7.

Table 4.1. Expression of identified proteins in *L. monocytogenes* strains SB92/844 and SB92/870

Spot No.(Shown on the reference gel)	Identified protein	SB92/844 [a)]	SB92/870 [b)]
0001	Major cold shock protein	Protein only present in SB92/844	
0002	Major cold shock protein, partial	↑(+1.8)	↑(+1.7)
0004	Major cold shock protein, partial	(+1.2)	↑(+1.7)
1001	Major cold shock protein, partial	(+1.1)	↑(+1.4)
1003	50S ribosomal protein L7/L12	↑(+2.0)	↑(+1.2)
1103	Regulatory protein SpoVG	(+1.0)	↑(+1.3)
2001	Co-chaperonin GroES	↑(+1.9)	↑(+1.8)
2402	CD4+ T-cell-stimulating antigen	Protein not present in V7, but present in SB92/870 and SB92/844	
3004	Phosphocarrier protein HPr	↑(+1.2)	↑(+1.5)
3005	Phosphocarrier protein HPr	Protein not present in V7, but present in SB92/870 and SB92/844	
5802	Glyceraldehyde-3-phosphate dehydrogenase	Protein not present in V7, but present in SB92/870 and SB92/844	
5803	Glyceraldehyde-3-phosphate dehydrogenase	Protein not present in V7, but present in SB92/870 and SB92/844	
6102	Superoxide dismutase	↓(-1.6)	↑(+2.0)
6205	Ribosome recycling factor	↑(+2.3)	↑(+1.7)
6502	Glyceraldehyde-3-phosphate dehydrogenase	↓(-3.0)	↓(-3.1)
6603	Glyceraldehyde-3-phosphate dehydrogenase	(+1.0)	↓(-2.6)
7102	PTS system, glucose-specific, IIA component, putative	Protein not present in V7, but present in SB92/870 and SB92/844	
7201	Phosphoglycerate mutase	↓(-1.9)	Protein not present in SB92/870
9203	Major cold shock protein	↑(+3.5)	↓(-1.5)

a) Relative protein expression values from Table 3.2 (a) Relative protein expression in *L. moncytogenes* SB92/844 to V7

b) Relative protein expression values from Table 3.2 (b) Relative protein expression in *L. moncytogenes* SB92/870 to V7

Some of the proteins identified were responsible for core metabolic functions under the same growth conditions. In this study, five spots (Spots 5802, 5803, 6502, 6603 and 201) have been identified (glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate mutase) and function in metabolic pathways correlated to carbohydrate metabolism. Phosphoglycerate mutase has a function involved in catalysing the isomerisation of phosphoglycerate substrates, a process essential for the metabolism of glucose and/or 2, 3-phosphoglycerate (Jedrzejas, 2000; Rigden et al., 2003). Cacace et al. (2010) observed a significantly higher level of enzymes in glycolysis under cold condition; however, this current study identified that these proteins were either down-regulated or missing from *L. monocytogenes* V7. These findings may suggest that local strains may differ from the overseas strain in the expression of proteins that were involved in glycolysis. Furthermore, the presence of similar proteins functioning as transport/binding proteins, lipoproteins and membrane bioenergetics indicated that the two local strains of *L. monocytogenes* utilised similar nutrients. Superoxide dismutase is the enzyme that functioned as protection from superoxide reactive oxygen species for organisms during their active metabolism (Archambaud et al., 2006). This enzyme is the major mediator of phagocytes' microbicidal activity. In this study, superoxide dismutase showed increased expression in strain SB92/870 and decreased expression in SB92/844, during the latter lag phase, which may suggest that this protein may play an important role in virulence and pathogenicity for those two local strains. Interestingly, several of the proteins identified were not detected in the international isolate V7, but which of these proteins may contribute to the key differences between the two local strains and international strain.

Additionally, although those proteins identified were expressed in both local strains, many of them had significantly higher expression in SB92/844 than in SB92/870, e.g. major cold shock proteins. Major cold shock proteins have been previously studied; they have functions as regulators of both cold and nutrient starvation (Schmid et al., 2009). These proteins identified may play an important role for in bacterial survival under stress conditions. One thing that must be stressed was that the absence of some proteins in SB92/870 and V7 may be due to detection and spot matching limitations in the particular software application used, and this would need to be confirmed by further investigation.

4.4 Implications of stress proteins to virulence and pathogenicity of *L. monocytogenes*

According to previous findings, strains from clinical cases were more virulent than strains from foods (Nørrung & Andersen, 2000). Even though the two local isolates may perform similar basic cellular processes there were differences in other characteristics. Differences in expression stress responses between these two local strains may help to determine the type of environment that was best suited for the bacteria or how the strain SB92/844 was able to interact with the host's immune response.

Expression of stress response proteins was very important for *L. monocytogenes's* survival under stress conditions such as cold, heat, osmotic pressure, acids, alkalines and high hydrostatic pressure. These stress proteins also contributed to the virulence and pathogenicity of *L. monocytogenes*. For example, by comparing the cold stress wild type and mutants lacking csp genes in the *L. monocytogenes* EDGe strain, it was shown that csp genes played an important role in cell invasion when grown at 37°C (Loepfe et al., 2010). The virulence genes and cell invasion of *L. monocytogenes* were controlled by expression of *prfA*. The decreasing expression of this gene during long-term cold storage at 4°C has shown that the expression of prfA was temperature-dependent and associated with pathogenicity (Duodu et al., 2010; Johansson et al., 2002; McGann et al., 2007). The above examples suggested that temperature-dependent virulence gene expression repression, as well as membrane damage and cell surface modifications in these organisms exposed at low temperatures, might lead to the phenotypic virulence defects observed in cold-adapted *L. monocytogenes*. Comparison of proteomes between *L. monocytogenes* cells grown in human THP-1 monocytes and those grown in TSB broth using 2D-DIGE, indicated that, in general, stress protein, Ctc, and oxidative stress protein, Sod, were playing an important role in the survival and adaptation for intracellular uptake (Van de Velde et al., 2009). Proteome analysis of the general response for the *fri* mutant and the wild type strain of *L. monocytogenes* was compared and exposed repression in Hly (Listeriolysin O) and the presence of stress response proteins, CcpA (Catabolite control protein A) and OsmC, which suggested that Fri protein functions in promoting virulence (Dussurget et al., 2005)?? Another general stress response modulating protein, Hfq, was a RNA binding regulatory protein. Previous work has shown that Hfq was able to protect *L. monocytogenes* from osmotic and ethanol stress as well as facilitate enhanced pathogenicity in bacteria-infected mice (Christiansen et al., 2004). Stack and his colleagues have found that the HrtA serine protease can protect *L. monocytogenes* from

various stresses conditions, such as acidic conditions. The function of this protein also acted as virulence capabilities?? (Stack et al., 2005). Recently, studies found that another general stress response protein, σB, was able to facilitate *L. monocytogenes'* adaptation to multiple,?? which suggested that this protein played an important role in promoting virulence and cell invasiveness in this bacterium (Garner et al., 2006; Reid et al., 2010).

Several major cold shock proteins were differentially?? expressed between the two local strains during the log phase, which may suggest that those major cold shock proteins may link to the temperature-depended virulence gene in *L. monocytogenes* and it also showed the key differences between the local strains and the international isolate. Another cold shock protein (Spot 9203) was highly expressed in the local clinical strain, SB92/844, and down-regulated in the food strain, SB92/870. This may indicate that this cold shock protein may contribute to the virulence and pathogenicity of *L. monocytogenes* strain SB92/844. It also suggested why strain SB92/844 tends to be more virulent than strain SB92/870. The cold shock proteins (Csp) functioned as nucleic acid chaperones, which can bind RNA and DNA. They played an important role in regulation of various microbial physiological processes, such as replication, transcription and translation in bacterial cells (Ermolenko & Makhatadze, 2002; Phadtare et al., 1999).

Chapter 5
Conclusion and Future Directions

Beyonddifferential protein expression in local and international *L. monocytogenes* strains, this study highlighted the possibility that stress response proteins of *L. monocytogenes* may have an impact on their clinical behaviour and pathogenesis. This study has provived an initial view of the protein expression of *L. monocytogenes* species in New Zealand strains. The two local isolates showed very similar growth patterns under the same growth conditions. This study found that the two local strains, SB92/844 and SB92/870, showed identical growth profiles in comparison to the overseas strain under the same growth conditions. The protein banding patterns of the three strains of *L. monocytogenes* showed no noticeable differences when using 1D SDS PAGE. Visual observations of the 2DE gels revealed that certain spots appeared to be expressed differently. Differences in protein expression of the three strains of *L. monocytogenes* were observed and certain proteins were identified that were expressed differently between two local strains, which may suggest that those proteins could be related to virulence and pathogenicity.

This study was unable to discriminate the strains investigated according to their origins: humans or food. Despite the great care taken in the choice of the three strains of bacteria, this study was unable to identify the specific virulence factors associated with the two local isolates. It was also unable to determine if the food strain of *L. monocytogenes* was able to causes disease in humans, since the development of listeriosis depended on the immune system and infection dose. Furthermore, the cultural conditions under which *L. monocytogenes* was grown in the laboratory, under optimal conditions at 37°, in a high nutrient environment, were very different from the natural environment bacteria grow in natural environment.

Further studies could be carried out to verify whether the differential expression of cold shock proteins may be associated with the virulence and pathogenicity of *L. monocytogenes* under various growth conditions, such as different media, temperatures, pH and stress responses. These further studies may result in variations in the patterns of protein expression. This study was based on one of the most commonly studied *L. monocytogenes* strains (V7), therefore, a similar approach could be carried out to other local *L. monocytogenes* strains to obtain a clear

view of whether certain stress proteins may contribute to the virulence factors and pathogenesis.

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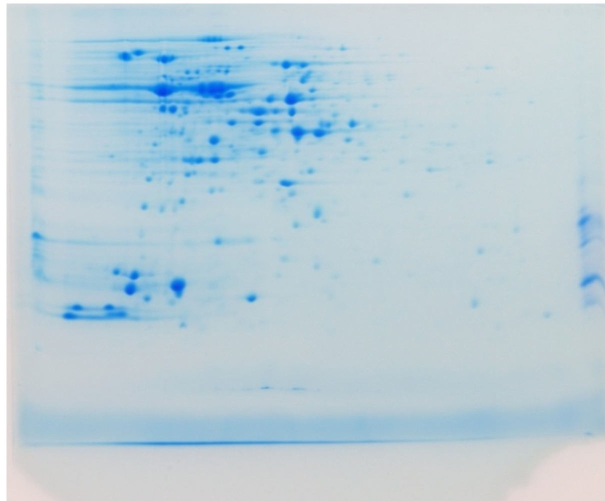
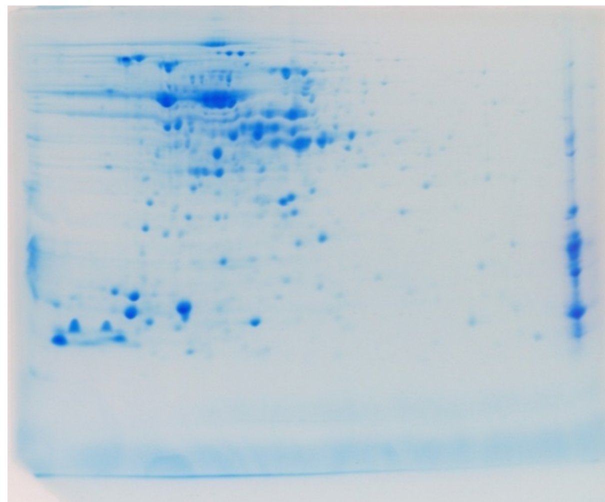
Appendix I

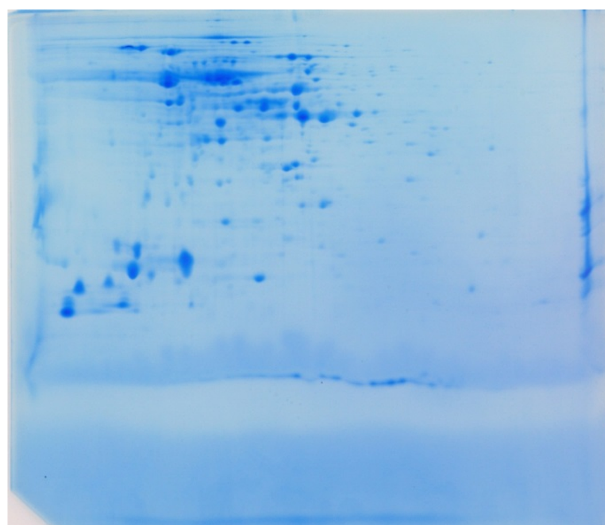
Hours	V7	Standard Deviation (SD)	SB92/870	Standard Deviation (SD)	SB92/844	Standard Deviation (SD)
0	0.09	0.008	0.10	0.004	0.10	0.010
1	0.12	0.003	0.11	0.006	0.14	0.015
2	0.24	0.023	0.23	0.011	0.30	0.034
3	0.47	0.023	0.46	0.025	0.62	0.047
4	0.97	0.059	0.92	0.045	1.19	0.086
5	1.77	0.190	1.77	0.053	1.53	0.046
6	2.03	0.185	1.88	0.046	1.84	0.031
7	1.93	0.456	1.94	0.026	1.85	0.057
8	1.98	0.032	2.00	0.060	1.91	0.040
9	2.07	0.067	2.09	0.078	2.04	0.042
10	2.00	0.049	1.98	0.061	1.95	0.076
11	1.99	0.056	1.93	0.061	1.98	0.040
12	1.86	0.069	1.88	0.057	1.91	0.056
13	1.83	0.126	1.86	0.015	1.82	0.078

Average of OD_{600} nm with standard deviations for three strains of *L. monocytogenes* (V7, SB92/870, and SB92/844) for 13-hour growth

Appendix II

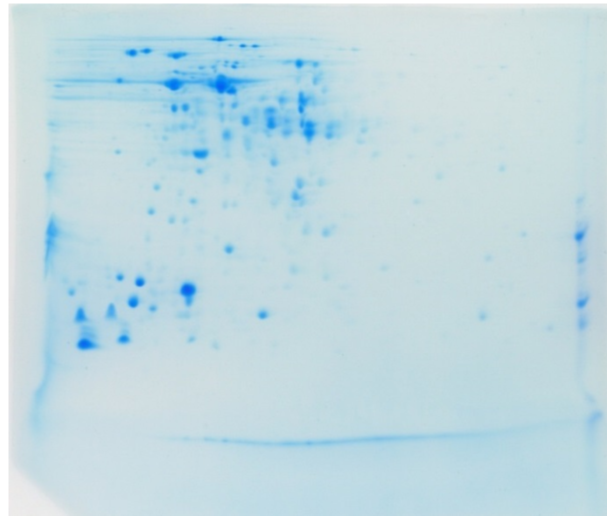


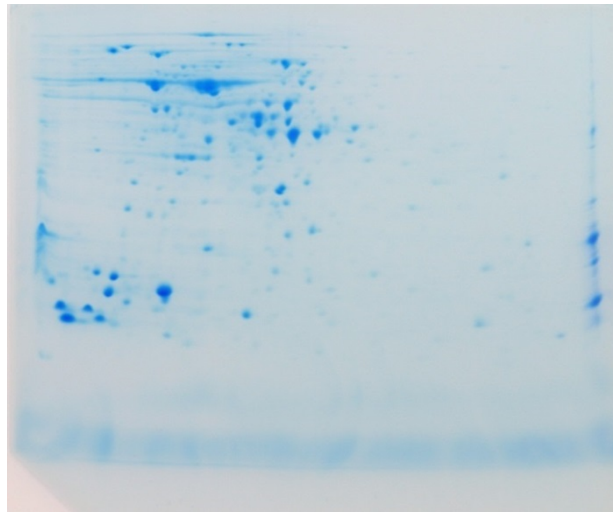


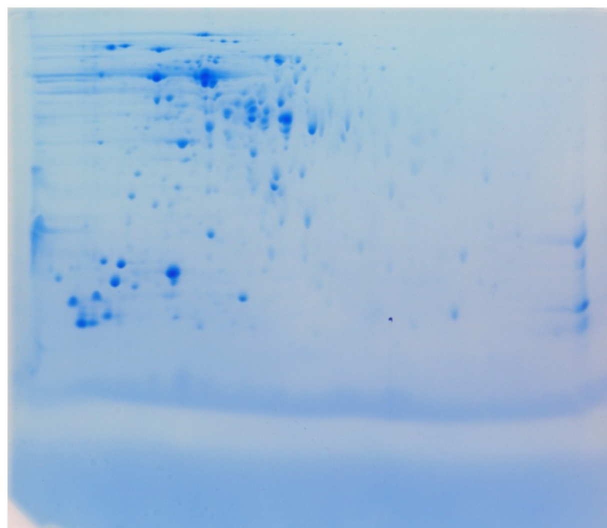


Original images of whole cell protein 2DE gels of *L. monocytogenes* isolate SB92/844 grown in BHI broth at 37°C for 6 h

Appendix III

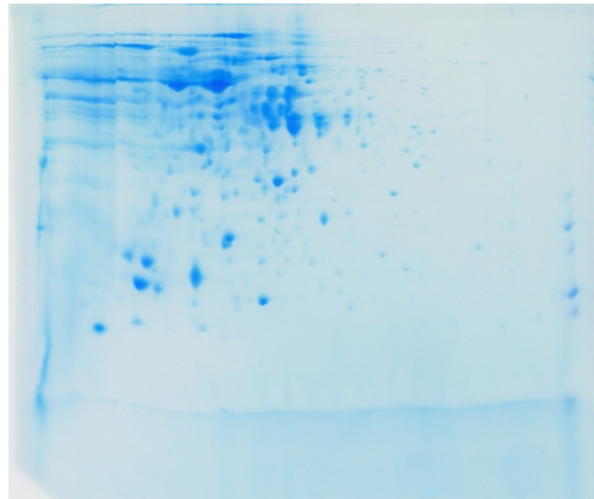


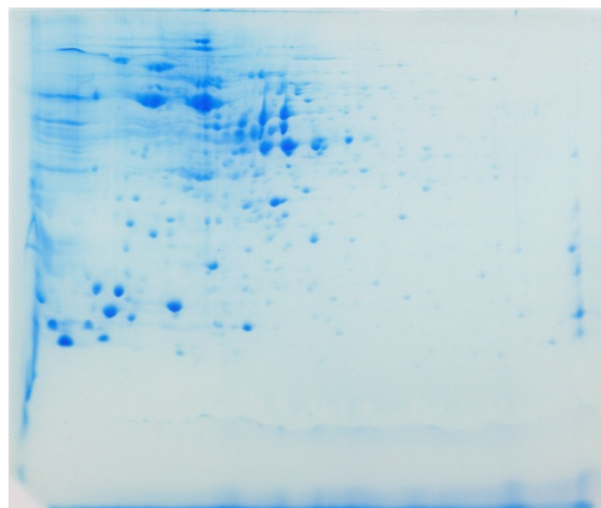


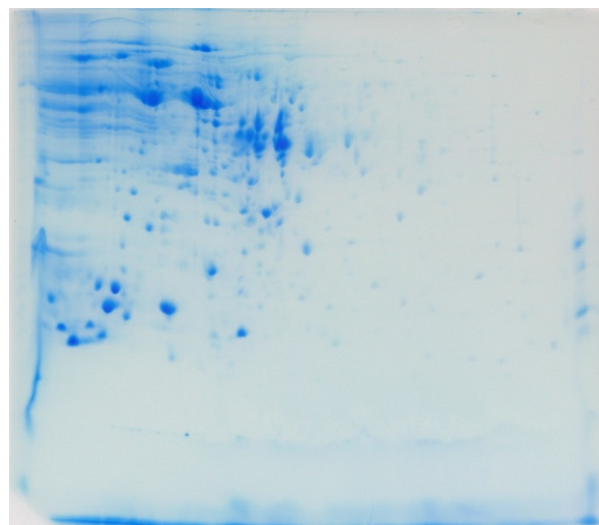


Original images of whole cell protein 2DE gels of *L. monocytogenes* isolate SB92/870 grown in BHI broth at 37°C for 6 h

Appendix IV







Original images of whole cell protein 2DE gels of *L. monocytogenes* isolate V7 grown in BHI broth at 37°C for 6 h

Appendix V

Spots analysis quantitation results for all spots identified by MALDI-TOF MS and Ion trap MS

Spot No.	V7-1	V7-2	V7-3	Average	844-1	844-2	844-3	Average	870-1	870-2	870-3	Average
1				0	11370.2							0
2	15882.9	12819	11376.2	13359.4	14230.8	27115.9	20191.6	23653.75	20772.8	23018.6	23223.5	22338.3
4	9497.5	3737.5	7895.5	8696.5	11653.6	10464.6	10100.4	10739.53	15547.4	14000	9992.6	14773.7
101				0	3170.9							0
102				0	8971.5		7829.9					0
201	2393.8		1974.4	2184.1	1186.1	2072.4		1629.25	2330.9	1778.8	2482.4	2197.367
501				0	1397.8				1484.1			
502	5637		5122.8	5379.9	3721.3	1840.5		2780.9				0
503				0	3432.2							0
504				0	1008.2							0
505				0	1991							0
506	6603.3	9298	9345.1	9321.55	9808.9		5606.6	7707.75	2661.5		8166.2	
507				0	2666.1			0				0
1001	11619		13350.6	12484.8	26745.8	12305.3	11086.9	11696.1	16134.9	16606.6	19414.7	17385.4
1003	20267.9	17682.6	37776.7	18975.25	19402.6	32218	42685.8	37451.9	14536.7	20159.5	25946.4	23052.95
1101	14095.2		8182.7		6116.5	5155.9	3999.4	5090.6	6841	7551.6	7890.5	7427.7
1103	11223.8	17803.1	10785.7	13270.87	13620.4	14674.8	12120	13471.73	17843.9	14745.8	19583.9	17391.2
1104	5502.2		6056.8	5779.5	3522.8				4713.1	3995.7	5069.8	4592.867
1301				0	1079.4					8114.8	768	
1501			11693.7		12069.5	3102.7						0
1502				0	5184.1				2143.1			
1503	3185.9		1695.9		2205.3	1688	5996.5					0
1701	3720.4				7412.5		2197.2			12223.3		
1802				0	6213.7	9808.3	5901.2	6057.45	8781.9		16291.2	
1804	7782.5	22871.9	8373.9	8078.2	11976.7	13421	13373.3		16462.9	7172.2	12331.7	14397.3
2001	6904		10468.7	8686.35	4264.8	5004.9	4527.4	4599.033	3976.8	4464.9	6068.2	4836.633
2101	3325.3	1569.1	3963	3644.15	2743	757.6			3158.9			
2103				0	1704.5					2994.9		
2201	1609.5			0	1669.4	1709.2	6308.6	1689.3	14817.9	1256.2	1772.4	1514.3
2301				0	897.9							0
2401				0	1135.9							0
2402				0	8204.2	5880.7	5478.6	5679.65	8511.8	5718	10705.3	9608.55
2501				0	3192.8							0
2502		37882.1			1905.2							0
2503	32801.9		8545.3		50842.5	26099.1	12206		37951.8	32033	12830.5	34992.4

2504				0	1397.9							0
2505				0	2427.7							0
2601	1996.8				727.1							0
2602	3436.6				2339.2	1864.7		2101.95	2967.1	1213.7	3795.1	3381.1
2701				0	3724.4					1589.5		
2702	12898.9			12898.9	10391.6	3308.8	15046.5	12719.05	14622	7961.3	18805	16713.5
3001				0	1002.5	2079.4				2110.1		
3003		1716	9944.7		2246.8	2398.3		2322.55	13060.9	3316.7	2944.6	3130.65
3004	31131.3	41728.3	17041.4	36429.8	38486	35585.1	53832.4	42634.5	63784.4	44957.5	53936.8	54226.23
3005				0	8228.9	7307.7	14704.2	7768.3	14765.3	12109.1	13062.4	13312.27
3101	4369.8				1008.9							0
3102	2553.4	3112.6	3648.6	3104.867	3067.2	2894.4	1705.1	2980.8	3412.6	2282.1	3325	3006.567
3201				0	2103.2					1965.2	3916.3	
3301				0	2045.5	1650.1	2124.5	1940.033	2593.1	1530.2	3917.8	2680.367
3302				0	2358.3	1772	1333.6			10855.3	622.2	
3303				0	1188.1							0
3401				0	10837.5	3641.1	3583.5	3612.3	8555.4	7188.5	9613.9	8452.6
3402		2731.7			1156							0
3403				0	2306.6		1662.9					0
3404				0	1884.6	1786		1835.3				0
3501		2731.7			1156							0
3601	2348.7				2458.6	2974.2		2716.4	2693.2			
3602		1238.9			584.1							0
3603				0	1552.3							0
3604				0	1596.6				4248		2074.5	
4101				0	2253.2							0
4201				0	3566	3141.6	2305.5	3353.8	2829.2	4488.2		
4202	26468.2		1223.9		5597	6215.2			23146.7			
4203	3518.5	4485.2	2387.2	4001.85	1589.4	1023.6		1306.5				0
4204	6794.6	2852			9591.4	8541.5				11004.6		
4205	6720.5	3674.3			4505.6	1499.7			10881.7	12176.8	9512.8	10857.1
4206				0	1686.2					6914.9		
4301				0	2291.3							0
4302				0	14543.6	15061.2	14204.7	14603.17	15592.6	9269.4	14014.9	14803.75
4401				0	970.1							0
4402		7614.3			2313.3	3210.6	4015.2	3612.9	2543.2		2381.3	
4501				0	5235.9							0
4502	37971.8	41031.4	18216.7	39501.6	10346.2	91362	8136.6					0
4503		96868.1			1027.1							0
4601				0	2007.9	2167.6		2087.75				0
4602			1747.7		423.9							0
4701				0	2652.7	797.5	22005					0

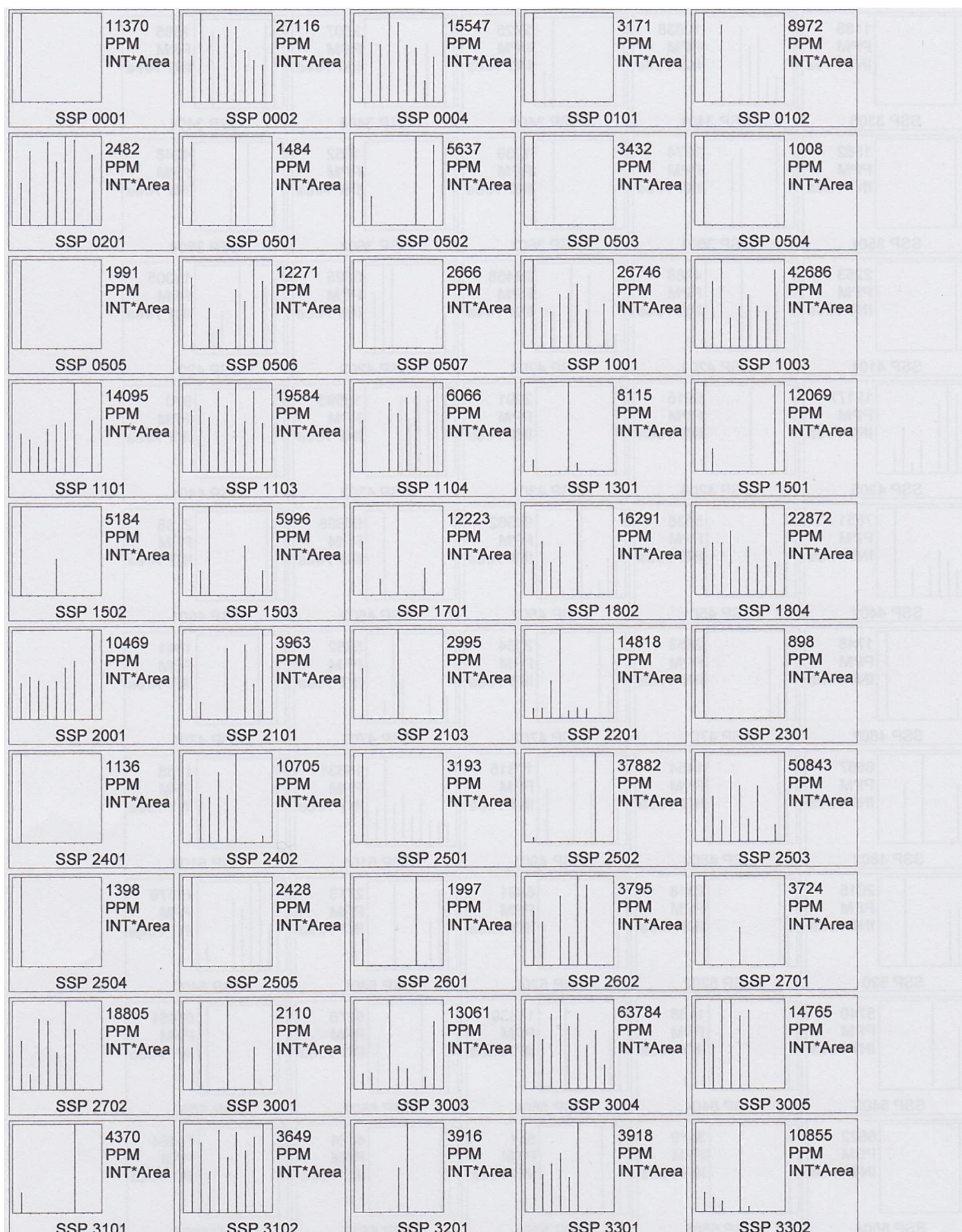
4702				0	1492	646.7	2264.3					0
4703	5461.6	1334.4			1656.2		1643.6				2715.7	
4704				0	1422.4		1940.6					0
4802	4440				4379.3					6686.6		
4803				0	909.4	1453.6						0
4901			11320.6		7505.9				10339.7		17814.7	
5101	8274.5	16831.1	7473.8	7874.15	4556.3	4632.5	4984.3	4724.367	8550.5	5828.7	9777	9163.75
5102				0	1168.5							0
5201	2015.1				1398.8	1258.6						0
5202				0	1387.9	2318.4						0
5203				0	6690.6							0
5401	2712.6				1998.2	2460.4	1474.8					0
5402				0	7845.4	6187.3	12578.9	7016.35	12403.5	9007.8	10373.3	10594.87
5403				0	3982				5739.6			
5404				0	1138.2							0
5501	12353.9				6879.9	9439.4						0
5502			1992.3		2029.3				5274.7			
5503			53358.3		33921.7		9636					0
5504				0	6621.6							0
5505				0	3169.8							0
5506				0	513.3		541.3					0
5507				0	2448.8	4024.4						0
5601				0	10464.4							0
5701		7252			2164.5	1294.6	2785.8	2475.15				0
5702				0	2911.2	2331.4	1941.7	2621.3	2567.9		2369.4	
5703	3115.4				3694.8	2729.8	3331.6	3252.067			4678.7	
5704			5146		1092.6		1485.3		2335.1			
5706				0	886.3	1462.2	934.4	910.35				0
5801				0	2689.6							0
5802				0	3626.7	4842	3582	4016.9	4369	3122.5	5206.2	4232.567
5803				0	4520.1	5340.3	3406.3	4422.233	3774.3	2938.4	5023.2	3911.967
6001	9870.2	10806.1	12677.9	10338.15	8608.2	9921.6	9023.6	9184.467	10646	10006.4	12869.5	11173.97
6002				0	1089.2							0
6101	911.5		5283		1395.8	1097.4	1171.2			7853.5	543.6	
6102	15941.5	17347.2	12005.5	15098.07	10333.9	9071	6928.2	9702.45	7824.3	4723.9	7428.4	7626.35
6103	1036.1	910.4			414.8	2010.4	7977.2			1437.1		
6104				0	5472.8							0
6105	6635.9	535.5	6790.5	6713.2	3484	3801.5	421.8	3642.75				0
6201				0	1183.7							0
6202	1849.5	2815.4			639				7655.3			
6203		1829.5	1719.4	1774.45	6166.6	3795.2	4359.3	4077.25		5100.1		
6204				0	1480.5							0

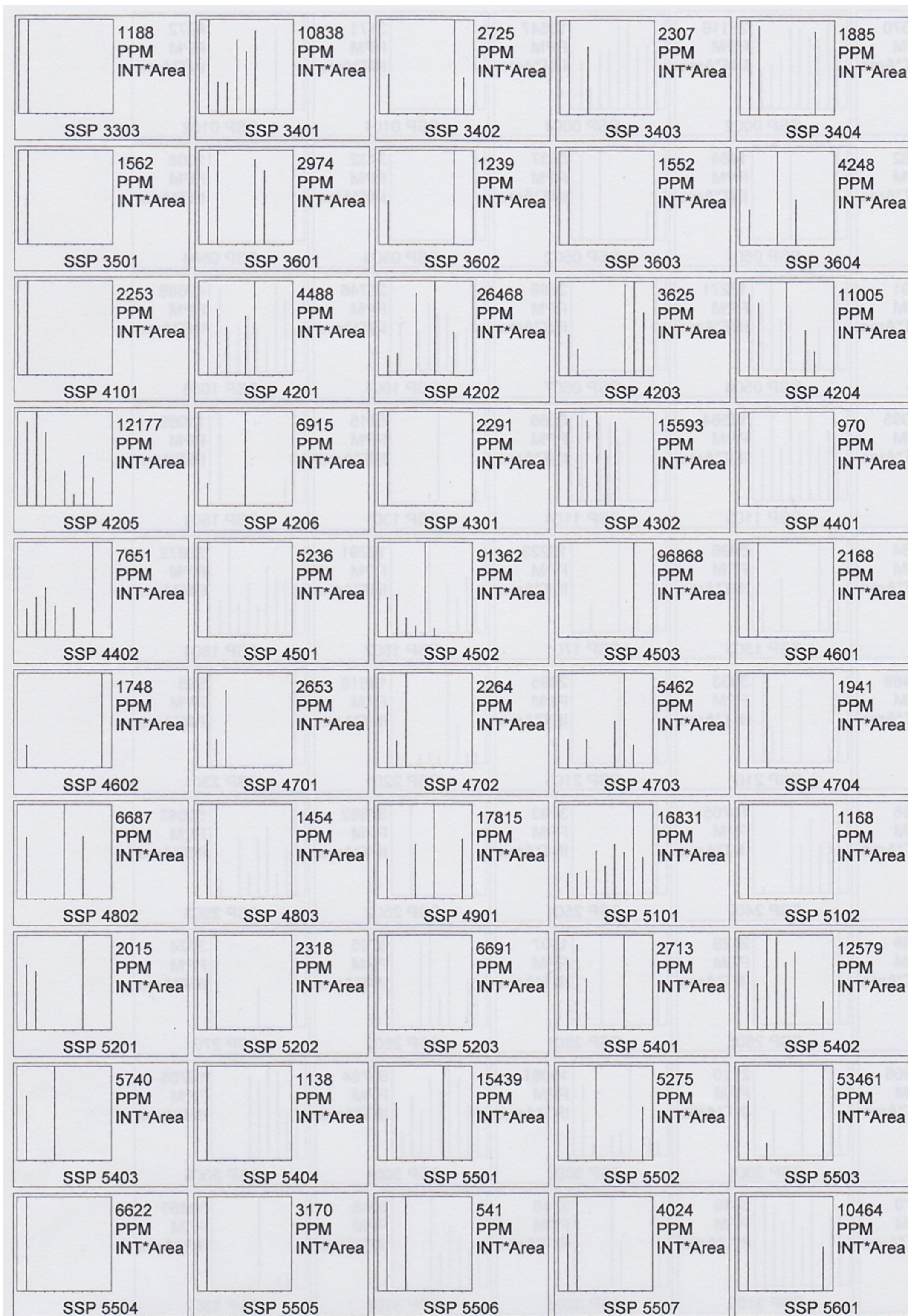
6205	3433.6	4950.1	3111.2	3272.4		7944.8	7444.8	7444.8	5921.6	12200	5421	5671.3
6301	8008.3		4192.7		1364.3							0
6302				0	430.1							0
6303	17572.4	17446.2	14037.5	17509.3	7862.8	14602.3	12647.7	13625	10568.4	11854.1	8793.3	11211.25
6401				0	8326.7	4214.4			13064.4		13936.6	13500.5
6402			17806.4		19969.7	16753.5	20937.6	20453.65	25289.4	7588.6		
6403				0	1940.7							0
6404				0	7072.3	13092.4						0
6502	27441.1	30173	27248.3	27344.7	12203	9907.3	5628.6	9246.3	3331.2	8179	9668.8	8923.9
6601		3705.4	1637		1382.3		1143.8			1964	6614	
6602	6441.1				693.5							0
6603	26845.5	26525.2	25304.2	26224.97	28484.8	22862.4	28910.7	26752.63	9040	19705.4	11253.4	10146.7
6701			23366		3789.7	3962				4212		
7102				0	2418.1	2666	2320.6	2468.233	12646.9	3801.8	8306.1	10476.5
7103	3736.4	9111.7	4376.2	4056.3	3965	6670.8	2945.7	3455.35	12319.8	4472.6	2767	3619.8
7104					4770.4	6180.5	5606	5518.967	1390.4	1631.1	8936.1	1510.75
7105		59.1			5512.1		446.9			477.8	526.7	502.25
7201	4104	4716.9	2059.2	4410.45	2186.2	2504.6		2345.4	1374			0
7202	1830.6		2578.3		865.9		8518.8			9272.9		
7301	35869.5	23184.7	57336.2	29527.1	34032.3	40315	41504	40909.5	54480.9	41559.7	42847.2	42203.45
7401				0	1047.1		7583.5			7877.9		
7402	16640				11026.6						17875	
7403				0	1300.1							0
7503				0	4508.2		1813.8					0
7504	2985.6	3643.8	1756.1	3314.7	3660.4	1332.6	3192.2	3426.3		2729	8804.5	
7601	5045.1				7096.5	2075.7	5477.9				4690.8	
7602		16254.7			2352.4	3046.1	1975.7	2164.05	2404.3			
7701			8077.4		945.4		800.9					0
7702		4021.9			8210.2	7642.1	8474.8	8109.033	3545.8	6926.2		
7703	1535.9				1386.1					1582.9		
8001	830.3		4025.4		253.5	318.1		285.8		1326.5		
8101	5796.9	1388.7	440.4		5229	256.2	6452.6	5840.8		5497.7	423.1	
9001	7515.5		4544.9		11522.2	18935.9	13879.4			21293		
9002				0	7544.8		7918.4	7731.6	2181.6	12418.9		
9003				0	6227.7					8344.8	119.1	
9101	1532		2180.7		1126.3	1697.3	733.2	1411.8		760.2	3466.8	
9102				0	10878.8	10409.9	22074.9	10644.35		25151.9		
9103	2271.4				2566.6	7774	9691.9			8355.5		
9104	4956.2	947.7	7360.8		7557.9	5054.3	2125			8478.5	464.6	
9105	396.3		771.6		5569.2					9093.1	2798.5	
9201	2118.2	1975.6		2046.9	1695.2	1421.2	837.4	1558.2		6572.1		

9202			1090		1202.7	1083.5	1820.7			249.3	5675.2	
9203	2259.1		6827.1	4543.1	1296.2		94	1296.2		7157.4	6736.9	6947.15
9204		4171			551							0
9301				0	6779.2	5391.1			16155.6			
9302	8854.8	6797.4	8989	8921.9	9290.4		20333.5			16945.8	7626	
9303				0	876.5							0
9304				0	2853.6	2151.7	2821.5	2608.933	869.6	2546.8	460.1	
9305				0	1997.5		4708.3					0
9306		1730.8			6989.9	1235.3	1399.2	1317.25		7627.8		
9307				0	3925							0
9308	4705.4	838.2			200	3125.5						0
9401				0	3232.2							0
9402	8512.4				6074							0
9403				0	1547.4	1465.6	2102.6	1506.5		1384.2		
9404	8150.6		2200.1		8924.8	6495.7	7364.5	7595		5227.1		
9405				0	5163.2	3193.4						0
9406	547.3				507.2	4613.2	887.4					0
9501	957.9				500.2	2553.9						0
9601				0	637.8							0
9602				0	991.5							0
9603			1539.7		1991.7							0
9604		4913.6			400.3	442.2		421.25				0
9605				0	2546.6							0
9606				0	2590.4	815.9						0
9701				0	2106.6	362.3						0
9702				0	844							0
9801				0	974.8	965.1		969.95				0
9802				0	1573.8	1406.4	1139.9	1373.367		1056.6	1695.9	1376.25

Appendix VI

Spots identification chats for all identified spots by MALDI-TOF MS and Ion trap MS

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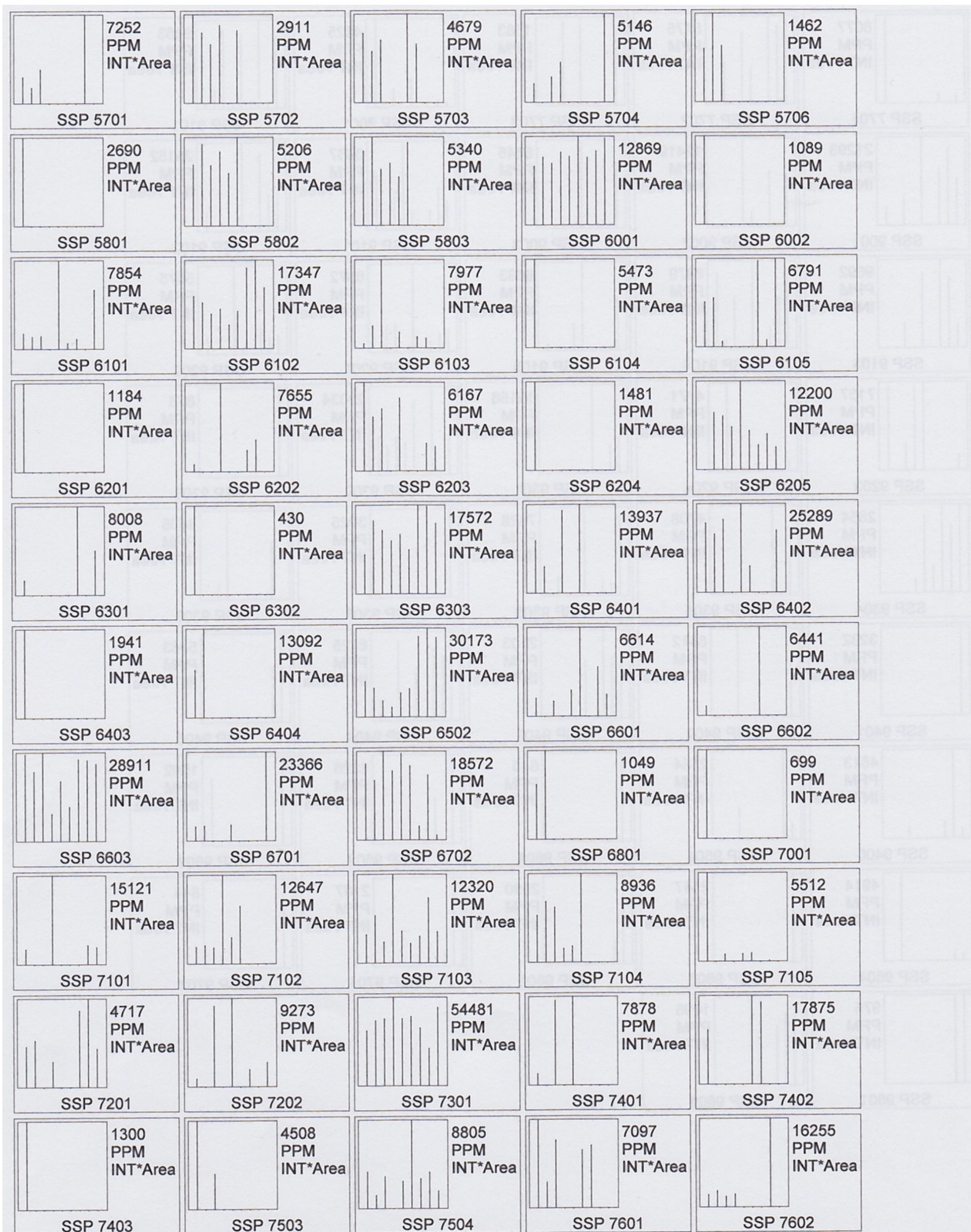
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1300 PPM INT*Area
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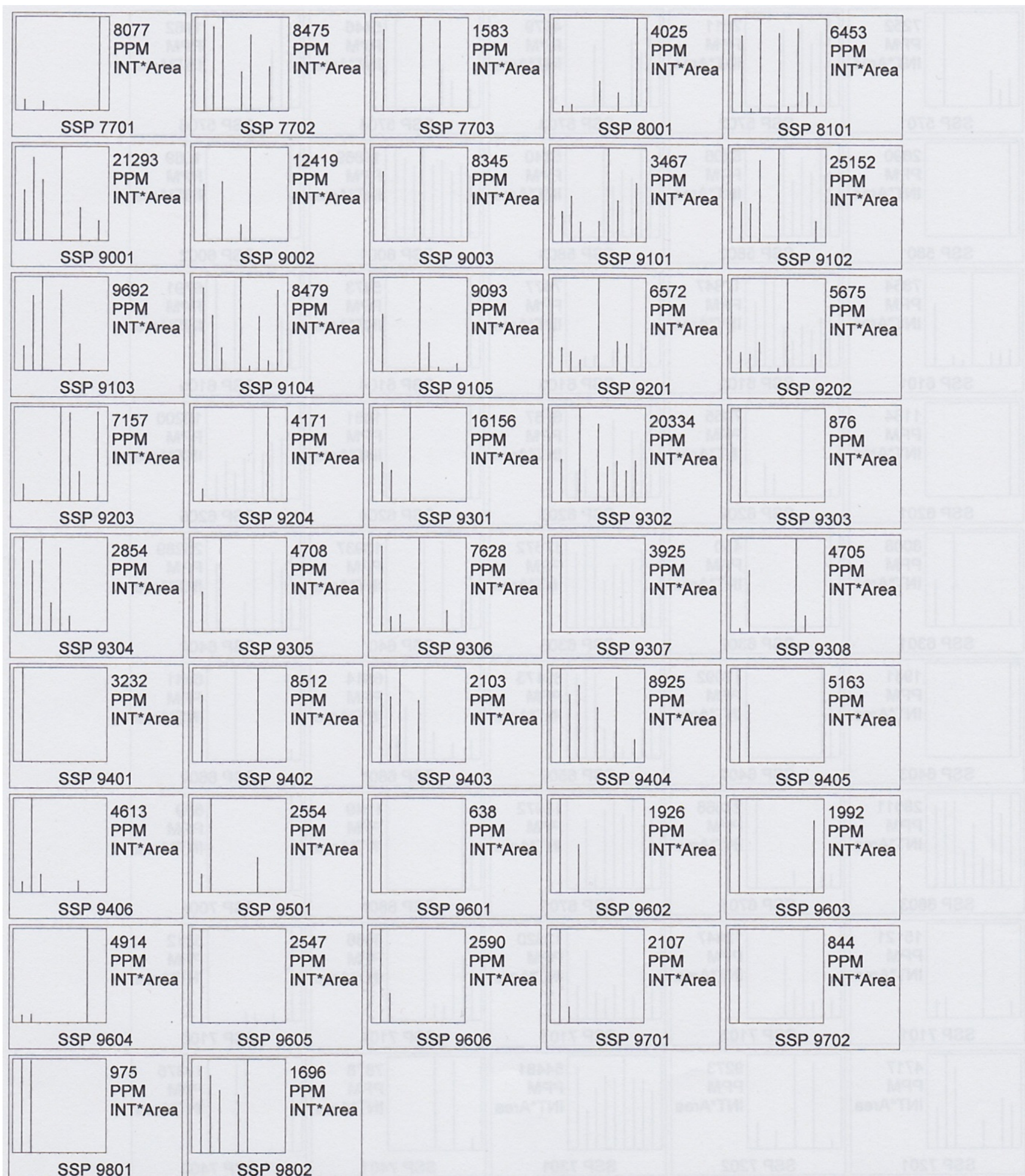

Note:

- SSP- represented by spot number
- The orientation of each for three strains of *L. monocytogenes* were SB92/844 (1), SB92/844 (2), SB92/844 (3), SB92/870 (1), SB92/870 (2), SB92/870 (3), V7(1), V7(2), V7(3).