IDENTIFICATION OF PUTATIVE SURFACE ADHESINS BY COMPARISON OF SURFACE-ADHERENCE VARIANTS OF *LISTERIA MONOCYTOGENES* USING LC-MS/MS AND RT-QPCR

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Listeria monocytogenes is a serious foodborne human pathogen that is known for biofilm formation, but the association of *Listeria* adherence in food environments and the resulting contamination of RTE products is not well characterized. A total of five methods for extraction of surface proteins from a strain of *L. monocytogenes* was evaluated for cytoplasmic protein availabilities in protein extracts using a LC-MS/MS (orbitrap) mass spectrometer. The surface protein profiles of weakly and strongly adherent strains of *L. monocytogenes* from food environments were examined to identify potential surface adhesins. Different subcellular localization tools were utilized for prediction of cytoplasmic protein association with cell envelop. The expression levels of the select genes that possessed higher expression in strongly than weakly adherent *L. monocytogenes* determined in this study were quantitated using real-time RT-PCR (RT-qPCR).

LC-MS/MS analysis of five surface extracts revealed that one of them showed the least cytoplasmic proteins using Tris-buffered urea extraction of hypotonic-stressed cells (UB-Ghost) among five extraction methods. Protein subcellular localization prediction revealed that many of the isolated cytoplasmic proteins may be 'moonlighting' proteins, suggesting that some cytoplasmic proteins may also moolight as surface proteins and adhesins.

Comparative analysis of surface proteins recovered from strains of weakly and strongly-adherent *L. monocytogenes* planktonic and sessile cells using these techniques revealed higher differential protein expression (i.e. >5-fold) in the strongly than in the weakly-adherent strain studied, hence suggesting the presence of other surface proteins acting as adhesins. Relative RT-qPCR analysis of 14 transcripts recovered from *L. monocytogenes* pre-incubated under planktonic or sessile condition at different temperatures revealed higher gene expression primarily for the strongly than the weakly adherent strain of *L. monocytogenes*. The analysis also showed higher gene expression for transcript extracts of both cells pre-incubated under conditions of sessile growth and higher temperatures.

Some surface adhesins in *L. monocytogenes* may be present as cytoplasmic proteins whereby expression is strongly influenced by growth as planktonic or adhered cells. Further studies may identify conditions to better eliminate *L.* monocytogenes from plant facilities where they can remain adhered and possibly contaminate manufactured foods.

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ABBREVIATIONS AND SYMBOLS

1. BRC ..... British Retailer Consortium
2. CDC ..... Centers for Disease Control and Prevention
3. CFU ..... Colony-Forming Unit
4. Cp ..... Crossing Point
5. Ct ..... Cycle Threshold
6. DAMP ..... Damage-Associated Molecular Pattern
7. DNA ..... Deoxyribonuleic Acid
8. FDA ..... Food and Drug Administration
9. FDOSS ..... Foodborne Disease Outbreak Surveillance System
10. FoodNet ..... Foodborne Diseases Active Surveillance Network
11. FSMA ..... Food Safety Modernization Act
12. HACCP ..... Hazard Analysis and Critical Control Points
13. ID ..... Infectious Dose
14. LC-MS/MS ..... Liquid Chromatography Coupled With Tandem Mass Spectrometry
15. MATS ..... Microbial Adherence To Solvent
16. mRNA ..... Messenger Ribonucleic Acid
17. PAMP ..... Pathogen-Associated Molecular Patterns

18. PCR ..... Polymerase Chain Reaction
19. PFGE ..... Pulsed-Field Gel Electrophoresis
20. PulseNet…….Public Health Laboratories For Subtyping Through The National Molecular Subtyping Network
21. RTE ..... Ready-to-Eat
22. RT-qPCR ..... Quantitative Real-time Reverse Transcription PCR
23. STEC ..... Shiga tocin-Producing *E. Coli*
24. SQF ..... Safe Quality Food
25. USDA ..... United States Department of Agriculture

CHAPTER I

INTRODUCTION

Listeria monocytogenes is an internationally well-known human pathogen responsible for a serious foodborne illness (De Noordhout et al., 2014). This Gram-positive, facultative anaerobic, intracellularly replicating pathogenic bacterium is identified as a major cause of foodborne deaths among pathogenic bacteria contaminants in foods in a variety group of human targets, including the elderly, neonates, fetus, HIV patients, and other immuno-deficient patients (Ramaswamy et al., 2007), as a result of the disease, listeriosis. In immune-deficient patients, the infection manifests as either corneal ulcer (Holland 1987), gastroenteritis (Dalton et al., 1997), meningitis (Gray 1966), pneumonia (Whitelock-jones 1989), or septicemia (Gray 1966). Pregnant patients suffering from intrauterine or cervical infection by this pathogen can result in a potential spontaneous abortion or stillbirths (Vázquez-boland et al., 2001a). The newborns surviving feto-maternal listeriosis can result in mental and physical retardation following sepsis and meningitis sufferings (Krigger, 2006).

This pathogen is ubiquitously found in food-associated contact surfaces in processing plants for both ready-to-eat (RTE) and raw products (Farber and Peterkin; 1991, Heymann, 2008). It is such a persistent organism in plants that no one standard sanitation method can completely eliminate it from recurring contamination of food

processing facilities. RTE products contaminated with *Listeria* are considered high risk to human consumption due to the fact that this type of food does not require cooking. As a result, the USDA and FDA have mandated a "zero-tolerance" policy against this food pathogenic bacterium, *L. monocytogenes*, in RTE products (Donnelly, 2001; FDA, 2008).

Listeria species and sub-species have been identified through continuous subtyping efforts performed on *Listeria* DNA using various tools include genotyping, MLST, and serotyping, which currently account for a total of thirteen serotypes of which 1/2a, 1/2b, and 4b are frequent vehicles of human listeriosis (FDA, 2012). Among them, serotype 4b is the most frequent isolate of *L. monocytogenes* from worldwide foodborne outbreaks, resulting in higher tolls of sickness and death (FDA, 2012). This trend has been tracked by Centers for Disease Control and Prevention (CDC) and research scholars using a variety of genotyping tools, including ribotyping (RT), multilocus sequence typing (MLST), and pulsed-field gel electrophoresis (PFGE), to determine the mechanisms of the distribution, transmission, and occurrence for implication in disease control and prevention (Giovannacci et al., 1999; Graves and Swaminathan, 2001; Salcedo et al, 2003; Swaminathan et al., 2001; Wang et al., 2012; Wiedmann et al., 1997).

This pathogenic bacterium has moderate-to-high resistance to processing stresses, including extreme osmolarity, salinity, pH, and temperature, that have been linked to known virulence genes identified through genetic in-silico analysis or experimental verification (Farber and Peterkin, 1991).

Several forms of adherent phenotype of *L. monocytogenes* strains are consistently found in food-associated environments, including utensils, floors, walls, and vehicles (Autio et al., 1999; Miettinen et al., 1999a,b, Tompkin et al., 1999; Hood and Zottola, 1995; Norton

et al., 2001a; Thimothe et al., 2004), causing cross-contamination to various food types of raw and RTE products at every food processing step (Giovannacci et al., 1999; Norton et al., 2001b). This can be due to less effective sanitation applied to processing areas and utensils or strains of strongly-adherent *L. monocytogenes* that are much more resistant to removal forces such as high water pressure rinses (Gravani, 1999). A few adhesion-associated surface proteins such as internalin A (InlA; lmo0433) (Chen et al., 2008), internalin B (InlB; lmo0434) (Chen et al., 2008), and biofilm-associated like protein (BapL; lmo0435) (Jordan et al., 2008) have been identified to date. These two internalin proteins are initially classified as members of *Listeria* virulence factors, which account for the biotic attachment of *L. monocytogenes* to host gastrointestinal epithelial cells and invasion or internalization (Jonquières et al., 1998; 2001; Pizarro-Cerdá et al., 2012). They are not found in other species of *Listeria,* as demonstrated in an in-silico-based investigation (NCBI) of genomic databases for *Listeria* spp. In the case of the gene *bap*L, it is also not present in all *L. monocytogenes* strains. In addition, both *bap*L and internalins are not the only factors possessed by the strong adherent phenotype of this pathogen, as demonstrated by mutagenesis studies (Chen et al., 2009; Jordan et al., 2008).

Following the elucidation of surface proteins involved in *Listeria* pathogenesis, many research efforts have been directed towards their surface identities. The emergence of high-performance tools in total protein identification have allowed such work to be carried out with less cost, labor, and time. This include the use of liquid chromatography coupled with tandem mass spectrometry (orbitrap) as a mass analyzer and the readily available mass identifier, the mascot protein database for identification (Kushnir et al., 2011). This technology is a routine application in molecular-based clinical testing labs.

CHAPTER II

REVIEW OF THE LITERATURE

Listeria monocytogenes - a foodborne pathogen of serious human health concern

Listeria monocytogenes is a worldwide historical pathogen of public concern (health and economy issues) causing loss of life in both humans and animals. This Gram-positive bacillus is an opportunistic foodborne pathogen that can cause the illness, listeriosis, in humans of all age groups with serious symptoms revealed in immuno-deficient patients such as neonates, HIV patients, and the elderly (Camejo et al., 2011; Jurado et al., 1993). Pregnant women are more susceptible to *Listeria* infection than other population groups (Southwick and Purich, 1996), leading to *Listeria* related abortion or stillbirth when care practices fail to eliminate this pathogen (Mateus et al., 2013). The manifestations of disease include encephalitis, septicemia, meningitis, pneumonia, and corneal ulcer in the general population. In surviving babies to fetomaternal listeriosis, they can experience listeriosis-related complications resulting in permanent retardation of mental and physical conditions in serious cases (Schlech, 2000). In the United States, listeriosis has been identified as the third leading cause of death and neonate meningitis among bacterial pathogens of foodborne concern since the 1950s when it was internationally recognized as a death-causing agent as a result of neonate sepsis and meningitis (Hof, 2003; Reiss et al., 1951). According to the CDC annual surveillance report for *L. monocytogenes*, the number of listeriosis cases increased in 2014 as opposed

to previous years, whereas 90% of the affected cases were primarily non-pregnancy-associated patients while the remaining 10% were pregnancy-associated patients (10%) (CDC, 2014a). According to the Foodborne Diseases Active Surveillance Network (FoodNet) report of "Incidence and Trends of Infection with Pathogen Transmitted commonly Through Food", listeriosis is consistently reported with higher fatality rates (10-30%) than *Salmonella*, *Campylobacter*, and six other pathogenic genera (bacteria and parasite) of national foodborne concern, which includes *Shigella*, Shiga toxin-producing *Escherichia coli* (STEC) O157, STEC non-O157, *vibrio*, *Yersinia*, *Cryptosporidium* (parasite), and *Cyclospora* (parasite) between years 2012 and 2013 (CDC, 2013a, 2014b). Scharff's 2010 model for the U.S. annual economic burdens (include medical, productivity cost, and others) caused by pathogen-related foodborne illness, estimates a total cost between $51 and $78 billion, with listeriosis contributed 2% and 4% of the total (Scharff, 2012). On the other hand, Scallan et al. (2011a, 2011b) estimates 48 million cases of foodborne illnesses are associated with pathogens with 128,000 hospitalizations and 3,000 result in death (Scharff, 2012). When compared to the numbers reported in 1999 (illness: 76 million, hospitalization: 325000, deaths: 5000), these numbers reveal decreased cases of pathogen foodborne illness and listeriosis as well (Mead et al., 1999). Nevertheless, this pathogen still remains one of the most fatal agents among worldwide bacterially-infected patients relative to other foodborne-pathogen related hospitalizations (De Noordhout et al., 2014). As a result of the seriousness of *L. mococytogenes* in foodborne illness, food safety regulations require a 'zero-tolerance' for this pathogen in RTE foods that still remains in effect today (Donnelly, 2001; FDA, 2008).

L. monocytogenes as an emerging foodborne pathogen

The first description of the bacterium *L. monocytogenes* was in diseased young rabbits by Murray et al. (1926; Gray and Killinger, 1966; Hof, 2003) takes place about three centuries after the start of the era of microorganisms (Hof, 2003; Murray et al., 1926; Pirie, 1940). This marks the first documented identification of the bacterium *L. monocytogenes* and its association with animal disease. Accumulatively, there are now 15 recognized species of *Listeria*, of which *L. monocytogenes* was the first identified *Listeria* species (Pirie, 1940), followed by *L. grayi* (Errebo Larsen and Seeliger, 1966), *L. innocua* (Seeliger, 1981), *L. welshimeri* (Rocourt and Grimont, 1983), *L. seeligeri* (Rocourt and Grimont, 1983), *L. ivanovii* (seeliger et al., 1984), *L. marthii* (Graves et al., 2010), *L. rocourtiae* (Leclrcq et al., 2010), *L. fleischmannii* (Bertsch et al., 2013), *L. weihenstephanensis* (Lang Halter et al., 2013) and, most recently, *L. floridensis* sp. nov., *L. aquatica* sp. nov., *L. cornellensis* sp. nov., *L. riparia* sp. nov. and *L. grandensis* sp. nov. (den Bakker et al., 2014). Besides the *Listeria* species *ivanovii* and *monocytogenes*, which are well-known for their major associations with animal and human listeriosis, respectively, the rest of the species have no documented associations with human and animal illness (Guillet, 2010; den Bakker et al., 2014). The bacterium species *mococytogenes* is readily found in natural environments such as soil, water, and on plants, primarily because of their distribution via animal feces, as well as in food and dairy production facilities and human cases of illness (Chambel et al., 2007; Farber and Peterkin, 1991; Fleming et al., 1985). The bacterium is a Gram-positive bacillus and other characteristics include facultative anaerobic growth, motile, 4-6 peritrichous flagella and non-spore forming bacterium (Lemon et al., 2007). To date, there are 13

serotypes of *L. monocytogenes* with 1/2a, 1/2b, and 4b being the most prevalently isolated strains in patients associated with *Listeria* infection, namely listeriolysis (FDA, 2012).

CDC's systematic documentation of *L. monocytogenes* as a pathogenic foodborne infectious agent: FoodNet, PulseNet.

L. monocytogenes is one of the internationally historical foodborne pathogens that remains under stringent surveillance by federal food safety and regulatory agents worldwide owing to the facts of high incidence, prevalence, and transmission capabilities (CDC; CDC, 2014a,b; Cartwright et al., 2013). Its earlier documented incidence in diseased rabbits takes place in 1926 in the U.K., reported by Murray et al. (1926; Hof, 2003). The incidence continues in Germany, where a connection between this bacterium and neonatal meningitis was documented, and in Canada, where human listeriosis outbreak due to consumption of contaminated coleslaw was reported (Schlech et al., 1983). Subsequently, the first large outbreak reported in the US that was attributed to foodborne vehicle as a source of listeriosis was the California Jalisco cheese outbreak (CDC, 1985). Since then, *L. monocytogenes* has been identified as a foodborne pathogen in immuno-deficient hosts, pregnant women, the elderly, and fetuses (CDC, 2014a; Silk et al., 2012). Following that, the U.S. CDC has started the programs Foodborne Disease Active Surveillance Network (FoodNet), to tract pathogen foodborne illnesses, as well as Public Health Laboratories for Subtyping Through the National Molecular Subtyping Network (PulseNet) to detect and define outbreaks using pulsed field gel electrophoresis (PFGE) since 1996 to systematically document DNA fingerprints of infectious pathogens involved in foodborne illness.

Incidence of listeriosis

A few years after the implementation of food safety HACCP programs (1998-2000), a decrease in listeriosis incidence was noted in 2002, as compared with the incidence documented between 1996 and 1998 (CDC, 2013a). The incidence, however, increased and fluctuated at lower levels in the following years (2000 – 2014) (CDC, 2013a, 2015). On the other hand, pathogens such as *Salmonella* and *Vibrio* still remained involved at high levels in foodborne illnesses than other foodborne genera; including *Campylobacter*, STEC *E. coli* O157:H7, and *Yersinia*, which are included among the "list of notifiable diseases" of FoodNet. An overall listeriosis incidence recorded between 1998 and 2008 revealed that listeriosis is becoming more prevalent in the younger group age between 1 and 49 despite previous reports of high incidence in elders beyond 50 years old (Cartwright et al., 2013; CDC, 2014a). This trend reverts back to the normal age group of listeriosis high risk, namely the elder population (≥65 years old), as documented in the (2009, 2011) annual summary report (CDC, 2014a). In 2014, the same source reveals that an estimated number of 1600 cases of listeriosis involving 250 deaths and more than 1400 hospitalized patients was expected each year in the U.S. (CDC, 2014c; Scallan et al., 2011b). According to the CDC estimation of total foodborne death in the U.S., done by Scallan in 2011, this death number represents an 8.3% of the estimated total death toll (3000 cases) caused by food-related illnesses (pathogen, non-pathogen, or unknown agent) (Scharff, 2012). Though these numbers decreased slightly compare to the previous years' CDC's "mobidity and mortality weekly report" summary 2009-2011 (CDC, 2013a), the estimated numbers were 1651 cases with 347 deaths, demonstrating that *Listeria* remained a life-threatening agent to high-risk groups with the

leading number of deaths from listeriosis patients compared to the other pathogens (*Salmonella* and *Campylobacter*) with higher incidences (CDC, 2014b). These estimated numbers are higher than the actual numbers of case reports received each year, because estimates are based on models that take into account cases that may go unreported for various reasons.

In 2013, the CDC estimated listeriosis incidence for hospitalization and death revealed a relatively low incidence compared with the other pathogens of FoodNet surveillance: *Campylobacter* (6621), *Salmonella* (7277), *Shigella* (2309), STEC E. coli O157 (552), STEC E. coli non-O157 (561), *Vibrio* (242), *Yersinia* (171), *Cryptosporidium* (1186), except for *Cyclospora* (14). However, listeriosis remains the leading cause of hospitalization (91%) and fatality (19.5%) among foodborne illnesses associated with bacterial pathogens, as compared with the other hospitalization (14%-38%) and death rates (0%-2.3%) (CDC, 2014b).

Prevalence of listeriosis *L. monocytogenes*

Although the presence of *Listeria* is ubiquitous in the environment (Sauders et al., 2012), the species *L. monocytoegenes* may be less well-represented in a given environment such as urban soil than *L. ivanovii* or *L. seeligeri* species (Bernagozzi et al., 1994; Beumer et al., 1996; Frances et al., 1991; MacGowan et al., 1994; Weis and Seeliger, 1975). In contrast, the pathogenic *L. monocytogens* is exceptionally common in food-associated environments (i.e. water, food contact surfaces, drains, air, personal protective equipments (PPE), vehicles, and utensils) (Ferreira et al., 2014; Gahan and Collins, 1991; MacGowan et al., 1994; Wendtland, 1994) and hence causes concern for contamination of high risk products (i.e. RTE foods) and listeriosis outbreaks after consumption of

contaminated food. *Listeria* detection rates of 1-5% (Pinner et al., 1992; Soriano et al., 2001; Wilson, 1995) and greater than 50% were reported in RTE and raw meats (Ryser et al., 1996), respectively. Ferreira and others reported that *Listeria* contamination of RTE foods even after harsh preparation conditions is mainly due to post-processing cross-contamination of the RTE food by *Listeria*-contaminated food contact surfaces as a result of their resistance against sanitizers and physical removal (Ferreira et al., 2014). In the US, much of the *Listeria*-contaminated RTE foods occurs at retail, unless prepackaged during manufacture (Pradhan et al., 2010; Endrikat et al., 2010). As a result, the national food safety regulators and food producers have jointly established food safety programs such as HACCP, BRC, FSIS, SQF, FoodNet, PulseNet, and FDOSS. In addition to that, the public is informed about food safety in weekly or monthly basis by the national food safety authorities (i.e. CDC, FDA).

Transmission of listeriosis

Listeriosis is a common disease in ruminant animals, contracted by eating animal feeds contaminated with the pathogenic *Listeria* species *ivanovii* or *monocytogenes* (more common), causing complications include encephalitis, late abortion, septicemia, and neurologic paralysis (Cordy and osebold, 1959; Hird, 1987; Hird and Genigeorgis, 1990; Low and Donachie, 1997; Schlech et al., 1983; Wang et al., 2012). The first case of animal listeriosis is reported by Murray et al. (1926) in diseased rabbits. On the other hand, the first documented human listeriosis transmitted by *L. monocytogenes* takes place in the year 1926 in Denmark (Nyfelt, 1929; Vázquez-boland et al., 2001a). Following that, a large foodborne listeriosis outbreak related to human is reported in the year 1981 in Canada (Evans et al., 1985; Schlech et al., 1983). The following years become the era

of worldwide human listeriosis, including the U.S. (Fleming et al., 1985), Switzerland (Büla et al., 1995), Sweden (Ericsson et al., 1997), France (Goulet et al., 1998), Italy (Aureli et al., 2000), Finland (Lyytikainen et al., 2000), Japan (Okutani et al., 2004), Canada (Gilmour et al., 2010), Malaysia (Adzitey et al., 2013), China (Feng et al., 2013), and others (De Noordhout et al., 2014). CDC and others reported that Listeriosis is the third leading cause of foodborne-mediated death in the US caused by pathogens (CDC, 2013b; Scallan et al., 2011b).

Since the 1980s, *L. monocytogenes* is considered a human foodborne pathogen due to the fact that it is acquired through ingestion of food contaminated with the pathogenic agent (Lorber 2000; Pinner et al., 1992; Schlech et al., 1983; Schuchat et al., 1992)., a similar transmission mechanism in animal scenario. In high risk groups (pregnant, elderly, HIV), infection by *Listeria* causes listeriosis manifestations include sepsis (Camejo et al., 2011), meningitis (Lecuit, 2005), pneumonia (García-Montero et al., 1995), gastroenteritis (Lecuit, 2005), corneal ulcer (Holland et al., 1987). Highly vurearable group is implicated in the HIV patients or patients with underlying immuno-suppressant therapy (Jurado et al., 1993; Lorber, 2000). In fetuses, the infections transit from their infected pregnant mother through blood streams or colonized genital tract (Bortolussi, 2008; Silver, 1998) and result in fetomaternal/perinatal listeriosis manifested by sepsis, meningoencephilitis, physical/mental impairment, premature delivery, miscarriage, and stillbirth (Lecuit, 2005; Lorber, 2000; Silver, 1998).

The minimal infectious dose for *L. monocytogenes* is unclear (Bortolussi, 2008; Farber et al.,1996; Lorber, 2000; Tauxe, 2001; FDA, 2012). However, a fairly high dose (in CFU), between 10^7 and 10^9, of viable cells is essential to cause illness in 10% of

healthy human hosts (infectious dose, ID_{10}), whereas immunosuppressed hosts may acquire disease (ID_{90}) at lower doses (i.e. 10^5-10^7) of cells (Farber et al., 1996). Nevertheless, the FDA reported a much lower infectious dose of cells, namely 1000 cells, to cause disease in susceptible individuals (FDA, 2012). Additionally, there is no experimental information about the onset period of listeriosis.

Virulence factors of *L. monocytogenes* pathogenesis

L. monocytogenes has become known as an infectious agent to mammalian species since the first deadly documented case in rabbits in 1926 from an unknown cause of death. After 25 years from the first documented incidence, the cause of death by listeriosis' meningitis is revealed (Hof, 2003; Murray et al., 1926). In later years, increased numbers of research-based pathogenesis studies have revealed more symptoms manifested in listeriosis such as sepsis, corneal ulcer, encephalitis, gastroenteritis, and pneumonia in immuno-compromised patients, while the pregnant women may experience miscarriage due to intrauterine or cervical infection by the pathogen (Vázquez-boland et al., 2001a; Mateus et al., 2013). Along with increased numbers of listeriosis cases, information on factors contributing to virulence started accumulating and the pathogenesis of listeriosis has become well established in a model of infection (Camejo et al., 2011; Lecuit, 2007; Tilney and Portnoy 1989).

Molecular mechanisms for *L. monocytogenes* survival in human gastrointestinal system

The host gastrointestinal system is made up of organic compounds including acids and bile salts, which together contribute to the first defense mechanism against pathogen acquired orally (Walter, 2008). *Listeria* infection starts with successful interaction

between this pathogen and the gastrointestinal environment after ingestion of viable cells surviving the first stress challenges in food processing sanitation settings before coming to colonization of the epithelial layer and subsequent invasive process initiated in the host.

In the first step of pathogenesis, the pathogen experiences extreme low pH stress in the stomach. Successful survival in the stomach environment has been connected with their genetic properties that establish its pH tolerance system namely glutamate decarboxylase system (GAD). This is carried out by the protein glutamate decarboxylase, composed of multiple subunits include two transporters and three GAD enzymes to uptake glutamate and perform glutamate decarboxylation, respectively (Glaser et al., 2001). Cytoplasmic protons accumulated from the acidic environment can be removed during glutamate decarboxylation and result in normal pH maintained in the *Listeria* cytoplasm suitable for cell viability and virulence (Cotter and Hill, 2003). This survival mechanism against low pH is common to enteric bacteria (Gorden and Small, 1993).

In the second stage of pathogenesis, the surviving bacteria cells encounter a high osmolarity condition in the small intestine. The ability of this pathogen to survive the high osmolarity condition is due to the presence of an osmolarity tolerance system or osmolyte tolerance system (Sleator et al., 2001). In this system, three proteins, OpuC, BetL and Gbu have been shown to be involved (Sleator et al., 1999; Ko and Smith, 1999; Fraser et al., 2000; Sleator et al., 2001). As in other enteric bacteria where this system is involved, it employs osmolytes of amino acid derivatives including carnithine and glycine betaine in the environment to maintain cellular osmotic pressure against hypertonic stress (Sleator et al., 2001).

In the next step, in the proximal small intestine, there are high levels of bile acids/salts at a level capable of inducing cytotoxic effects to some human Gram-positive pathogens, such as *S. pneumonia* (Atkin, 1926; Tuomanen, 2006). The bactericidal effect of bile on living cells is well documented. They include denaturation of cell membranes, DNA, RNA, and protein and can be attributed to pH, oxidative stress, and osmotic effects induced in that bile-rich environment (Begley et al., 2004; Bernstein et al., 1999a, b; Kandell and Bernstein, 1991; Lechner et al., 2002; Powell et al., 2001; Sokol et al., 1995). This pathogen is able to overcome this stressful environment by a variety of virulence determinants. Among 12 identified genes which are functionally involved in bile salt tolerance (Gahan and Hill, 2005), bile salt hydrolase, *bsh*, is implicated as a required factor for *Listeria* full virulence, verified in orally infected guinea pigs and mice (Dussurget et al., 2002). Another survival determinant in challenges with bile salts is a gene product encoded by bile exclusion locus (bilE). The protein promotes efflux of bile salts from the cell cytoplasm (Glaser et al., 2001; Sleator et al., 2005).

Molecular mechanisms for survival of *L. monocytogenes* in the lymphatic system

Following the colonization of *Listeria* cells at the host intestinal epithelium directed by InlA and InlB *Listeria* surface proteins, the cells become internalized into host phagosomes inside macrophages of the lymphatic system, as demonstrated in Figure 1 (Bierne et al., 2007; Bierne and Cossart, 2007; Gaillard et al., 1991; Jonquières et al., 1998; Lecuit, 2007; Mateus et al., 2013; Tilney and Portnoy, 1989). Like the hemolysin, listeriolysin O (Hly, LLO), the *Listeria* phospholipases C (PlcA and PlcB) are utilized by the pathogen to lyse the host phagosome, releasing the cells to escape host phago-lysosomal destruction (Alberti-segui et al., 2007). While the bacterium is inside the

macrophage cytoplasm, it can undergo rapid multiplication mediated by the bacterium hexose phosphate scavenger, encoded by hexose phosphate translocase (hpt), to uptake carbon in host cells (Chico-Calero et al., 2002). In addition to that, the cells residing in the cytoplasm are readily translocated within infected cells or to neighboring cells by actin-mediated motility, governed by ActA *Listeria* surface protein (Smith et al., 1996; Tilney and Portnoy, 1989). During transition into neighboring cells (secondary invasion), the *Listeria* are enveloped into two membranous layers of vacuole inside host cells. The same mechanism is utilized by this pathogen to evade from the vacuole, as was used to escape the phagosome, and readily promote ability of *Listeria* to invade deeper into host systems (Bierne et al., 2002). A greater extent of infection may result in infection of blood, cornea, central nervous system, spleen, brain, intrauterine and planceta in pregnant group, which can result in sepsis (Camejo et al., 2011), corneal ulcer (Holland et al., 1987), meningistis (Lecuit, 2005), encephalitis (Lecuit, 2005), miscarriage (Vázquez-Boland et al., 2001a), mental and physical impairment in surviving fetomaternal infants (Krigger, 2006).

Regulation of molecular mechanisms involved in virulence and pathogenesis

L. monocytogenes normal virulent abilities are controlled directly or indirectly by the key positive regulator factor (PrfA) protein. This protein is encoded by a monocistronic operon regulated by three promoters, namely *prfAp*1, *prfAp*2, and *prfAp*3, initiating transcription of its regulon by binding at the *prfA* box, a promoter region containing the highly conserved palindromic sequence tTAACanntGTtAa. Its overexpression is detected during growth in the host cytoplasm compared to growth in the extracellular environment. This is supported by the fact that many virulence factors

controlling viability and invasivibilty are part of the PrfA regulon. PrfA is capable of identifying a variety of effectors as signaling indications for a functional PrfA production. A number of four factors identified are phosphorylated glucose (Marr et al., 2006; Ripio et al., 1997; Chico-Calero et al., 2002), temperature ≥30°C (Johansson et al., 2002; Newman and Weiner, 2002), trans-acting riboswitch (Loh et al., 2009), and the alternative sigma factor, σ^B (Ollinger et al., 2009; Schwab et al., 2005). The uptake of the carbohydrate is mediated by the bacterial hexose-phophate translocase and is implicated in the rapid intracellular proliferation of *Listeria*. In the temperature-dependent regulation of *prfA* transcription, the mRNA thermosensor in the 5' untranslated region forms a stem-loop structure at temperatures below 37° C, causing the prevention of mRNA transcription. The transcriptional activity is reversed above 37°C, allowing the production of functional PrfA. The other form of mRNA-based regulation of PrfA expression is by a trans-acting riboswitch, which involves small-molecule metabolite binding of the mRNA. In addition, the transcriptional regulation of *prfA* by σ^B is implicated in the stress experience of *Listeria* during host infection, including stresses encountered in host's gastrointestine, lymphatic system, and blood. *Listeria* virulence genes are efficiently regulated by PrfA based on four currently known mechanistic factors, namely catabolite, temperature, small-molecule metabolite, and host biochemical stresses. These contribute implications to listeriosis control, prevention, and more importantly they enhance our knowledge of *Listeria* pathogenesis.

Model of pathogenesis for intracellular infection of *L. monocytogenes*

The first schematic model (Fig. 1) of pathogenesis for intracellular *Listeria* infection of the host (cell infection cycle), which describes the interaction between the

host internal cell environment and pathogenic *Listeria* for subsequent invasion and disease development was constructed by Tilney and Portnoy (1989).

In general, successful pathogenesis requires that *Listeria* survives against stresses in gastrointestinal environment when taken in orally, and colonizes the epithelial cells of distal ileum and proximal colon. This is the first step in listeriosis pathogenesis. The second step is evasion from phagolysosomal destruction upon engulfment into a phagosome, utilizing LLO and two forms of Plc. The following step is utilizing the actin nucleator of a *Listeria* surface protein ActA for movement promotion within infected cells or to neighboring cells. Lastly, the same proteins LLO and Plc promote continuous break-down of double membranes vacuoule when this pathogen performs a deeper invasion of the host cells. As a consequence, a disease readily develops in the immunocompromised patients.

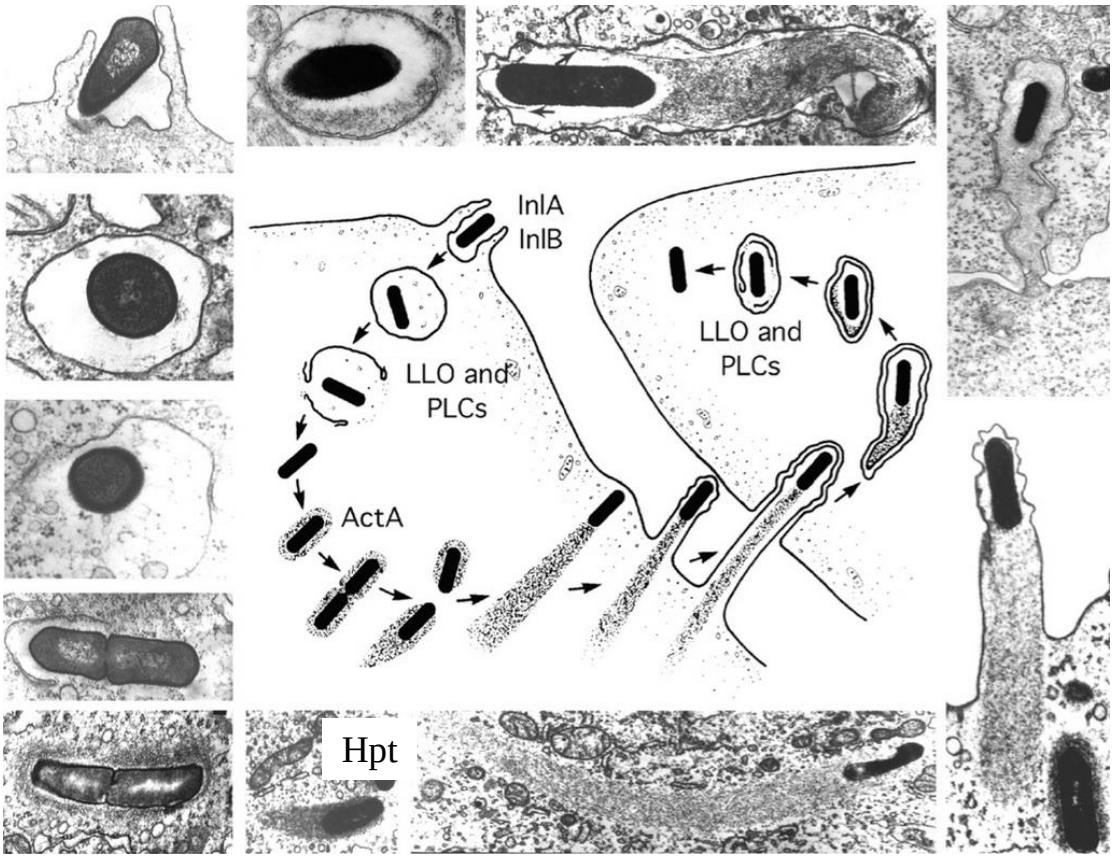

Figure 1. Intracellular infectious life-cycle of listeriosis. Stages of infection include entry, escape, actin nucleation, actin-based motility within infected cell, actin-based motility to neighboring cell. Scheme adapted from Chico-calero et al. (Jan, 2002), based on an original drawing produced by Portnoy et al. (2002).

Immune response against *L. monocytogenes*

In general, mammalian host defense system is naturally composed of two major immunity responses, namely innate and adaptive immunities, to contain and destroy the incoming foreign particles including foodborne bacteria (O'Neill, 2004). The former immunity is associated with the white-blood cells neutrophils and macrophages as the major phagocytes to kill pathogens (O'Neill, 2004). Adaptive immunity involves humoral and cellular immunity of B-cells and T-cells, respectively. Compromising infectious disease-causing agents during this process results in a number of disease manifestations including pneumonia (Pericone et al. 2000), meningitis (Pericone et al., 2000), corneal ulcer (Parmar et al., 2003), sepsis (Pericone et al., 2000), diarrhea (Zhang et al., 2003), ear infections (otitis media) (Alonso et al., 2013), and strep throat (Cunningham, 2000), which consequently cause kidney failure (Gadea, et al., 2012), death (Janakiraman, 2008; Mylonakis et al., 2002), and complications in mental and physical conditions if no proper medical care executed (Krigger, 2006). These symptoms are common in most bacterial-acquired infections except that *L. monocytogenes* is consistently leading in pathogen-acquired fatalities over other bacterial foodborne pathogens (CDC, 2014b; Farber and Peterkin, 1991; Gellin et al., 1991).

L. monocytogenes can elicit immunity responses in mammalian hosts after entering the lymphatic system through Peyer's Patch-dependent or Peyer's Patch-independent pathways (Kolb-Maurer et al., 2000; Pron et al., 2001). In the former, *Listeria* cells colonized to the intestinal epithelial layer are engulfed into the lymphatic system in phagosomes of macrophages, which then fuse with lysosome to form phagolysosomes, a site where the host attempts to eradicate bacteria. Unsuccessful

eradication of pathogenic *Listeria* is fairly common in this stage of immunity response due to the natural abilities of *Listeria* to evade host containment using the bacterium hemolysin (LLO), phospholipase C (PlcA/B) and actin-based motility (ActA). As a result, the bacterial cells are readily residing in the cytoplasm of host cells.

Innate immunity response

When the first defense line fails to eradicate *Listeria*, the innate immunity response along with other cytokine-independent killing pathways, namely ubiquitination and autophage, join in to improve elimination efficacy. A group of signals, including release of TNFα, IL-12, IFNγ, and type-1 interleukin are used during innate response to *Listeria* invasion in the cytoplasm in macrophage to elicit immune cells for eradication of the pathogen (Tripp et al., 1993; Hsieh et al., 1993). Specifically, the cytokines TNFα and IL-12 are secreted by macrophage following detection of *Listeria* cells by cytoplasm sensors, and account for induction of natural killer (NK) cells to produce IFNγ, a macrophage activating cytokine molecule. Activated macrophages in turn lead to bactericidal activity and *Listeria* clearance as well as formation of a group of pattern recognition receptors, for example a number of toll-like receptors or other pattern recognition receptors, on sentinel cell membranes, including macrophages and dendritic cells. This group of receptors is responsible for "pathogen-associated molecular patterns" (PAMPs) or "damage-associated molecular pattern" (DAMP) recognition (Kambayashi and Laufer, 2014), a conserved protein motif shared in a wide group of protinaceous foreign particles, and subsequent induction of specific and more efficient immunity responses. Often, the innate immunity response fails to contain *Listeria* and thus the final defense steps involving activation of T-cell mediated apoptosis of self and other infected

cells by antigen-presenting cells, such as macrophages or dendritic cells, join in. This marks the start of the adaptive immunity response. A group of signals, including pattern recognition receptors (protein), type I interferon, and IL-10 are used during transition into the adaptive immunity response. It is implicated that enhanced secretion of IL-10, an anti-inflammatory signaling molecule produced by T helper cells during this stage, decreases the innate immunity response, making the host susceptible to serious infection. Induction of apoptosis by infected cells, including T-cells, by type I interferon produced by activated macrophages following formation of PAMPs or DAMP on the membrane surface, is implicated in the host susceptibility to *Listeria* infection as well (Archer et al., 2014; Auerbuch et al., 2004; Carrero and Unanue, 2006; Ibrahim et al., 2013; O'Connell et al., 2004; Takaha et al., 2012). As a result, *Listeria* cells benefit and cause more significant infection in the host.

Adaptive immunity response

Macrophages and dendritic cells are thesignificant antigen-presenting cells in the previous immunity response (innate immunity). They are key determinants for induction of adaptive immunity, which improves the eradication efficacy of the host immunity system following the activation of T-cells through interaction between T-cell receptors (TCR) surrounding the membrane and the antigen-presenting cells Upon activation of T-cells by macrophages or dendritic cells, the B-cells get activated to produce antibodies and become antigen presenting cells, which result in increased number of activated T-cells to improve eradication efficacy. Two major types of T-cells, the $CD4^+$ and $CD8^+$ cells, are responsible for enhancing immunity responses to persistent foreigners, including *Listeria* and other pathogens. Specifically, the cytolytic inducer cytotoxic T

cells (T_C cells/$CD8^+$) and the T helper cells (T_H cells/$CD4^+$) stimulate maturation of B-cells as well as activate macrophage and cytotoxic T-cells. As agents involved in humoral immunity, B-cells produce antibody to neutralize invasion and target infectious agents for destruction (Moore et al., 2001). This eradication mechanism is relatively less effective than the other groups of adaptive immunity cells (T-cells) when dealing with intracellular bacteria such as *Listeria* (Shen et al., 1998). In T-cell-mediated immunity, infected cells are lysed to release intracellular *Listeria* cells for phagocytosis by macrophages. Subsequently, the cytokine IFNγ is secreted by both types of T-cells to activate macrophages for *Listeria* eradication. It is implicated that this type of immunity can be impeded by *Listeria* infection, thereby making the host more susceptible.

Interaction between *Listeria* and intestinal epithelial cells: mechanisms and implications of adhesion and invasion

Successful *Listeria* systemic infection in the host first requires colonization of the intestinal epithelial cells, namely the distal ileum and proximal colon (Jaradat et al., 2003a; Jonquières et al., 1998; Moroni et al., 2006). This step can be mediated by surface protein-protein interaction between the pathogen and the host intestinal epithelium.

Upon adhesion, the cells gain entry across the intestinal barrier into the host lymphatic system (Lecuit, 2007) by exploiting the host lymphoid tissue, namely the Peyer's Patch-dependent pathway for subsequent infection (Kolb-Maurer et al., 2000; Pentecost et al., 2006; Pron et al., 2001). Other than this pathway, *Listeria* is capable of gaining entry independent of the lymphoid tissue, by the Peyer's Patch-independent pathway, by exploiting the pathogen's virulence mechanisms and the host non-

phagocytic intestinal epithelial cells (Lecuit et al., 2001; Rácz et al., 1972; Pentecost et al., 2006).

Microfold (M) and dendritic cells are essential components of Peyer's Patches (PPs), responsible for internalization of pathogens (Gebert et al., 1996; Kucharzik et al., 2000; Mabbott et al., 2013). The interaction mechanism between pathogenic *Listeria* and PPs prior to infection is not successfully demonstrated with *inl*A, *inl*B, or *act*A mutants, because all exhibit a similar infection capacity as the wildtype strain (Daniels et al. 2000; Pron et al., 2001). On the contrary, a number of specific interactions of surface proteins between pathogen surface adhesins and the receptors on non-phagocytic intestinal epithelial cells are well elucidated such as InlA – E-cadherin (Mengaud et al., 1996; Jonquières et al., 1998), InlB – gC1qR (Braun et al., 2000; Shen et al., 2000), ActA – proteoglycan (Alvarez-Dominguez et al., 1997; Pandiripally et al., 1999), Ami – Hep-G2 (Milohanic et al., 2001), FbpA – fibronectin (Dramsi et al., 2004), Hsp60 – Lap (Pandiripally et al., 1999; Wampler et al., 2004), and Vip – Gp96 (Cabanes et al., 2005).

Molecular factors of adhesion and invasion

Three major modes governing localization of surface proteins in *Listeria* are the classical SecA(2), LPXTG, and GW cell wall-enchoring motifs. However, in-silico predictive localization of surface proteins using TAT localization tool reveal that the classical surface localization mode, TAT, in Gram-negative bacteria may be present in this pathogen (Natale et al., 2007). This is confirmed in a recent analysis of this pathway in pathogenic *Listeria* by Machado et al. (2013), which revealed no involvement in the bacterial survival or biofilm-forming ability.

Unlike SecA, which is implicated as an essential component for *Listeria* survival as observed in SecA-deficient mutants regulated by IPTG-promoter (Monk et al., 2008), the other secretion system of *L. monocytogenes*, SecA2 (Lenz and Portnoy, 2002), is implicated in the promotion of mice immunity response, namely T-cell activation, during intracellular infectious life-cycle (Rahmoun et al., 2011). In SecA2-negative *Listeria* mutant, it is implicated that the normal gene product is essential for intracellular multiplication and cell-to-cell spread in addition to the roles of cell adhesion and invasion and it is also required for virulence in mice (Camejo et al., 2011). Lenz et al. (2003) implicate that this system is involved in secretion of a majority of *Listeria* autolysis important for pathogenesis (Lenz et al., 2003).

LPXTG proteins are catalytically translocated to the bacterial cell wall surface by sortase A (SrtA) (Dhar et al., 2000; Marraffini et al., 2006; Paterson and Mitchell, 2004) transpeptidation, which involves covalent interaction between the LPXTG-enchoring motif and the cell wall. A study of the overall virulence role in SrtA-deletion mutant, reveals that the normal mechanism is crucial in this pathogen for host cell entry and colonization during a deeper cell invasion (liver or spleen) (Bierne et al., 2002).

The cell wall-enchoring motif GW repeats in a protein C-terminus and is implicated in the surface associated InlB and Ami protein of *L. monocytogenes*, which are accountable for *Listeria* internalization for systemic invasion and adhesion to hepatocytes, respectively. This group of surface proteins attach to lipotechoic acid in the cell wall upon secretion extracellularly (Braun et al., 1997, 2000). Another cell wall autolysin, Auto, is mediated by GW repeats. It is implicated in the *Listeria* entry into a

wide range of cell types. The normal Auto is necessary for cell viable during oral and intravenous inoculation of guinea pig and mice, respectively (Cabanes et al., 2004).

Altogether, *Listeria* has a group of translocation pathways that is crucial for successful virulence. Among 44 known virulence factors, approximately 8 surface adhesins are directly interacting with host intestinal epithelial cells (Camejo et al., 2011).

Internalins (Inl)

This group of genes is a primary step for screening between pathogenic and non-pathogenic members of the genus *Listeria* (Den Bakker et al., 2010), although a pathogenic strain can have more than one group of these virulence genes (Paula et al., 2014). To date, a total of eleven internalin derivatives include *inl*A, *inl*B, *inl*C, *inl*D, *inl*E, *inl*F, *inl*G, *inl*H(*inl*C2), *inl*I, *inl*J, i*nl*K has been documented in *Listeria monocytogenes* (Hamon et al., 2006). It is worth noting that none of these fully match the genome of the non-pathogenic species *L. innocua* or other species of *Listeria* available in NCBI. Among them, a total of four internalins (*inl*A, *inl*B, *inl*C, *inl*J) are synergistically contributing to the abilities for host cell adhesion and invasion (Bublitz et al., 2008; Mengaud et al., 1996; Sabet et al., 2005; Sabet et al., 2008; Lindén et al., 2008). Jonquières et al. (1998) report of a truncated form of InlA in an avirulent *L. monocytogenes* strain, LO28, isolated from culture stool of a healthy pregnant woman, indicates a strong obligation of InlA in *Listeria* virulence. Susceptibility of host immunity response to *Listeria* infection in the lymphatic system, takes place immediately after entry into a phagosome, can be partially attributed to down-regulation of host interleukin 6 (IL-6), a proinflammatory cytokine by the stress-induced virulence protein InlH (Lecuit, 2007; Personnic et al., 2010). In addition, autophagy escape of *Listeria*, responsible by InlK, renders the host more

vulnerable to *Listeria* infection (Dortet et al., 2011; 2012). In the case of *Listeria* cell-to-cell spreading activated by the protein ActA, InlC enhances *Listeria* movement into neighboring cells (Paula et al., 2014; Rajabian et al., 2009). The amino acid Leucine-rich repeats are noted in a group of surface associated internalins, namely InlA, InlB, InlJ, and InlH which are essential for improved interaction of surface proteins between *Listeria* and host cells during infection and invasion (Bierne et al., 2007; Lecuit et al., 1997; Lingnau et al., 1995; Personnic et al., 2010). The surface associated *Listeria* proteins InlA, InlJ, InlH, and InlK are led by the signature LPXTG motif that are acted upon by Sortase A to covalently bind to peptidoglycan (Mazmanian et al., 1999; Dortet et al., 2012). Another surface localization mechanism, that accounts for the surface localization of secreted InlB through association with lipotechoic acids in the cell wall, is led by the cell wall enchoring domain Glycin-Tryptophan (<90 amino-acid-repeats led by GW amino acids) (Braun et al., 1997; Braun et al., 2000; Jonquières et al., 2001).

Interaction between *Listeria* surface proteins and host cell surface is mediated by receptors of the host surface protein-based components. The surface proteins InlA and InlB are site-specifically important in *Listeria* adhesion and invasion through oral (intestinal epithelium route) and systemic (intracellular hepatic route), respectively (Braun et al., 1997). It is implicated that the interaction is species-specific, as documented by Disson et al., (2008), Khelef et al. (2006), and Lecuit et al. (1999). Collectively, they demonstrate that a positive interaction between each of the pair InlA:E-cadherin and InlB: tyrosine kinase Met (a receptor of hepatocyte growth factor) (Shen et al., 2000) or gC1qR (Braun et al., 2000) or glycosaminoglycans (GAGs) (Banerjee et al., 2004) that promotes *Listeria* entry into host cells of guinea pigs, rabbits, gerbils, mice,

and rats, selectively; positive InlA:E-cadherin interaction is involved in the hosts guinea pig, rabbit, and gerbil; whereas positive InlB:Met interaction includes the hosts mice, rats, and gerbils. Following these findings, a number of inlA-independent surface adhesins have been confirmed in pathogenic *Listeria* using mouse models (Jaradat and Bhunia, 2003b).

In summary, the story of listeriosis pathogenesis is still fragmented and evolving. More effort is necessary to explore the mechanisms of listeriosis pathogenesis and eventually connect the fragmented knowledge into a complete picture.

Invasion-associated protein (Iap)

The corresponding product of iap is p60, which possesses murein hydrolase activity, is responsible for cell wall hydrolization (Bubert et al., 1992a, b; Kuhn and Goebel 1989). The attenuated form of this product causes the deformation of *Listeria* cells. In a virulence perspective, it is a necessary factor for cell invasion of fibroblast and epithelial cells, internalization, and binding to enterocyte like cells (Bubert et al. 1992a, b; Kuhn and Goebel, 1989; Ruhland et al., 1993; Hess et al., 1995; Park et al., 2000). Attenuated virulence is demonstrated in iap-negative *Listeria* mutants when intravenously administered to mice (Pilgrim et al., 2003). This virulence determinant has been studied in an application at the national FDA lab (FDA, 2013) as a marker gene for real-time PCR identification and differentiation of pathogenic *Listeria* from other species (Bubert et al., 1992a, b). Currently, no report on its properties as an adhesin receptor is available (Camejo et al., 2011).

Listeria adhesion protein (Lap)

p104, a homolog of the protein alcohol acetaldehyde dehydrogenase, is the corresponding product of the gene lap, based on the protein weight in kilodalton. It is a SecA2 secretion protein (Burkholder et al., 2009), common in all *Listeria* spp except the species *grayi*. It promotes *Listeria* adhesion to host intestinal cells by interacting with the cell protein receptor Hsp60 (Pandiripally et al., 1999; Wampler et al., 2004; Jagadeesan et al., 2010; Burkholder and Bhunia, 2010). Attenuated virulence is evident in mice administered orally with p104-negative *Listeria* mutant, indicating p104 is one of the important virulence adhesion proteins in promoting *Listeria* extraintestinal dissemination (Burkholder and Bhunia, 2010). No direct involvement in infection of host cells has been observed.

Listeria adhesion protein B (LapB)

This in-silico revealed surface protein is exclusive in pathogenic *Listeria*. It is associated with the cell wall by LPXTG-anchoring motif. Its expression, promotes adhesion of *Listeria* and entry into a wide cell lines, is positively regulated by prfA. This protein is virulently important in orally and intravenously inoculated mice despite the mechanism of adhesion is unclear (Reis et al., 2010).

Amidase (Ami)

It is the 99-kDa surface protein amidase with a suggested function of recycling bacterial cell peptidoglycan (McLaughlan and Foster, 1998). The N-terminal domain of amidase activity is accountable for the cleavage of the bacterial cell wall substrate N-acetylmuramoyl-L-alanine during cell wall metabolism. Its association with the surface is mediated by interaction between the C-terminal GW cell wall-anchoring domain and the

lipotechoic acid in the cell wall (Oshida et al., 1995; Braun et al., 1997; Jonquières et al., 1999). Its involvement with bacterial virulence is implicated by the protein adhesion to Hep-G2 human hepatocytic cells and causes invasion in mice intravenously administered with wild-type and ami-negative mutant strains (Milohanic et al., 2000; Milohanic et al., 2001). The GW domain is implicated in the facilitation of *Listeria* adhesion to liver epithelium (Milohanic et al., 2001; Milohanic et al., 2004) and it is reported to promote growth in the liver.

Autolysin (Aut)

It is an autolytic protein composed by the domains signal peptide (GW repeats modules) and N-terminal N-acetylglucosaminidase, as revealed by a comparative genomics study. Optimum activity is governed by an acidic condition (Bublitz et al., 2009). In virulence studies, it is implicated that the cell viability and entry ability can be affected in aut-negative *Listeria* mutants inoculated intravenously (mice) and orally (guinea pig) (Cabanes et al., 2004). Additionally, it can promote *Listeria* entry into a wide type of epithelial cells in mammals (Camejo et al., 2011).

Immunogenic surface protein/autolysin (IspC)

This 86-kDa SecA2-independent surface adhesin (Ronholm et al., 2014) is up-regulated during infection and is a member of *Listeria* autolysins (Wang and Lin, 2007). The same group reveals that in strains in which SecA2 is absent, the activity of surface protein ActA is highly reduced, which is essential for adhesion and cell movement (Camejo et al., 2011). It is implicated as a key factor for *Listeria*-specific humoral immunity activation and reaction (Yu et al., 2007). As a result, it is a required component for complete *Listeria* virulence.

Actin-based motility (ActA)

Its first implication of intracellular virulence is the phenomenon of actin nucleation and polymerization on theapical end of *Listeria* cells providing movement within infected cells, or to neighboring cells, for deeper invasion (Alvarez-Domínguez et al., 1997; Suárez, et al., 2001). This also allows *Listeria* evasion from host destruction mechanisms, namely ubiquitination and autophagy (Perrin et al., 2004). It is implicated in the adhesion to the receptor of heparan sulfate proteoglycan on host cells, directing *Listeria* to the site of invasion (Alvarez-Domínguez et al., 1997).

Fibronectin-binding protein (FbpA)

It is a 570-amino-acid polypeptide, homologous to the fibronectin-binding proteins PavA, Fbp54, and FbpA of *Streptococcus pneumoniae* (Holmes et al., 2001), *S. pyogenes* (Courtney et al., 1996), and *S. gordonii* (Christie et al., 2002), respectively. In-vitro studies confirm a positive interaction between this protein and human fibronectin. It is localized on the bacterial surface without a classically known translocation mechanism (Dramsi et al., 2004). Its expression is implicated in the down-regulation (by co-immunoprecipitation) of other *Listeria* virulence factors, namely inlB and LLO, which are accountable for the intestinal adhesion/internalization of *Listeria* and vacuole escape during intracellular invasion, respectively (Dramsi et al., 2004). Additionally, inlB is implicated in the interaction between the cells and host liver epithelium to promote an adhesion and more extensive bacterial invasion (Bierne and Cossart 2002). As a result, it is an important virulence factor for the overall pathogenesis of *Listeria* taken up orally or intravenously in mice (Dramsi et al., 2004).

Additional adhesion factors identified

More adhesion factors, namely CtaP, DltA, and RecA, are included in appendix 4. They are indirectly involved in the overall adherent and invasive capacities of *L. monocytogenes* and hence are members of *Listeria* virulence factors.

Based on current information, a fuller picture of *Listeria* pathogenesis, after successfully surviving gastrointestinal stresses, revolves around four major abilities, namely initial colonization (host cell contact), gaining entry into cells, vacuole escape (phagosome), and cell-to-cell spread; no toxic agent has been reported in this deadly pathogen. Besides a hemolytic effect caused by pathogenic *Listeria*, there is no other information on alternative agents leading to the disease menifestations of meningitis, corneal ulcer, cervival infection, miscarriage, sepsis, and pneumonia is not readily available. Unlike hemolytic pneumococcus, high H_2O_2 production in this pathogen is another known agent that causes cytotoxic effects that leads to pneumonia, meningitis, and sepsis (Pericone et al., 2000). Additionally, there is no report on in vivo survival of *Listeria* in acidified phagolysosomal vacuoles whereas it is documented in the protozoan parasite Leishmania (Desjardins and Descoteaux, 1997) and the Q-fever causing agent Coxiella burnetii (Maurin et al., 1992). However, the implication of improved Auto (autolysin) and LLO (Beauregard et al., 1997) activities in acidic condition is documented and hence implicates a prospective research area to explain the mechanism of *Listeria* to survive in acidic phagolysosomal vacuole. This knowledge as well as the toxicity determinant can improve the whole picture of *Listeria* pathogenesis and application procedures in control and prevention of listeriosis.

Interaction between *Listeria* and processing plants: mechanism and implication of adhesion and cross-contamination

Listeria has been implicated as a fast colonizer upon surface contact in processing facilities and thereby establishing a sessile colony and biofilm entrapped cells (Lundén et al., 2000; Borucki et al., 2003). In a biofilm matrix, bacteria are more resistant to sanitation stresses, including heat, benzalkonium chloride, anionic acid sanitizer (Frank and Koffi, 1990), dessication, UV light (Borucki et al., 2003), and hypochlorite (Lee and Frank, 1991) introduced as antimicrobial interventions in processing plants. Currently, the genetic basis of biofilm formation in *Listeria* still remains elusive (Jordan et al., 2008). A group of biofilm determinants in Gram-positive bacteria have been proposed for this pathogen, however experimental analysis of these genes have not yet elucidated the molecular mechanism of biofilm formation in this pathogen (Götz, 2002; Lasa, 2006; Renier et al., 2011) (Fig. 2). Like other bacteria biofilm formers (Costerton et al., 1999; Fux et al., 2005), *Listeria* biofilm formers undergo an initial common step (i.e. colonization) to promote bacterial transition from the planktonic to the sessile phase. This is initiated by successful colonization of the bacterium after initial surface contacts. Surface contacts that may act as *Listeria* colonization substrates have been identified by several groups in independent studies; they are stainless steel, polystyterene, glass, rubber, plastic, and vegetagetion material (Gamble and Muriana, 2007; Chen et al., 2009; Norwood and Gilmour, 1999). It is implicated in a study by Gorski et al. (2003) that the adhesion strength is temperature-dependent, namely enhanced with increased temperature. However, their attempt to identify adhesion determinant by mutagenesis of differentially expressed genes between cells grown with cabbage and in broth medium was not successful (Palumbo et al., 2005). Recently, independent studies identified three

abiotic adhesins in *L. monocytogenes* that allowed attachment to glass surface contacts. They are the surface proteins InlA (Chen et al., 2008), InlB (Chen et al., 2008), and BapL (Jordan et al., 2008). Deletion of BapL had a negative effect on biofilm formation (Lasa and Penadés, 2006). This area requires further research to capture the full picture of the surface determinants essential for abiotic adherence that is largely responsible for cross-contamination of RTE product from surface-adherent *Listeria* in food processing facilities.

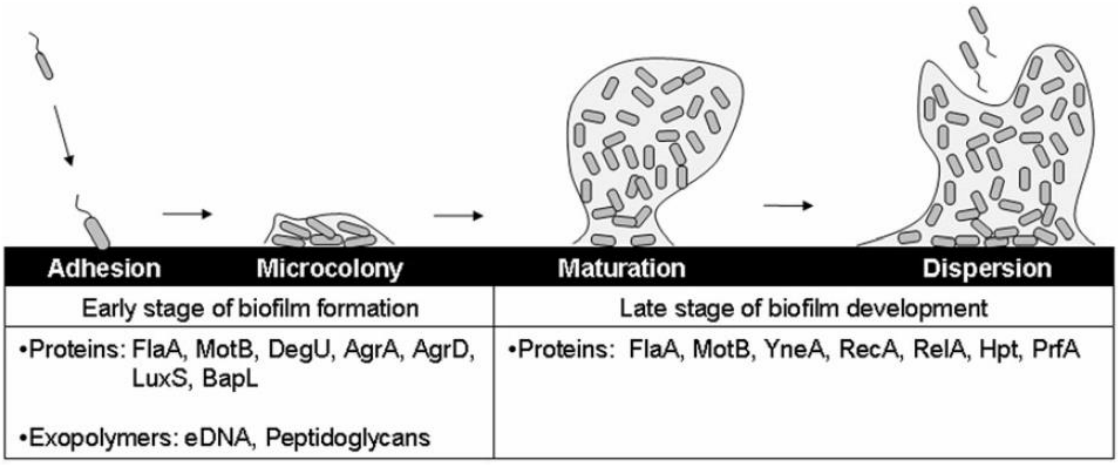

Figure 2. Presentation of *Listeria* molecular determinants, possibly account for biofilm formation, implicated in biofilm formation of other Gram-positive bacteria. This diagram is courtesy of Renier et al. (2011).

Method for high performance identification of surface proteins

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is an advanced product evolved from tools that serve a similar ultimate purpose, namely for specific identification of unknown proteins. Improved sensitivity and specificity of LC-MS/MS in mass detection, identification and quantification have positioned this tool as routine technique for clinical diagnostic practices in hospitals or clinical testing laboratories (Kushnir et al., 2011)

Liquid chromatography coupled with tandem mass spectrometry is a mass identifier

LC-MS/MS is designed to carry out two functions in stepwise order: 1) separate total proteins by their hydrophobicity and size, and 2) further separate charge-coated proteins by their ion masses with a liquid chromatography (LC) coupled with a tandem mass spectrometer (MS/MS), respectively. The latter gives a range of mass spectra constituted of protein mass-to-charge ratio and intensity. This represents quantitative fingerprints of proteins tested (McCormack et al., 1997; Link et al., 1999; Peng et al., 2002). Like other testing samples each protein has a mass based on the composing amino acids. Specific fragmentation of protein with a hydrolytic protease renders peptide fragments of specific sizes. Consequently, this increases the sensitivity and wide range of analytical peptide by the LC-MS/MS analysis. As a result, peptide fingerprints represented by spectra are generated (Eng et al., 1994; Link et al., 1999). The data generated is subsequently followed by a genome database search with one of the proteomic algorithms, X! Tandem, MASCOT, or SEQUEST to automatically generate peptide hypothetical (theoretical) spectra (Link et al., 1999; Peng et al., 2002). Peptide spectra are then matched to LC-MS/MS-generated actual spectra (same peptide) for

protein identification. In summary, LC-MS/MS is constituted of three modules, as described below with their respective functions:

a. Protein preparation – Urea-extraction of surface proteins is recommended for orbitrap compatibility at OSU (Tiong et al., 2015). PAGE-gel visualization and concentration measurements of protein samples are made for use of samples with a comparable amount of protein.
b. Ionization – Sample in solution is electroionized to form negatively-coated ions.
c. Sort ions – This is performed by separating/moving ions based on their size when applying electromagnetic charge.
d. Ion detector – ion signals are detected and converted into mass spectrum with quantitative meaning.

Quantitative application of LC-MS/MS (orbitrap)

Historically, the electrophoretic and calorimetric approaches are the only quantitative techniques for proteomic studies. Today, proteomic analyses have switched gear to the powerful mass analyzer, LC-MS/MS, that overcomes the problems of scarcity, environmental contamination by polyacrylamide gel wastes, intensive manual operation, and expensive costs. In LC-MS/MS, tryptic peptide fragments with variable masses are detected by mass spectrometer following chromatographic separation, ionization, and subsequently selection of ions and plotted as mass-to-ion ratio (m/z) and respective ion intensity in the form of peaks or spectra. MS intensity/abundance can be translated into two forms of quantitative data (1) absolute or (2) relative, by using isotope-labeled or isotope-free reactions, respectively. The former gives exact protein concentration; determined using known protein concentration and a standard curve (Han et al., 2001), while the latter gives relative differences of protein expression between samples by taking the ratio of ion intensities (ions) between samples compared. Depending upon the

experimental objective (absolute or relative quantitation), this can be prepared prior to injecting peptide fragments into the LC-MS/MS (Lau et al., 2007; Kline and Sussman, 2010). As a result, not only can protein MS get quantitated but also identified with the genomic databases that are available today based on tryptic peptide fingerprint analysis. In brief, LC-MS/MS quantitative proteomics can be achieved using Isotope labeling or Label-free methods. The former is limited by increased experiment time, high cost of reagents, incomplete labeling, and requiring specific quantitation software, underscoring the need of timely and costly effective quantitative methods in MS-based proteomics (Zhu et al., 2010). Recently, research groups represented by Ishihama et al. (2005) and Zhu et al. (2010) repeatedly demonstrated that the two types of data of (1) peptide number and (2) spectral count generated by the label-free quantitative method, respectively, can be transformed into absolute quantitative form (Ishihama et al., 2005; Zhu et al., 2010; Lau et al., 2007; Lu et al., 2007). As a result, this marks a significant move forward in the use of this technology entailing a reduced cost and time for carrying out a protein MS absolute quantitative experiment without using isotope labeling (Heroux et al., 2014; Zhu et al., 2010). This improved capability of MS-based label-free quantitative proteomics involves expected (theoretical data) and actual MS data of a protein sample studied. This can be derived from a proteomic algorithm such as SEQUEST (Eng et al., 1994; Link et al., 1999; Han et al., 2001; Li et al., 2003; MacCoss et al., 2003; Pan et al., 2006; Wang et al., 2006), X! Tandem (Craig and Beavis, 2004), MASCOT (Perkins et al., 1999; Schulze and Mann, 2004; Shadforth et al., 2005), or Phenyx (Hustoft et al., 2012) and the mass analyzer (LC-MS/MS), respectively (Pan et al., 2006; Lau et al., 2007).

There are two methods of mass detection by MS: targeted and non-targeted detection. The former allows proteomic studies for a selective mass, while the latter detects total proteins of various masses. This can be performed with a selective reaction monitoring (SRM) tool in the machine (Veenstra, 2007)

Quantitative measurement of proteins using the isotope-labeling method is carried out with one of these two processes either in vitro or in vivo. Several common *in vitro*-labeling strategies used for absolute quantitative LC-MS/MS proteomics are absolute quantitation (AQUA) and isobaric tags for relative and absolute quantitations (iTRAQ). Conversely, the in vivo form includes isotope coded affinity tagging (ICAT) and iTRAQ.

Table 1. List of quantitation methods for LC-MS/MS analysis

Absolute quantitation			Relative quantitation			
Stable isotope labeling	Label-free		Stable isotope labeling	Label-free		
Intensity, using AQUA peptide standard and calibration curve to determine endogenous peptide amount. QconCAT (Synthesize isotope labeled peptide by recombinant technology), Isotope dilution, SISCAPA (Use antibody to isolate specific isotope peptide and the same peptide prior to quantification by ESI-MS	Spectral counting, using Absolute Protein Expression (APEX), free open source calculation tool available at http://pfgrc.jcvi.org/, require additional experimentation (MudPIT, western blot, flow cytometry or prediction tool (to find expected peptides) for # of unique peptides estimation used in APEX scoring, (Zhu et al., 2010)	Number of peptide, identified using Protein Abundance Index (emPAI), *emPAI* = $10^{(Nobserved/Nobservable)}$-1. $N_{observed}$ = Number of observed peptides $N_{observable}$ = Number of observable peptides, (Ishihama et al., 2005) (Zhu et al., 2010)	Intensity, take ratio of light and heavy peptides for quantitation	Intensity, take ratio of peptides between samples for quantitation	Spectral counting, using normalized spectral abundance factor (NSAF). NSAF = (#Spectral count/protein's length)/(Σspectral count/protein's length), (Zhu et al., 2010)	Number of peptide, identified using emPAI, (Ishihama, et al., 2005)

Methods for extraction of *L. monocytogenes* surface proteins

The first phase of my research focused on comparing various protein extraction methods for improving the quality as well as quantity analysis of *Listeria* surface proteins by LC-MS/MS (orbitrap) and subsequent identification using the protein database MASCOT. The following methods of extraction (enzymatic and chemical approaches), documented in other Gram-positive bacteria by other researchers, were employed for surface protein preparation, detection and identification by LC-MS/MS and MASCOT protein database analysis, respectively. Subsequent comparison of quantity of cytosol and surface proteins was performed after predictive localization of each protein member in each extract using a variety of documented in-silico subcellular protein localization tools. Finally, a protein extraction method that minimized cytosolic contaminants was used for subsequent experiments to elucidate the molecular basis of the strong adherence phenotype in *L. monocytogenes* to abiotic contact surfaces.

Various methods, including ionic solution (LiCl), protein denaturants (urea buffer), and trypsin digestion (enzymatic shaving) were considered based on economical, practical and comparable perspectives. They were more economically manageable when compared to the affinity pull-down assay (Karhemo et al., 2012; Shin et al., 2003). Equally important, surface extracts produced by these methods could be readily used for subsequent LC-MS/MS detection, while extractions made with Laemmli's extraction buffer required subsequent purification steps to remove SDS (orbitrap incompatible) which results in high work load, time-consuming, and additional loss of sample (Valledor and Weckwarth, 2014; Issaq and Veenstra, 2008; Laemmli, 1970). Other than the mentioned properties of these extraction methods, their continuous applications in the

same type of work have resulted in a known mode of action and a number reports document their efficient application in surface protein extraction (Bøhle et al., 2011; Jeon et al., 2011; Yacoub et al., 1994).

Lithium Chloride extraction buffer (LiCl)

This agent is used as a chaotropically ionic denaturant for improved extraction of proteins located in the outer cell wall layer, while the treated cells remain intact (Voigt, 1985). The concentration of 5 M LiCl has been shown to successfully extract surface proteins from the Gram-positive bacterium *Staphylococcus epidermidis* and led to the identification of a surface adhesin responsible for the cell adhesion to polystyrene surface contact (Turner et al., 1997).

Tris-buffered urea (8M) extraction buffer (UB)

This is another form of chaotropic protein denaturant, frequently used in protein extraction that we employed in our study (Adams and Gallagher, 1992; Salvi et al., 2005). It is mentioned as a powerful denaturant due to the fact that it can break disulphite bonds rendering increased instability of surface proteins. This agent is known to cause carbamylation (Hummon et al., 2007; Roxborough and Young, 1995), namely protein deformation, but has been shown by Jeon et al. (2011) that this may be due to heating protein in urea extract. We did not use the normal total protein preparation method, which involved boiling process, in our study throughout the experiment because we were trying to avoid the recovery of cytoplasmic proteins in our extracts by avoiding the use of harsh treatments.

Hypotonic extraction buffer (UB-Ghost)

This method is devised to promote exclusion of cell cytosolic components prior to extraction of surface proteins using the extraction method UB. Specifically, cells are treated with hypotonic solution such as pure water to induce swelling and cell rupture as mentioned in a report by Boone et al. (1969). This way the contaminating cytosolic proteins can be minimized in surface extracts and hence increases the quality of surface proteomic extractions.

Trypsin extraction buffer (Tryp)

This proteolytic enzyme is readily applied in LC-MS-based proteomics technology mainly attributed to the properties of site specific cleavage, preferred mass range formation for sequencing, and the presence of a basic residue at the carboxyl terminus of a digested peptide (Hustoft et al., 2011). It is used for conversion of proteins to peptides, during the process of proteomics, before subjecting to mass spectrometry analysis (either LC-MS or Maldi-tof) and protein identification by database mining using a search engine (MASCOT, Sequest, or Phenyx). Combining ammonium bicarbonate (denature cell membrane into membrane sheets) (Fischer et al., 2006; Fujiki et al., 1982; Molloy et al., 2000), sucrose (i.e. 0.6 M concentration may cause swelling of bacteria cell) (Ventura et al., 2010), or DTT (protein denaturant) (Rodríguez-Ortega et al., 2006; Ventura et al., 2010) in trypsin digestion improves the reaction by exposing the restrictive site for trypsinolysis action. It is worth noting that they are also compatible with a LC-MS/MS analysis (Wu et al., 2003).

High performance quantification of mRNAs

Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) has been used to detect and quantitate gene expression (mRNA levels). A protein's availability or activity is based on its gene transcription level which previously was determined only by gel-based less sensitive methods such as the Northern blot (Bustin, 2000; Wang and Brown, 1999).

RT-qPCR comes into existence by employing the thought presented in molecular biology that a functional protein is an end product of genetic information, and RNA is an intermediate product between them (Crick, 1956, 1970). Conventionally, the two forms of quantitative conditions of this technique include absolute or relative. The former quantitative analysis involves a known mass of sample. On the other hand, the relative quantitative technique requires an internal control or reference gene of highly consistent expression in all circumstances, for example the inclusion of the house-keeping gene, 16S rRNA, for expression standardization in controls and samples that may have undergone PCR variation (Pfaffl, 2001; Pfaffl et al., 2002, 2005; Vandesompele et al., 2002). Two major mathematical models for determination of fold change in expression levels (relative expression ratio) of compared strains are demonstrated in Figures 3 and 4 and are used accordingly based on the cycle threshold (Ct) or crossing point (Cp), as mentioned by Livak and Schmittgen (2001), Pfaffl (2001, 2006) and Soong et al. (2000). The Ct or Cp is determined mainly by constant fluorescence measured from non-specific fluorescent dye that intercalate with any double stranded DNA immediately after each cycle of PCR amplification or sequence-specific DNA probes, made up of a

fluorescence-labeled short nucleotide sequence, that hybridize to its complementary sequence, detected by an RT-PCR fluorescence detection system.

$$R = 2^{-[\Delta C_P \text{ sample} - \Delta C_P \text{ control}]}$$

$$R = 2^{-\Delta\Delta C_P}$$

Figure 3. Calculation model for consistent amplification efficiency (E) among all Ct determinants (Pfaffl, 2006).

$$\text{ratio} = \frac{(E_{Ref})^{C_P \text{ sample}}}{(E_{target})^{C_P \text{ sample}}} \div \frac{(E_{Ref})^{C_P \text{ calibrator}}}{(E_{target})^{C_P \text{ calibrator}}}$$

Figure 4. Calculation model for inconsistent amplification efficiency (E) among all Ct determinants (Pfaffl, 2006).

$$E = 10^{[-1/\text{slope}]}$$

$$\% E = \{[10^{(-1/\text{slope})}] - 1\} \times 100$$

Slope = The slope of the standard curve, X-axis: serial dilution of DNA mass, Y-axis: ct number.

This technique was employed by researchers of *L. monocytogenes* to establish a positive correlation between *inl*A/B gene expression and the strength of cell attachment (Chen et al., 2009). In their work, they noticed some strains exhibiting opposite correlation between gene expression and their attachment strengths, namely the *inl*A/B gene was highly expressed in a number of strains with the property of reduced attachment

strength. They attributed this observation to the documented fact that this bacterium is genetically volatile or variable, namely they are in truncated forms (Jonquières et al., 1998; Martína et al., 2014; Wang et al., 2012).

In our study, we employed the technique of quantitative real-time reverse transcription polymerase chain reaction (real-time RT-PCR) to correlate between gene expression and phenotypic function. In our study, the bacterial reference gene 16S rRNA was used to respectively normalize the expression levels of a total of fourteen genes, presumably involved in *Listeria* surface adhesion, in the control and sample strains.

Conclusions and research phases

Overall, the scientific progress in characterizing and understanding the foodborne pathogen, *L. monocytogenes*, has provided a clearer knowledge in a variety of perspectives including genetic variability/DNA fingerprinting (to detect listeriosis agent, and define its relation to listeriosis outbreak, antibiotic resistance), molecular basis of biotic adherence, pathogenesis, persistent/prevalent determinants, detection and prevention systems. Currently increasing reports of antibiotic resistant strains to an increasing number of antibiotics suggests the need to follow up with research to find the reason for the increase in antibiotic ressitance that has been observed because of public health implications. Incidence of listeriosis still remained high in 2012 when compared to the incidence in about 10 years earlier (2004-2012). Taken together, there is no doubt that *Listeria* still remain a food threat of public health in this era and hence impliying the need of additional tools for *Listeria* detection, prevention, and elimination. For practical considerations, *Listeria* research should focus on functional genomics/proteomics to further understand its involvement in foodborne illness and disease.

To step in line with the current trend of listeriosis's progressive development and the aim of the FDA Food Safety Modernization Act of 2010 (FSMA) (FDA, 2011), which is preventing contamination of food instead of responding to it, as stated in the previous aim back in 2002 (Bioterrorism Act of 2002, title III) (FDA, 2002), our lab has carried out research towards improving or elucidating the knowledge of molecular basis of abiotic adhesion in *L. monocytogenes*, one of the tools of persistence in food-associated environments. The resulting work can have implications for the improvement of the current systems of detection and prevention, namely elimination. The research I have presented herein consists of three phases revolving around the objective:

(i) Phase I: To identify an extraction method for surface proteins that is compatible with LC-MS/MS (orbitrap).

(ii) Phase II: A high performance protein identification tool using orbitrap LC-MS/MS.

(iii) Phase III: A high performance gene expression assay to examine genes identified in II.

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CHAPTER III

COMPARISON OF FIVE METHODS FOR DIRECT EXTRACTION OF SURFACE PROTEINS FROM *LISTERIA MONOCYTOGENES* FOR PROTEOMIC ANALYSIS BY ORBITRAP MASS SPECTROMETRY

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INTRODUCTION

Listeria monocytogenes is an important foodborne pathogen associated with high mortality rates in large outbreaks. It is a known psychrotroph, capable of growing at refrigeration temperatures and is well known to be involved in biofilm formation leading to persistent environmental contamination of food processing facilities and possibly their manufactured food products. This is most serious in ready-to-eat (RTE) food plants whereby the consumed product is not required to be heated before consumption. In prior studies in our lab, we utilized a fluorescent microplate adherence assay to distinguish weakly- and strongly-adherent phenotypes among strains of *L. monocytogenes* isolated from several RTE meat processing plants (Gamble and Muriana, 2007; Kushwaha and Muriana, 2009). In subsequent studies, strains that were strongly-adherent to abiotic surfaces were also more invasive in tissue culture and live mouse assays leading to a greater significance of adherent isolates lingering around in meat processing plants (Kushwaha and Muriana, 2010a, 2010b).

Bacterial cells, by convention, are characterized into two major groups based on the Gram-stain reaction of their peptidoglycan layer, a major component of the outer cell wall surface of Gram-positive bacteria. In addition to other surface components, this layer serves as a template for anchoring both hydrophilic and hydrophobic surface proteins that mediate physiological and interactive roles of respective bacteria. These functions include nutrient uptake, cellular signal transduction by receptor-substrate reaction, movement by flagellin and actin, permeability properties involved in maintaining osmotic pressure and the morphological structure of bacteria, and adhesion to abiotic and biotic

targets (Bierne and Cossart, 2007; Lemon et al., 2007; Renier et al., 2011; Suarez et al., 2001). Due to the importance of this problem in the manufacture of RTE foods, characterizing the role of surface proteins from adherent variants of *L. monocytogenes* may help to understand the molecular basis of abiotic adherence in *L. monocytogenes*.

However, attempts to characterize the surface proteins of *L. monocytogenes* can be compromised by equipment-incompatible sample preparation methods or by experimental contamination from cytosolic, non-target proteins (Kline and Sussman, 2010). In conventional acrylamide gel analysis, Laemmli's protein extraction buffer is often used to easily prepare total proteins, and individual proteins are then isolated from gel-based separations for subsequent identification by mass spectrometry (Issaq and Veenstra, 2008; Laemmli, 1970). However, the sodium dodecyl sulfate (SDS) content requires purification steps prior to orbitrap mass spectrometry experiments, which is time-consuming, labor-intensive, and may result in additional loss of sample (Valledor and Weckwerth, 2014). Other extraction methods use chemicals of different specificities to allow the isolation of surface-expressed proteomes. One such method is the affinity pull-down assay to obtain surface-localized proteins prior to LC-MS/MS identification (Karhemo et al., 2012; Shin et al., 2003). More economical extraction methods include the use of ionic, denaturant chemicals, and trypsin enzyme to release surface proteins containing charges, membranous hydrophobic tails, and the enzyme reactive site, respectively (Jeon et al., 2011; Yacoub et al., 1994).

In this study, we evaluated the efficiency of various extraction methods including ionic solution (LiCl), protein denaturants (urea buffer), and trypsin (enzymatic shaving), using the strongly-adherent strain, *L. monocytogenes* SM3. Surface extracts from each

treatment were subjected to LC-MS/MS followed by protein identification, quantification, and prediction of subcellular localization. The combined use of an efficient extraction method together with a powerful protein identification tool enhances proteomic studies on the molecular basis for adherence in *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial culture and growth conditions.

L. monocytogenes SM3 was used as our phenotypic, strongly-adherent variant for comparing surface protein extraction methods. *L. monocytogenes* SM3 was a food isolate obtained from retail ground beef and has been shown to be strongly-adherent to abiotic surfaces (Gamble and Muriana, 2007) and more virulent during *in vitro* tissue culture and *in vivo* live mouse assays (Kushwaha and Muriana, 2010a, 2010b). *L. monocytogenes* SM3 was cultured overnight at 37^{o}C in Brain Heart Infusion (BHI; Becton-Dickenson) broth and transferred twice before use in experiments.

Reagents and chemicals.

Unless otherwise specified, electrophoresis and HPLC-grade chemicals were used throughout experiments. Nanopure water used throughout for the preparation of buffers and solvents, was generated using a Millipore RG/Siements LP1000 (Lowell, MA). The extraction materials, including LiCl, trypsin (TPCK treated), ammonium bicarbonate, sucrose, both urea and Tris, and EDTA were from Sigma-Aldrich (St. Louis, MO), Bio-Rad (Hercules, CA) and USB Corporation (Cleveland, OH, USA), respectively. The Pierce BCA Protein-Reducing Agent Compatible (BCA-RAC) assay kit was used for measuring protein concentration (Thermal-Fisher Scientific, Waltham, MA). Material for SDS-PAGE gels included Tris, TEMED, bis-acrylamide, and acrylamide (Bio-Rad, Hercules, CA); Coomassie blue R-250 (Hoefer, Holliston, MA); SDS and glycine

(Sigma-Aldrich, St. Louis, MO); beta-mercaptoethanol, methanol, and chloroform (Pharmco-Aaper, Louisville, KY).

Extraction protocols.

Five extraction methods were evaluated on intact cells of *L. monocytogenes* SM3. Overnight cells (18 h) from 50 ml of culture were pelleted by centrifugation (7000 rpm, 6 min) and subsequently washed three times with 10 mM phosphate buffered saline (PBS, pH 7.4) by centrifugation (7000 rpm, 6 min) prior to performing subsequent extraction protocols.

Lithium chloride extraction (LiCl).

The use of LiCl extraction solution was used to study the efficiency in the isolation of surface proteins from Gram-positive bacteria (Liang et al., 1995; Turner et al., 2004; Ventura et al., 2002). LiCl (5 M) was dissolved in nanopure water and filtered through a 0.45 µm filter (Pall Co., Newquay, Cornwall, UK). Washed cell pellets were resuspended in 300 µl LiCl containing 5 mM EDTA. After 30 min incubation at 37 °C with shaking (3000 rpm, Pulsing vortex mixer, VWR, Arlington Heights, IL) the supernatant was collected by centrifugation (11,000 rpm, 6 min) and filter-sterilized through a 0.45 µm filter. Proteins were precipitated by methanol-chloroform treatment (Wessel and Flugge, 1984), dried by speed-vac centrifugation, and resuspended in 8 M (Tris-buffered) urea (0.5 g urea, 130 µl of 8X Tris-HCl buffer; pH 8.5, 520 µl sterile nanopure water, and 25 µl of 100 mM beta-mercaptoethanol) containing 5 mM EDTA. Samples were stored at -80 °C until SDS-PAGE and LC-MS/MS analysis.

Tris-buffered urea (8 M) extraction (UB).

We also examined an extraction protocol used to extract membrane anchored surface proteins (Cordwell, 2008). The cell pellet was resuspended in 150 µl of 8 M (Tris-buffered) urea containing 5 mM EDTA. After incubation for 30 min at room temperature with shaking at 3000 rpm, the supernatant containing solubilized proteins was collected after centrifugation (11,000 rpm, 6 min), filter-sterilized through a 0.45 µm filter and stored at -80 °C, for subsequent analysis by LC-MS/MS and PAGE.

Hypotonic extraction buffer (UB-Ghost).

The same protocol as above was applied to a modified protocol for removal of intracellular components from intact cells by hypotonic treatment (Boone et al., 1969). Washed cell pellets were resuspended in 600 µl sterile nanopure water containing 5 mM EDTA and incubated for 1 h at 4 °C with shaking at 1200 rpm. After incubation the supernatant was decanted after centrifugation (7000 rpm, 6 min) and then the cells were washed three times with 10 mM PBS pH 7.4 and finally resuspended in 150 µl of 8 M (Tris-buffered) urea as mentioned above.

Trypsin extraction containing BICAM (Tryp-B+S).

Shaving reactions using trypsin were conducted in two ways to shed cell surface proteins, modified from a previously reported protocol (Bohle et al., 2011). Washed cell pellets were resuspended in 150 µl of 50 mM ammonium bicarbonate (BICAM) pH 8.5 containing 0.5 M sucrose. An amount of trypsin (2 µg trypsin/mg of cells, dry weight) was added to perform ‘surface shaving’ by incubation for 45 min at 37 °C with shaking at

1200 rpm. After incubation, the supernatant fraction was collected by centrifugation (11,000 RPM, 6 min), filtered through a 0.45 μm filter, and stored at -80 °C until subsequent analysis by LC-MS/MS and PAGE.

Trypsin extraction containing Tris (Tryp-T+S).

The same conditions as above were applied except that BICAM was replaced with 50 mM Tris pH 7.4.

Protein concentration assays.

Measurement of protein was carried out using the Pierce bicinchoninic acid (BCA) protein assay kit combined with the reducing agent compatible (RAC) reagent according to the manufacturer's instructions. The absorbance reading was obtained by the Genesys 20 spectrophotometer at a wavelength of 280 nm (Thermo Fisher Scientific, Rockford, IL).

SDS-PAGE analysis.

The concentration of protein mixtures was confirmed on 12.5 % SDS-PAGE gels run overnight at 50 V in an SE600 Vertical Electrophoresis System (Hoefer, Holliston, MA), according to the method described by Laemmli (1970). The proteins were visualized by Coomassie blue R-250 staining.

Digestion

Samples were denatured with urea (solution digests) or by SDS-PAGE (gel

bands/spots), reduced with Tris(2-carboxyethyl)phosphine (TCEP), alkylated with iodoacetamide (IAA), and digested overnight with trypsin (4 µg/ml), using Tris-HCl to buffer all solutions.

Liquid chromatography and tandem mass spectrometry (LC-MS/MS)

Samples were analyzed at OSU's DNA/Protein Core Facility on a hybrid LTQ-OrbitrapXL mass spectrometer (Thermo Fisher Scientific) coupled to a New Objective PV-550 nano-electrospray ion source and an Eksigent NanoLC-2D chromatography system.

MS data analysis

Centroided ion masses were extracted using the extract_msn.exe utility from Bioworks 3.3.1 and were used for database searching with Mascot v2.2.04 (Matrix Science) and X! Tandem v2007.01.01.1 (www.thegpm.org). Two protein databases, one representing 'bacteria' and another representing a smaller subset of 'all entries from SwissProt' were used, resulting in database sizes of 323,480 (bacteria) and 10,884 (small subset/all entries), respectively. Searches were conducted at fragment ion mass and parent ion tolerances of 0.80 Da and 10.0 ppm, respectively. Searches were permitted a max missed cleavages number of 1 and 2 with Mascot and X! Tandem, respectively. Other search parameters were set to include variable potential peptide modifications: pyroglutamate cyclization of N-terminal glutamines, oxidation of methionine, acylation of cysteine by acrylamide and iodoacetamide adducts, formylation and acetylation of the protein N-terminus.

Peptide and protein identifications were validated using Scaffold v2.2.00 (Proteome Software) and the Peptide Prophet algorithm (Keller et al., 2002). Probability thresholds were greater than 90% probability for protein identifications, based upon at least 2 peptides identified with 80% certainty. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The False Discovery Rate (FDR) was observed at 0.0% at such thresholds.

RESULTS

To evaluate the quality of the five extraction methods, surface extracts from a food isolate of *L. monocytogenes* SM3 were prepared. Once the quality of protein extracts were demonstrated by 1-dimensional polyacrylamide gels (1-D PAGE; Fig. 1), they were submitted for analysis by LC-MS/MS. The total number of peptides and proteins identified after each extraction method by LC-MS/MS was considerable (see Supplementary Data Tables 1A and B). The accumulative peptide and protein numbers were 1096 and 170 (1096, 170), respectively, resulting from different identification efficiencies in extracts obtained using LiCl (620, 119), UB-Ghost (509, 135), UB (653, 152) and Tryp containing sucrose and BICAM (640, 155) or Tris (655, 157) (Table 1 and 2). The protein members identified were from the ListiList functional codes of 1 (22), 2 (44), 3 (83), 4 (10), and 5 (11) representing “cell envelope and cellular processes”, “intermediary metabolism”, “information pathways”, “other functions”, and “similar to unknown proteins”, respectively (Tables 1 and 2) (Glaser et al., 2001; Moszer et al., 1995). The different tools of protein subcellular-location determinant Leger (listeria knowledge database), LocateP (genome-scale subcellular-location predictor for prokaryotic proteins), Psort, CW-PRED, PRED-LIPO, transmembrane segment, SignalP, PRED-TAT andhydrophobicity score were used to suggest the surface-association of non-ListiList envelop-associated proteins, based on sequence analysis and experimental characterization, as demonstrated in Supplementary Data Tables 1 & 2 or a summary table 2. A higher number of proteins potentially associated with the cell envelop was identified (98 proteins: 22 ListiList, 76 Others) as observed in the overview distribution of protein members in each associated method of extraction (Fig. 2). The efficiency of

each method in harvesting Listeria surface-associated proteins was summarized in Table 1.

Leger, LocateP, Psort, and CW-PRED were used to identify surface-associated proteins (cell wall, membrane, secreted, multiple layer locations) of 70, 78, 87, 89, and 90 proteins identified from LiCl, UB-Ghost, UB, Tryp-B+S, and Tryp-T+S extractions, respectively (Dieterich et al., 2006; Fimereli et al., 2012; Yu et al., 2010; Zhou et al., 2008).

For a total of 77 cytoplasmic proteins covering functional codes 2 to 5 (ListiList non-envelop related) identified, their physicochemical properties were elucidated using hydropathy scores and SignalP as well as PREP-TAT tools for potential association with the cell surface (Bagos et al., 2010; Bendtsen et al., 2004a, 2004b, 2005; Kyte and Doolittle, 1982; Natale et al., 2008; Nielsen et al., 1997; Nielsen and Krogh, 1998; Petersen et al., 2011). A total of five members containing a signal peptide was identified as secretory proteins (Supplementary Data 1), resulting the total number of 98 members of protein associating with the cell surface. Negative GRAVY values were determined in 66 members of internally predicted proteins from analysis of LiCl (46), UB-Ghost (50), UB (58), Tryp containing sucrose and BICAM (60) or Tris (61) extracts, suggesting hydrophilic property (Supplementary Data 1, 2). Averaged hydrophobicity was determined by an online ‘grand average of hydropathy’ (GRAVY) calculator for proteins identified in each extract to correlate between protein hydropathic and extraction properties (Fig. 3). Comparison of averaged hydrophobicity of proteins identified in each extract demonstrated no significant preference over the averaged hydrophobicity of proteins by each extraction method.

The number of transmembrane helices was determined for all suggested surface-associated and cytoplasmic proteins (Supplementary Data 1, 2) using PRED-LIPO and TMHMM V.2 (Bagos et al., 2008; Krogh et al., 2001; Moller et al., 2001; Sonnhammer et al., 1998). Following analysis, a total of 8 membrane proteins (7 ListiList envelop, 1 'other') were found in members of 1 transmembrane helix.

The demonstration of percent amino acid sequence recovered from proteins identified by each tryptic extract indicated that tryptic reaction was performed with approximately equal capacity with surface extracts prepared from LiCl as evident of highly similar distribution of protein number corresponding to the percent coverage (Fig. 4). Most proteins within all extracts were detected at 10 % sequence coverage by orbitrap.

An overview of the functions of the proteins identified in each extract indicated that the majority of identified proteins belonged to the member of ribosomal, RNA and DNA modification proteins, followed in order by proteins involved in metabolism, cell wall and membrane biosynthesis, unknown and other (chaperone, transporter) activities (Table 1).

DISCUSSION

L. monocytogenes is such a persistent contaminant of many RTE food products acquired from processing environments that it remains a major foodborne illness of concern in the United States (Chou et al., 2006; Destro et al., 1996; Hansen et al., 2006; Hu et al., 2006; Nightingale et al., 2004). Despite improved sanitation practices in areas where *Listeria* are commonly detected, the organism is difficult to completely eliminate from processing areas that handle raw food ingredients (Cole et al., 1990). The reasons are partly that this pathogen is equipped with capabilities of surviving stresses of temperatures, salinity, and osmotic levels for elimination of pathogenic and spoilage microorganisms, and more importantly, it is well known for the ability to adhere to surfaces and form biofilms (Chen and Hotchkiss 1993; Cole et al., 1990; Fleming et al., 1985).

The role of surface proteins in *L. monocytogenes* in remaining adherent even after conventional sanitation protocols has been demonstrated by several groups, which increases food contamination risks by this organism (Chen et al., 2009; Chen et al., 2008; Gamble and Muriana, 2007; Jordan et al., 2008). Gamble and Muriana (2007) identified surface-adherent variants of *L. monocytogenes* among isolates from raw meats and RTE processing plants by using microplate fluorescence assays for measurement of adherence abilities. Genotypic studies with adherent strains of *L. monocytogenes* involving deletion of the *bap*L, *inl*A, and *inl*B genes exhibited a reduced attachment level in each mutant construct, suggesting that other surface adhesins might act synergistically to provide ‘strong adherence’ to abiotic surfaces (Chen et al., 2009; Chen et al., 2008; Jordan et al., 2008). A continued effort to identify surface adhesins would ultimately improve

information on the molecular basis of attachment by *L. monocytogenes* and enhance prospects for the management of adherent bacterial contaminants (Kim and Silva, 2005).

In this study, we compared five commonly used surface protein extraction methods in preparation of a project to examine the cell surface proteome involved in adherence. Both *Listeria* cytoplasmic and surface proteins were identified in surface extracts recovered from either bleed (UB-Ghost) or non-bled (UB) cells with no obvious difference in numbers. The UB-Ghost method resulted in the identification of fewer peptides than any of the other extraction methods (Table 2; Supplementary Data 1, 2), suggesting the importance of 'bleeding the cells' of their cytoplasmic proteins prior to treatments with extraction reagents to allow for a cleaner surface protein preparation. Variability in the detection of peptides by LC-MS/MS has also been attributed to the use of different chemicals to solubilize proteins (Vaezzadeh et al., 2010). In this study, this was minimized by re-solubilization of all ethanol-precipitated protein extracts (from all of the various extraction methods) with the same re-solubilization buffer (i.e., Tris-buffered urea) before subjecting them to trypsin digestion and then LC-MS/MS analysis. The benefit of this approach in using the same re-solubilizing buffer is to minimize the variability of using different re-solubilizing solvents and is supported by similar profiles of peptide coverage recovered from each method of extraction.

Despite using surface-targeted extraction protocols, the ListiList protein functional classification codes suggested that many of the identified proteins originated from cytoplasm. This could be due to a 'moonlight phenomenon', whereby such proteins can be associated with multiple locations, functions, or both. The first bacterial moonlighting proteins were identified in the *Streptococcus pyogenes* (Campbell and

Scanes, 1995; Henderson and Martin, 2013; Jeffery, 1999; Pancholi and Fischetti, 1992) followed by an increasing number of proteins discovered in other species of *Streptococcus* (Hughes et al., 2002; Wu et al., 2008). Among the proteins identified within *Listeria*, elongation factor Ts (lmo1657), enolase (lmo2455), and elongation factor Tu (lmo2653) were described as moonlight proteins by sequence homology studies (Bergmann et al., 2001; Wilkins et al., 2003). The protein, alcohol acetaldehyde dehydrogenase, involved in adhesion to Caco-2 cells, was experimentally identified as a moonlight protein (Jagadeesan et al., 2010). Similar findings have been reported in other pathogenic bacteria (Bohle et al., 2011). Our in silico analysis of all the members of cytoplasmic protein using web-based tools, including Leger, LocateP, Psort, CW-PRED, PRED-LIPO, Transmembrane Segment, SignalP, and PRED-TAT revealed that >50% of the proteins, include one member of actA-like surface-associated membrane protein (lmo0392) (Kocks et al., 1992; Wehmhoner et al., 2005), were associated with the surface, thereby supporting our current findings of moonlighting properties by these proteins.

Higher numbers (approximately 1%) of surface-associated cytoplasmic proteins were obtained with the, UB and Tryp (B+S) extraction methods when compared with LiCl, UB-Ghost, and Tryp (T+S) extracts, which resulted in the percent increment in unique peptide numbers of 72% (LiCl), 74% (UB-Ghost), 79% (UB), 83% (Tryp B+S), and 81% (Tryp T+S). (Table 2; Supplementary Data 1, 2). The use of trypsin to gently cleave portions of surface proteins (i.e., 'proteolytic shaving') has still observed a significant level of contamination with cytoplasmic proteins (Bøhle et al., 2011; Olaya-Abril et al., 2012). The UB-Ghost extraction method exhibited a lower number of unique

peptides in the overall peptides identified than the other methods. This could be attributed to the harsh effect introduced by extraction solutions/procedures on the cells, which results in leakage of contaminating cytoplasmic and moonlight proteins in contrast to cells treated for removal of cytoplasmic contents prior to extraction (Cornett et al., 1979; Hebbani, et al., 2013; Hussain, et al., 1999; Ingram, 1981; Joseph and Shockman, 1974; Liang et al., 1995). As a result, a total of six members of moonlight proteins (non-ListiList envelop protein) were identified in the extracts independently obtained from LiCl, UB, and Tryp. extracts, indicating that cell lysis possibly occurred and pre-treatment for removal of cytoplasmic components is essential for a cleaner surface proteomics.

When extraction methods were verified for hydropathic specificity, a gravy score corresponding to each protein of the identifying method was determined. Much higher numbers of proteins with hydrophobic nature were represented in all methods used (Fig. 3), thereby confirming the reported specificity of these methods for extraction of hydrophobic proteins that includes surface-anchored proteins. These findings corroborate the hydrophobic nature of surface proteins in another Gram-positive bacteria as reported by Fischer and Poetsch (2006) who demonstrated the surface hydrophobic nature of surface proteins of *Corynebacterium glutamicum*.

CONCLUSION

We compared five extraction methods targeting surface proteins. All surface extracts demonstrated a majority composition (76) of so-called ‘moonlight proteins’, followed by cytoplasmic and surface proteins. The extraction method (UB-Ghost) that involved pretreatment for removal of cytoplasmic contents from *Listeria* cells preceding surface protein extraction in Tris-buffered urea, exhibited a lower number and percentage of unique peptides (identified by orbitrap for total protein members; Table 2) and moonlight proteins, respectively, than the remaining protocols. These results imply that UB-Ghost may improve quality of surface extracts which facilitates more specific enrichments of the targeted cell-surface sub-proteome fraction, and should enable deeper characterizations of cell-surface proteomes to identify adherent-specific proteins that govern *Listeria* risks in RTE production facilities.

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Table 1. ListiList functional classification of proteins identified from each extraction method.

	Bacterial Surface Protein Extraction Method				
Protein functional categories[1,2]	**LiCl**	**UB-Ghost**	**UB (Urea Buffer)**	**Tryp BICAM+ Sucrose**	**Tryp Tris+ Sucrose**
1. Cell envelop and cellular processes (cell surface proteins, membrane bioenergetics, etc)	15	17	18	18	19
2. Intermediary metabolism (Carbohydrates metabolism, etc)	26	32	37	39	42
3. Information pathways (Ribosomal proteins, elongation, transcriptional proteins, etc)	66	70	78	80	78
4. Other	6	6	8	9	9
5. Similar to unknown proteins	6	10	11	9	9
6. No similarity (Hypothetical protein)	0	0	0	0	0
Total :	119	135	152	155	157

The gel-less approach yielded total numbers of cytoplasmic proteins of 104, 118, 134, 137, 138 in LiCl, UB-Ghost, UB, Tryp BICAM+Sucrose and Tryp Tris+Sucrose, respectively.

[1] ListiList: http://genolist.pasteur.fr/ListiList/help/function-codes.html

[2] Leger Genome database: http://leger2.gbf.de/cgi-bin/expLeger.pl

Table 2. Rearrangement of protein members determined by various protein location prediction tools.

	Bacterial Surface Protein Extraction Method				
Protein Members	**LiCl**	**UB-Ghost**	**UB**	**Tryp/ BICAM+ Sucrose**	**Tryp/ Tris+ Sucrose**
Cytoplasm[1,2]	49 (190)	55 **(153)**	63 (219)	65 (211)	66 (231)
Moonlight	55 (309)	63 (**265**)	71 (342)	72 (355)	72 (343)
Surface[1,2]	15 (121)	17 (91)	18 (92)	18 (74)	19 (81)
Total :	119 (620)	135 (**509**)	152 (653)	155 (640)	157 (655)

Bracketed numbers represent total unique peptide numbers for each member of protein

[1] ListiList: http://genolist.pasteur.fr/ListiList/help/function-codes.html

[2] Leger Genome database: http://leger2.gbf.de/cgi-bin/expLeger.pl

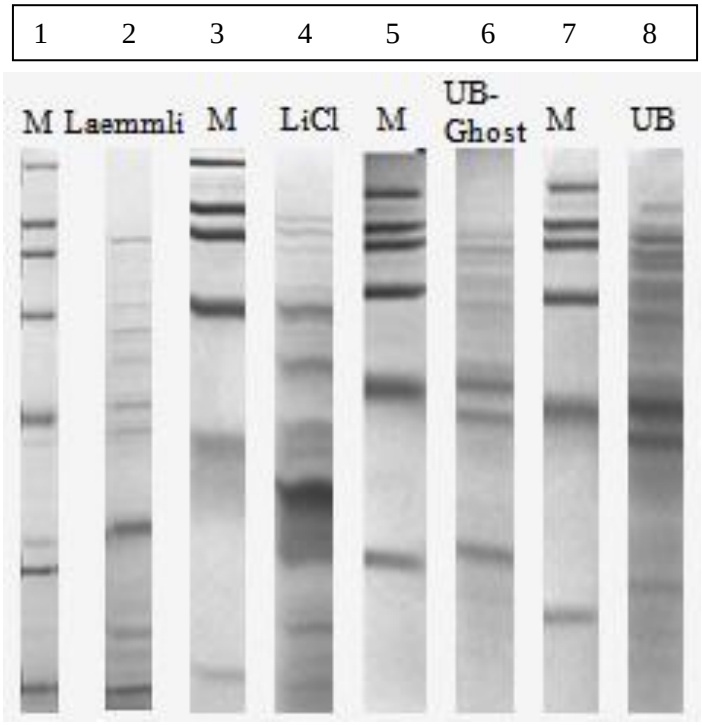

Figure 1. Comparison of protein profiles by 1-D PAGE from total and subcellular proteins prepared by various extraction methods. Lane 1, protein marker (M); lane 2, Laemmli's buffer; lane 3, protein marker (M); lane 4, LiCl; lane 5, protein marker (M); lane 6, UB-Ghost; lane 7, protein marker (M); lane 8, UB.

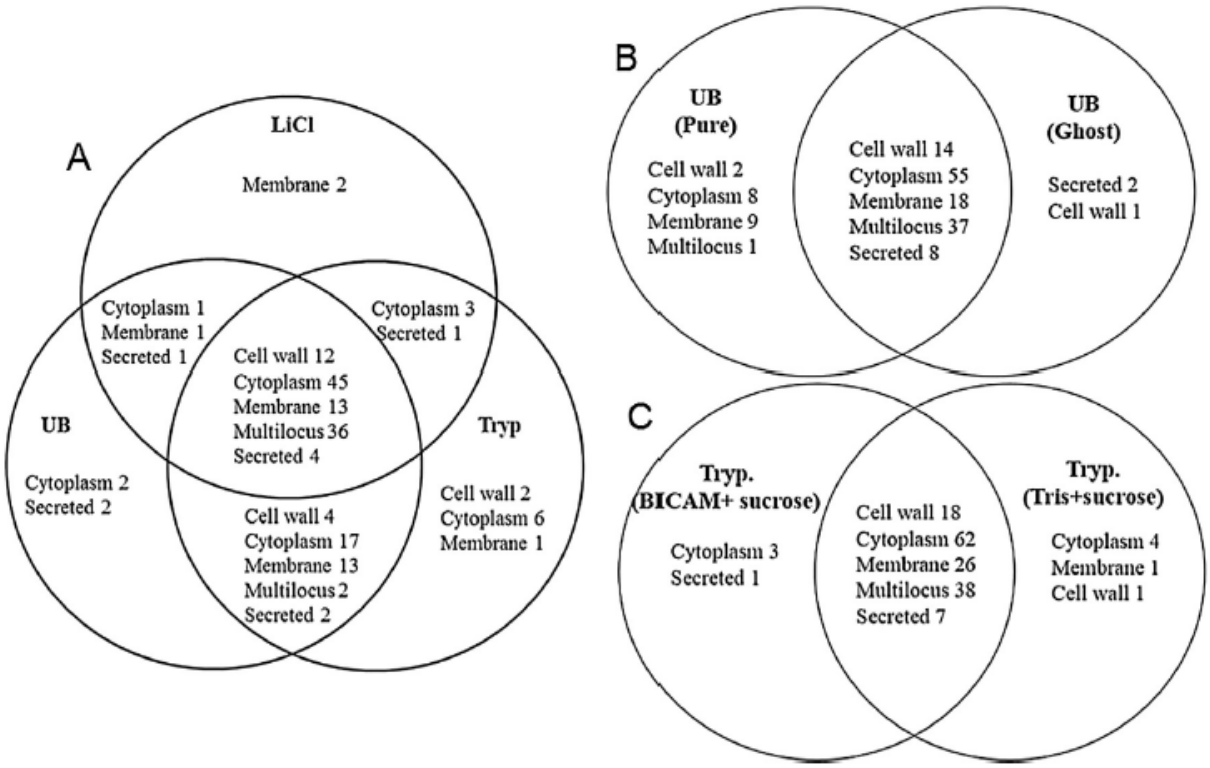

Figure 2. Distribution overlap of proteins identified from different surface extraction procedures. Panel A, LiCl (119) vs. UB (155) vs. trypsinolysis (161); Panel B, UB-Pure (152) vs UB-Ghost (135); Panel C, trypsinolysis B+S (155) vs. trypsinolysis T+S (157). Proteins were identified by LC-MS/MS based on ListiList and other subcellular protein localization tools

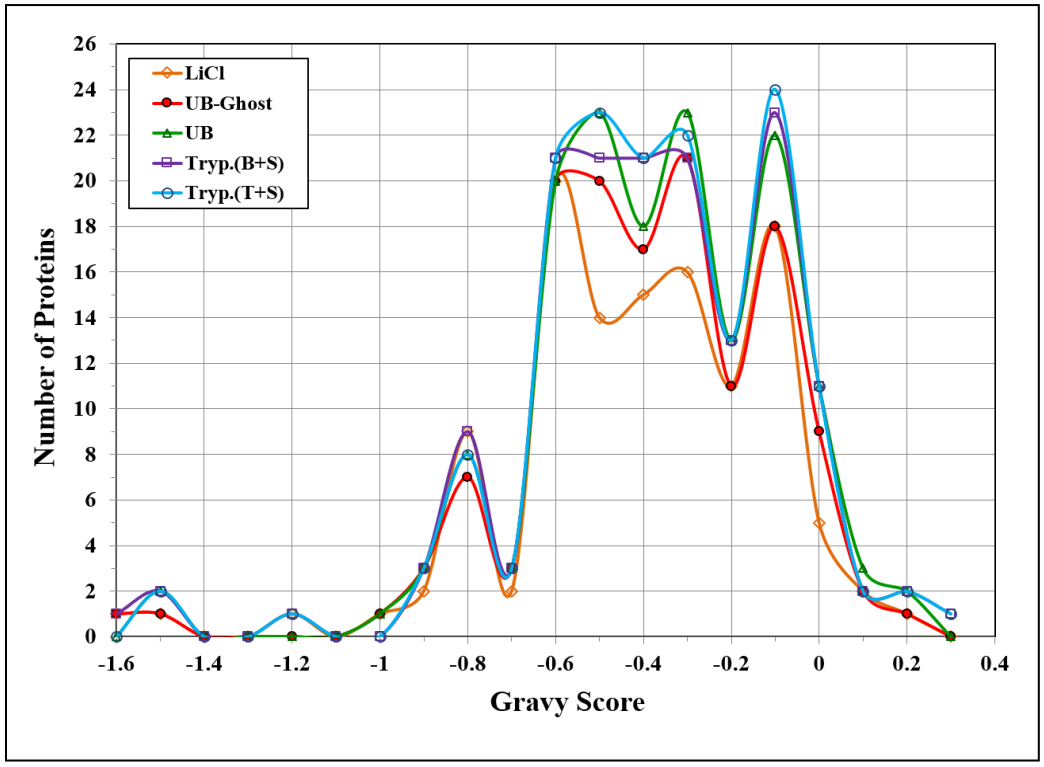

Figure 3. Overview of grand average of hydropathy (GRAVY) of proteins identified from each surface extraction by LC-MS/MS. Negative and positive values represent hydrophilic and hydrophobic proteins, respectively.

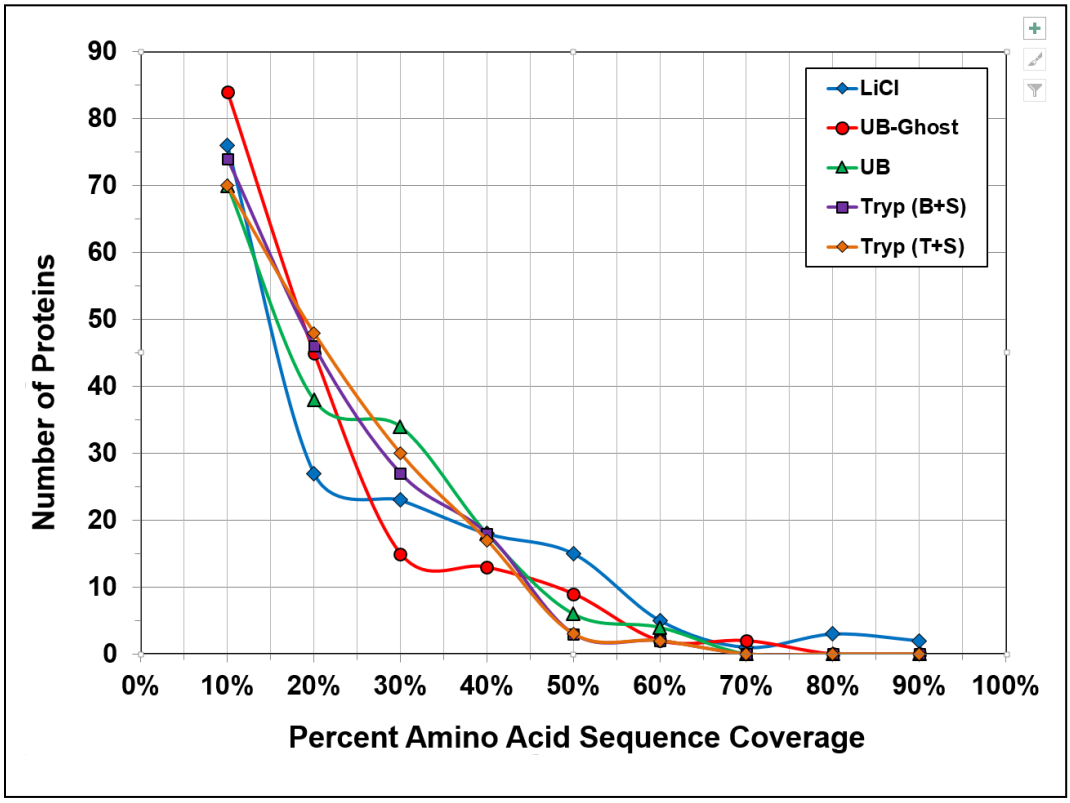

Figure 4. Amino acid sequence percentage demonstrates detection capacities of proteins recovered from each tryptic extract following conventional LC-MS/MS experiments.

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SUPPLEMENTAL DATA

The following are available at **Supplementary data**; http://www.sciencedirect.com/science/article/pii/S0167701215000056, **Supplemental Table 1A:** predicted localizations of surface proteins identified in each extraction method by LC-MS/MS and **Supplemental Table 1B:** predicted localization and physicochemical characterization of cytoplasmic proteins identified from each extraction method by orbitrap analysis.

CHAPTER IV

COMPARISON OF SURFACE PROTEOMES OF ADHERENCE VARIANTS OF *LISTERIA MONOCYTOGENES* USING LC-MS/MS FOR IDENTIFICATION OF POTENTIAL SURFACE ADHESINS

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INTRODUCTION

The cytoplasmic membrane and cell wall layers in Gram-positive bacteria are composed of phospholipids and peptidoglycan, respectively, where both serve as attachment platforms for surface proteins [1,2]. The localization of surface proteins is governed by their signal peptide and various forms of interaction with the cell layers [3-5]. Surface proteins surrounding the bacterial cell may be produced constitutively or facultatively, and may respond to signaling stimuli in the environment. They may aid in transporting organic substrates or inorganic factors, help to secrete metabolic products, assist in attaching to biotic and abiotic surfaces, form biofilms, and help in escaping host intracellular mechanisms of innate immunity [4,6,7]. Surface proteins also provide virulence functions in pathogenic Gram-positive bacteria such as autolysin (AtlE) corresponding to polystyrene adherence of *Staphylococcus epidermidis*, hemolysin (LLO/Hly) involved with phagocytic membrane cytolysis by *L. monocytogenes*, and pyruvate oxidase (SpxB) for hydrogen peroxide resistance in *Streptococcus pneumoniae* [8-11].

Identifying proteins has never been more sensitive, less laborious, and easier due to the availability of improved analytical technologies and biochemical kits [2,12]. However, sensitive analytical detection may be compromised by contaminating proteins/peptides introduced as artifacts during experimental processes, as has been observed when using lithium chloride, tris-buffered urea, and trypsin surface-shaving surface protein extraction methods whereby these methods allow cell leakage of cytoplasmic proteins [13-17]. The Matrix Assisted Laser Ionization Time-of-Flight Mass

Spectrometer (MALDI-TOF MS) analyzes individually-isolated proteins. However, recent advances linking liquid chromatography separation to mass spectrometry has produced powerful proteomic tools (*i.e.*, orbitrap LC-MS/MS) capable of making simultaneous identifications of proteins in complex mixtures [12,18].

Genomic databases are available for *in silico* identification of proteins with homolog function and homology identity of surface-associated proteins involved with pathogenesis. This facility enhances functional genomics by providing insight into pathogenic mechanisms using comparative protein homology studies. To date, many pathogenic protein determinants, separately involved in biofilm formation, cellular adhesion to biotic and abiotic surfaces, internalization in host cells, cellular escape from host defense mechanisms, and proteins involved in survival towards stress conditions have been identified in minimally characterized pathogenic bacteria using such computer-based search tools [3,19-24]. The aim of this study was to exploit cell surface proteomic techniques to identify and quantify recovered pure protein members extracted from the cell surface [16]. Surface-extracted proteins were identified by comparative analysis of cell surface proteomes recovered from strongly and weakly adherent phenotypic variants of *L. monocytogenes* using a gel-less approach of 2D nanoliquid chromatography coupled with an ion-trap mass spectrometry (2DnLC-MS/MS orbitrap) mass analyzer.

MATERIALS AND METHODS

Bacterial Cultures and Growth Conditions

Bacterial cultures were obtained from our laboratory culture collection. *L. monocytogenes* CW35 was isolated from retail RTE frankfurters [73] and *L. monocytogenes* 99-38 was isolated from retail raw ground beef. These strains were previously characterized in microplate adherence assays in comparison to other strains isolated from meat processing plants as weakly or strongly adherent, respectively, to abiotic surfaces [25,74]. Subsequent studies evaluating their interaction with tissue culture cells [75] or live mouse assay [76] demonstrated that the strongly adherent form was more invasive than the weakly adherent phenotype. Bacterial cultures were inoculated into brain heart infusion broth (BHI, Difco, Detroit, MI, USA) and incubated overnight at 30 °C and transferred twice before use in experiments.

Strain Characterization: Adherence Assays, Electron Microscopy, Molecular Typing, Serotyping, and Cellular Hydrophobicity

Microplate fluorescence assay and quantification of adhered cells. The microplate fluorescence assay was performed as described earlier [25,74]. Briefly, culture cells in fresh BHI broth medium were incubated overnight at 30 °C in 96-well microplates (Nunc, Denmark), aspirated and washed with a microplate washer (ELx405 Magna, BioTek Instruments, Winooski, VT, USA), replenished with sterile BHI broth and incubated again for another cycle of growth and washed again to remove loose, planktonic cells. A fluorescent substrate (5,6-carboxyfluorescein diacetate; 5,6-CFDA)

was added to the cells attached to the wells and incubated for 15 min to allow absorptive uptake and cytoplasmic hydrolysis of the substrate. The microplates were washed again on the plate washer to remove external fluorescent substrate and then read on a GENios fluorescence microplate reader (Phenix Research Products, Hayward, CA) with excitation at 485 nm and detection at 535 nm. Attached bacterial cell levels were confirmed by proteolytic release from attached surfaces as described previously [74]. Fluorescence and adherence data were analyzed by one-way analysis of variance (ANOVA) using the Holm-Sidak test for pairwise multiple comparisons to determine significant differences ($P < 0.05$) using the software program SigmaPlot 13.0 (SPSS Inc., Chicago, IL, USA). In prior studies, neither the fluorescent substrate (5,6-CFDA) nor the proteolytic treatment affected the viability of cells as determined with cells in liquid culture.

Scanning electron microscopy. Overnight cultures were diluted in sterile BHI broth and incubated in eight-well CultureSlides (Becton-Dickinson, Franklin Lakes, NJ, USA). The glass slides were washed and submitted for scanning electron microscopy (SEM) analysis by technicians at the Oklahoma State University Electron Microscope core facility.

Molecular typing. Bacterial strains were genotyped using the RiboPrinter Molecular Characterization System (DuPont Qualicon, Wilmington, DE, USA) to generate ribotypes using the restriction enzyme, *Eco*R1 and following the manufacturer's instructions. A phylogenetic tree was developed based on cluster analysis of data by the unweighted pair group method with arithmetic averages (UPGMA).

Serotyping. Serogroup determination of *L. monocytogenes* CW35 and 99-38 was performed by multiplex PCR as described by Doumith *et al.* [77] in comparison to strains of known serotype (*L. monocytogenes* EGDe and V7, 1/2a; ScottA, 4b). Genomic

extraction was prepared on washed, overnight cells by the bead collision method [78] and PCR reactions were performed as multiplex PCR reactions with primer sets of lmo0137, lmo1118, ORF2110, ORF2819, and prs as described [77]. Amplicons were examined on 2% agarose gels after staining with EtBr for documentation.

Microbial adherence to solvent (MATS) assay for cell surface hydrophobicity characterization of *Listeria* strains. Microbial adherence to solvent is based on the surface hydrophobicity of cell envelope components [79]. Briefly, a suspension of 10^8 cfu/mL bacterial cells (prewashed 3x in the same NaCl) in 2.4 mL of 0.15 M NaCl were mixed with 0.4 mL of a solvent (chloroform or hexadecane) with a vortex mixer. The homogenate was allowed to form two phases, and 1 mL of the aqueous phase was removed for absorbance reading at 400 nm. The percentage of microbial affinity to a solvent is evaluated using a formula as follows: $100 \times [1 - (A_S/A_0)]$ = % Affinity; A_0 indicates the optical density at 400 nm of the cell suspension (without solvent added) and A_S is the absorbance of the aqueous phase of the homogenate (with solvent added). Pairwise multiple comparisons for statistical significance ($P < 0.05$) were performed for data within each group of solvents by ANOVA using SigmaPlot 13.0 (SPSS), as described above.

Extraction of Surface Proteins from *Listeria Monocytogenes*

Cells grown in broth. The UB-Ghost method for extracting surface proteins was derived from previous protocols reported by Boone *et al.* [80] and Cordwell [81] and modified by Tiong *et al.* [16]. Cells were grown overnight at 30 °C in 50 mL BHI broth (Difco). Pelleted cells were incubated for 1 h at 4 °C with 600 μL of sterile nanopure water

containing 5 mM EDTA (USB Co., Cleveland, OH), with agitation at 1200 rpm (Pulsing Vortex Mixer, VWR Intl., Atlanta, GA). After incubation, the cells were pelleted by gentle centrifugation (3000× *g*, 6 min, 4 °C), decanted, and the cell pellet was washed three times in 10 mL of 10 mM phosphate-buffered saline (PBS, pH 7.4). After three washes, the final pellet was resuspended in 100 µL 8 M urea buffer and incubated for 30 min at ambient temperature with agitation at 3000 rpm. After incubation in urea buffer, the cells were pelleted by gentle centrifugation (3000× *g*, 4 °C, 6 min) and the supernatant was collected, filtered through 0.45 µm syringe filters (Pall Newquay, Cornwall, UK), and stored at −80 °C.

Cells adhered to glass beads. In an initial overnight incubation with glass beads, *L. monocytogenes* was incubated overnight (~20 h) at 30 °C in screw cap tubes containing 60 mL BHI broth and 80 g of 5-mm diameter soda lime glass beads (VWR Scientific). The spent culture was removed and replaced with 10 mL of fresh BHI broth and the beads were allowed to slowly turn on a rotisserie for 10 min before being replaced again with another 10 mL of fresh BHI broth; after the third replacement with fresh BHI, the tubes were allowed to incubate statically overnight. This process was repeated daily for seven days to promote attachment and enrich for attached cells. After seven days, the spent broth was removed and cells (attached to glass beads) were washed three times (10 min each) in 1x PBS (10 mL, pH 7.4) on a rotisserie; this washing procedure was then repeated with sterile nanopure water containing 5 mM EDTA. The cells/beads were washed one last time by incubating for 1 h at 4 °C in 10 mL sterile water on the rotisserie and the wash fluid was then discarded. Surface proteins of *L. monocytogenes* attached to glass beads were then extracted by rotating the glass beads with 10 mL buffered urea

solution (8 M urea, pH 8.5, 5 mM EDTA, 5 mM β-mercaptoethanol) for 45 min at ambient temperature. The recovered buffered urea solution with extracted proteins was centrifuged (12,000 rpm, 4 °C) to remove contaminating cells and filter sterilized using a 0.45-µm filter. Proteins in the extracted buffered urea solution were precipitated with absolute ethanol (1 volume protein extract:4 volumes absolute ethanol) [82]. Air-dried ethanol-precipitated protein samples were rehydrated in 8 M urea containing 5 mM EDTA and 5 mM β-mercaptoethanol.

Protein Concentration Measurement

Protein extracts were quantified using the Pierce bicinchoninic acid (BCA) protein assay kit (ThermoFisher Scientific, Waltham, MA, USA) combined with the reducing agent compatible (RAC) reagent, as instructed by the manufacturer. Absorbance readings were obtained with a Genesys 20 spectrophotometer at a wavelength of 280 nm (ThermoFisher).

SDS-PAGE analysis

Protein extracts were resolved on 1-D SDS-PAGE gels (12.5%) for visual validation and run overnight at 50 volts in an SE600 Vertical Electrophoresis System (Hoefer, Holliston, MA, USA), as described by Laemmli [83]. Protein extracts were loaded at three different concentrations (1x, 2x, 4x) from 7.2–38.3 µg per well (or lane). The resolved proteins were stained with Coomassie blue R-250 for visualization (Figure 4).

Orbitrap Mass Spectrometry (LC-MS/MS)

Protein samples, accompanied by SDS-PAGE analyses and concentration readings, were analyzed for protein identities and quantities at the Oklahoma State University DNA/Protein Core Facility. A hybrid LTQ-OrbitrapXL mass spectrometer (Thermo Fisher Scientific) coupled to a New Objective PV-550 nano-electrospray ion source and an Eksigent NanoLC-2D chromatography system (Eksigent, Framingham, MA, USA) was used. Protein sample digestion, liquid chromatography, and MS analyses were performed as previously described [16,84] with minor modifications. Briefly, protein fragments were prepared by overnight trypsinization of protein samples in the presence of denaturing (urea), reducing (Tris-2-carboxyethyl-phosphine), and alkylating (iodoacetamide) agents before subjected to liquid chromatography and tandem mass spectrometry (LC-MS/MS). Proteome samples were normalized against total protein by physical loading of equal amounts of protein (0.4 μg of total protein) into the column [85-87]. All reagents were prepared in a Tris-HCl buffer. Peptides were analyzed by using chromatography columns packed with 20 cm of 3-micron Magic C18 AQ particles (Bruker) and eluted using a 3–34% acetonitrile gradient over a period of 105 minutes.

MS Data Analysis, Protein Identification, and Proteomic Analysis

Ion masses were used to identify proteins, as described previously, with minor modifications [16,76]. Briefly, searches were performed with Mascot (Matrix Science, London, UK; v. 2.2.04) and X! Tandem (thegpm.org; CYCLONE, ver. 2010.12.01.1) [88] using the *L. monocytogenes* EGD-e database (5939 protein sequences), downloaded from NCBI on 01/26/11, and supplemented with 112 sequences of common protein

contaminants. Mascot and X! Tandem were searched with fragment ion mass and parent ion tolerances of 0.80 Da and 5.0 PPM, respectively. An allowance of max missed cleavage numbers of 1 and 2 was set for Mascot and X! Tandem, respectively. The searches also included parameters for variable peptide modifications elicited by pyroglutamate cyclization of N-terminal glutamines, oxidation of methionine, acylation of cysteine by acrylamide and iodoacetamide adducts, and formylation and acetylation of the protein N-terminus.

The Scaffold program (Proteome Software, Portland, OR, USA; ver. 2.2.00) and the Peptide Prophet Algorithm were used for validation of peptide/protein identities and construction of Venn diagrams. The identifications were conducted with a protein threshold of 95%, two minimum peptides, and a peptide threshold of 80% [89]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The observed protein false discovery rate (FDR) rate was one percent. Additional tools for proteomic analysis (ListiList, LocateP, PSORT, Cell Wall Predictor, Lipoprotein Predictor, Transmembrane Prediction, SignalP Identification, Hydropathy, and GRAVY values) were used as described previously [16].

Statistical Test for Determining Significant Differential Expression

The Fisher Exact Test [90,91] was performed on spectral count data collected by Multidimensional Protein Identification Technology (MudPIT) analysis of bacterial surface protein digests to validate the significant difference of comparative quantification

by total spectrum counts. The significant threshold and difference, *p*-values, were generated using Scaffold (Proteome Software).

RESULTS AND DISCUSSIONS

Adherence Analysis and Molecular Typing of *L. Monocytogenes* CW35 and 99-38

L. monocytogenes 99-38 was confirmed as a strongly adherent strain using the same microplate assay that was originally used to characterize multiple strains in our collection by showing significantly higher relative fluorescence levels than the weakly adherent CW35 strain (Figure 1A). In spite of using the same cell culture levels (~10^9 cfu/mL) to initiate attachment, strain 99-38 demonstrated a 30-fold greater level of cellular attachment than strain CW35 (Figure 1B). Scanning electron microscopy (SEM) analysis under similar conditions of attachment as in the microplate adherence assays also confirmed low adherence yields by strain CW35 (Figure 1C), while demonstrating an abundance of adhered cells of strain 99-38 (Figure 1D). Genotyping by RiboPrint patterns indicate that although strains CW35 and 99-38 (~86% similarity) do not show identical typing patterns, other strains show a greater disparity in genotype comparison (Figure 2). Serotype examination by multiplex PCR demonstrated that *L. monocytogenes* CW35 typed to serogroups 4b, 4d, and 4e, and strain 99-38 typed to serogroup 1/2a, 3a, both from lineage I (data not shown). Examination of cellular solvent affinity using polar (chloroform) and non-polar (hexadecane) solvents was inconclusive as strain CW35 (weakly adherent) showed a greater propensity to partition into the non-polar/hydrophobic phase than the strongly adherent 99-38 strain (Figure 3). Also, both adherence variant strains showed the least partitioning disparity between polar/non-polar solvents compared to other strongly or weakly adherent strains tested (Figure 3).

Total surface protein identification

Planktonic cells

The adherence of *Listeria* on equipment and food contact surfaces is one of the factors for the high incidence of contamination of foods produced in ready-to-eat (RTE) food manufacturing facilities. Previously, our lab differentiated various adherent phenotypes among strains of *L. monocytogenes* isolated from raw/processed meats and RTE meat processing facilities and classified them as weakly, moderately, or strongly adherent [25]. We have deployed these isolates as a platform into studies on the molecular basis of adherence in *L. monocytogenes* leading to its persistence in food processing environments. Analysis of the published genome of the type strain, *L. monocytogenes* EGD-e [26], revealed a total of 605 protein species associated with the cell wall (132), membrane (335), and secretions (138). However, only three adherence-associated surface proteins (lmo0433, lmo0434, and lmo0435) involved with attachment to abiotic surfaces have been validated to date in this pathogen [27-29]. In the current study, we applied the Ghost urea method for surface protein extraction that reduces contamination by cytosolic proteins, normalized samples based on total protein content (Figure 4), and performed peptide identification by high resolution LC-MS/MS (orbitrap) [16]. We identified a total of 619 protein species in the recovered protein extracts from planktonic cells of two *L. monocytogenes* food isolates, *L. monocytogenes* 99-38 (strongly adherent; 590 proteins) and CW35 (weakly adherent; 408 proteins) and categorized them as surface proteins or cytosolic proteins based on predicted localizations using online prediction tools described previously [16]. The 619 proteins detected from both planktonic strains represents ~22% of the protein species predicted

from genomic analysis of the type strain, *L. monocytogenes* EGD-e (2846 gene coding sequences) [30]. These proteins were repeatedly detected in three separate analyses performed on each of two independently prepared surface extracts (biological reps) from planktonic cells. These replications gave reproducibility rates of 81% and 85% in detecting the same proteins between biological reps for CW35 and 99-38, respectively (Figure 5; Table S1). A total of 92 protein species (of the 619 total detected) exhibited a 99-38 (planktonic)/CW35 (planktonic cells) relative total spectrum count ratio of ≥5-fold, in which 11 protein species were detected in extracts from both strongly adherent planktonic and sessile cells (Table 1) while the remaining 81 species were detected in planktonic cells alone (Supplemental Table S1), of which 11 protein species demonstrated a 99-38/CW35 peptide ratio of ≥10-fold relative expression (Table 2).

Sessile cells

Enrichment of sessile cells was carried out by sub-culturing the strongly adherent *L. monocytogenes* strain 99-38 in media containing glass beads to increase surface attachment area and then extracting proteins from washed adhered cells. A total of 107 proteins were identified, of which 21 proteins were exclusively found in sessile/attached cells and not detected in planktonic surface protein extracts (Table S1).

Envelope protein identification

A total of 124 protein species detected among all cell preparations, including 99-38 planktonic (109 proteins), CW35 planktonic (67 proteins), and 99-38 sessile (14 proteins) surface extracts, were categorized according to the *Listeria* genome database, ListiList [31,32] (Table 4, Supplemental Table S1). The ListiList functional category “cell

envelope and cellular processes" (code 1) includes subcodes for cell wall (1.1), transport/binding (1.2), signal transduction (1.3), membrane bioenergetics (1.4), mobility (1.5), secretion (1.6), cell division (1.7), cell surface (1.8), and cell transformation (1.10). Proteins responsible for signal transduction (1.3) and cell transformation (1.10) were not detected in all cell extracts. This may be due to low abundance of competence-related proteins due to DNA prophage insertions [47], the fact that cell signaling proteins may be membrane bound, or the result of phase-dependent gene regulation [48,49]. Soluble internalin (1.9) was not detected in all cell extracts and may be attributed to the effective washing process used prior to and during surface protein extraction, hence producing much cleaner surface extracts. Unlike the study of Calvo *et al.* [2], this work identified a total of 38 protein species responsible for ListiList cell wall (28 in this study *vs.* 4 in Calvo) and surface (10 in this study *vs.* 15 in Calvo) in planktonic surface protein extracts that are separately responsible for invasion, metabolism, cell envelope biosynthesis, penicillin binding, peptidase, transportation, and rod shape determination. A smaller number of such protein species (cell wall, 5; cell surface, 4) were detected in sessile surface extracts, indicating enrichment of specific proteins in sessile incubated cells that may be responsible for adhesion, invasion, motility, and cell wall biosynthesis. It is noteworthy that the ListiList-identified cell wall proteins lmo0275 (lmo0275, DNA uptake, not validated), and lmo0394 (P60-like invasion protein homolog, not validated) and the cell surface proteins lmo0204 (ActA, cell-to-cell motility), lmo0434 (InlB, adhesion and invasion) [50], and lmo2713 (internalin-like protein, not validated) were exclusively detected in surface extracts from sessile cells (Table 3), as compared with the

more abundant planktonic protein extracts in this study and as observed by Calvo *et al.* [2].

To date, only three surface adhesins responsible for *Listeria* attachment to abiotic surfaces have been identified. Of these, two are known *Listeria* invasins of human hosts (internalins A and B) [27,28]. The data mentioned above suggest that bifunctional ('moonlighting') proteins may be involved in the process of *Listeria* attachment to abiotic surfaces and in virulence. Recently, Piercey *et al.* [51] attributed regulatory functions to internalins A and B, further strengthening the involvement of a protein in multiple functions such as virulence, attachment, and biofilm formation. Bae *et al.* [52] demonstrated that a *L. monocytogenes* locus *lcp* (*Listeria* cellulose binding protein, LCP) may be involved with binding to carbohydrates on the surface of both host cells and vegetables. Quorum sensing may also be a mechanism by which attachment to select surfaces regulates the expression of certain genes that impact both adherence and virulence. For instance, toxin production by *Staphylococcus aureus* is pronounced when adhered, but not when in a planktonic state [53]. This may be explained by the ready availability of secreted self-regulating molecules on neighboring high-density adhered cells as opposed to dilution of secreted regulatory molecules among low-density/scattered planktonic cells.

Among all the ListiList cell wall and surface protein species identified in this study, the gene expression levels of many of them have been shown to be upregulated (lmo0434, lmo2713, lmo0204), downregulated (lmo2505, lmo2691), or neutral (lmo2558) when investigated by intracellular infection assay [50,54] (Table 5, Supplemental Table S1). *In silico* analysis of all the identified ListiList envelope proteins

for detection of surface-associated signal peptides such as LPXTG/NXZTN [33], GW [55], Lipoprotein [36], SecA [44], and TM [36] by web-based tools revealed that among planktonic/sessile surface extracts, 0/0, 11/1, 0/0, 13/0, 6/0, 28/8, and 57/5 protein species were envelope proteins bearing LPXTG/NXZTN, GW, LIPO, SecA, TM, multiple signals, and unknown signal pathway, respectively (Table 6).

Protein subcellular localization prediction

A total of 503 and 93 protein species were detected in extracts from planktonic (99-38: 481; CW35: 341) and sessile (99-38: 93) cells were designated as non-envelope associated proteins by the *Listeria* genome database using multiple protein subcellular localization tools to analyze for prediction of cell surface-associated proteins. These tools included Leger, LocateP, Psort, CW-PRED, LIPO-PRED, transmembrane segment, SignalP, TAT-PRED, and hydropathy plot in which each of them detects a specific surface-associated signal peptide in a protein for prediction of surface protein localization [16]. A total of 389 surface proteins, identified and distributed among sessile (82) and planktonic (377) surface proteins (non-ListiList surface proteins), were mainly ribosomal and hypothetical/unknown proteins, respectively, and are represented in the ListiList functional category (see Supplementary Table S1).

Moonlight proteins have been identified in bacteria, including *L. monocytogenes*, as proteins demonstrating multiple locations and functions [52,57-63]. A number of such proteins include the gene product responsible for the ListiList functional group elongation (lmo1657; lmo2653, translation elongation factor) [64], main glycolytic pathways (lmo2455, enolase) [65], and specific pathways (lmo1634, alcohol

acetaldehyde dehydrogenase) [66]. Burkholder *et al.* [67] reported a surface localization pathway (*i.e.*, SecA2) used by *Listeria* cells to localize lmo1634 on the cell surface. In addition, Chen and others documented the attachment of InlA and InlB surface adhesins/invasins to an abiotic contact surface (*i.e*., glass) [27,28]. These studies confirm multiple locations and functions for some proteins. So many moonlighting proteins have been identified that a database has been established [68].

In our study, both the lmo1634 and lmo2653 gene products were detected in all cell surface extracts (planktonic and sessile) with the total spectrum count ratio of planktonic extracts 99-38/CW35 <2-fold. Other main glycolytic pathway proteins, including glyceraldehyde-3-phosphate dehydrogenase (lmo2459), were identified in all surface extracts. A ribosomal protein adhesin homolog such as L12 (RplL), reported in *Neisseria gonorrhoeae* [69], was exclusively detected in the *L. monocytogenes* (lmo0251) planktonic surface extracts (99-38 and CW35) in this study. However, nothing is known about the adherence-related functions of lmo2459, lmo0251, and the rest of the ListiList functional categories (Table 5). Remarkably, this work exhibited cytoplasmic proteins that were either surface-associated with known or unknown signal peptide, but 16S rRNA proteins were not detected in the extracts, suggesting that the extraction method was reasonably effective in eliminating intracellular proteins.

Variation in the ListiList cell surface and cell wall protein species detected in this work was comparable to the findings reported by Calvo *et al.* [2] and differences may be attributed to different incubation temperatures (30 °C *vs.* 37 °C) as reported by Gorski *et al.* [70], McGann *et al.* [71], and Peel *et al.* [72]. Calvo *et al.* [2] reported a positive correlation between temperature and mRNA levels of *inl*A/*inl*B in a member of *L.*

monocytogenes 1/2a, which is commonly associated with foodborne illness outbreaks. McGann *et al.* [71] observed increased attachment levels (fewer planktonic cells were recovered and hence a lower plate count) in unspecified strains of *L. monocytogenes* pregrown at higher temperatures, and applied separately to radish tissue as compared to the same strains pregrown at lower temperatures, suggesting that different molecular factors were involved in attachment at various temperatures. Peel *et al.* detected increased levels of flagellin when incubated at temperatures <37 °C. Altogether, the various conditions used explain the different protein findings among these separate groups of researchers in the identification of surface protein species.

CONCLUSION

To our knowledge, the current work reveals the first comparative identification of total surface proteins detected in surface extracts of two different adherence phenotypes of *L. monocytogenes* (strongly-adherent 99-38 and weakly-adherent CW35). The key purification step in the UB-Ghost method involved bleeding off cytosolic components prior to surface protein extraction. We consider this as critical for enhanced recovery of surface proteins, as opposed to other proteomic standard protocols involving trypsin, LiCl, or urea extraction buffer where cytosolic contaminants are readily present in the resulting extract [16]. A greater variety of surface-associated proteins identified by LC-MS/MS were represented by moonlight proteins (389), ListiList "envelope and cellular processes" (124), and analyses of surface proteins, as reported by other groups [2,92,93].

Different protein species as well as total spectrum counts were detected in surface extracts of the *L. monocytogenes* adherence variants enriched by planktonic or sessile conditions. A higher number of protein species was detected in the planktonic cell surface extracts (619) as compared with the sessile extract (107). This could be explained by the greater abundance of planktonic cells grown in culture media than sessile cells attached to glass beads. In spite of this imbalance, a group of five ListiList envelope protein species (lmo0275, lmo0394, lmo0204, lmo0434, and lmo2713) were exclusively detected in the 99-38 *L. monocytogenes* surface extract enriched by sessile incubation, as compared with the planktonic extracts in this study or the Calvo group [2]. This may indicate that there were specific protein factors required for sessile activities such as biofilm formation, cell wall maintenance, and cell attachment.

Among the protein species identified, a total of 141 surface-associated proteins (ListiList and non-ListiList, identified by subcellular localization tools) were without signal peptides, suggesting that other signal determinants of protein surface transportation may yet be discovered. Currently, there is no singular all-in-one, up-to-date protein localization tool that includes biochemically validated locations of proteins. For instance, lmo0202 (hly) [50] and lmo1634 (lap) [66,67] have been validated as surface proteins for years but this information has not been updated in the *Listeria* genome database, ListiList [32], or Leger [26,31]. As a result, many web-based protein localization tools were deployed in this study and hence the process was time-consuming and complicated. This information is useful in functional characterization of protein homologs in close bacterial relatives and expedites targeted analysis of virulence factors such as surface adhesins in foodborne pathogenic bacteria.

We feel the data presented herein offer compelling validation towards the use of LC-MS/MS to the detection of bacterial surface proteomes that are expressed under select conditions. Our data show a difference in expression on abiotic surfaces (glass beads) relative to planktonic cells and their adherence phenotypes and warrant further studies in this area. We suspect that additional adhesins may be involved/expressed when *L. monocytogenes* is attached to vegetable surfaces and hope to apply similar methods for extracting surface proteins from *L. monocytogenes* directly attached to produce. These are important food safety issues as more *Listeria* outbreaks have been linked to fruits and vegetables that are often consumed without cooking.

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Table 1. Isolated surface proteins detected in both attached and planktonic cells where expression fold change of planktonic cells 99-38 (strong)/CW35 (weak adherence) ≥5-fold, or not present in planktonic cells (CW35)**;** data are the average of three technical replicates for each of two biological replicates. C: cell wall, CY: cytoplasm, CM: cytoplasmic membrane, E: Extracellular, M: membrane, S: secreted, TM: transmembrane, TMH: transmembrane helix. A star (*) indicates a relative significant difference of total spectrum count between the same proteins detected in planktonic 99-38 and CW35 cells with a *P*-value threshold of <0.02.

Gene (ListiList) [a]	Protein Function Homolog (kDa)	Leger [b]	LocateP [c]	Psort [d]	CW-PRED [e]	PRED-LIPO [f]	Trans [g]	SignalP [h]	PREP-TAT [i]	Hydropathy Score [j]	GRAVY Score [k]	Total Spectrum Count Detected by LC-MS/MS		
												99-38 Attached	99-38 Plank.	CW35 Plank.
lmo0199 * (2.3)	Phosphoribosyl pyrophosphate synthetase (35)	M	CY	0.03 (E,C) CY	No	No	0	No	No	3	−0.03	4	15	2
lmo0415 * (2.1.1)	Endo-1,4-beta-xylanase (52)	CY	M	0.33 (E,C,CM) E	No	Yes (TM)	1	No	Yes (TM)	1	−0.41	3	17	0
lmo0978 * (2.2)	Amino acid aminotransferase (37)	CY	CY	2.5 (CM,E,C) CY	No	No	0	No	No	3	−0.22	4	9	2
lmo1067 * (3.7.4)	GTP-binding elongation factor (69)	M	CY	1.22 (CY,E,C) CM	No	No	0	No	No	3	−0.39	5	16	3
lmo1072 * (2.1.2)	Pyruvate carboxylase (128)	M/S	CY	2.5 (CM,E,C) CY	No	No	0	No	No	3	−0.26	4	22	1
lmo1325 * (3.7.3)	Translation initiation factor IF-2 (85)	CY	CY	0.03 (E,C) CY	No	No	0	No	No	3	−0.45	3	15	1
lmo1504 * (3.7.2)	Alanyl-tRNA synthetase (98)	S	CY	0 CY	No	No	0	No	No	3	−0.37	7	24	4
lmo1519* (3.7.2)	Aspartyl-tRNA synthetase (66)	M	CY	0 CY	No	No	0	No	No	3	−0.29	6	8	0
lmo1663 * (2.2)	Asparagine synthetase (72)	M	CY	2.5 (CM,E,C) CY	No	No	0	No	No	3	−0.43	4	6	0
lmo2558 * (1.8)	Autolysin amidase (102)	C/M/S	Sec	0.02 (C) E	No	Yes (Sec)	1	Yes	Yes (Sec)	3	−0.48	4	70	1
Lmo2608 (3.7.1)	30S ribosomal protein S13 (14)	CY	CY	0.03 (E,C) CY	No	No	0	No	No	3	−0.75	11	5	1

[a] ListiList functional/location classification code [31,32].

[b] *Listeria*'s post-genome database (LEGER) [26]; Updated information to genome database ListiList, agreed upon by the *Listeria* Genome European Consortium [31].

[c] LocateP [33] Distinguish cytoplasmic from other subcellular proteins by identifying the no-N-terminal signal sequence: tat/sec.

[d] PSORTb v3.0.2 protein subcellular localization prediction tool; values represent surface localization score [34].

[e] Cell wall predictor by Fimereli, 2012 [35].

[f] Lipoprotein predictor by Bagos 2008 [36].

[g] Transmembrane segment/helix v. 2.0 prediction [37-39].

[h] SignalP identification of secreted protein by identifying signal peptide and cleavage site [40-43].

[i] Sec and TAT driven secretion system [44,45] ;

[j] Hydropathy plot [46]; Gravy values = Negative indicates hydrophilic protein, >1.8 at each window size of 9 and 19 indicates transmembrane region in a protein (1), <1.8 at each window size of 9 and 19 indicates surface protein (2), <1.8 at window size 19 indicates cytoplasmic protein (3) [46];

[k]Average gravy calculator (http://www.gravy-calculator.de/).

Table 2. Isolated surface proteins found in planktonic 99-38 cells but not in attached 99-38 cells where the 99-38/CW35 ratio ≥10-fold (average of three technical replicates for each of two biological replicates from planktonic cells). C: cell wall, CY: cytoplasm, CM: cytoplasmic membrane, E: Extracellular, M: membrane, S: secreted, TM: transmembrane, TMH: transmembrane helix. A star (*) indicates a relative significant difference of total spectrum count between the same proteins detected in planktonic 99-38 and CW35 cells with a *P*-value threshold of <0.02.

Gene (ListiList) [a]	Protein Function (kDa)	Leger [b]	LocateP [c]	Psort's Protein Localization [d]	CW-PRED [e]	PRED-LIPO [f]	Trans [g]	SignalP [h]	PREP-TAT [i]	Hydropathy Score [j]	GRAVY Score [k]
lmo0220 * (1.7)	ATP-dependent zinc metalloprotease (76)	C/M	M	0.01 (CY) CM	No	Yes (Lipo)	2	Yes	Yes (Sec)	1	−0.37
lmo0392 * (5.2)	Hypothetical protein (34)	M	M	2.5 (CM,E,C) CY	No	Yes (TM)	1	No	No	3	0.16
lmo0723 * (1.5)	Methyl-accepting chemotaxis protein (66)	CY	M	0.04 (CY) CM	No	Yes (TM)	2	No	Yes (Sec)	1	−0.2
lmo1068 * (6)	Hypothetical protein (31)	M/S	Extracellular (Lipid anchored)	(Equal score to all) Unknown	No	Yes (Lipo)	0	Yes	Yes (Sec)	1	−0.67
lmo1076 * (1.1)	Autolysin (64)	S	Sec	0.02 (C) E	No	Yes (Sec)	1	Yes	Yes (Sec)	1	−0.61
lmo2033 * (1.7)	Cell division protein (46)	M	CY	2.5 (CM,E,C) CY	No	No	0	No	No	3	−0.06
lmo2157 * (5.2)	Hypothetical protein (71)	CY	CY	2.5 (CM,E,C) CY	No	No	0	No	No	3	−0.35
lmo2206 * (4.1)	Clp protease subunit B (98)	M	CY	0.03 (E,C) CY	No	No	0	No	No	3	−0.37
lmo2414 * (2.2)	Aminotransferase (48)	C,M	CY	2.5 (CM,E,C) CY	No	No	0	No	No	3	−0.23
lmo2510 * (1.6)	Preprotein translocase subunit (95)	M	CY	0.03 (EC) CY	No	No	0	No	No	3	−0.46
lmo2748 * (5.2)	Hypothetical protein (16)	CY	CY	2.5 (CM,E,C) CY	No	No	0	No	No	3	−0.40

[a] ListiList functional/location classification code [31,32].

[b] *Listeria*'s post-genome database (LEGER) [26]. Updated information to genome database ListiList and agreed upon by the *Listeria* Genome European Consortium [31].

[c] LocateP [33]. Distinguish cytoplasmic from other subcellular proteins by identifying the no-N-terminal signal sequence: tat/sec.

[d] PSORTb v. 3.0.2 protein subcellular localization prediction tool; values represent surface localization score [34].

[e] Cell wall predictor by Fimereli, 2012 [35].

[f] Lipoprotein predictor by Bagos 2008 [36].

[g] Transmembrane segment/helix v. 2.0 prediction [37-39].

[h] SignalP identification of secreted protein by identifying signal peptide and cleavage site [40-43].

[i] Sec and TAT driven secretion system [44,45].

[j] Hydropathy plot [46]. Gravy values = Negative indicates hydrophilic protein, >1.8 at each window size of 9 and 19 indicates transmembrane region in a protein (1), <1.8 at each window size of 9 and 19 indicates surface protein (2), <1.8 at window size 19 indicates cytoplasmic protein (3) [46].

[k]Average gravy calculator (http://www.gravy-calculator.de/).

Table 3. Isolated surface proteins detected in attached 99-38 cells but not in planktonic 99-38 cells (average of three technical replicates for each of two biological replicates from planktonic cells). C: cell wall, CY: cytoplasm, CM: cytoplasmic membrane, E: Extracellular, M: membrane, S: secreted, TM: transmembrane, TMH: transmembrane helix.

Gene (ListiList) [a]	Protein Function (kDa)	Leger [b]	LocateP [c]	Psort's Protein Localization [d]	CW-PRED [e]	PRED-LIPO [f]	Trans [g]	SignalP [h]	PREP-TAT [i]	Hydropathy Score [j]	GRAVY Score [k]
lmo0046 (3.7.1)	30S ribosomal protein S18 (9)	CY	CY	0.33 (E,C,CM) CY	No	No	0	No	No	2	−0.63
lmo0055 (2.3)	Hypothetical protein	CY	CY	0.03 (E,C) CY	No	No	0	No	No	2	−0.25
lmo0186 (5.2)	Hypothetical protein (44)	CY	M	(Equal score to all) Unknown	No	Yes (TM)	1	No	Yes (TM)	1	−0.47
lmo0202 (4.6)	Listeriolysin O precursor (59)	C/M/S	S	0.28 (C,CM,CY) E	No	Yes (Sec)	0	Yes	Yes (Sec)	1	−0.47
lmo0204 (1.8)	Actin-assembly inducing protein (70)	C/M/S	M	CM	Yes	Yes (Sec)	1	Yes	Yes (Sec)	1	−0.80
lmo0214 (3.2)	Transcription-repair coupling factor (135)	CY	CY	0.03 (E,C) CY	No	No	0	No	No	3	−0.33
lmo0241 (5.2)	Hypothetical protein (28)	CY	CY	1.84 (CY,C,E) CM	No	No	0	No	No	3	−0.18
lmo0275 (1.10)	Hypothetical protein (30)	CY	M	(Equal score to all) Unknown	No	Yes (Sec)	0	Yes	Yes (Sec)	1	−0.22
lmo0394 (1.1)	Listeria extracellular P60 protein (25)	CY	S	0.27 (C,CM) E	No	Yes (Sec)	1	Yes	Yes (Sec)	1	−0.33
lmo0434 (1.8)	Internalin B (71)	S	S	0.79 (CM, E) C	No	No	1	No	Yes (sec)	3	−0.46
lmo0707 (1.5)	Flagellar hook-associated protein 2 FliD (validated) (46)	CY	CY	0.28 (C,CM,CY) E	No	No	0	No	No	3	−0.33
lmo0724 (5.2)	27 Hypothetical protein (27)	CY	M	0.45 (CY,C,E) CM	No	Yes (Sec)	1	No	Yes (Sec)	1	−0.17
lmo1272 (5.2)	Ribosomal biogenesis GTPase (33)	CY	CY	1.84 (CY,C,E) CM	No	No	0	No	No	3	−0.44
lmo1480 (3.7.1)	30S ribosomal protein S20 (9)	CY	CY	0.33 (E,C,CM) CY	No	No	0	No	No	2	−0.83
lmo1784 (3.7.1)	50S ribosomal protein L35 (8)	CY	CY	0.33 (E,C,CM) CY	No	No	0	Yes	No	2	−1.22
lmo2156 (5.1)	Hypothetical protein (13)	S	M	(Equal score to all) Unknown	No	Yes (Sec)	1	Yes	Yes (Sec)	1	−0.44

Table 3 (Cont.). C: cell wall, CY: cytoplasm, CM: cytoplasmic membrane, E: Extracellular, M: membrane, S: secreted, TM: transmembrane, TMH: transmembrane helix.

Gene (ListiList) [a]	Protein Function (kDa)	Leger [b]	LocateP [c]	Psort's Protein Localization [d]	CW-PRED [e]	PRED-LIPO [f]	Trans [g]	SignalP [h]	PREP-TAT [i]	Hydropathy Score [j]	GRAVY Score [k]
lmo2505 (1.1)	Peptidoglycan lytic protein P45 (43)	C/M/S	S	0.27 (C,CM) E	No	Yes (sec)	1	Yes	Yes (Sec)	3	−0.55
lmo2619 (3.7.1)	30S ribosomal protein S14 (7)	CY	CY	0.33 (E,C,CM) CY	No	No	0	No	No	2	−0.61
lmo2656 (3.7.1)	30S ribosomal protein S12 (validated) (15)	C/M	CY	0.03 (E,C) CY	No	No	0	No	No	2	−0.76
lmo2691 (1.1)	Autolysin, N-acetylmuramidase (64)	C/S	M	2.18 (C,CM) E	No	Yes (Sec)	1	No	Yes (Sec)	3	−0.74
lmo2713 (1.8)	GW repeat-containing cell wall binding repeat protein (35)	CY	S	0.13 (C,E) CM	No	Yes (Sec)	0	Yes	Yes (Sec)	1	−0.54

[a] ListiList functional/location classification code [31,32].

[b] *Listeria*'s post-genome database (LEGER) [26]. Updated information to genome database ListiList agreed upon by the *Listeria* Genome European Consortium [31]. [c] LocateP [33]. Distinguish cytoplasmic from other subcellular proteins by identifying the no-N-terminal signal sequence.

[d] PSORTb v. 3.0.2 protein subcellular localization prediction tool; values represent surface localization score [34].

[e] Cell wall predictor by Fimereli 2012 [35].

[f] Lipoprotein predictor by Bagos 2008 [36].

[g] Transmembrane segment/helix v. 2.0 prediction [37-39].

[h] SignalP identification of secreted protein by identifying signal peptide and cleavage site [40-43].

[i] Sec and TAT driven secretion system [44,45].

[j] Hydropathy plot [46]. Gravy values = Negative indicates hydrophilic protein, >1.8 at each window size of 9 and 19 indicates transmembrane region in a protein, <1.8 at each window size of 9 and 19 indicates surface protein, <1.8 at window size 19 indicates cytoplasmic protein [46].

[k] Average gravy calculator (http://www.gravy-calculator.de/).

Table 4. Functional classification of surface proteins identified by LC-MS/MS (orbitrap).

Code–Functional Group [a]	Attached *Listeria* Strain 99-38	Planktonic *Listeria* Strain 99-38	Planktonic *Listeria* Strain CW35	*Listeria* Strain EGD-e
1—Cell envelope; cellular processes	14 (2.3%)[b]	109 (17.6%)	67(13.1%)	620
2—Intermediary metabolism	26 (4.3%)	152 (24.9%)	94 (18%)	611
3—Information pathways	59 (13.1%)	139 (30.8%)	106 (26.1%)	452
4—Other	2 (1.3%)	34 (22.8%)	23 (18.1%)	149
5—Similar to unknown proteins	6 (0.8%)	136 (18.2%)	100 (14.3%)	746
6—No similarity	0	20 (7.7%)	18 (8.1%)	260
Total	107(3.8%)	590 (20.8%)	408 (14.4%)	2838

[a]Functional groups of proteins were assigned according to the classification codes of the *Listeria* genome and the LEGER proteome databases [26,31,32].

[b]The number or proteins identified and the percentage relative to total EDG-e proteins in each code category.

Table 5. Cell envelope proteins (surface proteins) expressed in planktonic cells at a 99-38/CW35 peptide ratio ≥ 10-fold, or found in attached cells, identified by ListiList[1] or protein localization tools. A star (*) indicates a relatively significant difference of total spectrum count between the same proteins detected in planktonic 99-38 and CW35 cells with a *P*-value threshold of <0.02.

#	Gene ID	Gene Name	ListiList Functional Category	ListiList Code	Presence In Sessile cells	Expression In Planktonic cells	*In Vivo* Regulation (literature) [a,b]
1	lmo0002 *	dnaN	DNA replication	3.1	---	>10	---
2	lmo0055	purA	Metabolism of nucleotides and nucleic acids	2.3	√	---	---
3	lmo0186	lmo0186	Unknown	5.2	√	---	---
4	lmo0202	hly	Miscellaneous	4.5	√	---	↑
5	lmo0204 [1]	actA	Cell surface proteins protein precursor	1.8	√	---	↑
6	lmo0241	lmo0241	Unknown	5.2	√	---	---
7	lmo0275 [1]	lmo0275	Transformation/competence	1.10	√	---	---
8	lmo0394 [1]	lmo0394	Cell wall	1.1	√	---	---
9	lmo0434 [1]	inlB	Cell surface proteins	1.8	√	---	↑
10	lmo0705 *,[1]	lmo0705	Mobility and chemotaxis	1.5	---	>10	---
11	lmo0707 [1]	lmo0707	Mobility and chemotaxis	1.5	√	---	---
12	lmo0724	lmo0724	Unknown	5.2	√	---	---
13	lmo0898 *	lmo0898	Unknown	5.2	---	>10	---
14	lmo1072 *	pycA	Main glycolytic pathways	2.1.2	√	>10	Neut
15	lmo1272	lmo1272	Unknown	5.2	√	---	---
16	lmo1325 *	infB	Initiation (translation)	3.7.3	√	>10	---
17	lmo1360 *	folD	Metabolism of coenzymes and prosthetic groups	2.5	---	>10	---
18	lmo1544 *,[1]	minD	Cell division	1.7	---	≥10	↓
19	lmo1699 *,[1]	lmo1699	Mobility and chemotaxis	1.5	---	>10	---
20	lmo1784	rpmI	Ribosomal proteins	3.7.1	√	---	---
21	lmo1860	msrA	Protein modification reductase A	3.8	---	≥10x	---
22	lmo1953	pnp	Metabolism of nucleotides and nucleic acids	2.3	---	>10	---
23	lmo2156	lmo2156	Unknown	5.1	√	---	---
24	lmo2415 *,[1]	lmo2415	Transport/binding proteins and lipoproteins	1.2	---	>10	---
25	lmo2505 [1]	spl	Cell wall	1.1	√	---	↓
26	lmo2525 *,[1]	mbl	Cell wall	1.1	---	≥10	---
27	lmo2558 *,[1]	ami	Cell surface proteins	1.8	√	≥10	Neut
28	lmo2656	rpsL	Ribosomal proteins	3.7.1	√	---	---
29	lmo2691 [1]	lmo2691	Cell wall	1.1	√	---	↓
30	lmo2713 [1]	lmo2713	Cell surface proteins	1.8	√	---	↑

NA: No information available in the literature; [a] Chatterjee *et al.*, 2006 [54]; [b] Camejo *et al.*, 2011 [50].

Table 6. Distribution of different signal types of the ListiList envelope protein species identified by LC-MS/MS (orbitrap).

Surface Extract from:	LPXTG/NXZTN [a]	GW [b]	LIPO [c]	Sec [d]	TM [c,d]	GW & Sec	GW, LIPO & Sec	LIPO & Sec	Sec & TM	GW & TM	LPXTG, GW, Sec	LPXTG & Sec	Unknown signal	Total
Planktonic cells	0	11	0	13	6	8	5	8	3	1	1	2	57	115
Sessile cells	0	1	0	0	0	1	2	5	0	0	0	0	5	14

[a] LPXTG was identified by LocateP [33].

[b] GW domain identification tool [55,56].

[c] Lipoprotein predictor by Bagos 2008 [36].

[d] Sec and TAT driven secretion system [44,45].

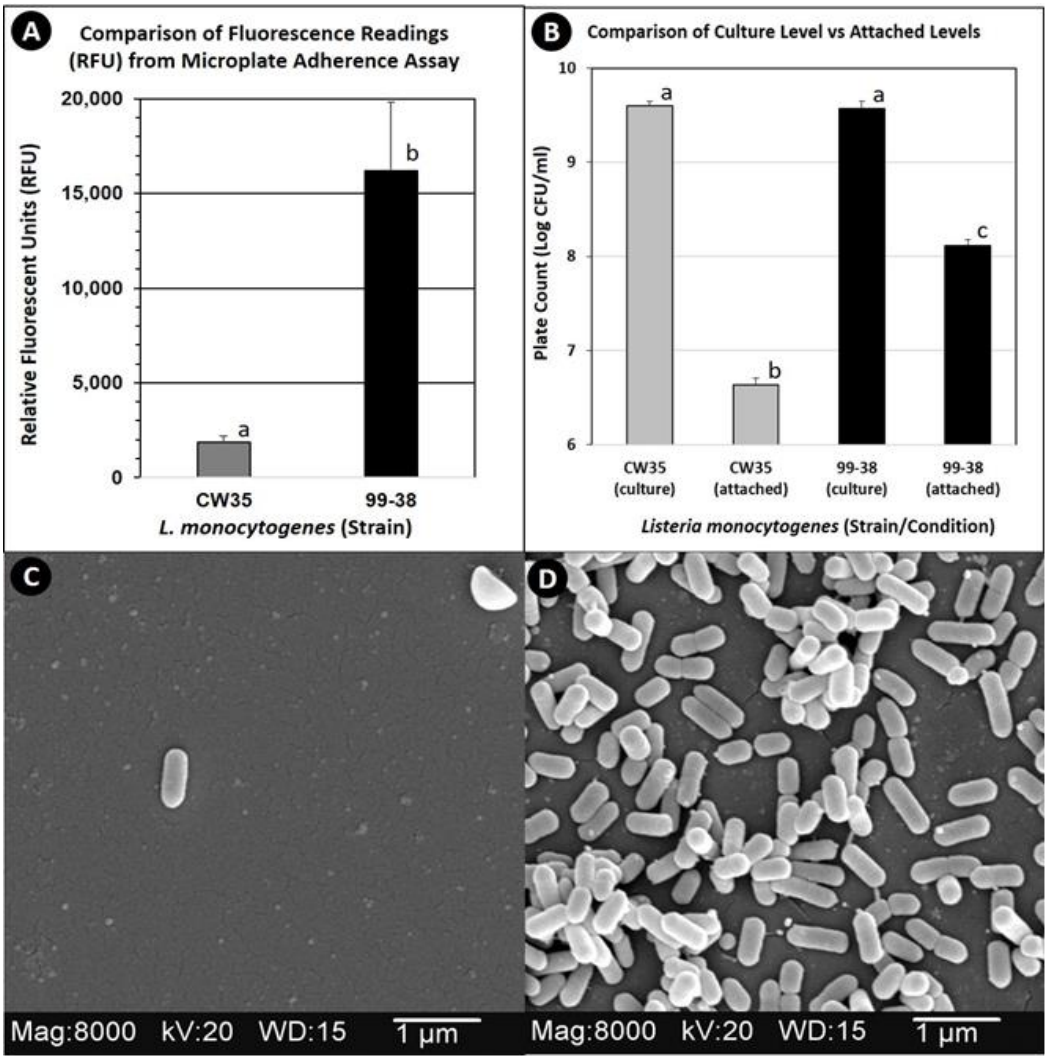

Figure 1. Comparison of weakly adherent *L. monocytogenes* CW35 and strongly adherent *L. monocytogenes* 99-38 by microplate fluorescence adherence assay (panel **A**), enzymatic detachment from biofilms on microplates (panel **B**), and scanning electron microscopy (panels **C** and **D**: CW35 and 99-38, respectively). Graphical data represent the average of triplicate replications and error bars represent the standard deviation from the mean. Bars with the same lowercase letter are not significantly different ($P > 0.05$); bars with different lowercase letters are significantly different ($P < 0.05$).

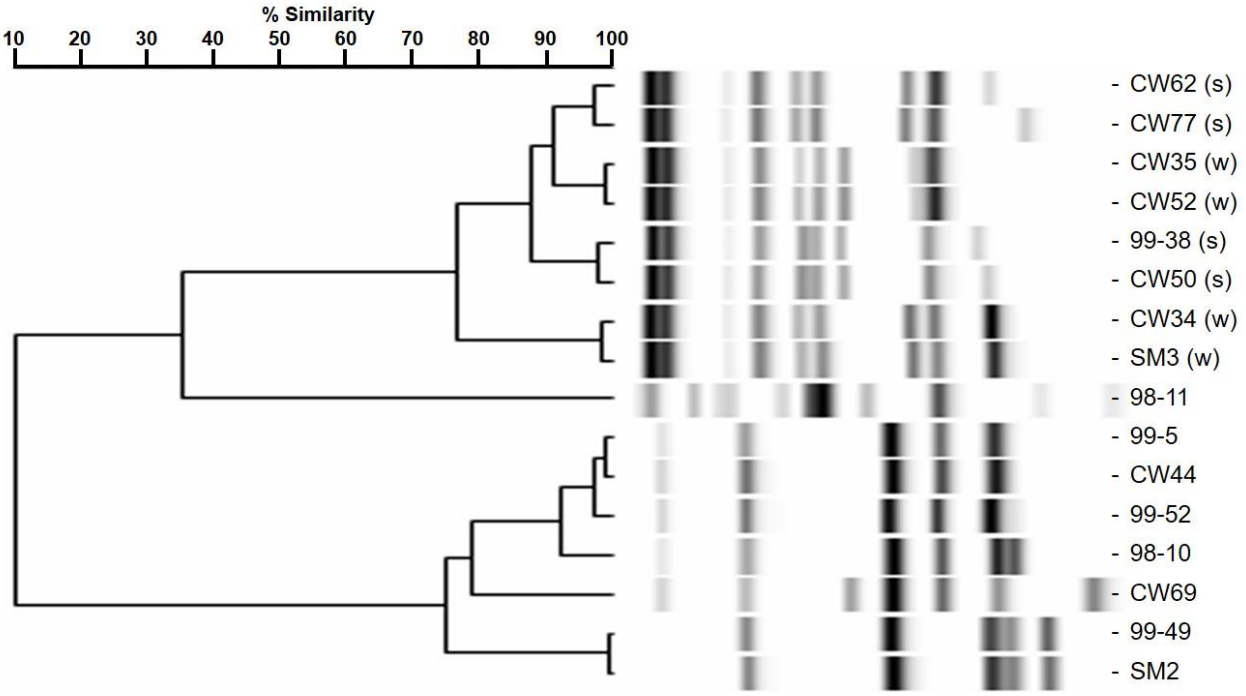

Figure 2. RiboPrint patterns and dendrogram analysis of relatedness for various strains of *L. monocytogenes*, notably CW35 and 99-38 ('s' and 'w' refer to strong and weak adherence, respectively).

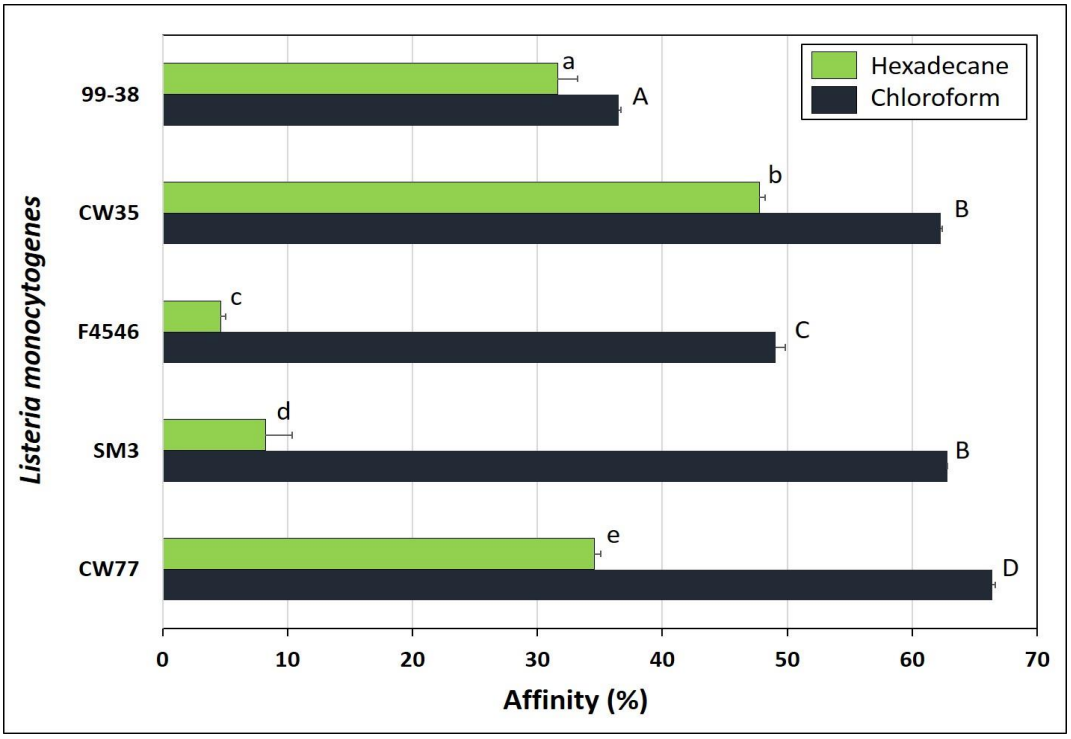

Figure 3. Hydrophobic affinity assays for strains of *L. monocytogenes*. High values indicate hydrophobic tendencies; low values indicate non-hydrophobic (hydrophilic) tendencies. Data bars represent the mean of duplicate samples and replications, and error bars represent the standard deviation from the mean; 's' and 'w' refer to strong or weak adherence. Within a solvent grouping, data bars with the same lowercase or uppercase letter are not significantly different ($P > 0.05$); bars with different lowercase or uppercase letters are significantly different ($P < 0.05$).

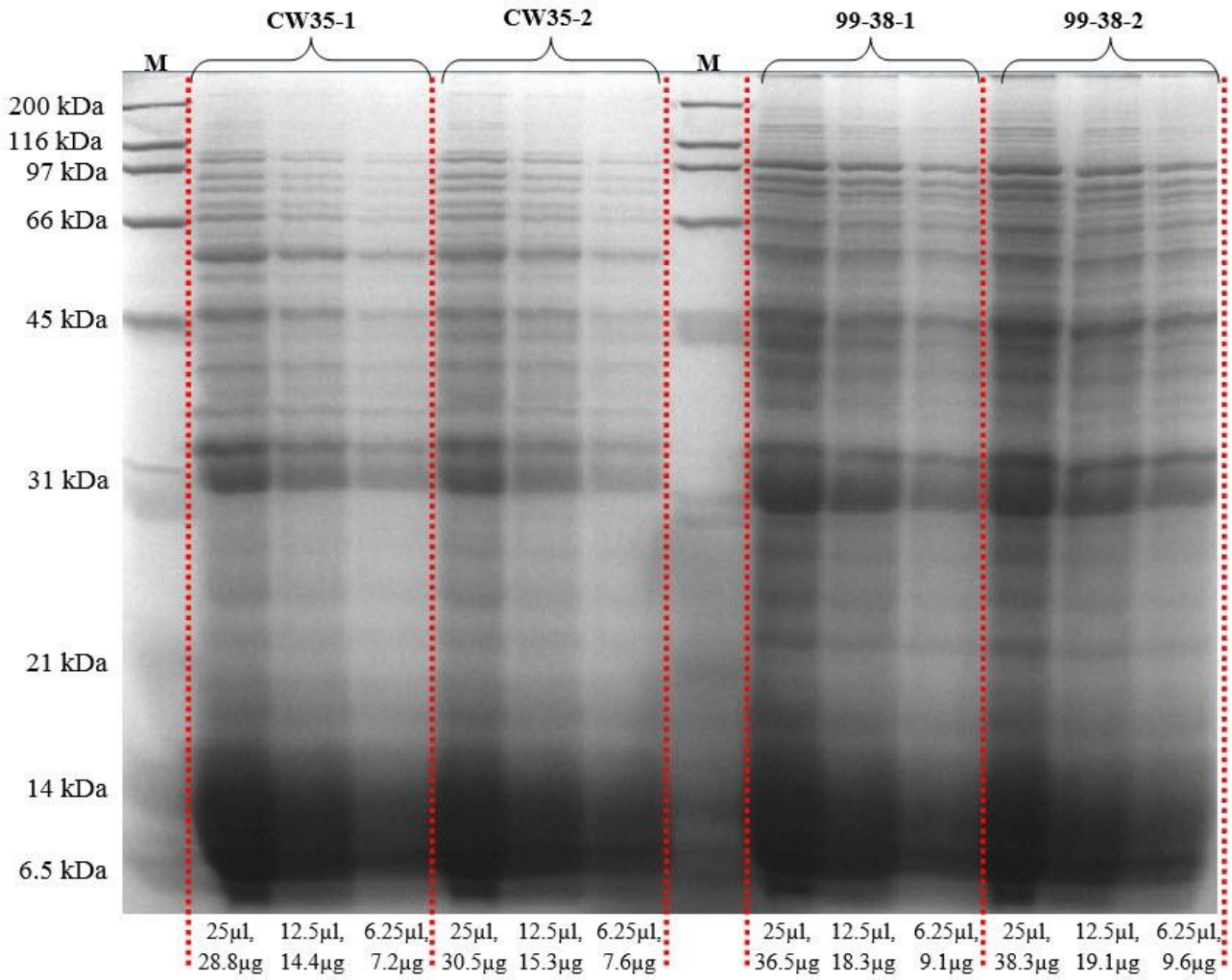

Figure 4. Comparative protein profiles from subcellular proteins prepared by the UB-Ghost protein extraction method examined by 1D SDS-PAGE. Extracts from two biological replications from weakly adherent (CW35) and strongly adherent (99-38) strains of *Listeria monocytogenes.* Protein marker, M; protein amounts below the figure are for total protein loaded per lane.

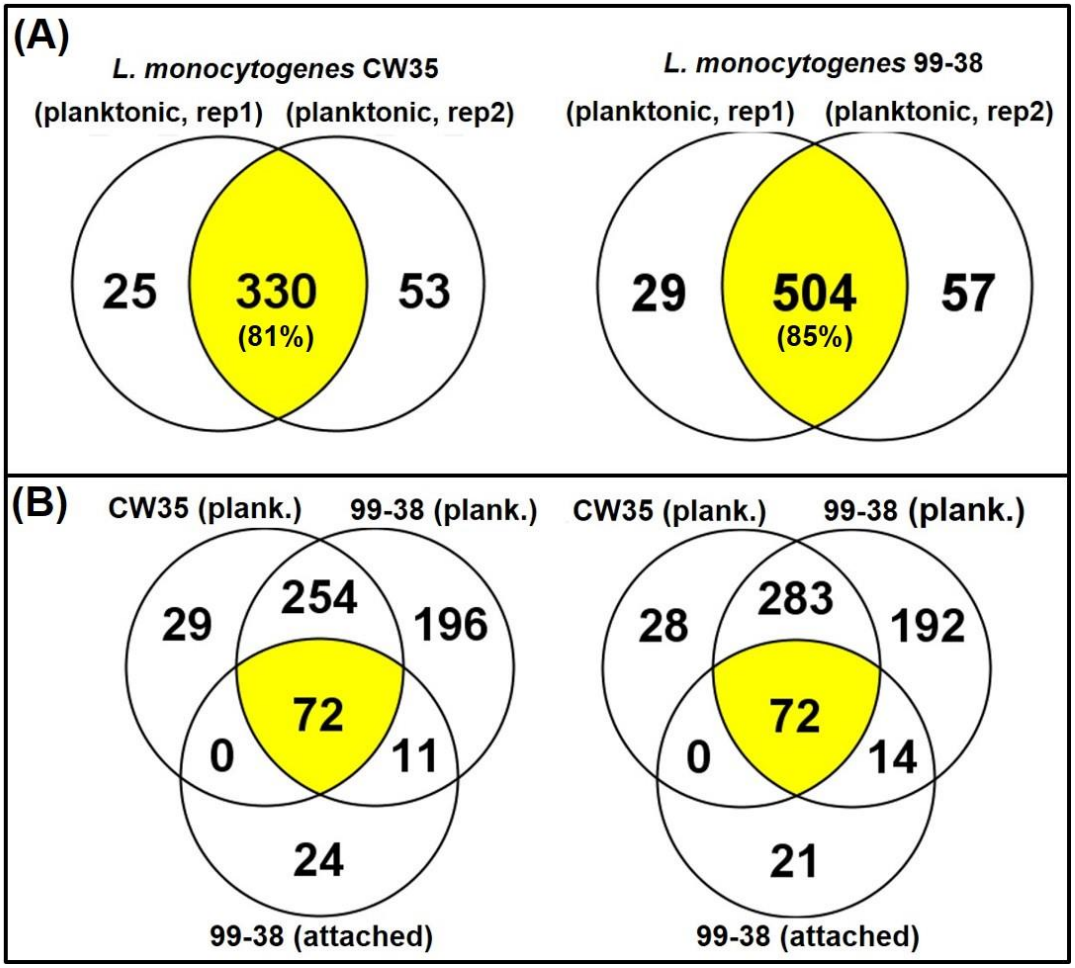

Figure 5. Venn diagrams of identified protein distributions among strains (*i.e.*, CW35, 99-38), biological reps (rep 1 & 2), and cell treatments (planktonic *vs.* attached). Panel **A**, comparison of proteins between biological replications of extractions with *L. monocytogenes* CW35 (**left**) and 99-38 (**right**). Panel **B**, three-way comparison of proteins identified from planktonic cells of CW35, planktonic cells of 99-38, and attached cells of 99-38 in biological replication 1 (**left**) and 2 (**right**). Each biological replicate represents the average of triplicate technical/analytical replications.

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SUPPLEMENTAL DATA

The following are available online at **Supplementary material**;

http://www.mdpi.com/2076-0817/5/2/40, Table S1: Complete MS spectrum count data.

CHAPTER V

RT-QPCR IDENTIFICATION OF GENES ENCODING SURFACE-ASSOCIATED PROTEINS FOR ADHERENCE OF *LISTERIA MONOCYTOGENES* TO ABIOTIC SURFACES

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INTRODUCTION

L. monocytogenes is a Gram-positive, intracellular foodborne human pathogen, capable of surviving antimicrobial hurdles such as limiting oxygen [1], degenerative agents associated with immunological response (phagocytosis) [2], bile salts (10%) [3], and extreme temperatures (-0.4 –50°C) [3]. The systemic disease it causes is termed listeriosis, and it has a multitude of diagnostic manifestations such as miscarriage, muscle pain, stillbirth [4], meningitis [5], septicemia [2], pneumonia [6], corneal ulcers [7], fever, and gastroenteritis [8] in patients. In large outbreaks it has among the highest mortality rates (20-25%) as compared to other foodborne pathogens reported by the Center of Disease Control and Prevention [9].

These stress tolerant characteristics have been linked to the pathogen's molecular defense mechanisms contributed by proteins with essential roles such as biofilm-associated protein (BapL) [10], general stress-response regulation by sigma factor B (SigB), and membrane lysis by Listeriolysin O (Hly) and phospholipases during phagocytosis for cell sustainability/viability to intracellular stresses [2]. Other *Listeria* virulence factors include adhesins for attachment and invasins to gain entry into host cells (InlA, lmo0433; InlB, lmo0434; Vip, lmo0320; Ctap, lmo0135; FbpA, lmo1829; IspC; Ami, lmo2558; LapB, lmo1666; Iap 60, lmo0582) and the cell-to-cell movement mediator actin (ActA, lmo0204) [2].

Persistence of this ubiquitous bacterium in food products manufactured under standard sanitation protocol, especially with ready-to-eat (RTE) foods such as dairy products, processed meats, vegetables, and fish [11,12] have been reportedly caused by cross-contamination of the products with the contacting utensils and food contact surfaces carrying the contaminant *L. monocytogenes*. Biofilm formation following initial adherence increases the cell's resistance to

elimination and removal by the standard sanitation protocols [13-16]. Muriana and others have demonstrated that isolates of *L. monocytogenes* from raw and processed meats and food processing facilities were capable of adhering to numerous substrate surfaces such as stainless steel, polystyrene, rubber, plastic, and glass, and that different strains displayed different degrees of adherence classified as weak, moderate, or strong [17,18]. They also demonstrated that although the weak and strongly-adherence variants adhered equally well to biotic cells, the strongly-adherent strains were more invasive as demonstrated in virulence assays in Caco-2 tissue culture and live mouse assays [19]. Studies by other investigators have demonstrated that adherence strength may be correlated to incubation temperature [3,20]. To date, four surface-associated adhesins, including *inl*A, *inl*B, *bap*L, and the Staphylococcus epidermidis *ami* (lmo2558) homolog *atl*, have been characterized by different groups for attachment to abiotic surfaces [10,21,22]. However, single mutants (*inl*A, *inl*B, *bap*L, *ami*) or double deletions (*inl*A and *inl*B) did not abolish abiotic attachment completely.

In this study, mRNA levels of gene transcription were evaluated for 15 genes encoding cell surface proteins identified in previous experiments as potentially involved with attachment to abiotic surfaces. These results are compared to those of Chen and others who evaluated two genes, *inl*A and *inl*B [23] to establish positive correlations between gene expression and attachment strength of two adherent phenotypes of *L. monocytogenes*. Gene targets were determined based on multiple LC-MS/MS comparative analysis of surface sub-proteome extracts of adherence variants of *L. monocytogenes* (CW35/weak vs 99-38/strong) [24]. A group of 15 genes, including a 16S rRNA reference gene [25], *inl*A [23], and 13 other target genes suggested in LC-MS/MS data were utilized for this purpose [24].

MATERIALS AND METHODS

L. monocytogenes strains.

Initial adherence assays were carried out with eight strains of *L. monocytogenes* (weakly adherent strains: CW34, CW35, CW52, SM3; strongly adherent strains: CW50, CW62, CW77, JAG167, 99-38). Two adherent forms of *L. monocytogenes* (CW35, 99-38) were chosen for further analysis (real-time RT-PCR). All 'CW' strains originated from RTE retail frankfurters whereas strains 99-38 and SM3 were isolated from retail ground beef while JAG167 was isolated from an RTE meat processing plant [9,17,19]. The bacterial strains were cultured by transferring 100 µl of thawed frozen culture suspension into 9 ml of brain heart infusion (BHI) broth (Difco; Becton-Dickinson, Franklin Lakes, NJ), incubated overnight (18 to 24 h) at 30 °C and subcultured twice before experimental tests. Frozen culture stocks were prepared from 9 ml of overnight culture, centrifuged, resuspended in 2 ml of sterile BHI broth (containing 10% glycerol) and stored at -76 °C.

Fluorescent microplate adherence assay.

An adherence ability was characterized as described by Gamble and Muriana [18,19]. A consistent positive correlation between cell adhesion abilities and the viable count has been validated by many groups [17,19,26]. Briefly, each *Listeria* strain was cultured at 30 °C and diluted 5-log in fresh BHI broth, and 200 µl was transferred into designated wells of a sterile 96-well black polystyrene untreated microplates (Nunc, Roskilde, Denmark) with a clear lid, wrapped with Parafilm (Alcan Packaging, Neenah, WI), and incubated at 30 °C for 24 h. Subsequently, the plate was washed three times with Tris buffer (pH 7.4, 0.05 M) in a Biotec

Elx405 Magna automated plate washer (Ipswich, Suffolk, UK) to remove loosely adhered cells, and the plate washer was afterwards sanitized with 200 ppm of sodium hypochlorite (pH 6.5) after each use. The cells were subjected to another cycle of incubation in fresh BHI broth (200 µl), which was followed by washing. After the final incubation and washing, the cells were suspended in 200 µl of 5,6-carboxy-fluorescein diacetate (5,6-CFDA; Invitrogen, Carlsbad, CA) fluorescent substrate solution, incubated at 25 °C for 15 min, washed (as mentioned above), and suspended with the same Tris buffer (200 µl). The plate was then read from above in a Tecan GENios fluorescent plate reader (Phoenix Research Products, Hayward, CA) using a fixed signal gain of 75% (unless otherwise specified) with an excitation wavelength of 485 nm and a detection wavelength of 535 nm.

Extraction, purification and evaluation of chromosomal DNA

Chromosomal DNA was extracted using the glass bead collision method of Coton and Coton with minor modifications [27]. Briefly, pelleted overnight cells of *L. monocytogenes* were resuspended with sterile DI water and spun down twice before subjected to bead collision in Tris buffer (10 mM, pH 8) to shear the cells and release cytosolic components. Chromosomal DNA and cell debris were spun to form supernatant and pellet, respectively. Supernatant containing DNA was aspirated into sterile Eppendorf tubes and stored at -20 °C. The quality of DNA was verified using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific) and PCR.

PCR, DNA agarose gel electrophoresis and sequencing analysis.

PCR mixtures for amplification of genes were prepared according to the manufacturer's directions for GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA). Briefly, each

amplification contained 0.2 mM deoxynucleoside triphosphate mix (Fisher Scientific, Fair Lawn, NJ, USA), 1.5 mM $MgCl_2$ (Promega), 1.25 U GoTaq polymerase (Promega), and 0.4 µM of primers (IDT, Coralville, IA) (Table 2). The reaction conditions were programmed as follows: initial denaturation of 5 min at 95 ºC, followed by 40 cycles of 1 min denaturation at 95 ºC, annealing for 40 s (primer-dependent temperature; Table 1), extension for 60 s at 72 ºC (Table 1), and a final extension cycle of 72 ºC for 10 min before holding at 4 ºC in a PTC-200 thermal cycler (MJ Research, Bio-Rad, Hercules, CA, USA). All nucleotide oligomers used in this study were generated from the specific DNA sequences of the *L. monocytogenes* type strain EGDe (NCBI) type strain by Integrated DNA technology (IDT).

PCR products were examined by agarose gel electrophoresis and purified using a Wizard SV Gel and PCR clean-up kit (Promega), and submitted to the Department of Biochemistry and Molecular Biology Recombinant DNA and Protein core facility (Oklahoma State University, Stillwater, OK) for sequence identification with a ABI 3730 DNA analyzer.

Total RNA extraction, purification, cDNA synthesis, evaluation, and real-time reverse transcription PCR.

Bead attached cells. Strains of *L. monocytogenes* were grown in screw cap bottles containing glass beads (5 mm, 80 g) immersed in BHI broth for 18 hr at 30 °C or 42°C. Each day (for 6 days), bottles of *L. monocytogenes* incubated with glass beads were decanted, washed (1x PBS) on a rotating machine (10 min per wash), and followed by another six daily cycles of incubation in fresh BHI prior to cell harvesting for total RNA extraction. At the end of incubation and washing, attached cells were harvested by gentle shaking with a reciprocating

vortex shaker (MRC, Cincinnati, OH, USA) using RNAzol®RT solution and transferred into sterile Eppendorf tubes.

Planktonic cells. Pelleted cells of various strains of *L. monocytogenes* in sterile Eppendorf tubes were prepared from 1 ml of overnight cultures in BHI broth at 30 °C or 42 °C, and washed 3 times by suspension with 1x PBS prior to total RNA extraction.

Both washed adhered and pelleted planktonic cells were lysed by repeated pipetting in 1 ml of RNAzol®RT solution (MRC) for total RNA extraction, as instructed by manufacturer. Residual DNA was removed with gDNA wipe-out reagent included in the QIAGEN QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA, USA) as instructed by the manufacturer. A 2.8 µl reaction mixture of genomic DNA (gDNA) wipe-out solution contained 0.15 µg of total RNA, 0.4 µl of gDNA Wipeout Buffer (7x), and RNase-free water. This reaction mixture was subsequently incubated in a water bath at 42 ºC for 2 min. The degradation of DNA was verified by PCR amplification of one of the genes to be assayed (lmo0202, *hly*) using RNA extract containing 1 µg of RNA as the potential PCR template. RNA purity and integrity were verified with UV absorbance ratio (260/280) and denaturing agarose gel (1.5%) analysis, respectively. The RNA concentration was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA) measured at 260 nm. RNA samples were kept at -80 °C for storage.

Synthesis of cDNA was performed using the same Qiagen kit above (QuantiTect Reverse Transcription Kit) as described by manufacturer. A 4 µl volume of cDNA synthesis buffer contained 0.2 µl Quantiscript Reverse Transcriptase, 0.8 µl of Quantiscript RT Buffer (5x), 0.2 µl of RT Primer Mix, and approximate 2.8 µl of the remaining reaction product. The reaction was then carried out at 42 ºC for 30 min and finally at 95 ºC for 3 min to inactivate reverse

transcriptase enzyme. The formation of cDNA in the synthesis buffer was verified by PCR amplification of the *hly* gene with 0.5 µl of cDNA synthesis product as the template and agarose gel electrophoresis. The concentration was determined with a NanoDrop ND-1000 spectrophotometer measured at 260 nm. Samples of cDNA were stored at -20 ºC.

Real-time reverse transcriptase quantitative PCR (real-time RT-qPCR) of first-strand cDNA was prepared using the QuantiTect SYBR Green PCR Kit (QIAGEN) and performed in a MyiQ Real-Time PCR Detection System (Bio-Rad) as described by Xiao et al. [28]. Briefly, 10 µl of PCR reaction mixtures contained 5 µl of QuantiTect SYBR Green PCR Master Mix, 0.2 µg of the first-strand cDNA, and 0.3 µM of gene-specific primers as listed in Table 2. The real-time PCR reactions were carried out in 96-microwell plates (Axygen) for production of ~150 bp amplicons: initial denaturation at 95 ºC for 10 min, and 40 cycles of denaturation at 94 ºC for 15 s, annealing at 50–60 ºC (based on individual PCR thermal gradient analysis) for 20 s, and extension at 72 ºC for 1 min. The specificity of PCR amplifications were verified by melting curve analysis and agarose gel electrophoresis of real-time PCR products (between 50–60 ºC and 95 ºC). The relative expression ratios of specific genes of one strain of *L. monocytogenes* to the other were measured based on the crossing point and amplification efficiency (E) values normalized to a reference gene (16S rRNA). Expression ratio analysis used the following relative quantification method, delta Ct [29-31] as derived from Pfaffl's and Livak's $2^{-\Delta\Delta CT}$ method for relative quantification of gene expression to accommodate different PCR amplification efficiencies of a gene:

Relative Expression Ratio = $[(E_{Ref})^{C_{TTest}} / (E_{Target})^{C_{TTest}}] / [(E_{Ref})^{C_{Tcalibrator}} / (E_{Target})^{C_{Tcalibrator}}]$

Expression was relative to a reference gene:

Expression for calibrator or control = $[(E_{Ref})^{C_{Tcalibrator}} / (E_{Target})^{C_{Tcalibrator}}]$

Expression for test = $[(E_{Ref})^{C_{TTest}} / (E_{Target})^{C_{TTest}}]$

PCR amplification efficiency was obtained using the following formula as described [30,32]. The amplification efficiency of primer sets can be found in Table 3.

$E = 10^{[-1/slope]}$

% E = $\{[10^{(-1/slope)}] - 1\} \times 100$

E = Amplification efficiency.

Slope = The slope of the standard curve, X-axis: serial dilution of DNA mass, Y-axis: ct number. Identities of a subset of PCR products (i.e. lmo0202, lmo0723, lmo1293, lmo2505, lmo2656, lmo1076 amplicons) were verified by DNA sequencing at the OSU core facility.

Statistical significant measurement

Comparison studies (attachment strength or expression values) either within each strain or between strains yielded pairs of mean bars with respective standard deviation (error bars). Student's t-test in Sigmaplot 13 was used to analyze each pair of means for determination of significant difference. Statistically significant differences between means compared were called at $P < 0.05$.

RESULTS

Adherence properties of various strains of *L. monocytogenes*.

Eight isolates of *L. monocytogenes* from raw and RTE meats and environmental surfaces were evaluated for their attachment to polystyrene microplate surfaces using the fluorescent microplate adherence assay [18]. Adherence assays of eight previously characterized adherence phenotypes of *L. monocytogenes* confirmed two adherently-variant *L. monocytogenes* groups (Figs. 1, 2A). Strongly-adherent strains (CW50, CW62, CW77, 99-38) gave greater than 10-fold higher RFU signals than weakly-adherent strains (CW34, CW35, CW52, CW72) in the microplate adherence assay, agreeing with previous published findings [17-19].

Gorski noted that *L. monocytogenes* cells exhibited increased adherence to vegetative surfaces when higher incubation temperatures were used [3]. In the microplate adherence assays, both adherence phenotypes of *Listeria* revealed higher adherence at 42 °C incubation temperature than at 30 °C. The findings suggest that temperature may be an important factor impacting adherence of *L. monocytogenes* in food manufacturing facilities (Fig. 3) [3].

Morange et al. [33] and Kushwaha and Muriana [19] further reported that the virulence (i.e., invasiveness) of *L. monocytogenes* was dependent upon incubation temperature and the strong adherence phenotype in *L.* monocytogenes. Thus, the current findings suggest a correlation between virulence and adherence abilities when the *Listeria* cells are exposed/incubated at higher temperatures.

Differential gene expression of two adherence-variant strains of *L. monocytogenes*.

A subset of transcripts of each of *L. monocytogenes* total RNA of weakly (CW35) and strongly (99-38) adherent phenotypes, recovered from various growth conditions such as sessile (bead attached cells), planktonic plus 42 ºC and 30 ºC (control), was quantitated using RT-qPCR in relative to their 16S rRNA transcripts. The growth condition settings were adapted from Hong et al. [34] and McGann et al. [20] for the reasons that the beads used for sessile cells preparation rendered more surface area of growth than a 96-well microplate and the incubation temperature, 42 ºC, was the highest temperature used that rendered significant differential expression of the surface adhesins corresponding genes, *inl*A and *inl*B. Relative transcripts of both strains were obtained using a published relative expression quantification method for analysis of data containing inconsistent amplification efficiencies [30] (Table 4) and the normalized data was plotted in figure 4. Overexpressed genes (expression fold ≥2 or detected only in single strain) were primarily attained in 99-38 cells recovered from planktonic plus 30 °C growth (7 vs 3), plus 42 °C growth (7 vs 3), and sessile plus 30 °C growth (10 vs 2) (Table 5). On the other hand, four overexpressed genes (lmo0202, lmo1293, lmo2505, lmo2656) of both CW35 and 99-38 strains recovered from abnormal growth conditions (Table 6).

Nightingale [35], Chen and others [23] reported that truncated forms of *inl*A/B are common among *L. monocytogenes* food isolates. Our preliminary works indicate that CW35 chromosomal DNA possessed an alterated form of *inl*A gene (3-codon deletion detected in the C-terminus) and thus producing truncated form of InlA protein in all conditions tested (as relative to 16S rRNA mRNA levels).

In addition to expression variations caused by the external factors, the gene of interest might have mutations at the binding regions of primers, which could reduce the PCR

amplification efficiency of gene of compared strains, and hence causing false expression detections [36]. As demonstrated in table 3, the amplification efficiencies of each gene varied among strains tested (Table 2). Consequently, the amplification differences were corrected [29,30,37]. Thus, the expression results were validated.

PCR amplification of genes.

Of six genes with no detectable mRNA levels, two genes (lmo1076, lmo2558) have been reportedly absent in both *L. monocytogenes* serotypes 4a and 4b strains (Tables 4, 5, 6) [2]. PCR analysis of these genes (6) in CW35, 99-38, and EGD type strain genomes prepared by the bead collision method [27], with gene specific primers listed in Table 2, revealed normal (lmo0434, lmo0587, lmo0723) and alterated (lmo1068, lmo1076, lmo2558) genes (results not included). All alterated non-lethal genes were only observed in CW35 strain. Further PCR assays of alterated genes with different set of primers (Table 2) suggested that that the alteration was due to deletion (lmo1076) and nucleotide alteration (lmo1068, lmo2558) (Figure 5), and hence suggesting that CW35 strain possesses alterated lmo1068, lmo1076, and lmo2558 genes. Thus, alterations of lmo1076 and lmo2558 genes agree with the current results and report by Camejo et al. [2].

The function and virulence information of overexpressed genes of *L. monocytogenes*.

The functions of 5 genes were determined by using Leger [38] and ListiList [39] post-genome database for *Listeria* research and functional classification tools, respectively, as they are currently unrevealed [2,40]. They were secreted proteins (2), ribosomal protein S12-like protein (1), methyl-accepting chemotaxis-like protein (1), and unknown protein (1) (Table 7). Of the ten members of gene studied, seven were experimentally characterized as virulence (6) and

non-virulence (1; lmo2713) [41] factors, whereas two were Iap-like proteins (lmo0394, lmo2505), *Listeria* virulence factor [42], and one was not virulence-related as implicated in intracellular down regulation (lmo2691) [43].

DISCUSSION

L. monocytogenes is often detected in food processing plants and its persistence is related to its ability to survive in environments of low temperature, pH, and water activity, and especially the ability to form bioflms.

Molecular factors involved in bacterial adherence to various abiotic surfaces has been documented by various groups [17,18,21,22,44-48]. Researchers have noted that *L. monocytogenes* may have multiple surface adhesins (i.e. InlA, InlB, and BapL) that participate in surface adherence [10,21,23]. It is worth noting that the *bap*L gene is not present in all strongly adherent *L. monocytogenes* isolates [49]. In the current study, a subset of 15 surface-associated gene-specific transcripts overexpressed primarily in the strongly-adherent *L. monocytogenes* strain, 99-38. This could suggest characterizations of a group of potential adhesins.

Listeria strains investigated in this study exhibited more adherence than previous reports [18,19]. This could be caused by the high temperature incubation (42 °C) of *L. monocytogenes* which could result in the high expression of InlA and InlB surface adhesins, as noted by McGann et al. [20]. Chen et al. [21] confirmed that the adherence of *L. monocytogenes* cells on glass surfaces may be enhanced by a synergistic activity of these surface proteins and that it may be positively correlated to their expression levels [23]. Gorski et al. [3] noted that adherence of *Listeria* cells to contact surfaces was independent of flagella, and hence this gene was not analyzed in this study.

When relative gene expression levels were compared between *L. monocytogenes* CW35 and 99-38 strains, the latter strain possessed more overexpressed genes (Table 5). This could suggest that the strongly-adherent *L. monocytogenes* 99-38 expressed more specific proteins that

are potentially involved in surface adherence. The expression profiles of these genes (i.e. lmo0202, lmo0434, lmo1293, lmo2505, lmo2656, lmo2713) were consistent with the protein profiles attained with LC-MS/MS for surface extracts of the 99-38 *Listeria* cells attached to beads (Table 5A) [King, Hartson, and Muriana, 2016]. Of four abundant proteins detected in surface extracts from planktonic cells of 99-38 (30 °C), only one member (lmo0723) correlated with its expression profiles. This inconsistent profiles could partly due to the competitive detection of abundant protein of LC-MS/MS and smaller cell mass used for real-time RT-PCR, as explained by others [50]. Chen and others observed that *L. monocytogenes* attached more strongly when the transcript levels of *inl*A/B were abundant [21,23]. Surprisingly, strain of CW35 demonstrated adherence on beads even though its relative *inl*A transcripts were similar to the control (planktonic, 30 °C) (Table 4). This observation could suggest the presence of other adhesins [19].

Chen et al. [23] and Gorski et al. [3] reported that other surface adhesins are considerable and that attachment is temperature-regulated, respectively. Gene expression analysis of other select surface-associated gene products recovered from sessile or planktonic cells pregrown at 42 °C revealed that most of the genes tested were differentially up-regulated in one strain or another (Tables 4, 5, 6). Of four genes that appear to be upregulated in both strains when held at sessile cells at 42 °C (Table 4), two of the products (lmo0202, lmo1293) have been implicated in *Listeria* adaptation of host intracellular stresses whereas the function of lmo2656 is unknown [41,43,51]. On the other hand, two strain-specific upregulated genes (lmo2691, lmo2713) exhibited intracellular upregulations, as reported by the same groups. Camejo and et al. [2] report of the *Listeria* virulence factors lmo0202 (*hly*), lmo1076 (*aut*), lmo2558 (*ami*), and lmo2691

(*mur*A) involved in vacuole escape, invasion, adhesion, and autolysis, respectively [2]. However, none of them have been reportedly associated with *Listeria* adhesion to abiotic surfaces.

Chen et al. reported that both surface-associated InlA and InlB proteins of *L. monocytogenes* promote equally to mammalian epithelial cell as well as abiotic surface adherence [2,21]. Various groups have revealed that attached cells of *L. monocytogenes* to different substrate surfaces can be easily removed with protein denaturants [18,52], suggesting the proteinaceous nature of adherence factors. A mammalian epithelial cell adhesin, Ami, has been known to have high level of amino acid sequence homology to *Staphylococcus aureus* major autolysin (*atl*E) that contributes to cell adherence to polystyrene and hence suggesting that this gene may also be involved in abiotic attachment [22,53-56].

The genes used in this study were primarily surface-associated proteins (10), unknown (3), and cytoplasmic related (2) (Table 7). The detection of cytoplasmic-surface related proteins by LC-MS/MS analysis of surface extracts of *L. monocytogenes* suggests the involvement of moonlighting proteins that have multiple functions and locations [24,57-62]. Cytoplasmic protein lmo1293 shows considerable involvement in *Listeria* adherence as indicated by overexpressed levels of mRNA under all conditions and strains tested (Table 6). The data presented herein suggests that these genes are worthy of further investigations for potential roles as surface adhesins.

CONCLUSION

Adherence of *L. monocytogenes* to abiotic surfaces is a serious problem impacting sanitation in food manufacturing industries affecting persistence of the organism that may result in contamination of RTE products and human listeriosis transmitted through ingestion of contaminated foods. The ability to adhere promotes initial attachment that can lead to more fully-developed biofilms that are difficult to remove and can resist sanitization regimens. Attachment can be attributed to a group of genes encoding surface adhesins. The current relative mRNA expression study suggested new suspect adhesins based on observations with strain-specific gene expression profiles and inducible gene expression profiles, supported by current literature on the function of closely related proteins. The proteins we examined consisted of 5 functionally unknown proteins (lmo0723, lmo0585, lmo0587, lmo1068, lmo2656), 4 virulence proteins (lmo0202, lmo1076, lmo1293, lmo2558), 2 that were similar to other virulence proteins (i.e., Iap: lmo0394, lmo2505) and 2 that were not associated with virulence (lmo2691, lmlo2713). The additional roles for potential adhesins would additionally qualify them as moonlighting proteins. Knowledge of different conditions that are capable of regulating a group of adhesin genes and understanding the mechanisms leading to *Listeria* attachment, may help prevent facility contamination with the pathogen by manipulating physical and biological conditions. These results imply that more than one surface proteins may regulate the adherence property (jointly or independently) and the role of overexpressed genes in *Listeria* adherence should be further investigated as to whether they contribute to persistent biofilms.

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Table 1. *L. monocytogenes* strains used in this study

Strain[a]	Serotype	Adherence phenotype[b]	Origin of isolation	Reference
L. innocua CLIP 11262	6a	ND[c]	Cheese	[63]
CW34	ND	Weak	RTE retail frankfurters	[9,18,19]
CW35	ND	Weak	RTE retail frankfurters	[9,18,19]
CW50	ND	Strong	RTE retail frankfurters	[9,18,19]
CW52	ND	Weak	RTE retail frankfurters	[9,18,19]
CW62	ND	Strong	RTE retail frankfurters	[9,18,19]
CW72	ND	Weak	RTE retail frankfurters	[9,18,19]
CW77	ND	Strong	RTE retail frankfurters	[9,18,19]
EGDe	1/2a	Strong	Animal (EGD derivative)	[64]
F2365	4b	ND	Cheese	[65]
F6854	1/2a	ND	Frankfurter	[65]
H7858	4b	ND	Meat	[65]
Jag48	ND	Strong	RTE meat processing facilities	[17]
Jag167	ND	Strong	RTE meat processing facilities	[17]
Scott A-2	4b	Weak	Milk (Scott A derivative)	[18,66,67]
99-38	ND	Strong	Retail ground beef	[18,19]

[a]*L. monocytogenes* strains 99-38, CW and Jag were our lab collection.

[b]Determined by microplate adherence assay [18].

[c]ND, not determined.

Table 2. Gene-specific primers used in this study

Gene	Primer sequence[a]	Amplicon size (bp)	Reference
16S rRNA	F: CGGAGCAACGCCGCGTGTATGAAGAA R: TATTACCGCGGCTGCTGGCACGTAGTTA	146	[25,68] This RT-PCR study
lmo0202	F: ACGGAGATGCAGTGACAAATG R: TGGATAGGTTAGGCTCGAAATTG	146	This RT-PCR study
lmo0394	F: GGAAAGTTGGTTATGTTTCAGG R: AAACAGCTTGGGCCAGTAG	145	This RT-PCR study
lmo0433	F: TGTTACAAGAACCTACGGCACCAACAA R: TTGGCGCTATATTGGGCATATAAGGTGATG	145	This RT-PCR study
lmo0434	F: AACCTTTCCTTAGACCGATACG R: TTGGTAGACCGATAGCTTATTCAC	150	This RT-PCR study
lmo0585	F: TGGAACTTCAATCGTGAGTGTTG R: AGTGTTGCGCTTCCTGCTG	147	This RT-PCR study
lmo0587	F: ACAATAGCGTCCGTTGTATCTGG R: TTACTTCAGCCGTTCCACCAC	148	This RT-PCR study
lmo0723C	F: TGGTTTCGCAGTCGTAGCCGAAGAA R: GCTTCGGATTCGGAAAGACCTGTGTTCA	150	This RT-PCR study
lmo1068A	F: TTCTTGGTGGAGATGTAACAACGACGTATT R: ACTTTCTGGGTTACTCGCACTTACTTCTTT	149	This RT-PCR study
lmo1076C	F: CTAATGGTTTATGGTCTGAGGTTCCAGGT R: ACCGCCTACTTGGAATTGATAGTAAGTTCG	146	This RT-PCR study
lmo1293	F: TTAGAAGAAGGCCGTGAGATGG R: GCTTCATGTTGAATTGAGTAGCGTAG	146	This RT-PCR study
lmo2505	F: ATCACGTTCACTTACAAGACCAG R: GAAGATCAAGCAACAGCAATTC	150	This RT-PCR study
lmo2558C	F: AGCTCTAACACTCCAACGAGAAGCTACGA R: TGACGCGACTATATGCAGTGATGGCTTTG	149	This RT-PCR study
lmo2656	F: CACTATGTTCTTGTAAGTTGTGACC R: AACGTGGCGTATGTACTCG	147	This RT-PCR study
lmo2691	F: AATGCAACAAGCTCTTCTACACC R: CATGACAGATGCGTACAGGTC	150	This RT-PCR study
lmo2713	F: AAGGCACGTGAGTCAATCC R: GTAGTAGTGTTAAGTACCTCGGTTCAG	145	This RT-PCR study
1mo1076B	F: CGTTATGCAACGGACAACAC R: ACCATGCCCATCTGCTTTA	150	This PCR study
lmo1076A	F: TATGGCTGCTTTAGTCGTGCCTCA R: TGTCCGTTGCATAACGTCCCTGTA	470	This PCR study
lmo1076D	F: TATGGCTGCTTTAGTCGTGCCTCA R: ACCGCCTACTTGGAATTGATAGTAAGTTCG	991	This PCR study
lmo2558B	F: TTA GGC GGAACAACCCATAC R: AGGCAGTGATTGCTTTATCATATT C	148	This PCR study
lmo2558A	F: TTGCTTCGCGCAACAACAGGATAC R: ACTGTTCCTTTGCCATCACTGTGC	458	This PCR study
lmo2558D	F: TTGCTTCGCGCAACAACAGGATAC R: TGACGCGACTATATGCAGTGATGGCTTTG	1129	This PCR study

Table 2 (Cont.).

Gene	Primer sequence[a]	Amplicon size (bp)	Reference
lmo1068C	F: TAAGTGCGAGTAACCCAGAAAG R: CCCGCCGACAGATTTACTT	149	This PCR study
lmo1068B	F: CTTGGTGGAGATGTAACAACGACG R: TGGATCTGGTACGCCTATTTGCGA	438	This PCR study
lmo1068D	F: TTCTTGGTGGAGATGTAACAACGACGTATT R: TGGATCTGGTACGCCTATTTGCGA	440	This PCR study
lmo1068E	F: CTTGGTGGAGATGTAACAACGACG R: ACTTTCTGGGTTACTCGCACTTACTTCTTT	147	This PCR study
lmo0723A	F: CGCCGTGCTAATTTCCTTATTC R:GCCCAGTTCATCTCTACCATT	148	This PCR study
lmo0723B	F: TGATGGGCGAACAAATCCAAACCC R: AACAGCAAGACGTGATTGTTCCGC	416	This PCR study
lmo0723D	F: TGATGGGCGAACAAATCCAAACCC R: GCTTCGGATTCGGAAAGACCTGTGTTCA	505	This PCR study

[a] F, forward; R, reverse.

Table 3. Amplification efficiency (E) and the percent efficiency (%E) of each pair of primers used in the RTqPCR transcript quantitation. The CW35 and 99-38 genomic DNA was used as template DNA.

Gene	CW35[a] (E)	99-38[a] (E)	CW35[a] (%E)	99-38[a] (%E)
16S rRNA	1.77	1.84	77.18	84.33
lmo0202	1.72	1.82	72.36	81.67
lmo0394	1.66	1.61	66.50	61.16
lmo0433	1.88	1.99	88.00	99.00
lmo0434	1.56	1.52	55.83	51.69
lmo0585	1.64	1.58	64.32	57.66
lmo0587	1.71	1.87	71.41	86.64
lmo0723	NA	1.70	NA	70.20
lmo1293	1.66	1.82	65.75	82.41
lmo1068	NA	1.77	NA	77.27
lmo1076	NA	1.59	NA	59.24
lmo2505	1.71	1.61	71.36	61.37
lmo2558	NA	1.73	NA	73.24
lmo2656	1.72	1.69	72.38	69.31
lmo2691	1.69	1.88	69.17	88.44
lmo2713	1.78	1.53	78.51	52.57

[a] CW35, weakly-adherent phenotype; 99-38, strongly-adherent phenotype.

NA, not available due to no signal (Ct).

Table 4. Relative mRNA levels of 15 genes as compared to the reference gene (i.e. 16S rRNA gene).

Gene name	*L. monocytogenes* CW35			*L. monocytogenes* 99-38		
	Bead-sessile + 30 °C[a]	Planktonic + 30 °C[a]	Planktonic + 42 °C[a]	Bead-sessile + 30 °C[a]	Planktonic + 30 °C[a]	Planktonic + 42 °C[a]
lmo0202	2.73E-04 (1.46E-04)	9.72E-05 (2.74E-05)	4.38E-03 (9.49E-04)	5.69E-04 (2.74E-04)	1.90E-04 (4.93E-05)	3.63E-03 (1.45E-03)
lmo0394	1.29E-05 (1.50E-05)	1.83E-04 (1.51E-04)	0.00E+00 (0.00E+00)	2.28E-03 (1.01E-03)	1.93E-05 (9.66E-06)	8.50E-05 (4.69E-05)
lmo0433	5.19E-06 (3.83E-06)	5.18E-06 (6.96E-07)	1.12E-05 (4.97E-06)	3.63E-06 (1.94E-06)	6.07E-06 (1.32E-06)	5.55E-06 (3.60E-06)
lmo0434	0.00E+00 (0.00E+00)	0.00E+00 (0.00E+00)	0.00E+00 (0.00E+00)	4.46E-03 (1.70E-03)	3.14E-04 (1.10E-04)	3.26E-03 (8.72E-04)
lmo0585	6.00E-04 (1.94E-04)	7.59E-06 (4.86E-07)	0.00E+00 (0.00E+00)	3.73E-04 (2.07E-04)	4.04E-05 (2.42E-05)	1.21E-04 (3.57E-05)
lmo0587	3.33E-04 (1.02E-04)	4.49E-06 (2.55E-06)	3.25E-05 (2.04E-05)	0.00E+00 (0.00E+00)	0.00E+00 (0.00E+00)	0.00E+00 (0.00E+00)
lmo0723	0.00E+00 (0.00E+00)	0.00E+00 (0.00E+00)	0.00E+00 (0.00E+00)	3.04E-04 (1.06E-04)	1.91E-05 (1.33E-05)	6.34E-05 (2.72E-05)
lmo1068	0.00E+00 (0.00E+00)	0.00E+00 (0.00E+00)	0.00E+00 (0.00E+00)	9.11E-05 (2.94E-05)	0.00E+00 (0.00E+00)	0.00E+00 (0.00E+00)
lmo1076	0.00E+00 (0.00E+00)	0.00E+00 (0.00E+00)	0.00E+00 (0.00E+00)	2.92E-03 (5.94E-04)	0.00E+00 (0.00E+00)	0.00E+00 (0.00E+00)
lmo1293	2.70E-04 (1.21E-04)	2.71E-05 (1.60E-05)	3.48E-04 (2.32E-04)	2.76E-04 (1.20E-04)	5.16E-05 (2.08E-05)	1.14E-04 (4.65E-05)
lmo2505	3.38E-03 (9.97E-04)	8.83E-05 (3.77E-05)	2.91E-04 (7.61E-05)	3.81E-02 (9.96E-03)	4.05E-04 (1.49E-04)	2.45E-03 (9.39E-04)
lmo2558	0.00E+00 (0.00E+00)	0.00E+00 (0.00E+00)	0.00E+00 (0.00E+00)	1.01E-05 (8.12E-06)	0.00E+00 (0.00E+00)	0.00E+00 (0.00E+00)
lmo2656	4.11E-02 (1.70E-02)	7.51E-04 (1.55E-04)	3.88E-03 (3.34E-04)	8.70E-02 (2.57E-02)	8.57E-04 (2.69E-04)	3.75E-03 (2.09E-03)
lmo2691	1.03E-04 (7.16E-05)	2.46E-05 (1.79E-05)	0.00E+00 (0.00E+00)	9.67E-05 (2.43E-05)	3.31E-06 (2.46E-06)	7.67E-06 (4.59E-06)
lmo2713	3.79E-07 (1.19E-07)	0.00E+00 (0.00E+00)	0.00E+00 (0.00E+00)	1.07E-02 (2.28E-03)	2.40E-04 (9.88E-05)	1.82E-03 (6.59E-04)

Key: ND = No detectable levels of transcripts.

[a] Expression data represents an average of 2 technical replicates for each of 3 biological replicates with their standard deviation of the mean given in the brackets.

Table 5. Select transcriptional expression comparisons (fold-differences) of *L. monocytogenes* 99-38 and CW35 cells under different conditions

Locus Tag	Planktonic (30ºC)		Sessile (30ºC)		Planktonic (42ºC)	
	99-38	CW35	99-38	CW35	99-38	CW35
lmo0202	1.95	--	2.1	--	--	--
lmo0394	--	9.48	176.7	--	√	--
lmo0433	--	--	--	--	--	2
lmo0434	√	--	√	--	√	--
lmo0585	5.3	--	--	1.6	√	--
lmo0587	--	√	--	√	--	√
lmo0723	√	--	√	--	√	--
lmo1068	--	--	√	--	--	--
lmo1076	--	--	√	--	--	--
lmo1293	1.9	--	--	--	--	3.1
lmo2505	4.6	--	11.3	--	8.4	--
lmo2558	--	--	√	--	--	--
lmo2656	--	--	2.1	√	--	--
lmo2691	--	7.4	--	--	√	--
lmo2713	√	--	28232.2	--	√	--

Key:

-- : Neutral fold-expression; expression not detected in both strains.

√ : Not determined; gene expression was not detected in the other strain.

Table 6. Expression fold differences of 15 genes in sessile (30 ºC) or planktonic (42 ºC) condition compared to their planktonic equivalent at 30ºC

Gene annotation	Sessile (30 ºC)		Planktonic (42 °C)	
	99-38[a]	CW35[a]	99-38[a]	CW35[a]
lmo0202	3.0	2.8	19.1	45.1
lmo0394	118.1	0.1	4.4	NA
lmo0433	0.6	1.0	0.9	2.2
lmo0434	14.2	NA	10.4	NA
lmo0585	9.2	79.1	3.0	NA
lmo0587	NA	74.2	NA	7.2
lmo0723	15.9	NA	3.3	NA
lmo1068	NA	NA	NA	NA
lmo1076	NA	NA	NA	NA
lmo1293	5.4	10.0	2.2	12.8
lmo2505	94.1	38.3	6.1	3.3
lmo2558	NA	NA	NA	NA
lmo2656	101.5	54.7	4.4	5.2
lmo2691	29.2	4.2	2.3	NA
lmo2713	44.6	NA	7.6	NA

[a]Expression fold difference; a ratio of treatment/control.

NA, not available, due to expression levels were not detectable.

Brackets, group of genes that were overexpressed in both *L. monocytogenes* 99-38 and CW35 strains when each condition of sessile and 42 °C was used, as compared to growth at 30 ºC.

Table 7. Functional and virulence information of 15 gene targets

Locus Tag	Gene Name [38]	[a]Subcellular Localization	Function	Virulence Determinant
lmo0202	*hly*	Extracellular [69]	Listeriolysin, vacuole escape [2].	Yes. Validated [2].
lmo0394	--	Extracellular[P]	*Listeria* extracellular P60 protein, Iap-like protein, reduced invasion in mutant [42].	Yes. Not validated [42].
lmo0433	*inl*A	Cell wall[70]	Internalin, promote adhesion to and invasion into host intestinal epithelial cells [2]. Promote adhesion to glass surface [21,23].	Yes. Validated [2,21,23].
lmo0434	*inl*B	Cell wall[70]	Internalin, promote adhesion to and invasion into host liver cells. Involved in placental invasion [2] and adhesion to glass surface [21,23].	Yes. Validated [2,21,23].
lmo0585	--	Unknown[LP]	Putative secreted protein [38,39].	Not studied.
lmo0587	--	Unknown[LP]	Putative secreted protein [38,39].	Not studied.
lmo0723	--	Cytoplasm[P]	Methyl-accepting chemotaxis-like protein [38,39].	Not studied.
lmo1068	--	Unknown[LP]	Unknown function [38,39].	Not studied.
lmo1076	*aut*	Cell wall [2]	Promote entry into different mammalian epithelial cell lines. Virulence factor [2,71].	Yes. Validated [71].
lmo1293	*glp*D	Cytoplasm[P]	Glycerol-3-phosphate dehydrogenase. Promote intracellular virulence [51].	Yes. Validated [51].
lmo2505	*spl*	Cell wall[L]	Peptidoglycan lytic protein P45 [72]. Iap-like protein, reduced invasion in mutant [42].	Yes. Not validated [42].

Table 7 (Cont.).

Locus Tag	Gene Name [38]	[a]Subcellular Localization	Function	Virulence Determinant
lmo2558	*ami*	Extracellular [2]	Autolytic amidase, promote adhesion to mammalian epithelial cells. Virulence factor [2,53-55].	Yes. Validated [53-55].
lmo2656	*rps*L	Cell wall^{L}	Ribosomal protein S12 [38,39].	Not studied.
lmo2691	*mur*A	Cell wall^{L}	Autolysin, N-acetylmuramidase, promote cell separation [73].	No. Not validated [43].
lmo2713	--	Cell wall [43]	Unknown, secreted protein with 1 GW repeat [38,39]. Internalin-like protein [43].	No. Validated [41,43].

[a]Subcellular localization of the gene products were determined using in-silico prediction tools [Leger (L); Psort (P)] as described [40] and experiments.

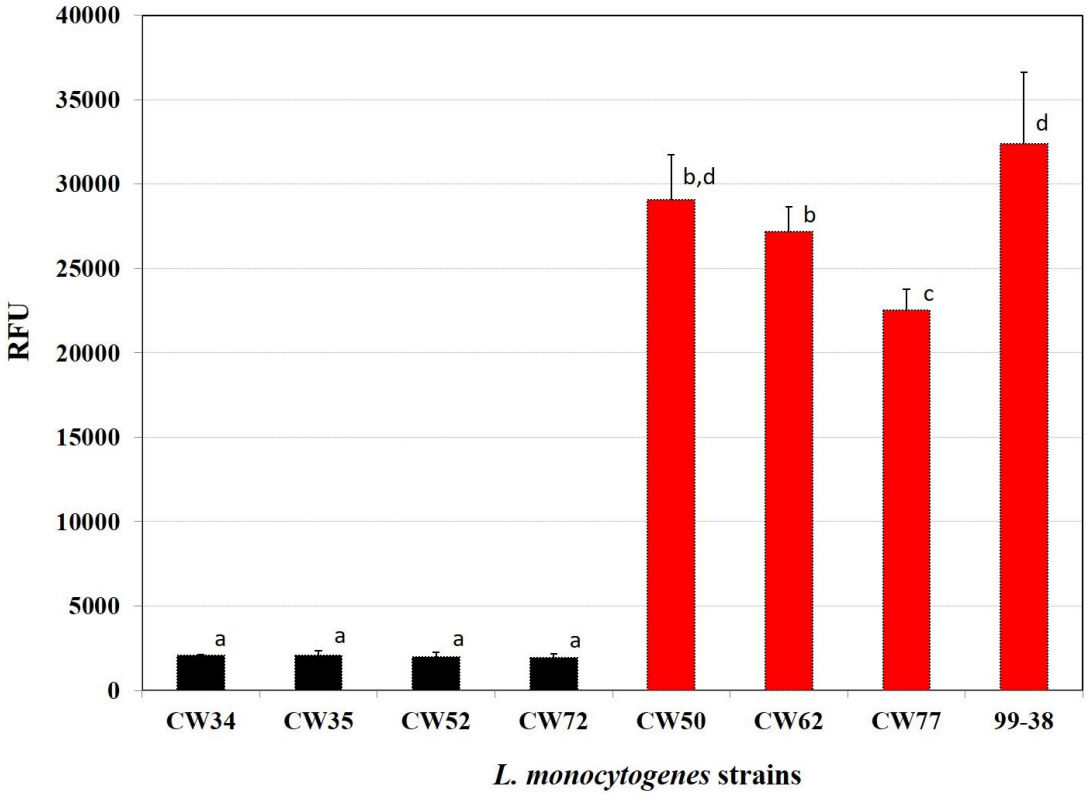

Figure 1. Measurement of relative adherence of various strains of *L. monocytogenes* using the microplate fluorescence (5,6-CFDA) adherence assay. Data bars represent the mean of triplicate replications. Means with the same lowercase letters are not significantly different; means with different letters are significantly different ($P < 0.05$). The error bars indicate standard deviation from the mean. RFU, relative fluorescence units.

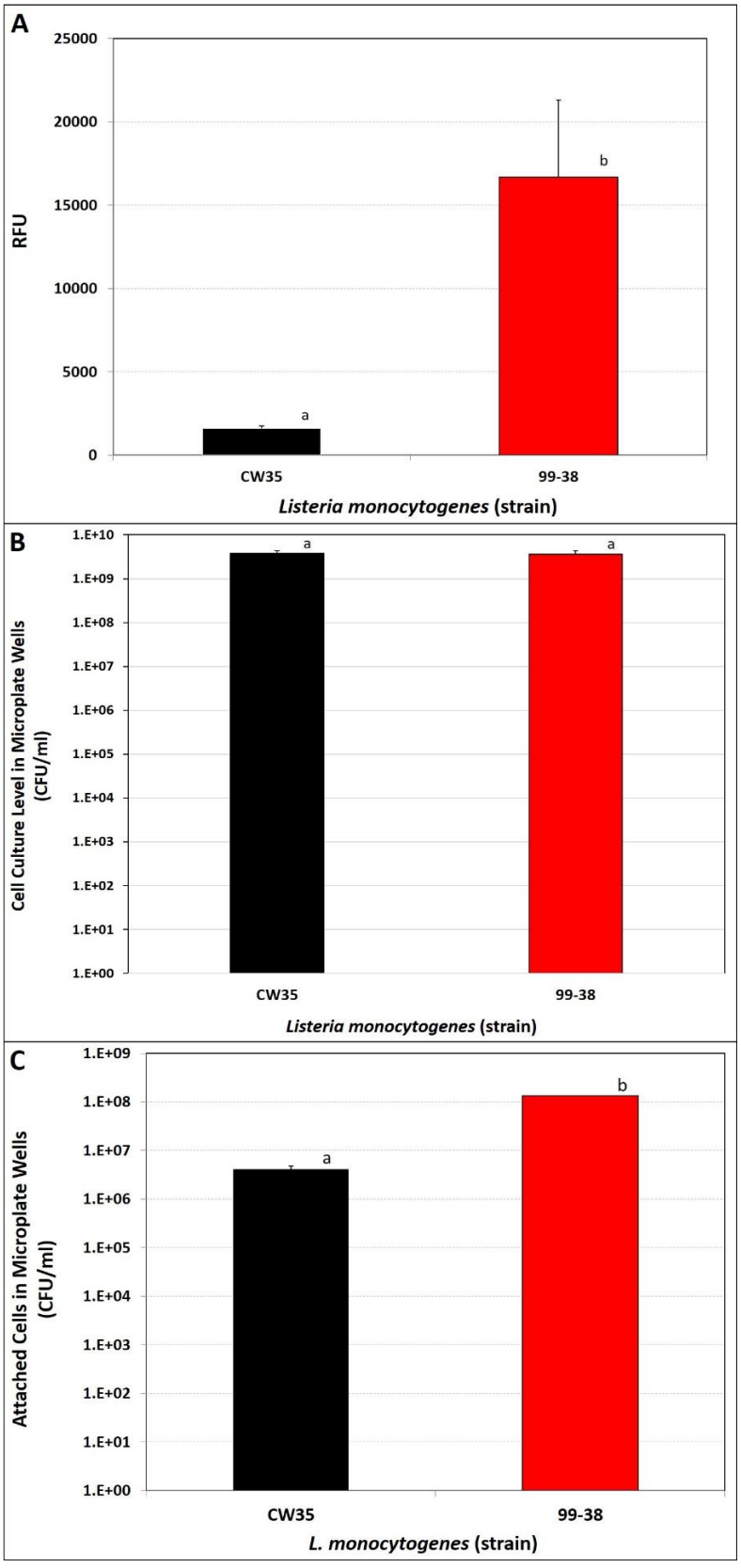
A
25000
20000
15000
10000
5000
0
RFU
a
b
CW35
99-38
Listeria monocytogenes (strain)
B
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
Cell Culture Level in Microplate Wells (CFU/ml)
a
a
CW35
99-38
Listeria monocytogenes (strain)
C
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
Attached Cells in Microplate Wells (CFU/ml)
a
b
CW35
99-38
L. monocytogenes (strain)

Figure 2. Comparison of attachment characteristics of *L. monocytogenes* CW35 (weakly-adherent) and 99-38 (strongly-adherent). Panel A, comparison of attachment by the microplate fluorescence adherence assay. Panel B, enumeration of cell cultures used in Panel A. Panel C, enumeration of attached cells after release by treatment with BAX protease. All data represent the means of triplicate replications. Means with the same lowercase letters are not significantly different from each other; means with different letters are significantly different ($P < 0.05$). The error bars indicate standard deviation from the mean. RFU, relative fluorescence units; CFU, colony forming unit.

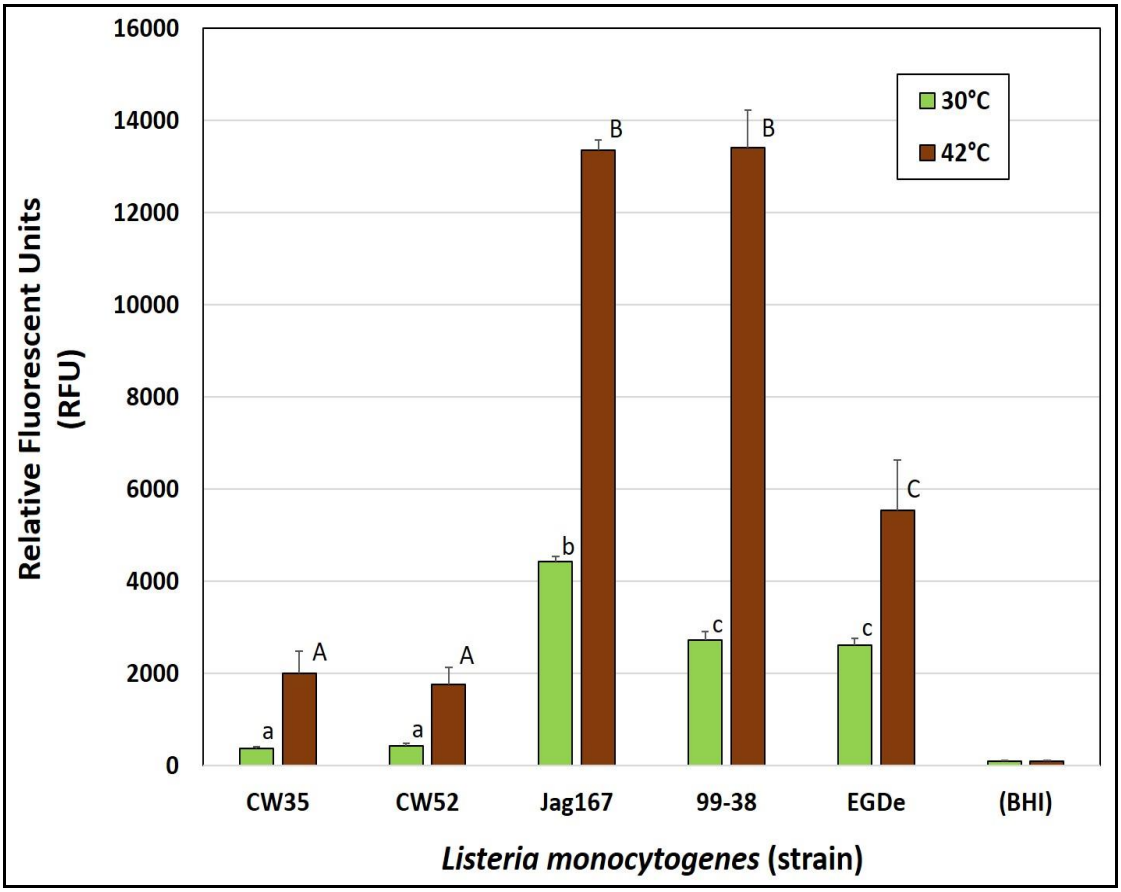

Figure 3. Effect of temperature (30 ºC vs 42 ºC) on attachment of different adherence-variant strains of *L. monocytogenes* (strongly adherent: Jag167, 99-38, EGDe; weakly adherent: CW35, CW52) as determined by the microplate adherence assay. Cultures were allowed to grow overnight in microplates at the respective temperatures after which planktonic (loose) cells were removed by plate washer and attachment was examined by the 5,6-CFDA-based adherence assay. RFU signals were obtained using a fixed manual gain of 65%. All data represent the means of triplicate replications. Means with the same lowercase letters are not significantly different; means with different letters are significantly different ($P < 0.05$). The error bars indicate standard deviation from the mean. RFU, relative fluorescence units.

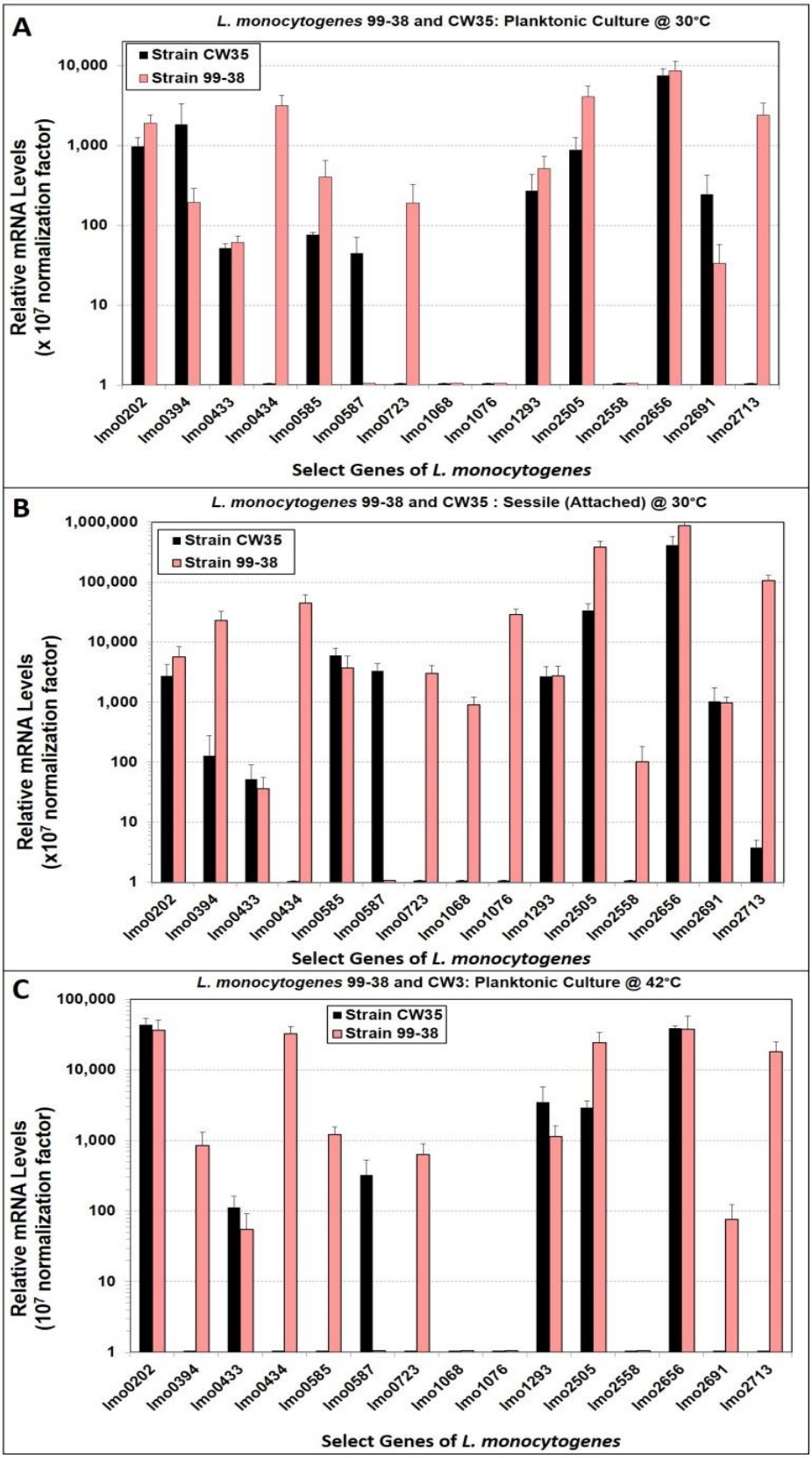
A
L. monocytogenes 99-38 and CW35: Planktonic Culture @ 30°C
Strain CW35
Strain 99-38
Relative mRNA Levels
(x 10⁷ normalization factor)
10,000
1,000
100
10
1
lmo0202
lmo0394
lmo0433
lmo0434
lmo0585
lmo0587
lmo0723
lmo1068
lmo1076
lmo1293
lmo2505
lmo2558
lmo2656
lmo2691
lmo2713
Select Genes of L. monocytogenes
B
L. monocytogenes 99-38 and CW35 : Sessile (Attached) @ 30°C
Strain CW35
Strain 99-38
Relative mRNA Levels
(x10⁷ normalization factor)
1,000,000
100,000
10,000
1,000
100
10
1
lmo0202
lmo0394
lmo0433
lmo0434
lmo0585
lmo0587
lmo0723
lmo1068
lmo1076
lmo1293
lmo2505
lmo2558
lmo2656
lmo2691
lmo2713
Select Genes of L. monocytogenes
C
L. monocytogenes 99-38 and CW3: Planktonic Culture @ 42°C
Strain CW35
Strain 99-38
Relative mRNA Levels
(10⁷ normalization factor)
100,000
10,000
1,000
100
10
1
lmo0202
lmo0394
lmo0433
lmo0434
lmo0585
lmo0587
lmo0723
lmo1068
lmo1076
lmo1293
lmo2505
lmo2558
lmo2656
lmo2691
lmo2713
Select Genes of L. monocytogenes

Figure 4. Relative transcript expression profiles of select genes from weakly-adherent (CW35) and strongly-adherent (99-38) strains of *L. monocytogenes*. Panel A, from cells recovered from planktonic growth at 30 ^{o}C. Panel B, from cells attached to glass beads during growth at 30 ^{o}C. Panel C, from planktonic cells grown at 42 ^{o}C. Expression is relative to that of the reference gene, 16S rRNA. All data bars represent the means of triplicate replications for gene expression RT-qPCR assays. The error bars indicate the standard deviation from the mean. Expression was normalized (x10^{7} factor) to eliminate negative expression levels.

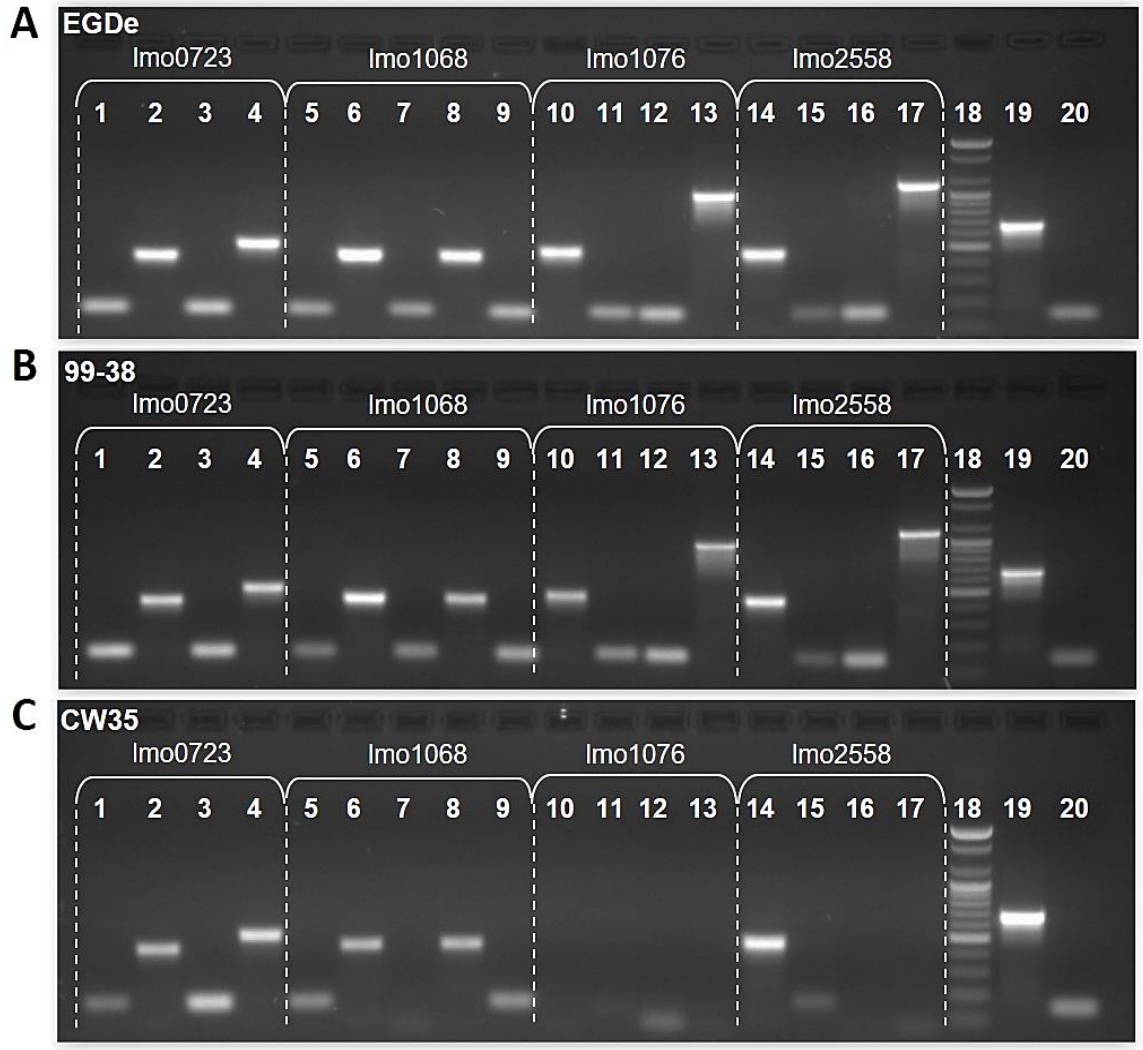

Figure 5. PCR products from genomic DNA of *L. monocytogenes* EDGe (Panel A), 99-38 (Panel B), and CW35 (Panel C) for PCR evaluation of lmo0723, lmo1068, lmo1076, and lmo2558. Multiple (different) gene-specific primer pairs were used to target each gene for PCR amplification and subsequent agarose gel analysis of products. PCR primer combinations were based on *L. monocytogenes* type strain EGDe (Panel A) and tested on 99-38 (Panel B) and CW35 (Panel C). Gene lmo0723: Lane 1, 0723A (148bp); 2, 0723B (416bp); 3, 0723C (150bp); 4, 0723D (505bp); lmo1068: 5, 1068A (149bp); 6, 1068B (438bp); 7, 1068C (149bp); 8, 1068D (440bp); 9, 1068E (147bp); lmo1076: 10, 1076A (470bp); 11, 1076B (150bp); 12, 1076C (146bp); 13, 1076D (991bp); lmo2558: 14, 2558A (458bp); 15, 2558B (148bp); 16, 2558C (149bp); 17, 2558D (1129bp); 18, 100bp DNA ladder; 19 and 20, positive controls.

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APPENDIX

Summarized information for *L. Monocytogenes*

1. A list of virulence factors identified in *L. monocytogenes* (Adapted from [1]Camejo et al., 2011; [2]Wang and Lin, 2008).

Adhesion (biotic)

ActA: Attach and entry by recognition of the receptor protein heparan sulfate...(1)

Ami: Bind to liver epithelial cells.....(1)

CtaP: Promote bacterial membrane integrity and host cell adhesion .....(1)

DltA: Maintain cell wall lipotechoic acid for proper binding to murine macrophage, epithelial cells .....(1)

FbpA: Bind to host fibronectin .....(1)

InlF: Bind to and entry into host cell in host kinase ROCK-deficient mutant.....(1)

InlJ: Bind to the intestinal mucus component MUC2.....(1)

Lap: Bind to host intestinal receptor protein Hsp60 .....(1)

LapB: Bind to and entry into mammalian epithelial cell lines .....(1)

RecA: Contribute to the ability of *Listeria* to bind to and entry into human intestinal epithelial cells .....(1)

Invasion

ActA: Promote invasiveness in macrophage and epithelial cells .....(1)

Aut: Autolytic protein, control cell surface components for pathogenesis, important

for entry into various mammalian epithelial cell lines and cell viability.....(1)

FlaA: Implicated in invasion.....(1)

GtcA: Promote entry into intestinal epithelial cells.....(1)

Iap: Perform murein hydrolase activity for cell division, important for cell internalization and binding to human enterocyte-like cells.....(1)

InlA: Promote intestinal epithelium and placental invasion.....(1)

InlB: Promote hepatocyte and placental invasion.....(1)

Lgt: Invasion and intracellular survival.....(1)

LLO: Vacuole evasion and promote invasion by inducing influx of host Ca^{2+}....(1)

LpeA: Entry into murine hepatocyte andintestinal epithelial cells. Promote survival in macrophage.....(1)

MprF: Promote invasiveness in epithelial cells and macrophage.....(1)

RecA: Implicated in invasion.....(1)

Vip: Control efficient entry into several epithelial cell lines.....(1)

Vacuole escape

ActA: Promote vacuole lysis.....(1)

GAPDH: Delay phagocytic vacuole maturation by inhibiting Rab5a GTPase activity.....(1)

LLO: Cytolysin toxin.....(1)

Lsp: Promote maturation of lipoprotein for successful phagosomal escape.....(1)

PlcA: Cooperate with LLO in the lysis of 1° and 2° vacuoles.....(1)

PlcB: Cooperate with LLO in the lysis of 1° and 2° vacuoles.....(1)

PrsA2: Stabilize LLO and PlcB.....(1)

SipZ: Promote vacuole lysis.....(1)

SvpA: Promote escape from phagosome .....(1)

Intracellular survival and multiplication

Fri: Modulate free iron and H_2O_2, essential for optimum growth .....(1)

Hpt: Uptake host glucose-1-phosphate for *Listeria* energetic requirement .....(1)

LLO: Promote vacuole replication.....(1)

LplA1: Modify pyruvate dehydrogenase for self growth requirement .....(1)

OppA: Uptake oligopeptides for intracellular survival in macrophage and growth in organs.....(1)

PrsA2: Promote viability in host cytosol .....(1)

PycA: Replicate in macrophage and epithelial cells.....(1)

RelA: Uptake amino acid.....(1)

sRNAs (small non-coding RNA): Promote efficient growth in macrophage .....(1)

Intracellular motility and cell-to-cell spread

ActA: Promote intracellular motility .....(1)

InlC: Promote bacterial dissemination by deforming epithelial cells.....(1)

IspC: Promote ActA exposure to *Listeria* surface .....(2)

P60: Promote formation of actin through successful cell-cell division after septum formation.....(1)

Regulators for virulence genes

DegU: Activate GmaR, flagella production (positive regulator).....(1)

GmaR: Inhibit MogR repression, flagella production (antirepressor).....(1)

Hfq: RNA-regulatory protein, confer efficient growth in macrophages, stress tolerance, and virulence .....(1)

MogR: Repress flagella production (negative regulator).....(1)

PrfA: Positive regulatory factor A, global virulence regulatory system, control

expression of virulence genes, important for intracellular viability .....(1)

σ^B: Sigma factor, stress regulatory response, join with PrfA in contribution to intracellular growth and virulence .....(1)

VirR: Control liver colonization, maintain cell wall and membrane for defense.(1)

2. A list of surface adhesins responsible for attachment of *L. monocytogenes* to abiotic surfaces.

BapL..... (Jordan et al., 2008)

InlA .....(Chen et al., 2008)

InlB .....(Chen et al., 2008)

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