Identification of genetic determinants associated with biofilm formation capacity of *Listeria monocytogenes*

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Abstract

Persistence of *Listeria monocytogenes* in food processing plants is a huge health and economic burden. Biofilms are considered to be one of the major mechanisms by which this pathogen persists within these environments. Studies so far have mostly used optimal growth conditions in their investigations which may not provide a realistic understanding of the biofilm forming abilities of *L. monocytogenes* in food processing plants. Therefore the aim of this study was to 1) establish a model (12 ºC, Beef Broth) that closely relates to the food processing environment 2) screen 66 isolates of *L. monocytogenes* from food and clinical sources and determine their biofilm forming phenotypes (non-, weak, moderate and strong formers) and 3) analyze the correlation between biofilm formation phenotypes and biofilm associated genes detected using polymerase chain reaction (PCR) and Basic Local Alignment Search Tool (BLAST) for whole genome sequences. Biofilm formation established at 12 ºC in Beef Broth was the most consistent and quantifiable at day 9 of incubation. Subsequently, 66 isolates were screened using this model, resulting in 60 isolates being identified as strong biofilm formers, 5 isolates as moderate biofilm formers and 1 isolate as a weak biofilm former. Twenty biofilm associated genes were analyzed using PCR in 27 representative isolates. Out of the 20 genes, at least 17 of them were detected in all the tested isolates. Out of the 106 biofilm associated genes analyzed using BLAST, all the isolates were found to show the presence of at least 92 genes. In conclusion, there was no obvious correlation between the presence/absence of the genes selected for analysis and the ability to form biofilms using approaches performed in this study. However, the model established in the study will be useful in further analysis (transcription and translation studies) of genetic markers responsible for biofilm formation of *L. monocytogenes* under food processing conditions.

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List of Abbreviations

A

agrA – Accessory Gene Regulator A

agrB – Accessory Gene Regulator B

agrC – Accessory Gene Regulator C

agrD – Accessory Gene Regulator D

Ami – Autolytic Amidase

AO – Acridine Orange

B

bapL – Bioflm Associated Protein of *L. monocytogenes*

BB – Beef Broth

BHI – Brain Heart Infusion

BLAST - Basic Local Alignment Search Tool

C

CDC – Centre for Disease Control and Prevention

CFIA – Canadian Food Inspection Agency

CFU – Colony Forming Unit

CTC-5-Cyano-2,3-ditoyl Tetrazolium Chloride

D

DAPI - 4'6- Diamidino-2-Phenylindole

DNA - Deoxyribonucleic Acid

E

eDNA – Extracellular DNA

EU - European Union

F

flaA – Flagellin

flgL – Flagellar hook associated protein FlgL

fliD – Flagellar capping protein FliD

fliF – Flagellar MS-ring Protein FliF

fliI – Flagellar Protein Export ATPase FliI

fliP – Flagellar biosynthesis protein FliP

G

gad2 – Glutamate Decarboxylase 2

H

hly – Hemolysin

hpt – Hexose Phosphate Transport

I

iap – Invasion Associated Protein

InlA – Internalin A

InlB – Internalin B

inlJ – Internalin J

L

LLO - listeriolysin

luxS – S-risosylhomocystein lyase

M

MLST – Multi Locus Sequence Typing

motA – Flagellar Motor Protein MotA

motB – Flagellar motor rotation MotB

mpl – Zinc Metalloproteinase

MQ – Milli Q

MWB – Modified Welshimer's Broth

N

NESP – National Enteric Surveillance Program

O

OD- Optical Density

P

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PFGE – Pulsed Field Gel Electrophoresis

PHAC – Public Health Agency of Canada

plcA – Phosphatidylinositol specific phospholipase – C

plcB – phosphatidylcholine-specific phospholipase C

(p)ppGpp – Guanosine Pentaphosphate

prfA – Positve Regulatory Factor A

PVC – Polyvinyl Chloride

Q

QS – Quorum Sensing

R

RAPD – Random Amplified Polymorphic DNA

relA – (p)ppGpp synthetase

RNA – Ribonucleic Acid

rpoB – RNA polymerase B

rrn – 16S rRNA

RTE – Ready-To-Eat

S

SEM – Scanning Electron Microscopy

SigB – Sigma B

T

TSB – Tryptic Soy Broth

TSB-YE – Tryptic Soy Broth- Yeast Extract

U

UK – United Kingdom

USA – United States of America

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Chapter 1: Introduction

1.1 Research Rationale

Listeria monocytogenes is a food borne pathogen that causes a severe illness known as listeriosis in humans (Farber & Peterkin, 1991). Though rare, the high mortality rate of 20-30% associated with infection has placed it on the list of nationally notifiable diseases in Canada (Bortolussi, 2008). *Listeria* is known to inhabit a wide variety of niches including food processing plants. Its persistence within the food processing environment acts as a potential source of contamination of food products, such as ready-to-eat (RTE) meat (Farber and Peterkin, 1991). The ability of *L. monocytogenes* to form biofilms has been thought to significantly contribute to its survival in the food processing environment, where rigorous cleaning and disinfection routines are implemented (Blackman & Frank, 1996; Pan et al., 2006). This has been substantiated by reports that indicate that persistent strains tend to be stronger biofilm formers when compared to their sporadic counterparts (Borucki et al., 2003; Ochiai et al., 2014). The identification of the biofilm formation capacity of *L. monocytogenes* isolates from food and food related environments would provide valuable risk information regarding the potential of persistence to public health regulatory agencies and food industry for further mitigation strategies.

For years, studies have been aimed at understanding biofilm formation by *L. monocytogenes* and identifying the underlying genetic factors that contribute to this phenomenon. However, the results obtained from these studies were sometimes contradictory. All the isolates of *L. monocytogenes* used in the published studies so far have shown the ability to form biofilms and it is believed that any strain of *L. monocytogenes* can form biofilms if it is given the right conditions (Carpentier and Cerf, 2011). However, differences in biofilm forming capacities have been observed depending on

the lineages (lineage I or lineage II), serotypes (4b or 1/2a), source of isolation (food or clinical) and the surface (stainless steel, polystyrene and glass) of attachment tested (Borucki et al., 2003; Di Bonaventura et al., 2008; Djordjevic et al., 2002; Harvey et al., 2007).

Mutagenesis studies have identified numerous genes that influence biofilm formation, illustrating that it is a complicated phenomenon that involves multiple genetic determinants (Chang et al, 2012; Ouyang et al., 2012). However as a result of experimental variation in phenotype studies, a clear link between biofilm forming phenotypes and their genetic determinants are not well established. This variation could be a result of different growth conditions used in every study (Folsom et al., 2006; Milanov et al., 2009; Takahashi et al., 2009). Moreover the use of optimal conditions for biofilm formation renders the outcomes from these studies less relevant to food processing conditions. Therefore the aim of the study was to identify the genetic determinants responsible for varying capacities of biofilm formation of *L. monocytogenes* by correlating phenotypes of biofilm formation using a model (growth condition) that relates to the food processing environment with genotypes of various isolates.

1.2 Research Hypothesis

Certain genetic determinants or a combination of them are associated with biofilm formation capacity of *L. monocytogenes*.

1.3 Research Objectives

1. To study the influence of different growth conditions on biofilm formation and to standardize a biofilm assay protocol in a condition that relates to the food processing environment.

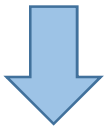
2. To screen isolates of *L. monocytogenes* using the standardized biofilm assay protocol and to divide the isolates into groups based on their biofilm forming potential.
3. To identify genetic determinants responsible for biofilm forming phenotypes using the following approaches:
    a. Analysis of biofilm-associated genes identified in published studies within selected isolates with PCR assays.
    b. Analysis of the presence of biofilm-associated genes identified in previously published studies with BLAST.

Work plan to complete the objectives

Step 1: Establishment of biofilm bioassay mimicking food processing conditions

1. Influence of different growth conditions including food processing conditions on biofilm formation

2. Determination of optimum period of incubation for biofilm formation in food processing conditions



Step 2: Establishment of biofilm phenotypes of various field isolates using procedure established in step 1

1. Screening of food and clinical isolates in food processing conditions

2. Biofilm phenotype classifications of isolates based on biofilm forming potential

Step 3: Investigation of the correlation between biofilm phenotypes and presence/absence of various biofilm formation related genes

Plan A: Identification of correlation between various biofilm forming capacities and a small numbers of selected genes (n=20) using PCR analysis

Plan B: Identification of correlation between various biofilm forming capacities and large numbers of biofilm formation related genes (n=106) based on whole genome sequences

Fig 1: Work plan to complete objectives: Step 1) Establishment of biofilm bioassay mimicking food processing conditions. In this section the aim was to study the effect of four different growth conditions including food processing conditions (12 ºC, Beef Broth) followed by determining optimum period of incubation for biofilm formation in food processing conditions in this section. **Step 2) Establishment of biofilm phenotypes of various field isolates using procedure established in step 1**. In this step the objective was to screen 66 isolates of food and clinical origin in food processing conditions and classify them based on their biofilm forming potential. **Step 3**) **Investigation of the correlation between biofilm phenotypes and presence/absence of various biofilm formation related genes**. This step involved determining genetic determinants for representative isolates from each phenotype with PCR using 20 genes published in literature. In order to supplement PCR a more involved search for genetic determinants were to be carried out by performing whole genome sequencing of all the test isolates and BLAST searches for 106 biofilm associated genes.

Chapter 2: Literature Review

2.1 *Listeria* and *L. monocytogenes*

The genus *Listeria* consists of gram-positive, facultatively anaerobic, rod shaped bacteria that are 0.5 to 2 µm in length and 0.5 µm in diameter. It possesses peritrichous flagella and exhibits the characteristic tumbling motility at 25 ºC (Farber & Peterkin, 1991). There are 14 species recognized under this genus: *L. monocytogenes, L. innocua, L. welshimeri, L. seeligeri, L. ivanovii, L. marthii, L. rocurtiae, L. weihenstephanensis, L. fleischmannii, L. floridensis, L. aquatic, L. cornellensis, L. riparia* and *L. grandensis,* out of which *L. monocytogenes* and *L. ivanovii* are pathogenic in nature (Bertsch et al., 2013; den Bakker et al., 2014; Halter et al., 2013; Orsi et al., 2011).

L. ivanovii is primarily associated with infections in ruminants although there has been a few reports of human cases (Alexander et al., 1992; Cummins et al., 1994; Guillet et al., 2010; Şahin & Beytut, 2006). *L. monocytogenes* on the other hand is a virulent food borne pathogen that can cause a serious infection in ruminants and humans called listeriosis (Farber & Peterkin, 1991; Nightingale et al., 2004). At least 13 serotypes of *L. monocytogenes* have been identified to date; 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e and 7. However only serotypes 4b, 1/2a, 1/2b and 1/2c have been implicated in 98% of human cases of listeriosis in Canada and worldwide (Chenal-Francisque et al., 2011; Kathariou, 2002). These 13 serotypes are further grouped into 4 lineages (I, II, III and IV) using molecular typing methods such as ribotyping, pulsed field gel electrophoresis (PFGE) and multi locus sequence typing (MLST) (Orsi et al., 2011; Valderrama and Cutter, 2013).

2.2 Physiological characteristics of *L. monocytogenes*

L. monocytogenes is a ubiquitous organism, known to inhabit a wide range of ecological niches as evidenced by its isolation from plants, animals, soil, water, silage and sewage (Farber & Peterkin, 1991). Owing to its ability to survive in temperatures ranging from -0.4 to 50 ºC, pH 4.7 to 9.2 and high concentrations of sugar (39.4% sucrose) and salt (10% NaCl) it is acknowledged to be an organism that truly knows how to survive (Farber & Peterkin, 1991; Gandhi & Chikindas, 2007; McClure et al., 1989)

2.3 Listeriosis

Transmission of listeriosis to humans occurs through consumption of food contaminated with the bacterium (Swaminathan and Gerner-Smidt, 2007). While high risks are often associated with the consumption of RTE meat and soft cheese, it is not uncommon for occurrence of *L. monocytogenes* in fresh produce (fruits, sprouts and other vegetables), fresh and RTE sea food, and other dairy products (Cartwright et al., 2013; Mritunjay and Kumar, 2015; Todd and Notermans, 2011; Warriner and Namvar, 2009). In order to develop an infection 10 to 100 million colony forming units (CFU) are needed for a healthy individual and 0.1 to 10 million CFU are required for individuals in the risk group (pregnant women, children, immunocompromised and elderly) (Bortolussi, 2008). Unlike other food borne illnesses, listeriosis can have long incubation periods ranging from 2 to 70 days (Goulet et al., 2013).

The infection itself can occur in two forms: adult and neonatal listeriosis. In adults a wide variation of the illness has been reported ranging from febrile gastroenteritis in healthy individuals to serious infections such as meningitis and septicemia in the immunocompromised and elderly and abortions and still births in pregnant women. Neonates on the other hand may

acquire listeriosis from their mothers during birth and based on the time of development postpartum it can be divided into early or late onset listeriosis (Bortolussi, 2008; Vázquez-Boland et al., 2001). In a majority of the cases, clinical presentation of early onset listeriosis is bacteremia and late onset listeriosis is meningitis (Okike et al., 2013). Even though listeriosis is not as common as other food borne illnesses it has an alarming mortality rate of 20-30% despite antimicrobial treatment (Mead et al., 1999). Furthermore based on the underlying medical condition of the patient, the outcome of the illness could be severe and can last for life (Maertens De Noordhout et al., 2014)

2.4 Incidence of listeriosis

Majority of the sporadic and outbreak cases are said to be caused by strains of serotypes 4b, 1/2a, 1/2b and to a lesser extent 1/2c. Interestingly, isolates of serogroup 1/2 is highly prevalent in food processing environments compared to isolates of serotype 4b (Cartwright et al., 2013; Kathariou, 2002). *L. monocytogenes* was first identified as a food borne pathogen in 1981, when contaminated coleslaw caused a major outbreak in the Maritime Provinces in Canada leading to the death of 41 people (Schlech et al., 1983). Subsequently, there was an increase in the number of listeriosis outbreaks reported in Canada and worldwide (Donnelly, 2001; Farber & Peterkin, 1991). In 1986, an average of 2.3 cases per million were reported in Canada as opposed to 7 cases per million reported in the United States of America (USA) (Clark et al., 2010; Farber & Peterkin, 1991). Between 1987 and 1994 the occurrence of listeriosis had climbed from 1.7 to 4.5 cases per 100,000 people in Canada (Farber & Peterkin, 1991). Around the same period of time, the annual number of listeriosis cases were also on the rise in the United Kingdom (UK) along with other member states of the European Union (EU) (Farber & Peterkin, 1991).

In 1995, in order to ensure improved population based monitoring of major illnesses caused by food borne pathogens, the Center for Disease Control and Prevention (CDC) in the USA established the Food-borne Diseases Active Surveillance Network (FoodNet) (Henao et al., 2015). Even though a major outbreak occurred in 1999 due to contaminated deli meat causing 500 deaths in the USA, increased surveillance helped in overall reduction of invasive listeriosis between 1996 and 2003 by 24% (Cartwright et al., 2013). Besides there was also a marked decrease in listeriosis outbreaks caused by RTE meat and poultry from 8.1% in the 1990s to 0.3% in 2010 in the USA (Cartwright et al., 2013).

In an effort to enhance laboratory based surveillance in Canada, the National Listeriosis Reference Service was established in 2001. During 1995 and 2004 an annual incidence rate of 2.8 cases /million were recorded (Clark et al., 2010). In contrast, a lower incidence rate of 0.3 cases /million was reported between 2001 and 2006 in the EU (Denny and McLauchlin, 2008). Canada experienced a listeriosis outbreak in 2008, causing 57 cases of illnesses and 23 deaths. The major outbreak caused by tainted daily meat led to extensive changes in directives for registered meat and poultry sector (Farber et al., 2011). According to the National Enteric Surveillance Program (NESP) in Canada the incidence rate of listeriosis between 2010 and 2013 had remained at 0.33 cases/million (NESP, 2012).

Though recent years have witnessed an overall reduction in the number of annual cases of listeriosis, reports of food contamination followed by recall continue to occur (CFIA, 2016). This only goes to showcase the tenacity of the organism and difficulties associated with eliminating it from the food production process.

2.5 Virulence and Pathogenesis

L. monocytogenes has the ability to switch between a harmless saprophyte and a virulent intracellular pathogen. The transition between these two lifestyles is orchestrated by the master virulence regulator, positive regulatory factor A (PrfA) - a deoxyribonucleic acid (DNA) binding protein that regulates the transcription of most of the genes that have been implicated in the intracellular life cycle of this pathogen (Freitag et al., 2009). *L. monocytogenes* is internalized by both phagocytic and non-phagocytic cells (Drevets et al., 1992; Ireton, 2007). Bacterial surface proteins, internalin A (InlA) and internalin B (InlB) aid in the entry of this pathogen by interacting with a corresponding ligand on the host cell. While the interaction of InlA is restricted to E-Cadherin (calcium dependent cell-cell adhesion molecule) (Jacquet et al., 2004), InlB has the ability to bind to the mesenchymal-epithelial transition factor (a receptor for hepatocyte growth factor), the globular portion of the receptor that binds to the first component of complement (C1q) and glycosaminoglycan (Cossart et al., 2003). As the bacteria force their entry through the plasma membrane of the host cell, they get enveloped in a single membraned vacuole. Listeriolysin O (LLO), a pore forming toxin along with phosphatidylinositol-specific phospholipase C (PlcA) and phosphatidylcholine-specific phospholipase C (PlcB) enable the bacterial cell to escape from the vacuole into the cytosol. In the cytosol, ActA (surface protein required for actin assembly) promotes the mobilization of host actin proteins followed by polymerization of an actin tail. With the help of the actin tail the bacterial cell rapidly moves within the cytosol and spreads to neighboring cells resulting in a double membraned vacuole. Upon entry into the adjacent cell, LLO and PlcB causes the lysis of the vacuole and the cycle is repeated (Cossart and Toledo-Arana, 2008; Hamon et al., 2007).

It is evident from the clinical manifestation of listeriosis discussed earlier, that *L. monocytogenes* has the ability to breach three host barriers namely, the intestinal barrier, the fetoplacental barrier and the blood brain barrier, leading to gastroenteritis, maternofetal infections and meningoencephalitis, respectively (Cossart et al., 2003). InlA interacts with E-cadherin that is present in the enterocytes thereby allowing bacterial translocation across the intestinal epithelium (Lecuit et al., 2001). During the transit to the intestine, the alternative stress response factor Sigma B (SigB) enables the bacteria to survive the acidic environment of the stomach and the high osmolality in the bile (Fraser et al., 2003; Wemekamp-Kamphuis et al., 2004). *L. monocytogenes* also uses the InlA–E-cadherin interaction for infringing the placental barrier. The bacteria are considered to gain access to the syncytiotrophoblast via the apical membrane (Lecuit et al., 2004). Three mechanisms are suggested for the entry of *L. monocytogenes* into the brain: 1) entry of bacteria from the blood stream through infected monocytes; 2) direct invasion of the brain vascular endothelial cells; and 3) entry through the axons of the nervous system (Drevets & Bronze, 2008).

2.6 Persistence of *L. monocytogenes* in food processing plants

2.6.1 Definition of persistence

Persistence, may be defined as the ability of a pathogen to survive in any environment (e.g. soil, water systems, human hosts, medical devices, food processing facilities) for an extended period of time (Donlan, 2001; Garzoni & Kelley, 2009; Jamieson et al., 2002; Thévnot et al., 2005). Bacteria persist in stressful environments with the help of several physiological and genetic mechanisms including capsule formation, endospore formation, viable but non-culturable (VBNC) forms and biofilm formation (Colwell, 2000; Costerton et al.,1995; Gibson et al., 2006;

Heyndrickx, 2011). These adaptive mechanisms have facilitated their successful persistence in their respective habitats (Roszak and Colwell, 1987).

2.6.2 Characterization of *L .monocytogenes* persistence

Typically in a food processing facility, persistence of *L. monocytogenes* is characterized by repeated isolation of a particular subtype on different occasions during the entire period of investigation (Ferreira et al., 2014). *L. monocytogenes* has been shown to persist in the food processing environments for few months to several years (Dauphin et al., 2001; Miettinen et al., 1999; Vogel et al., 2001).

Persistence has been observed across various food processing facilities involved in the production of meat and poultry, milk and other dairy products, sea-food and chilled RTE foods like pizza (Berrang et al., 2002; Gudmundsdóttir et al., 2006; Hayes et al., 1986; Keto-Timonen et al., 2007; Lappi et al., 2004; Lundén et al., 2003; Miettinen et al., 1999). One study isolated strains belonging to a particular random amplified polymorphic DNA (RAPD) subtype from cooked poultry products, 1 year after the sampling period was over. The same subtype was consistently isolated from food contact surfaces, floor drains and floors during the 6-month study indicating persistence of this subtype and the potential cross contamination that had occurred between the food processing environments to cooked food products (Lawrence and Gilmour, 1995). Another study observed the persistence of a particular clone of *L. monocytogenes* characterized by PFGE to be present in a dairy processing plant for over 7 years despite attempts to eliminate sources of contamination (Unnerstad et al., 1996). Even though a certain subtype may be initially introduced to the food processing environment through raw materials, in most of the cases repeated contamination is primarily shown to occur during processing and post processing (Lundén et al., 2002; Miettinen et al., 1999; Vogel et al., 2001).

2.6.3 Fitness and persistence of *L. monocytogenes*

Researchers indicate persistence of certain strains of *L. monocytogenes* to be a culmination of bacterial fitness, as there is evidence to suggest, that persistent strains may be better at adapting to changes in the environment when compared to sporadic strains (Aase et al., 2000; Autio et al., 2003; Lundén et al., 2000). For instance, persistent isolates were found to upregulate many genes including the ones responsible for stress response and peptidoglycan (PG) synthesis in the presence of benzethonium chloride (BZT - a commonly used quaternary ammonium compound disinfectant in the food processing environment) (Fox et al., 2011).

Bacterial fitness with respect to serotypes have also been observed in *L. monocytogenes* (Donaldson et al., 2009). Strains of serotypes 1/2a and 1/2c that belong to lineage II are found to be more prevalent in the food processing environments compared to strains of lineage I (4b and 1/2b) (Latorre et al., 2007; Thévnot et al., 2005; von Laer et al., 2009). The reasons for the differences in prevalence of different serotypes in food processing environments are not very clear. However, it is speculated that certain serotypes might be pre-disposed to being vulnerable at certain environmental temperatures (Avery and Buncic, 1997). For instance, one study reported that isolates of serotype 1/2a might have a natural tendency to thrive in food processing conditions unlike isolates of serotype 4b. While isolates of serotype 1/2a were found to be resistant against bacteriocins after cold storage (4 ºC) but susceptible when exposed to mild heat treatments (37 ºC), the exact opposite was observed for isolates of serotype 4b (Buncic et al., 2001). Additionally, isolates that belong to lineage II are believed to have far superior adhesion properties to materials (stainless steel, plastic and glass) commonly used in food processing plants compared to isolates of lineage I (Borucki et al., 2003; Harvey et al., 2007; Lundén et al.,

2000). However this theory remains contentious as others have reported the exact opposite (Djordjevic et al., 2002; Takahashi et al., 2009).

Nevertheless, persistent isolates have not been found to have an increased pathogenic potential when compared to their sporadic counterparts (Jensen et al., 2008a, 2008b; Nightingale et al., 2005). However, the risk of food contamination and the likelihood of listeriosis spreading to humans increases with isolates that tend to persist longer in food processing plants (Fox et al., 2012; Gilbreth et al., 2005; Lambertz et al., 2013). In fact there are a few reports that have found a link between persistence of isolates in food processing plants and outbreaks of listeriosis (Nocera et al., 1990; Olsen et al., 2005; Orsi et al., 2008). For instance, the Maple Leaf establishment (97B) detected positive listeria samples from environmental sources for continuously four months leading to the major outbreak in 2008 (PHAC, 2009).

2.6.4 Factors that contribute to persistence

Overall two main factors are hypothesized to contribute towards persistence of a pathogen within the food processing facility.

1. Environmental factors such as poor infrastructure (improper compartmentalization between processing lines) and equipment design (hard to access sites) resulting in inadequate cleaning and disinfection that promote the retention of bacteria (Lundén et al., 2002; Miettinen et al., 1999; Senczek et al., 2000).
2. The ability of certain subtypes of the bacteria to capitalize on these factors and establish persistence (Keto-Timonen et al., 2007; Lundén et al., 2003).

As one of the major food borne pathogens and an organism that gets frequently isolated from food processing plants, *L. monocytogenes* is considered to persist mainly by exercising the

ability to form biofilms for surviving within these environments (Møretrø & Langsrud, 2004; Norwood & Gilmour, 2001; Tompkin, 2002). Hence over the past two decades studies have been directed towards understanding the phenomenon of biofilm formation in *L. monocytogenes* with a hope to design approaches to tackle its persistence within food processing establishments (Blackman & Frank, 1996; Harvey et al., 2007; Piercey et al, 2016).

Due to consideration of its major role in persistence, biofilm formation of *L. monocytogenes* will be the main focus of this thesis. In the following section the definition and process of biofilm formation and biofilm formation of *L. monocytogenes* in relevance to the food processing environment will be discussed.

2.7 Definition and process of biofilm formation

Biofilms are microbial communities attached to biotic or abiotic surfaces with the help of self-produced extra cellular polymeric substances (EPS) (Costerton et al., 1995). The EPS is made up of constituents such as polysaccharides, proteins and extracellular DNA (eDNA) that hold the bacterial cells together in a biofilm (Davey and O'Toole, 2000; Donlan, 2002). The communities of microbes residing within biofilms can either be comprised of a single species or multiple species (Davey and O'Toole, 2000). Biofilms are known to provide protection against many stresses such as ultra-violet (UV) radiation, pH shifts, osmotic shock and desiccation, the microbial cells may encounter in their inhabited environment (Elasri & Miller, 1999; Hingston et al., 2015; McClure et al., 1989). Due to these reasons, biofilms are considered as one of the major mechanisms that allow bacteria to colonize habitats formidable to higher life forms (Flemming, 1993).

The process of biofilm formation occurs in five major steps: 1) Reversible attachment of single cells to surface; 2) Irreversible attachment followed by multiplication of cells; 3) Formation of microcolonies with the production of EPS; 4) Mature biofilm formation with channels for transfer of nutrients; and 5) Dissociation of cells and colonization of a new surface (Donlan, 2002; Harvey et al., 2007; Lasa, 2006).

2.7.1 Biofilm formation of *L. monocytogenes* in food processing plants

While biofilms are considered favorable in bioconversion processes such as in the production of ethanol and acetic acid and in the treatment of wastewater, its presence is often hindering and unwelcome in other processes like drinking water purification systems (Ashbolt, 2004). Furthermore biofilms found in medical devices can be life threatening as bacterial cells can detach from the biofilm causing serious infection, a process best described using the term bio-transfer potential (Hood and Zottola, 1995; Qureshi et al., 2005). The same concept can be extrapolated to a food processing facility. Biofilms formed on food contact surfaces can get dislodged during processing and can contaminate food products such as RTE meat (Chung et al., 1989; Hood & Zottola, 1997b). Given the resilient nature of biofilms it is commonly believed to be one of the major contributors for the observed persistence of *L. monocytogenes* within food processing environments (Blackman & Frank, 1996; Helke et al., 1993; Tompkin et al., 1999). Though there is no direct evidence to support the claim, the increasing volume of evidence resulting from *in-vitro* studies strongly suggest that it might actually be the case (Kadam et al., 2013; Norwood & Gilmour, 2001; Pan et al., 2006).

An understanding of biofilms has occurred primarily by means of studying a number of natural ecosystems (Costerton et al., 1995). However, the factors that influence biofilm formation on

abiotic surfaces within a food processing facility might be different (Todhanakasem, 2013). Therefore factors relevant to biofilm formation in these environments are discussed below.

2.7.2 Harborage sites

Harborage sites are hard to reach sites in equipment (e.g. hollow parts), buildings (places that are inaccessible for cleaning through mechanical aid) and food contact surfaces (with pits and crevices) that collect soil during food processing and serve as a good niche for surface-attached bacterial growth or biofilm formation (Carpentier and Cerf, 2011; Giaouris et al., 2014). Microbial cells in harborage sites are able to covertly multiply and evade the harsh effects of cleaning and disinfection (Carpentier and Cerf, 2011; Holah and Thorpe, 1990). Many food borne pathogens including *L. monocytogenes* are known to form biofilms on stainless steel, aluminum, glass, Buna-N and Teflon seals and nylon materials that are commonly used in food processing environments (Blackman & Frank, 1996; Herald & Zottola, 1988; Notermans et al., 1991). Reports on which of these materials facilitate strong biofilm formation by *L. monocytogenes* however remain disputed (Borucki et al., 2003; Djordjevic et al., 2002; Takahashi et al., 2009). Nevertheless from a food processing standpoint, bio-transfer potential is considered to be the lowest with stainless steel as it does not get abraded easily with use (Wirtanen et al., 1996). Moreover, the crevices formed due to repeated use on stainless steel was also found to retain much less bacteria compared to other materials like polycarbonate, mineral resin and enameled steel (Holah and Thorpe, 1990).

2.7.3 Conditioning film

Any surface in an aqueous medium will get coated by constituents from that medium (Donlan, 2002). As a result, the physio-chemical properties of the surface get modified, which in turn

affects the attachment of bacterial cells to the surface (Donlan, 2002). In the food processing environment food contact surfaces can get conditioned by food residues during processing. However surface conditioning by food constituents can either have a positive or a negative effect on biofilm formation. While milk and milk proteins were shown to reduce adherence of *L. monocytogenes* to stainless steel surfaces (Barnes et al., 1999), residues from turkey or beef frankfurters were reported to enhance long term survival of cells by rendering a buffering effect on them (Somers and Wong, 2004). Furthermore, the effectiveness of antimicrobial treatments on *L. monocytogenes* was also observed to be compromised in the presence of food residues on conveyer belts (Chaitiemwong et al., 2010). Additionally biological soiling of surfaces are also believed to enhance survival of *L. monocytogenes* in food processing environments (Allan et al., 2004a, 2004b).

Apart from food residues, surfaces in the food processing environment can also be coated by biofilms of resident microorganisms. The adherence of *L. monocytogenes* to surfaces can either be enhanced or prevented depending on the microorganism it coexists with (Leriche and Carpentier, 2000; Sasahara and Zottola, 1993). Even though *L. monocytogenes* was found to adhere in very small numbers to a multispecies biofilm, the bacterial cells within, were found to be more resistant against disinfection compared to their counterparts in a monospecies biofilm (Norwood and Gilmour, 2000). Additionally, strain dependent adherence abilities of *L. monocytogenes* in dual species biofilms have also been documented (Rieu et al., 2008b).

2.7.4 Physio-chemical interactions between the bacterial cell and the environment

The nature of interaction between the cell and the substratum determines the extent of adhesion of cells to a surface. In general hydrophobic interactions are shown to enhance adhesion of bacterial cells to a surface (Liu et al., 2004). However the nature of interaction greatly depends

on the environment within which the bacterial cells and the substratum exist (Houdt and Michiels, 2010). The surface chemistry of the substratum may be modified by conditioning of the surface thereby influencing adhesion (Chmielewski and Frank, 2003). One study found that treatment of silica surfaces with bovine serum albumin reduces hydrophobicity of the surface as a result of which less number of bacterial cells were found to bind to the surface (al-Makhlafi et al., 1995). Even though Buna-n and stainless steel are hydrophobic in nature, increase in pH of the aqueous solution surrounding the material imparts a negative charge and reduces the hydrophobicity of the substratum and the bacterial cell surface. This reduction in bacterial adhesion is thought to be a result of electrostatic repulsion between the bacterial cell and the substrate (Smoot and Pierson, 1998). As previously mentioned, the cell surface properties of the bacterium also changes in response to its environment. For instance, *L. monocytogenes* cultivated in TSB was shown to be more hydrophobic than in BHI (Briandet et al., 1999). Negative charge of the cell surface was found to be increased at 15 ºC and 20 ºC making them more hydrophilic and less likely to bind to polymeric materials at such lower temperatures (Briandet et al., 1999; Chavant et al., 2002; Smoot and Pierson, 1998). It is believed that the bacterial cell may produce proteins to get acclimatized to the growth condition leading to changes in the cell wall composition (Briandet et al., 1999).

2.8 Methods used for the study of biofilms of *L. monocytogenes*

2.8.1 Biofilm models

In general there are four models that are used in the investigation of biofilms. They are closed systems, open systems, microcosms and *ex vivo* models (Lebeaux et al., 2013). It is important to choose the appropriate model based on the purpose of the study. In closed or static systems biofilms are allowed to grow in conditions where the amount of nutrition (or growth media) is

fixed [e.g. Microtiter plate] (Djordjevic et al., 2002). Open or dynamic systems, on the other hand, have a continuous supply of fresh media [e.g. CDC biofilm reactor] (Donlan et al., 2004). Microcosms can either be closed or open systems but are more specialized, as they are designed to simulate real life conditions [e.g. addition of saliva to dental composite discs to mimic dental biofilms] (Rudney et al., 2012). *Ex vivo* models are mainly used to study biofilms that cause clinical infections. The progression of microbial biofilms can be studied by using an organ or a tissue placed in an artificial environment [e.g. use of trachea extracted from mice to study tracheal infection] (Simmons and Dybvig, 2009) .

For the investigation of biofilm formation of *L. monocytogenes* in food processing environments, microtiter plates (static model) has been one of the commonly used methods (Djordjevic et al., 2002; Merritt et al., 2005). Also microtiter plate assays are the method of choice if a large number of isolates are screened for their biofilm forming abilities (Merritt et al., 2005; Ouyang et al., 2012). Alternatively biofilms are also grown on coupons of desired choice of material (stainless steel, glass, and polystyrene), and biofilm formation is quantified microscopically (Folsom et al., 2006; Norwood and Gilmour, 2001). The following sections will discuss the use and limitations of commonly used methods for studying biofilm formation in *L. monocytogenes*.

2.8.2 Quantitative analysis of biofilms

2.8.2.1 Crystal violet staining

Crystal violet is a basic dye commonly used for the assessment of biofilms (Djordjevic et al., 2002; Li et al., 2003). Biofilms grown on microtiter plates are stained using crystal violet following which they are rinsed and solubilized using a solvent like ethanol or acetic acid. The amount of dye bound to the biofilms is analyzed by measuring the de-stained solution

spectrophotometrically at 500-600 nm. The optical density (OD) value obtained indirectly corresponds to the biofilm mass (Merritt et al., 2005).

It is a simple, easy and a high throughput method to assess the abilities of attachment of bacterial cells to an abiotic surface (Merritt et al., 2005). It is also an effective technique for screening large number of mutants for their abilities to attach to surfaces before their precise biofilm forming potential can be determined (Alonso et al., 2014; Chang et al, 2012). In addition, this method can also be used to measure the influence of different parameters such as nutrition, temperature and pH on biofilms (Di Bonaventura et al., 2008; Nilsson et al., 2011). Crystal violet stains not only bacterial live or dead cells but also other matrix components such as eDNA, proteins and other negatively charged constituents of the cell (Popescu and Doyle, 1996). Hence there is a drawback of not being able to measure viable bacteria within a biofilm (Pantanella et al., 2013). Therefore several methods have been used as discussed below to determine live or dead bacteria in a biofilm and for visualization of biofilm structures as it would be important for confirmation of true biofilm formation.

2.8.2.2 Enumeration of bacteria in biofilms

2.8.2.2.1 Enumeration using plate counts

This technique allows the estimation of viable bacteria in a biofilm (Merritt et al., 2005). Biofilms grown on the test surfaces are detached with the help of mechanical methods like sonication or enzymes that degrade the extracellular matrix. The detached biofilms are then plated to enumerate the colony forming units (CFU) (Allan et al., 2004a; Gamble & Muriana, 2007). This technique can be used to study the effect of disinfectants on biofilms (Pan et al., 2006). By making use of selective media, the cell numbers of individual bacterial species within

a multispecies biofilm can be enumerated (Norwood and Gilmour, 2000). However use of mechanical and enzymatic aids to detach the biofilms may not be able to completely remove the adhered bacteria and the viability of the cells can also be affected during detachment (Welch et al., 2012). Furthermore, the cells in the biofilm may remain aggregated after detachment, as a result of which there could be a bias in determining the viability of cells in biofilms (Pan et al., 2006).

2.8.2.2.2 Fluorescent stains

2.8.2.2.2.1 Acridine Orange

Direct enumeration of bacteria in biofilms can also be performed by staining the cells using flourophores like acridine orange (Pan et al., 2006) and 4'6- diamidino-2-phenylindole (DAPI) (Poulsen et al., 1993) which can then be visualized using epifluorescence microscopy.

Acridine orange (AO) has the ability to bind to both DNA and RNA. Live cells tend to accumulate higher amounts of RNA and fluoresce orange-red when bound to AO. Dead cells or slow growing cells have relatively lower quantities of RNA and fluoresce green (Foong and Dickson, 2004; Mah and O'Toole, 2001). Due to the ability of AO to bind to organic molecules it is often used for analyzing the formation of biofilms on food contact surfaces that are conditioned with residues from food (Poimenidou et al., 2009). However it has been shown that the fluorescence of cells stained with AO can vary based on conditions like growth medium, contact time and species. In some cases the surfaces of the polymers may pick up the dye and fluoresce; there therefore could be difficulties in differentiating between background fluorescence and bacterial cells (Verran et al., 2008).

2.8.2.2.2.2 CTC-DAPI

DAPI is a non-intercalating DNA specific stain. When bound with DNA it fluoresces bright blue while in the unbound state with DNA or non-DNA particles it fluoresces yellow (Porter and Feig, 1980). As staining properties of DNA in nonviable cells remain intact, DAPI cannot be used to differentiate between live and dead cells in a biofilm but the direct total cell counts in a biofilm can be obtained (Gagnon and Slawson, 1999; Kepner and Pratt, 1994).

Therefore both AO and DAPI allow direct total viability count and direct total count of cells to be determined, respectively. However the physiological state of the cells within a biofilm cannot be determined using these dyes (Kepner and Pratt, 1994).

This draw back can be overcome with the use of 5-cyano-2,3-ditoyl tetrazolium chloride (CTC). CTC is a redox dye that can be reduced by bacteria via electron transport activity to produce fluorescent insoluble CTC-formazan crystals that accumulates within the cell and fluoresces in the red range when excited (Rodriguez et al., 1992; Yu & McFeters, 1994). The fluorescence is a direct measure of physiological activity (respiration) of bacteria (Rodriguez et al., 1992). CTC used often in conjunction with DAPI allows for the analysis of heterogeneity within a biofilm; the distribution of both physiologically active cells and total number of cells can thus be enumerated (Winkelstroter and Martinis, 2015). It is also useful for studying the spatio-temporal patterns of bacteria that are treated with biocides (Huang et al., 1995). The advantage is that CTC allows for the visualization of cells without destroying them (Schaule et al., 1993). However due to the nature of the dye, it can be used only for aerobic and microaerophilic systems (Rodriguez et al., 1992).

2.8.2.2.2.3 LIVE/DEAD *Bac*light bacterial viability kit

The kit obtained from Molecular Probes consists of two nucleic acids stains SYTO 9 and Propidium iodide (PI) (Molecular Probes Handbook). SYTO 9 binds to cells having intact and damaged membranes while PI only binds to cells having damaged membranes. Therefore live cells are stained green and dead cells are stained red but surface remains non-flourescent (Maukonen et al., 2000; Schwab et al., 2005). This kit can be used to obtain total and viability counts of bacteria within biofilm (Rodrigues et al., 2009). Live and dead cells can be distinguished even within a multi-species biofilm (Shen et al., 2010). Sometimes there is a chance for bacteria with damaged membrane to be mislabeled as dead cells even though it might be able to recover once transferred to a nutrient medium. Similarly bacteria with intact membrane may not be able to become metabolically active when transferred to a nutrient medium and may be mislabeled as live cells. Hence confirmation with another viability assay is often recommended (Molecular Probes Handbook). Another disadvantage is that the fluorescence is quenched quickly, as a result of which the samples will have to be visualized within in the same day of sample preparation (Maukonen et al., 2000).

2.8.2.2.2.4 Fluorescence *in situ* Hybridisation (FISH)

Even though stains such as AO and CTC-DAPI allow visualization and quantification of viability, they are non-specific. This limitation can be overcome with the help of FISH as it makes use of a nucleic acid specific-labelling with the use of a fluorescent probe (Amann, 1995). It helps in the visualization and quantification of individual species of bacteria within a multispecies biofilm (Amann and Fuchs, 2008). This technique is based on 16S or 23S rRNA and hence the image outcome is not influenced by growth conditions (MacDonald and Brözel,

2000). Additionally, when used in conjunction with confocal laser scanning microscopy (CLSM), spatial organization of cells in biofilms can be viewed without any loss in their structure (Almeida et al., 2011).

2.8.3. Microscopical Visualization of Biofilms

2.8.3.1 Scanning Electron Microscopy (SEM)

SEM provides excellent 3D visualization of structures at varying resolutions (Marsh et al., 2003). However with dense biofilms, quantification of cells using EM is limited (Harrison et al., 2006). Also, an extensive sample processing (fixation, dehydration, critical point drying, an coating with a nonconductive material) is required which makes the whole process time consuming (Borucki et al., 2003; Moltz & Martin, 2005; Zameer et al., 2010). As the samples have to be dehydrated, the cell structure can be distorted or compacted (Borucki et al., 2003). The EPS can appear as fibers rather than as the thick gelatinous matrix that surrounds the cells (Herald and Zottola, 1988; Zameer et al., 2010) There is also the problem of increased artifacts that is introduced during sample processing. In addition, if the samples are not coated properly with the conducting material (for e.g. sputter coated with gold) charging effect may occur resulting in images that are unusable (Chavant et al., 2002; Little et al., 1991).

2.8.3.2 Epifluorescence Microscopy

This technique allows visualization of bacterial cells attached to opaque surfaces (Carpentier and Chassaing, 2004). It can be used for quick enumeration of total viable counts and total counts of metabolically active cells when used with stains such as AO and CTC-DAPI respectively (Poimenidou et al., 2009; Whitehead et al., 2009). It is also suitable for studying the effects of sanitization where distribution of cells and organic residues on surfaces like stainless steel before

and after treatment can be assessed and presented as percentage coverage on surface (Pan et al., 2006). Sample preparation is very quick and also allows the cells to be visualized in a nondestructive manner (Monk et al., 2004). However this technique may not be suitable for thick biofilms (Schwab et al., 2005). The images may sometimes be hazy and out of focus (Kim et al., 2001). The fluorescence of stains can be quenched quickly hence samples ought to be processed immediately (Harmsen et al., 2010; Rodrigues et al., 2009). The surface coverage of cells may sometimes get overestimated for e.g. when imaging is performed with AO, as it can stain the organic residues giving background fluorescence (Blackman and Frank, 1996; Djordjevic et al., 2002; Foong and Dickson, 2004) .

2.8.3.3 Confocal Scanning Laser Microscopy (CLSM)

High resolution of images can be obtained with CSLM and the problem of hazy out of focus images obtained by epifluorescence microscopy can be overcome with this technique. It allows for the visualization of even thick biofilms with 2D and 3D optical sectioning of films (Chae and Schraft, 2000). The transition of a single cell within a biofilm from the time it is planktonic to the time when it multiplies and forms biofilms can be tracked (Habimanaa et al., 2011). This technique is suitable for analyzing spatio-temporal patterns of cells within a biofilm that are treated with biocides (Guilbaud et al., 2015). The sample preparation is quick and the cells within a biofilm can be quantified without causing destruction to its structure (Rieu et al., 2008a).

2.8.3.4 Atomic Force Microscopy (AFM)

AFM allows the study of microbial structure and behavior in association with its environment (Wright et al., 2010). Apart from visualizing the structure of biofilms the effect impacted by the

biofilms on the substratum can also be analyzed (for e.g. corrosion of stainless steel surface) (Steele et al., 1994). The feature of force measurement helps in gaining an insight into the developmental stages of biofilms, measure the physical properties of planktonic and biofilm cells and the effect of environmental stressors such as humidity, pressure and heat on biofilms (Oh et al., 2007; Rodriguez et al., 2008; Volle et al., 2008). However this technique has the limitation of not being able to scan a large surface area. Also the soft gelatinous nature of biofilms can get lost during the imaging process (Mendez-Vilas et al., 2004).

2.8.4 Genetic approaches

Identification of determinants involved in biofilm formation largely relies on mutagenesis studies. Random mutagenesis is carried out in a strain that generates thousands of mutations within the genome. These mutants are then screened rapidly using the microtiter plate assay with crystal violet (Alonso et al., 2014; Chang et al., 2012; Ouyang et al., 2012). In silico analyses of genomes are also carried to identify if genes responsible for biofilm formation in other species are present in the desired species [e.g. Bap in *Staphylococcus*] (Jordan et al., 2008). Mutation of identified genes is then carried out and analyzed phenotypically to assess changes in biofilm formation (Cucarella et al., 2001). Additionally, virulence gene profiles generated using PCR are also used to assess the correlation between the presence/absence of genes and biofilm forming phenotypes (Meloni et al., 2012).

2.9 Evaluation of biofilm formation capacities of *L. monocytogenes* and research gaps

The ability of *L. monocytogenes* to form biofilms is considered to be one of the major factors for its persistence within food processing plants (Harvey et al., 2007; Møretrø & Langsrud, 2004; Norwood & Gilmour, 2000). However epidemiological investigations reveal strains of this

bacterium display varying abilities to establish persistence, as some subtypes tend to be isolated more frequently than others from food processing plants (Autio et al., 2003; Dauphin et al., 2001; Lawrence and Gilmour, 1995; Miettinen et al., 1999). Researchers have suggested that perhaps, understanding differential biofilm formation in *L. monocytogenes* could be a key in decoding the basis of ecological diversity among these strains in food processing plants (Kumar & Anand, 1998; Møretrø & Langsrud, 2004; Valderrama & Cutter, 2013; Wong, 1998). Therefore extensive studies have been carried out in the laboratory setting to study variation in biofilm formation in this food borne pathogen.

Early research on *L. monocytogenes* showed that the ability of this bacteria to form biofilms to be much lower than many other gram positive and gram negative bacteria (Hood & Zottola, 1997a, 1997b; Jeong & Frank, 1994a, 1994b). Nonetheless biofilm formation has been shown to be influenced by various factors including serotype/lineage, growth media, temperature and surface of attachment (Di Bonaventura et al., 2008; Djordjevic et al., 2002; Doijad et al., 2015; Harvey et al., 2007; Kalmokoff et al., 2001; Lundén et al., 2000). Therefore differences that have been documented in biofilm formation of *L. monocytogenes* in relevance to these factors are discussed in the following sections.

2.9.1 Serotype and Lineage

Strains that belong to lineage I (serotypes 4b and 1/2b) and lineage II (serotypes 1/2a and 1/2c) are often implicated in human cases of listeriosis (Chenal-Francisque et al., 2011; Kathariou, 2002). Hence differences in biofilm formation that may arise as a result of lineage or serotype diversity are of prime interest to researchers (Borucki et al., 2003; Djordjevic et al., 2002; Harvey et al., 2007; Lundén et al., 2000). To date, the correlation between serotypes, lineage and

biofilm formation have remained fairly inconclusive. While some studies have found the biofilm forming ability of strains of lineage II to be higher than the strains of lineage I (Borucki et al., 2003; Harvey et al., 2007; Lundén et al., 2000), others have observed the exact opposite (Djordjevic et al., 2002; Takahashi et al., 2009) There are also reports that suggest that no significant correlation exists between lineage and biofilm formation (Milanov et al., 2009; Tresse et al., 2007). However in some cases either serotype dependent or strain dependent correlation with biofilm formation in response to changes in the growth conditions have been documented (Di Bonaventura et al., 2008; Folsom et al., 2006; Kadam et al., 2013).

2.9.2 Growth Media

Biofilm formation of *L. monocytogenes* is usually assessed in nutrient rich media (Tryptic Soy Broth [TSB] and Brain Heart Infusion [BHI]), less nutrient rich or minimal media (Modified Welshimer's Broth [MWB] and Hsiang-Ning Tsai medium [HTM] and in nutrient limiting conditions (diluted versions of TSB [dTSB]). In general biofilm formation has been reported to be increased in less-nutrient rich and in nutrient limiting conditions (Combrouse et al., 2013; Harvey et al., 2007; Kadam et al., 2013; Zhou et al., 2012). However isolates that belong to 1/2 serogroup have been reported to be able to form strong biofilms in all types of growth conditions [nutrient rich, nutrient poor and nutrient limiting conditions] (Folsom et al., 2006; Kadam et al., 2013). This ability of serogroup 1/2 isolates to form biofilms in various conditions is thought to be one of the reasons for its increased prevalence in food processing plants where the availability of nutrients could be in a continuous state of flux (Folsom et al., 2006).

However it is important to note that the media used in most of the studies contain nutrients that will promote the optimal growth of biofilms. However this may not be the case under natural

conditions in food processing plants (Roszak and Colwell, 1987). One study reported less biofilm formation in meat broth (less-rich) compared with TSB and BHI (Stepanović et al., 2004). However this condition may reflect the true biofilm formation of different isolates of *L. monocytogenes* under natural conditions.

2.9.3 Temperature

Majority of the studies evaluate biofilm forming abilities of *L. monocytogenes* in optimal temperatures [30-37 ºC] (Piercey et al., 2016). Biofilm formation is usually reported to increase with increase in temperature While the highest amount of biofilm formation is observed at 37 ºC, the lowest is observed at refrigeration temperatures like 4 ºC or 5 ºC (Kadam et al., 2013; Moltz & Martin, 2005; Nilsson et al., 2011; Pan et al., 2009; Smoot & Pierson, 1998). This was further corroborated by microscopy, where only a rudimentary biofilm was observed at lower temperatures such as 4 ºC and 12 ºC while a mature biofilm, with a three dimensional structure with channels was evident at 37 ºC irrespective of the test surface used (stainless steel, glass and polystyrene) (Di Bonaventura et al., 2008). However the ambient temperatures in food processing plants are usually between 10 to 20 ºC (Møretrø et al., 2013; Piercey et al., 2016). Also regulatory agencies such as the Canadian Food Inspection Agency (CFIA) recommends 10 ºC as the ambient temperature and temperatures above or below that point is allowed only with proper justification (CFIA, 2014). Therefore temperatures used for assessment of biofilm forming capacities of *L. monocytogenes* isolates should be relevant to the *in-situ* conditions.

2.9.4 Surface of attachment

The nature of the test surface has been shown to influence *L. monocytogenes* biofilm formation. Djordjevic et al. (2002) observed biofilm formation on polyvinyl chloride (PVC) to be higher

than on stainless steel. This was in direct contrast to the observations made by Takahashi et al. (2009) as they observed the surface coverage of biofilms on stainless steel to be much higher compared to PVC. Studies by Borucki et al. (2003) was in line with the previous study as they found a clear difference in attachment between stainless steel and PVC in isolates that displayed low adherence abilities. Temperature dependent biofilm formation on surfaces have also been documented. Lower incubation temperatures (4 ºC, 12 ºC and 22 ºC) seem to favor biofilm formation on glass compared to polystyrene and stainless steel (Di Bonaventura et al., 2008). In addition to temperature, biofilm formation was also found to be influenced by periods of incubation. Biofilm formation on glass by cells at 5 ºC seem to require longer period of contact unlike the cells at 25 ºC and 37 ºC as they were able to attach to glass at the end of 3 hours (Milanov et al., 2009). It is evident that not all the isolates respond equally when they are grown on a particular test surface. As a result there is no conclusion as to which of these surfaces facilitate maximum biofilm formation under laboratory conditions. Therefore it is important to choose a test surface that is commonly used in the food processing environment and is prone to retaining maximum number of bacterial cells after repeated cleaning and disinfection.

2.9.5 Period of incubation

Persistent isolates have been found to be able to adhere to a test surface over a much shorter incubation period compared to sporadic isolates (Lundén et al., 2000). In fact few studies have found the ability of *L. monocytogenes* to attach to surfaces under 3hrs and form biofilms at the end of 24 hrs (Borucki et al., 2003; Chae and Schraft, 2000; Doijad et al., 2015; Milanov et al., 2009). One study that looked at biofilm formation over a period of 14 days observed that biofilm forming ability of some sporadic isolates were able to reach the same level as persistent isolates implicating biofilm formation capacity can increase if the biofilms are left undisturbed for an

extended period of time (Harvey et al., 2007). Interestingly in the same study, there were some isolates whose biofilm forming abilities remained unchanged throughout the period of investigation (Harvey et al., 2007).

2.9.6 Isolate origin

The correlation between the origin of isolates (environmental, clinical, food and animal sources) and biofilm formation have also been tested and found to be nonexistent in many of the studies (Harvey et al., 2007; Kalmokoff et al., 2001; Milanov et al., 2009; Norwood and Gilmour, 1999). However few studies have documented a positive correlation between the source of isolation and biofilm formation (Barbosa et al., 2013; Nilsson et al., 2011). Interestingly one study found that isolates from animal clinical cases, human clinical cases and meat sources only displayed weak to moderate biofilm forming potential as opposed to the isolates from milk and milk processing environments which showed strong biofilm forming abilities (Doijad et al., 2015).

It is evident that *L. monocytogenes* can attach at varying levels to industrially important surfaces. The ability of certain subtypes of this bacterium to respond according to changes in environment indicates that a concerted regulation of genes could be at play (Gandhi and Chikindas, 2007). Hence an understanding of biofilm formation of *L. monocytogenes* at a molecular level could shed light on special characteristics that render certain subtypes to become persistent in food processing environments (Møretrø & Langsrud, 2004). In the following section the molecular determinants identified so far with respect to biofilm formation will be discussed.

2.10 Molecular determinants of biofilm formation

The process of biofilm formation is known to be complicated involving a myriad of pathways (Davey and O'Toole, 2000; Luo et al., 2013). Mutagenesis studies have revealed that a number

of genes including those involved in virulence, stress response, cell wall synthesis, motility, and metabolism have a role to play in biofilm formation of *L. monocytogenes* (Chang et al., 2012; Ouyang et al., 2012). The following section will touch upon some of the molecular determinants identified to date and their role in biofilm formation of this pathogen.

2.10.1 Flagella

The presence of extracellular appendages like flagella are known to influence bacterial attachment to surfaces and have been documented to contribute to biofilm formation of other gram positive and gram negative bacteria (Davey and O'Toole, 2000). Therefore mutational analysis of genes including those that encode for the production of flagella (*flaA, fliF, fliI, flgL, fliP and fliD*) and motility (*motA* and *motB*) have been performed to understand the role of flagella in biofilm formation in this pathogen (Ouyang et al., 2012). Initial adherence and biofilm formation have been shown to be impaired in mutants that either carry paralyzed flagella or are completely devoid of flagella (Lemon et al., 2007). One study reported that flagella per se and not motility, facilitated early attachment of cells to stainless steel by acting as an adhesive structure (Vatanyoopaisarn et al., 2000). This observation was substantiated by many studies as no correlation was reported between motility and biofilm formation in static biofilm assays (Di Bonaventura et al., 2008; Djordjevic et al., 2002; Takahashi et al., 2010). In contrast another study reported that even though the absence of flagellar motility slowed down the initial adherence of cells, a hyper biofilm phenotype was displayed by the cells following a 24-hr incubation in flow systems (Todhanakasem and Young, 2008) .

2.10.2 Extracellular Polymeric Substances

Quantification of EPS of *Listeria* biofilms revealed that proteins were the most predominant components followed by eDNA and finally very small amounts of polysaccharides (Combrouse et al., 2013). In accordance to that, addition of trypsin lead to 99.9% reduction in adherence of cells to Buna-N rubber and stainless steel (Smoot and Pierson, 1998). In contrast to the previous report one study suggested that eDNA was entirely responsible for initial attachment and early biofilm formation as addition of DNase I and not RNase A nor proteinase K led to the disintegration of surface attached structures. In addition it was theorized that high molecular weight DNA together with *N*-acetylglucosamine (NAG) was able to form a polymer similar in nature to poly-*N*-acetylglucosamine (PNAG) of *Staphylococcus* and facilitated cells in adhesion and biofilm formation. The theory was based on the fact that addition of both the components were able to restore attachment to normal levels in eDNA free cultures (Harmsen et al., 2010). One study documented the production of an extracellular polymer composed of β-1,4-linked N-acetylmannoseamine with α-1,6-linked galactose by cells forming aggregates in minimal medium. However the reported polysaccharide was found not to a play a role in biofilm formation on abiotic surfaces (Chen et al., 2014).

2.10.3 Quorum Sensing (QS)

QS systems enable bacteria to regulate gene expression in a cell density dependent manner and they have been documented to play a role in biofilm formation in many bacterial species (Miller and Bassler, 2001; Swift et al., 2001)

L. monocytogenes has been found to have a QS system much similar to the *agr* system found in *Staphylococcus aureus* (Autret et al., 2003). The *agr* system is an operon consisting of genes that

code for AgrD - an auto inducing peptide, AgrB - a protein involved in processing the peptide, AgrC - a two – component histidine kinase, and AgrA - a response regulator (Miller and Bassler, 2001). The *agr* system in *L. monocytogenes* is shown to influence biofilm formation as mutations in *agrA* and *agrD* display reduction in their ability to form biofilms compared to the wild type strains under both static and dynamic biofilm formation conditions (Riedel et al., 2009; Rieu et al., 2007; Rieu et al., 2008a). However the expression of *agr,* between static and dynamic settings, is shown to vary. While the expression remained minimal (1%) but unchanged during sessile growth, it changed from 15% to 80% in the dynamic condition at the end of 40 hr. incubation period (Rieu et al., 2008a). Hence if *agr* system in *L. monocytogenes* enables biofilm formation via quorum sensing mechanism remains to be deciphered (Garmyn et al., 2009).

Quorum Sensing can also occur via the production of autoinducer (AI-2) molecules (Garmyn et al., 2009). The gene *luxS* codes for an enzyme involved in the catalysis of precursor molecules of AI-2 that are shown to play a role in biofilm formation of many bacteria (Daines et al., 2005; Surette et al., 1999). In *L monocytogenes*, mutation of *luxS* leads to enhanced biofilm formation compared to the parental strain under batch and continuous biofilm conditions (Belval et al., 2006; Sela et al., 2006). The culture supernatant of *luxS* mutants were shown to accumulate copious amounts of AI-2 precursor molecules, S-adenosyl homocysteine (SAH) and S-ribosyl homocysteine (SRH). However addition of artificially synthesized AI-2 molecules did not change the phenotype of luxS mutants, but addition of its precursor SRH reverted the cells back to the wild type – phenotype. Hence the role of *luxS* in *L monocytogenes* is suggested to be confined to the detoxification pathway of SAH rather than acting as a signal molecule (Belval et al., 2006).

2.10.4 Other genes related to biofilm formation

2.10.4.1 Virulence

The virulence regulator PrfA plays a positive role in biofilm formation. In PrfA deletion mutants, the flagellar motility remains intact and the cells are able to attach. However the maturation of biofilms are severely compromised (Lemon et al., 2010). A transcriptional study on the PrfA deletion mutant of the strain EGDe revealed that 175 genes were regulated exactly opposite to that of the wild type. These genes were shown to be associated with various functions required for the metabolism of nucleic acids and lipids, cell wall synthesis, mobility and chemotaxis. Thus it was suggested that PrfA might play an indirect role and more than one pathway could be involved in biofilm formation of *L. monocytogenes*. (Luo et al., 2013).

Some isolates carry mutations in *inlA* that code for InlA proteins that are reduced in length. Isolates that produce truncated InlA demonstrate enhanced biofilm forming abilities but decreased virulence compared to the isolates that have full length InlA (Franciosa et al., 2009). Interestingly it was reported that the mutation that codes for truncated InlA tend to occur more commonly among food isolates but are rare among human clinical cases (Nightingale et al., 2005).

L. monocytogenes cultures treated with serratiopeptidase show marked reduction in their ability to form biofilms. It is suggested that the effect observed on biofilm formation, could be due to the reduction of surface proteins such as InlB, Ami, ActA and N-acetyl muramoyl-L-alanine amidase (Longhi et al., 2008).

2.10.4.2 Response regulator

An orthologue of the *Bacillus subtilis*, two component system DegU/DegS, has been identified in *L. monocytogenes*. However *L. monocytogenes* lacks the gene that codes for the cognate kinase DegS. DegU solely regulates itself by repressing its own synthesis. *degU* mutants were shown to lack flagella, possess reduced biofilm forming potential and virulence. Increasing levels of DegU phosphorylation is thought to be required for cells metamorphosing from planktonic motile state to a sessile biofilm state (Gueriri et al., 2008).

2.10.4.3 Cell wall associated proteins

Biofilm associated protein (Bap), is a cell wall anchored protein in *S. aureus* important for the biofilm formation of this pathogen on abiotic surfaces (Cucarella et al., 2001). The homolog of the protein in *L. monocytogenes*, called BapL, was found to not to be crucial for biofilm formation as some isolates without BapL formed biofilms equal to isolates in which the protein was present (Jordan et al., 2008).

dltABCD operon is involved in the addition of D-alanine residues into lipoteichoic acids making it negatively charged. The inability of *dltABCD* mutant to form biofilms could be due to loss of these D-alanine residues changing the surface hydrophobicity of the cell or the thickness of the teichoic acids (Alonso et al., 2014).

2.10.4.4 Stress response

In-frame deletion of the alternative stress response gene *sigB* affects the ability of strains to form biofilms in both static and continuous biofilm formation systems (van der Veen and Abee, 2010a). Mutants of guanosine pentaphosphate synthetase (*relA*) and hexose phosphate transport protein (*hpt*) were also shown to be affected in biofilm formation. The transcription of *relA* was

shown to increase after initial adherence indicating cells in surface attached growth require stringent response to cope with nutrient starvation (Taylor et al., 2002)

2.11 Summary

2.11.1 Conclusion

Persistence of *L. monocytogenes* in food processing plants is a huge problem as it poses a serious health risk, especially to people in the susceptible group. Bacterial cells within a biofilm are known to be protected against sudden changes in the environment and from the harsh effects of cleaning and disinfection. As a result, biofilms are considered to be one of the major factors for the prolonged survival of this pathogen within food processing plants. However this ability of persistence does not seem to be widespread among all the strains of *L. monocytogenes*. Instead, only few subtypes of this bacterium are reported to be prevalent ranging from few months to even years within the food processing establishments. Extensive studies have been carried out to understand the process of biofilm formation in *L. monocytogenes*. However, majority of these studies have evaluated biofilm forming abilities mostly under optimal conditions. While that being said, not all the isolates of *L. monocytogenes* are able to form strong biofilms even when provided with ideal conditions. Therefore the ability to form strong biofilms seem to be exclusive to certain isolates of *L. monocytogenes*, very much like the ability to persist in food processing plants. Studies investigating the genetic basis of biofilm formation in this pathogen have identified a number of genes that play a role in this complicated microbiological process. However the genetic determinants that contribute towards varying capacities of biofilm formation remains to be deciphered.

2.11.2 Research gaps

The need of the hour is to find ways to effectively control the prevalence of *L. monocytogenes* in food processing plants. To that end it is essential to understand the major factors that contribute to the persistence of this pathogen in the food processing environment. Biofilm formation is one such factor, however a major drawback of the biofilm studies performed thus far is that they are performed under optimal biofilm forming conditions. It is essential however to assess biofilm forming abilities in a model that closely relates to the food processing environment. Furthermore, there are no studies that have reported a link between genotype and differences in biofilm forming capacities. The proposed model in this study will allow establishing a link between phenotypic and genotypic characteristics leading to an understanding of variation in biofilm forming abilities of *L. monocytogenes* within food processing establishments. The genetic determinants identified could then potentially serve as markers that may indicate the risk of persistence of *L. monocytogenes* isolates within food processing plants. Furthermore this data could also be utilized by the CFIA to recommend necessary control measures if a risk is identified.

Chapter 3: Materials and Methods

3.1 Bacterial strains

All strains of *L. monocytogenes* used in the study were stored in BHI broth containing 25% (v/v) sterile glycerol (Catalogue# G5150, Sigma, Mississauga, Ontario, Canada) at – 80 ºC until use. Sixty six isolates of *L. monocytogenes* from food and clinical sources and one isolate from American Type Culture Collection (ATCC) were used in the study. Details of all the strains are provided in Table 1.

3.2 Media preparation for overnight culture and biofilm assay

All overnight cultures were performed in TSB supplemented with 0.6% (w/v) yeast extract (YE) (Cat# LP0021, OXOID, Nepean, Ontario, Canada) [TSB-YE]. To determine the influence of growth conditions on biofilm formation and to identify optimum period of incubation for biofilm formation under food processing conditions, MWB (CM813856, International Laboratory USA, California, USA) and in – house prepared 1% (v/v) beef broth (BB) in sterile Milli-Q (MQ) water were used.

BB was prepared by stomaching (Stomacher 400 circulator, Seward, Worthing, West Sussex, U.K) commercial medium ground beef (23% fat) with sterile MQ water in the ratio of 1:10 (w/v) at 300 rpm for 2 min in a Stomacher® closure bag (Cat# BA6141/CLR, Seward). The beef juice was then centrifuged (Allegra X-15R Centrifuge, Beckman Coulture, Brea, California, USA) at 232.32 × g for 10 min to spin down fat and cellular debris. Suspended particles were further removed by passing the beef juice through filters of Stomacher® filter bag (Cat# BA6141/STR, Seward). Beef juice was then heated at 72 ºC in a water bath for an hour. The BB was then aseptically aliquoted into 15-ml centrifuge tubes and was stored at -20 ºC until use.

Table 1. *L. monocytogenes* isolates used in the study

Lab.No	Isolate ID	Status	Origin
S1	LI0521[b]	Clinical	-
1	OLF13036[a]	Food	Dairy
2	OLF13037-1[a]	Food	Fresh smoked Atlantic salmon
3	OLF13038-1[a]	Food	Environmental
4	OLF13039[a]	Food	RTE meat
5	OLF13040[a]	Food	RTE meat
6	OLF13041[a]	Food	Environmental
7	OLF13042- 1[a]	Food	RTE Fish
8	OLF13043-1[a]	Food	RTE fish
9	OLF13046[a]	Food	RTE fish
10	OLF13047[a]	Food	Environmental
101	13LS-01 Lm 4e HPB 1848[b]	Food	-
102	13LS-02 Lm 1/2a HPB 4705[b]	Food	-
103	13LS-03 Lm 4a HPB 3501[b]	Food	-
104	13LS-04 Lm 4ab HPB 1265[b]	Food	-
105	13LS-05 Lm 4c HPB 4497[b]	Food	-
106	13LS-06 Lm 3b HPB 4909[b]	Food	-
107	13LS-07 Lm 4d HPB 18[b]	Food	-
108	13LS-08 Lm 1/2c HPB 1869[b]	Food	-
109	13LS-09 Lm 3a HPB 2768[b]	Food	-
110	13LS-10 Lm c HPB G1 [b]	Food	-
111	13LS-11 Lm 1/2b HPB 4857[b]	Food	-
112	13LS-12 Lm 4b PHB 5816[b]	Food	-
113	13LS-13 Lm 4b PHB 5906[b]	Food	-
114	13LS-14 Lm 4b PHB 6024[b]	Food	-
115	Listeria Lm ATCC 7644[e]	Clinical	-
121	GTA-L7[c]	Food	Environmental : dairy plant (cheese)
122	GTA-L13[c]	Food	Environmental :meat plant
123	GTA-L20[c]	Food	Environmental :meat plant
124	GTA-L37[c]	Food	Environmental :meat plant
125	GTA-L38[c]	Food	Environmental :meat plant
126	GTA-L44[c]	Food	Environmental: dairy plant (cheese)
127	GTA – L8[c]	Food	Maple leaf roast beef slices
128	OLF11077[a]	Food	-
130	OLF14031[a]	Food	-
131	OLF14036[a]	Food	-
132	OLF14040-1[a]	Food	-
133	OLF14048[a]	Food	-
134	OLF14078[a]	Food	-
135	OLF14105-1[a]	Food	-
136	OLF14144-1[a]	Food	-
137	OLF15016[a]	Food	-
138	15C0632302[d]	Clinical	Blood Culture
139	15P0055120[d]	Clinical	Blood Culture
140	15P0055120[d]	Clinical	Blood Culture
141	15A0156303[d]	Clinical	Blood Culture
142	15A0156522[d]	Clinical	Blood Culture
143	15C0744362[d]	Clinical	Blood Culture
144	15C0789637[d]	Clinical	Peritoneal fluid
145	15C0789737[d]	Clinical	CSF
146	15H0131100[d]	Clinical	Blood Culture

Lab.No	Isolate ID	Status	Origin
147	15C0789836[d]	Clinical	Blood Culture
148	15C0789846[d]	Clinical	Blood Culture
149	15C0789885[d]	Clinical	Blood Culture
150	15A0160894[d]	Clinical	Blood Culture
151	15A0160895[d]	Clinical	Blood Culture
152	15C0789928[d]	Clinical	Blood Culture
153	15A0168003[d]	Clinical	Blood Culture
154	15A0168004[d]	Clinical	Blood Culture
155	15H0131283[d]	Clinical	Pleural
156	15C0870347[d]	Clinical	Blood Culture
157	15H0131731[d]	Clinical	Blood Culture
158	15C0870710[d]	Clinical	Blood Culture
159	15C0870937[d]	Clinical	Blood Culture
160	15S0017664[d]	Clinical	Blood Culture
161	15C1045605[d]	Clinical	Blood Culture
162	15C1045621[d]	Clinical	Blood Culture

[a] - Pulsed Field Gel Electrophoresis Lab, Ottawa laboratory - Fallowfield (OLF), Canadian Food Inspection Agency (CFIA)
[b] - Health Canada, Ottawa
[c] - Greater Toronto Area Laboratory (GTA), CFIA
[d] - Public Health, Ontario
[e] - ATCC

3.3 Biofilm culture and assay procedure

Overnight cultures were grown in 10 to 12 ml of TSB-YE by inoculating 1/8th of a 10 µl loop from BHI plates. The culture was incubated at 37 °C for 22 hrs. The overnight culture was diluted in phosphate buffered saline (PBS, 0.01M, pH 7.2) to the required OD_{600} value (refer Sections 3.3.1 and 3.3.2). The culture was then further diluted to obtain a final concentration of 10^6 CFU per milliliter (CFU/ml) in the media used for biofilm assay. The concentration of the diluted bacterial culture was confirmed to be 10^6 CFU/ ml by plating on BHI agar plates for each experiment.

All biofilm assays were performed using 24 well non-treated tissue culture plates (Cat # 08772-51, Falcon, through Fisher Scientific, Nepean, Ontario, Canada). Biofilm assays were performed based on the method detailed by Djordjevic et al. (2002) with modifications. The wells of a 24

well plate were seeded with 2 ml of the bacterial suspension, and wells containing media alone served as a negative control. Plates were incubated at appropriate temperature for required number of days (refer Sections 3.3.1 and 3.3.2 for details on growth media, temperature and period of incubation). Biofilms of *L. monocytogenes* isolates formed in the wells of the 24 well plates at a specific time point were rinsed three times after removing the culture media with ~ 4 ml of water and allowed to air dry. Biofilms were then stained by adding 2 ml of 0.1% (v/v) Gram's crystal violet (Cat# 4312526, BD, through Fisher Scientific, Nepean, Ontario, Canada) and incubated at ambient temperature for 30 min. The dye was removed and the wells were rinsed three times with MQ water. Visual confirmation of crystal violet stained biofilms were obtained using inverted light microscopy (refer Section 3.3.3.1). For representative isolates, confirmation using scanning electron microscopy was also performed in addition to crystal violet staining and inverted light microscopical observation (refer Section 3.3.3.2). The bound crystal violet was then de-stained by adding 0.5 ml of 95% (v/v) ethanol (de-staining solution) to the wells with shaking on a titer plate shaker (Model # 4625, Lab – Line Instrument Inc., Melrose Park, Illinois, USA) for 30 min. Crystal violet dissolved in the de-staining solution (200 µl) was then transferred to wells of a 96 well plate and read at 595 nm (Cat # 168-1135, Bio-Rad i-Mark Microplate Reader, Hercules, California, USA).

3.3.1 Influence of different growth conditions on biofilm formation and determination of optimum period of incubation for biofilm formation in food processing conditions

Strains S1 and 115 were used in this section. Strain 115 (ATCC 7644) served as the positive control since it has been shown to be able to form biofilms in many published studies (Di Bonaventura et al., 2008; Longhi et al., 2008; Moltz and Martin, 2005). Overnight culture was standardized to an OD_{600} 0.120 – 0.130 (~ 10^7 CFU/ ml) before performing the final dilution (~

10^6 CFU/ ml) for biofilm assay. The growth conditions used in this section are listed in Table 2. Biofilm formation in the mentioned growth conditions was assessed everyday over a period of 14 days. The raw data was subtracted with a cut-off OD value (OD_c) that was determined for every growth condition separately by taking the average of the negative control (media) plus 3 standard deviations to remove background.

Subsequently, statistical analysis (refer Section 3.5.2) of biofilm formation at 12 ºC in BB was performed, to identify the best time point for screening a large number of *L. monocytogenes* isolates for their biofilm forming potential in this growth condition.

Table 2. Growth conditions used for biofilm formation

Growth Media	Temperature	Rationale
1% BB	12 ºC	Related to food processing environment
MWB	37 ºC	Optimal condition
1% BB	37 ºC	Comparative
MWB	12 ºC	Comparative

3.3.2 Screening food and clinical isolates of *L. monocytogenes* for biofilm forming potential in food processing conditions

The biofilm forming ability of 67 *L. monocytogenes* isolates including the positive control was assessed in BB at 12 ºC following 9 days of incubation based on the results in the preliminary experiments in this study. Strain 115 served as the positive control. Overnight culture was standardized to an OD_{600} 0.180 – 0.230 (~ 10^8 CFU/ ml) before performing the final dilution (~ 10^6 CFU/ ml) for biofilm assay. Each isolate was screened for their biofilm potential in three independent experiments and each experiment had four technical replicates per isolate.

Biofilm formation of the isolates were assessed and classified in to non-formers, weak biofilm formers, moderate biofilm formers and strong biofilm formers based on the method published by Stepanović et al. (2004). Briefly, If $OD \leq OD_C$ = no biofilm formation, $OD_c < OD \leq (2 \times OD_C)$ = weak biofilm formation, $(2 \times OD_C) < OD \leq (4 \times OD_C)$ = moderate biofilm formation, $(4 \times OD_C) < OD$ = strong biofilm formation.

3.3.3 Microscopy

3.3.3.1 Inverted Light Microscopy

Crystal violet stained biofilms in the wells of the 24 well plate were viewed using Zeiss Axiovert 10 inverted microscope (Oberkochen, Germany) at x20 magnification every day to verify potential formation of biofilms.

3.3.3.2 Scanning Electron Microscopy

3.3.3.2.1 Sample preparation for SEM

The polystyrene coupons used for SEM were prepared by cutting uncoated polystyrene cell culture microscope slides (Cat. #71888, Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) into approximately 11 mm squares using a scalpel and plyers. The polystyrene coupons were then labelled with a diamond marking pencil (Fisherbrand) and laboratory marker (VWR). Coupons were sterilized with 100% ethanol for 20 mins followed by 70% ethanol for 20 minutes and rinsed in sterile MQ water prior to being placed into a sterile polystyrene 24-well plate containing bacterial culture in BB. After biofilm formation, the coupons were gently rinsed 2 times with 0.1 M phosphate buffer (pH 7.4) then fixed with 2.5% glutaraldehyde (EM Grade 70% solution, Cat. #16365, Electron Microscopy Sciences) in 0.1 M phosphate buffer (pH 7.4).

The coupons were stored in fixative at 4 ºC (minimum 24 hours) till further processing for SEM. Note that only during the fixation and rinsing time the coupons were allowed to gently rotate on a shaker for continuous contact with fresh solution.

3.3.3.2.2 SEM Examination

After fixation, the coupons were rinsed in 4 changes of 0.1 M phosphate buffer for a minimum of 30 minutes each and then post-fixed in 2% osmium tetroxide (Cat. #19134, Electron Microscopy Sciences) in MQ water for 1 hour. The coupons were rinsed in sterile MQ water for 15 minutes to remove excess osmium tetroxide then dehydrated in graded series of ethanol 30%, 50%, 70%, 85%, 95%, 15 minutes each followed by 3 changes of 100% ethanol (Anhydrous Ethyl Alcohol, Commercial Alcohols, Brampton, Ontario, Canada) 1 hour each. Critical point drying was carried out using the Autosamdri – 814 (Rockville, Maryland, USA) critical point dryer. The coupons were mounted on 12.7 mm slotted stubs (Cat. #75210, Electron Microscopy Sciences) using silver conductive adhesive (Cat. #12684-15, Electron Microscopy Sciences) and 12 mm Pelco™ carbon conductive tabs (Cat. #16084-1, Ted Pella Inc., Redding, California, USA). Mounted samples were sputter coated with gold using Emitech K550X Sputter Coater (Ashford, Kent, U.K) and viewed on FEI Quanta 600 SEM (Hillsboro, Oregon, USA).

3.4 Identification of genetic determinants responsible for varying capacities of biofilm formation

3.4.1 Analysis of biofilm associated genes using PCR

Representative isolates from each phenotype were screened for 20 target genes associated with biofilm formation (selected based on published studies) as listed in Table 3 using a combination of singleplex and multiplex PCR procedure developed in house. Also, singleplex PCR was used

for confirmation of presence/absence of amplicons if they could not be detected in multiplex PCR reactions. A total of 28 isolates including the positive control (115) were used in this study. The test isolates included: 2, 3, 4, 7, 9, 101, 102, 103, 104, 108, 111, 123, 127, 128, 132, 133, 137, 140, 148, 158, 161 and 162 identified as strong biofilm formers, 106, 121, 122 and 126 identified as moderate biofilm formers and 107 identified as weak biofilm former based on the biofilm assay results obtained in Section 3.3.2. Bacterial strains were grown overnight in 10 ml of BHI broth at 37 ºC. DNA extraction was performed using the Qiagen DNeasy Mini Prep kit for Gram positive bacteria (Cat. #74106, Qiagen Inc. Canada, Toronto, Ontario). Primers for 20 genes (Table 3) were synthesized by Integrated DNA technologies (IDT) (Coralville, Iowa, USA). Each group of the multiplex PCR was arranged in house based on the size of the end products and annealing temperatures (Table 3) with extensive verification to make sure optimal amplification of each target gene was achieved. PCR reactions were performed using the Qiagen Multiplex PCR kit (Cat. #206143, Qiagen Inc.). Amplification reactions were carried out in a Biometra TPersonal 48 thermocycler (Cat. #846-050-551, Gottingen, Germany). Initial denaturation was carried out at 94 ºC for 30 sec, extensions were carried out at 72 ºC for 90 sec, and a total of 35 amplification cycles were performed. The final extension was done at 72 ºC for 10 min. PCR products were resolved by electrophoresis on 1.5% agarose gel in 1X TAE buffer stained with SYBR Safe DNA gel stain (Cat. #S33102, Invitrogen, Carlsbad, USA) and visualized using a Bio-Rad Universal Hood (S. No - 75S/03383) and Life technologies E-Gel Imager (Carlsbad, California, USA).

Table 3. Primers used for analysis of biofilm associated genes using PCR

Reaction	Target	Primers	Forward primer Sequence	Reverse Primer Sequence	Anneal temp (ºC)	Reference
	luxS	luxS	ATGGCAGAAAAAATGAATGTAGAAA	TTATTCACCAAACACATTTTTCCA		Ali, 2011
1	*actA2*	actA2	GACGAAAATCCCGAAGTGAA	CTAGCGAAGGTGCTGTTTCC	49 ºC	Jaradat et al., 2002
	prfA	prfA	GATACAGAAACATCGGTTGGC	GTGTAATCTTGATGCCATCAGG		Kérouanton et al., 2010
	inlB	inlB	AAAGCACGATTTCATGGGAG	ACATAGCCTTGTTTGGTCGG		Meloni et al., 2012
	rrn	rrn	CAG CAG CCG CGG TAA TAC	CTC CAT AAA GGT GAC CCT		Meloni et al., 2012
	iap	list1	CAAACTGCTAACACAGCTACT	TTATACGCGACCGAAGCCAAC		Bubert et al., 1999
	agrC	agrC	ATTAATACGGCAACCAACGAAC	AAATCGGTGGCATATTTACTGG		Riedel et al., 2009
2	*gad2*	gad2	AATACCTTGCCCATGCAGTC	GGCTTGGAAATCTTGGATGA	49 ºC	Karatzas et al., 2010
	plcA	plcA	CGA GCA AAA CAG CAA CGA TA	CCG CGG ACA TCT TTT AAT GT		Meloni et al., 2012
	intJ	intJ	TGTAACCCCGCTTACACAGTT	TTACGGCTGGATTGTCTGTG		Liu et al., 2003
3	*rpoB*	rpoB	TCGCAGTTATCTCAGTTCATGG	TAGCGCACGGTTACTATCATCG	51 ºC	Makariti et al., 2015
	sigB	sigB	TCATCGGTGTCACGGAAGAA	TGACGTTGGATTCTAGACAC		Bae et al., 2011
	plcB	plcB	CTGCTTGAGCGTTCATGTCTCATCCCCC	ATGGGTTTCACTCTCCTTCTAC		Osman et al., 2014
4	*actA*	actA1	CGCCGCGGAAATTAAAAAAAGA	ACGAAGGAACCGGGCTGCTAG	55 ºC	Jallewar et al., 2007
	hlyA	hlyA	GCAGTTGCAAGCGCTTGGAGTGAA	GCAACGTATCCTCCAGAGTGATCG		Osman et al., 2014
	iap	iap1	ACAAGCTGCACCTGTTGCAG	TGACAGCGTGTGTAGTAGCA		Osman et al., 2014
5	*agrA*	agrA	CGAATGCCTACACATCAAGGTA	TCACCACACCTTTTGTCGTATC	50 ºC	Riedel et al., 2009
6	*agrB*	agrB	AAAGTCCCTTTGTCAGAAAGAATG	CACCTGAAACAAAGATCCTACCA	50 ºC	Riedel et al., 2009
7	*agrD*	agrD	TCGCCTTAGTAACAGGGCTTT	CGTGCAATGTTTTGG	51 ºC	Ali, 2011
8	*inlA*	inlA	AGCCACTTAAGGCAAT	AGTTGATGTTGTGTTAGA	43 ºC	Almeida & Almeida, 2000
9	*mpl*	mpl	TTG TTC TGG AAT TGA GGA TG	TTA AAA AGG AGC GGT GAA AT	46 ºC	Meloni et al., 2012
10	*bapL*	bapL	TGCTCCAGCGAAAATCAA	TGCTTCCCAGTAATACAACG	48 ºC	Jordan et al., 2008

3.4.2 Analysis of biofilm associated genes using BLAST

Whole Genome Sequences of all the isolates were provided by the CFIA laboratory, Carling except for the isolate S1, which was obtained from NCBI published by Pightling et al. (2014). Subsequently, BLAST analysis was performed at a 30% cut-off value using the Gene Seeker script for all the isolates. The gene sequences of 106 biofilm associated genes were obtained from the NCBI website using six reference strains (EGD-e, 10403S, F2365, LL195, L312 and M7). The list of genes and their reference strains can be found in *Appendix A2*. These genes were used to BLAST an *L. monocytogenes* database containing roughly 700 genomes from NCBI and Ottawa Laboratory – Carling (OLC), CFIA. In order to ensure the reliability of the results generated, the script utilized a set of genes (*abcz, bglA, cat, dapE, dat, idh*, and *lhkA*) in Multi Locus Sequence Typing (MLST), as a means of quality control (QC). The genomes that showed absence for any one of these QC genes were not included in the study. Query sequences of isolates that showed an absence of one or more genes with the reference sequence for the aforementioned biofilm associated genes were considered exclusive. Ten strains as such from the database were selected for phenotype (Biofilm Assay) analysis. BLAST analysis was then extended to the rest of the isolates in the study to identify the presence/absence of the genes and correlate them with the biofilm forming phenotype

3.5 Statistical Analysis

All analyses were performed using GraphPad Prism Version 6.01. (La Jolla, California, USA)

3.5.1 Influence of different growth conditions on biofilm formation:

Biofilm formation was analyzed individually for each strain. Biofilm formation (OD value) between different growth conditions and incubation periods (37 ºC - MWB, 12 ºC - BB, 12 ºC -

MWB and 37 ºC - BB) was analyzed using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test ($\alpha = 0.05$). Results were considered significant if $P < 0.05$.

3.5.2 Determination of optimum period of incubation for biofilm formation in food processing conditions

Student's paired-*t*-test was used to compare biofilm formation between different days of incubation at 12 ºC in BB. Results were considered significant if $P < 0.05$.

3.5.3 Determination of biofilm formation based on the source of isolation

Student's unpaired-*t*-test was used to compare biofilm formation between clinical and food isolates. Results were considered significant if $P < 0.05$.

Chapter 4: Results

4.1 Influence of different growth conditions on biofilm formation

Two strains (S1 and 115) were tested to investigate the influence of different growth conditions on biofilm formation. The conditions included two broth media (BB and MWB) and incubation at two temperatures (12 and 37 ºC) for the period of 14 days. All the results in the sections 4.1 (Fig. 2A and 2B) are the average of three separate experiments with 3 replicates in each experiment. The OD_c values determined for each growth condition separately to remove the background are as follows; 0.056 for 12 ºC BB, 0.063 for 12 ºC MWB, 0.040 for 37 ºC BB and 0.086 for 37 ºC MWB.

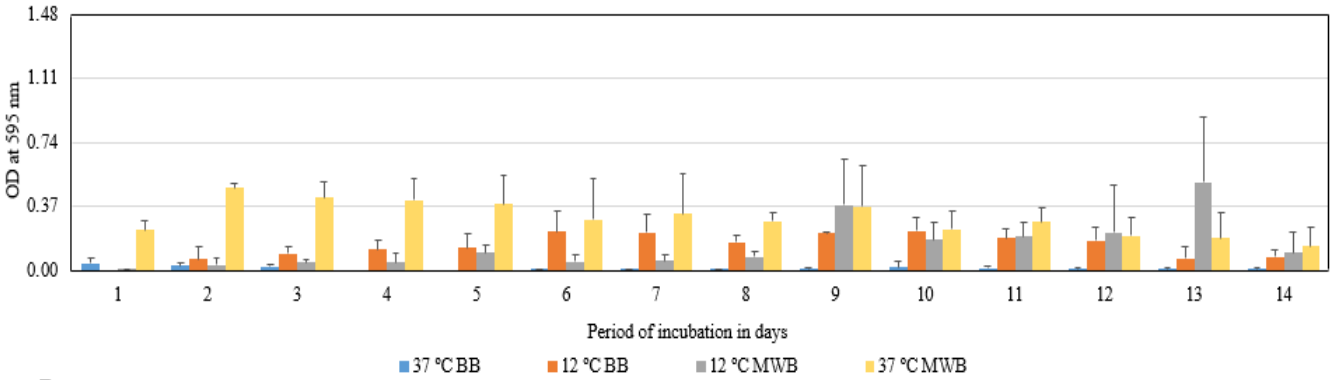
Significant biofilm formation was observed in all the growth conditions ($P<0.0001$, one–way ANOVA). However biofilm formation varied based on the growth media, temperature and the tested strain. When incubated at 12 ºC, strains S1 (Fig. 2A) and 115 (Fig. 2B) showed very similar biofilm formation pattern in both media with slow biofilm growth followed by moderate to strong biofilm formation observed after day 6 for incubation in BB and day 9 for incubation in MWB. On the other hand, biofilm formation pattern for both the strains were different for the incubation at 37 ºC. When incubated in MWB, both the strains showed moderate biofilm formation starting from day 1 and the OD_{595} values continued to remain in the moderate to strong range throughout the 14 day period. However in BB, there was no biofilm formation for the strain S1 and the moderate biofilm formation observed for strain 115 on day 1 slowly declined and became undetectable after day 4.

A comparison of the biofilm formation using different growth conditions showed that significantly higher biofilm formation ($P < 0.001$, one-way ANOVA) was observed when MWB was used at 37 ºC irrespective of the strain being considered. No significant difference was observed in the

biofilm OD_{595} values obtained for the two strains when tested at 12 ºC in BB and MWB ($P > 0.05$, Tukey's multiple comparison test). The OD_{595} values obtained for strain S1 at 12 ºC in both media were significantly higher than those obtained for BB at 37 ºC ($P < 0.005$, Tukey's multiple comparison test) (Fig. 2A). However the same was not true for strain 115 as only a slightly higher OD value ($P = 0.03$, Tukey's multiple comparison test) was observed in BB at 12 ºC when compared to 37 ºC (Fig. 2B).

Therefore, the effect of growth media on biofilm formation was minimal for both the strains at 12 ºC. Moreover biofilm formation was observed to be more consistent at this temperature. However for the incubation at 37 ºC, a medium dependent biofilm formation was observed.

A


1.48
1.11
0.74
0.37
0.00
OD at 595 nm
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Period of incubation in days
37 °C BB
12 °C BB
12 °C MWB
37 °C MWB

B

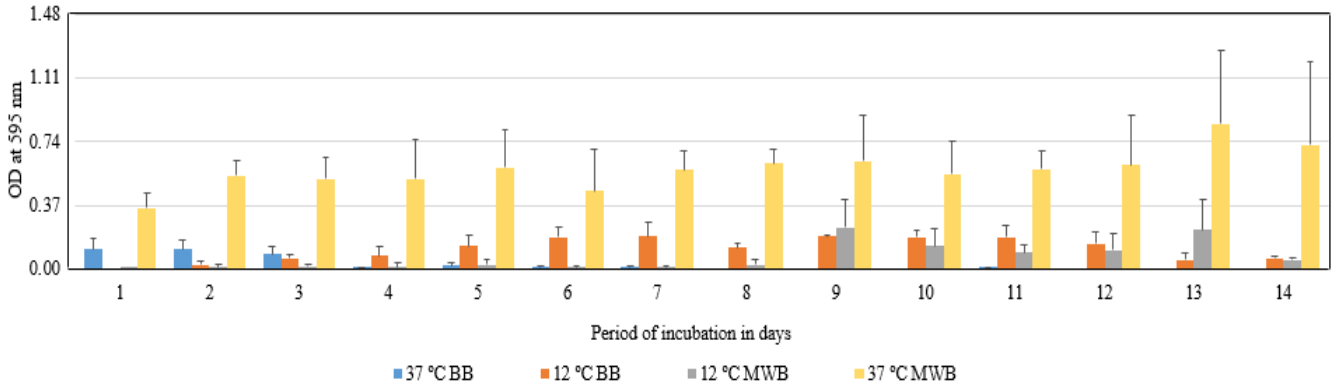

1.48
1.11
0.74
0.37
0.00
OD at 595 nm
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Period of incubation in days
37 °C BB
12 °C BB
12 °C MWB
37 °C MWB

Fig 2: Influence of different growth conditions on biofilm formation: Biofilm assay performed over a period of 14 days in four growth conditions: 37 ºC in MWB, 12 ºC in BB, 12 ºC in MWB and 37 ºC in BB. (A) Biofilm formation of strain S1 in four growth conditions; (B) Biofilm formation of strain 115 in four growth conditions. The numbers presented in this graph represent the average of 3 experiments with standard deviation.

4.1.1 Determination of optimum period of incubation for biofilm formation in food processing conditions

Following the study in section 4.1 that showed consistent quantifiable biofilm formation at 12 ºC in BB, biofilm formation between different days were compared using paired Student's *t*-test in order to obtain a single consistent and optimal incubation time for screening large number of isolates in the next section of the study.

Early time points, 1, 2, 3 and 4 and very late time points 13 and 14 had significantly (P<0.05, Student *t*-test) lower biofilm growth compared to days 6, 7, 9, 10 and 11. However biofilm formation between days 6, 7, 9, 10 and 11 were not significantly different (P > 0.05, Student *t*-test) from one another. Biofilm OD_{595} values at day 8 were lower than day 9 (P < 0.05, Student *t*-test) but was not significantly different than days 6, 7, 10 and 11 (P > 0.05, Student *t*-test). Since day 9 gave the most consistent results, it was selected as the time point for screening *L. monocytogenes* isolates for their biofilm forming potential in this growth condition (Fig 3).

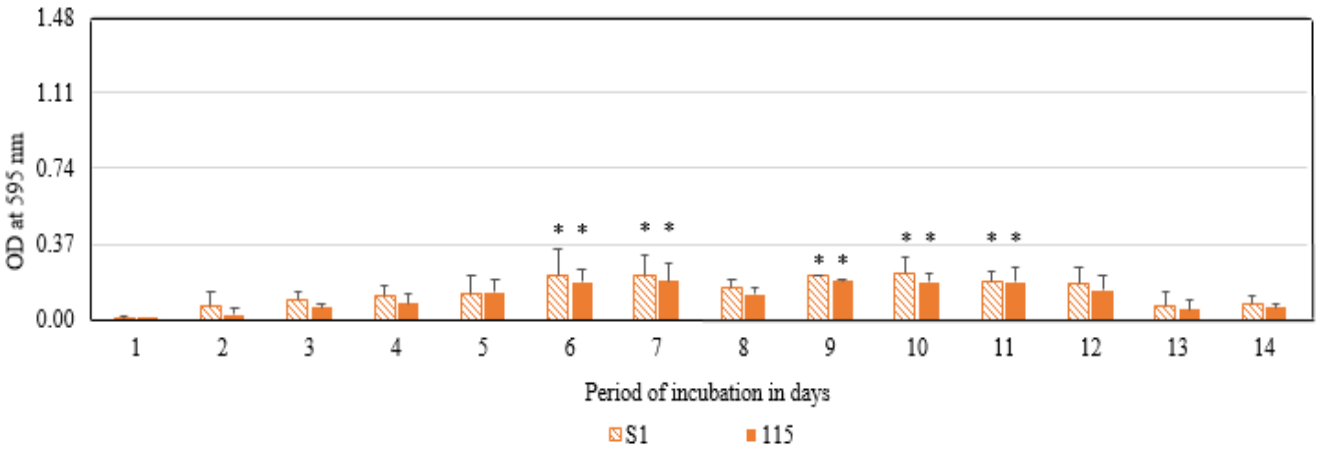

1.48
1.11
0.74
0.37
0.00
OD at 595 nm
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Period of incubation in days
S1
115

Fig 3: Determination of optimum period of incubation for biofilm formation in food processing conditions. Biofilm formation by strains S1 and 115 at 12 ℃ in BB is presented separately in this graph. * represents significant OD values ($P < 0.05$) obtained on days 6, 7, 9, 10 and 11 compared to early and late time points based on paired Student's-*t*-test.

4.1.2 Microscopical confirmation of biofilm formation

Light microscopy images showed that the accumulation of bacterial cells stained with crystal violet correlated with increasing incubation times and temperature on days 1, 4 and 9 at 12 ºC in BB (Fig. 4. A-C) and 37 ºC in MWB (Fig. 4. D-F) for strain 115.

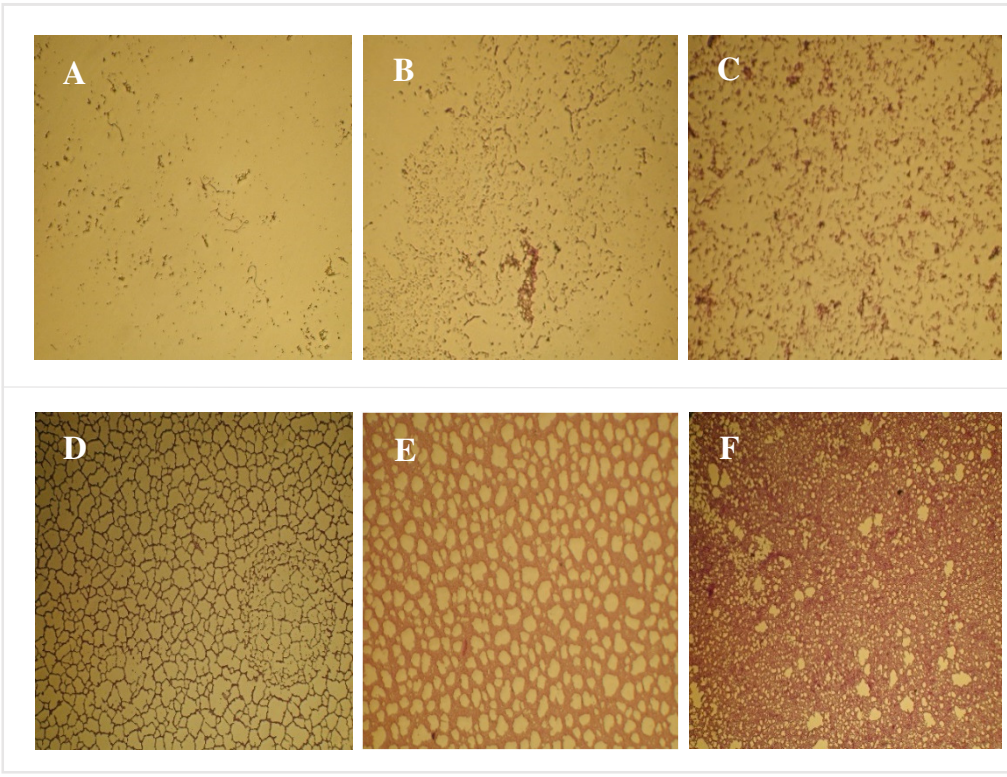

A
B
C
D
E
F

Fig 4: Inverted Light microscopy images of strain 115. Images taken at a magnification of ×20 show clear differences in crystal violet staining between different incubation times and temperature. Biofilm formation at (A) Day 1 (B) Day 4 and (C) Day 9 at 12 ℃ in BB. Relatively high biofilm formation observed at (D) Day 1 (E) Day 4 and (F) Day 9 when incubated at 37 ℃ in MWB.

4.2 Screening of *L. monocytogenes* isolates in food processing conditions

Sixty seven isolates including the positive control (115) were screened for biofilm formation capacities by testing in BB at 12 ºC for 9 days. Biofilm OD_{595} values of the isolates varied from OD_{595} 0.076±0.035 to 0.517±0.027 (Fig. 5). The OD_C value was determined to be 0.056. Accordingly, isolates with OD_{595} values < 0.056 were considered non biofilm formers, OD_{595} values between 0.056 and 0.112 were considered weak biofilm formers, OD_{595} values between 0.112 and 0.224 were considered moderate biofilm formers and OD_{595} values > 0.224 were considered strong biofilm formers. Out of 66 test isolates there was only one isolate (107) that showed a weak biofilm forming ability and five isolates (S1, 121, 122, 126 and 106) with moderate biofilm forming ability, but the rest of the isolates were grouped under strong biofilm forming phenotype under the tested condition. Furthermore, the positive control isolate 115 also displayed a moderate biofilm forming phenotype.

The strong biofilm forming group consisted of 60 isolates and a range of biofilm OD_{595} values were observed within this phenotype. Twenty eight (46.7%) out of 60 isolates were found in the lower range displaying OD_{595} values between 0.230 ± 0.105 and 0.336 ± 0.038 ($4\times OD_C - 6 \times OD_C$), 40% (24 out of 60) of the isolates were found in the mid-range with OD_{595} values between 0.338 ± 0.207 and 0.437 ± 0.157 ($6 \times OD_C - 8 \times OD_C$) and 13.3% (8 out of 60) of the isolates were in the upper range with OD_{595} values between 0.456 ± 0.034 and 0.517 ± 0.027 ($> 0.8 \times OD_C$).

Near half of the clinical isolates (12 out of 26 clinical isolates) were found in the lower range. The remaining were found to be almost equally distributed with 7 isolates in the mid-range and 6 isolates in higher range of the strong biofilm forming spectrum. Isolates of food origin were found to be mainly concentrated in the lower range to mid-range of the strong biofilm forming spectrum

with 16 and 17 isolates respectively in each range. While the upper range of the strong biofilm forming phenotype were poorly represented by food isolates with only two strains, the moderate biofilm forming phenotype consisted 4 out of 5 isolates that originated from food. The only weak biofilm former was also of food origin. When the biofilm formation of isolates based on their source was considered, the average absorbance value of clinical isolates (OD = 0.369) was found to be slightly higher ($P < 0.04$, unpaired Student's-*t*-test) than food isolates (OD = 0.324) under the tested condition.

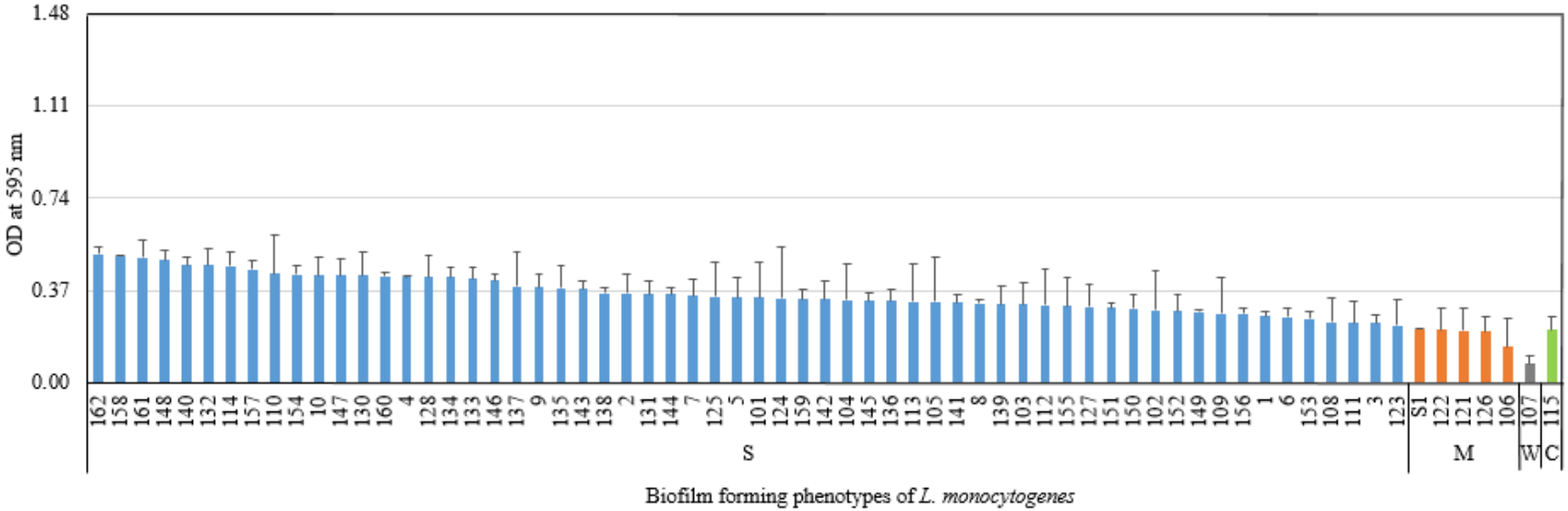

1.48
1.11
0.74
0.37
0.00
OD at 595 nm
162 158 161 148 140 132 114 157 110 154 10 147 130 160 4 128 134 133 146 137 9 135 143 138 2 131 144 7 125 5 101 124 159 142 104 145 136 113 105 141 8 139 103 112 155 127 151 150 102 152 149 109 156 1 6 153 108 111 3 123 S1 122 121 126 106 107 115
S
M
W
C
Biofilm forming phenotypes of L. monocytogenes

Fig 5: Biofilm forming phenotypes of *L. monocytogenes*. Varying biofilm forming capacities obtained when 66 isolates were tested at 12 ºC in BB. Based on the OD_{595} values, isolates were divided into weak (W), moderate (M) and strong (S) biofilm formers. C represents the positive control, strain 115. The numbers presented in this graph represent the average of 3 experiments with standard deviation.

4.2.1 Microscopical confirmation of biofilm formation

The images from selected strains with crystal violet staining (Fig.6-1. A-D) were in line with images obtained through EM [Fig. 6-2 (A-B) – 6-5 (A-B)], as strains with higher OD_{595} values displayed a three dimensional layer of cells in large clusters in comparison to strains with lower OD_{595} values which had cells clustering in smaller groups. Furthermore strong biofilm formers (Fig.6-2 – 6-4) were able to adhere in high numbers to the polystyrene coupon surface unlike the weak biofilm former (Fig. 6-5) which had very few adherent cells.

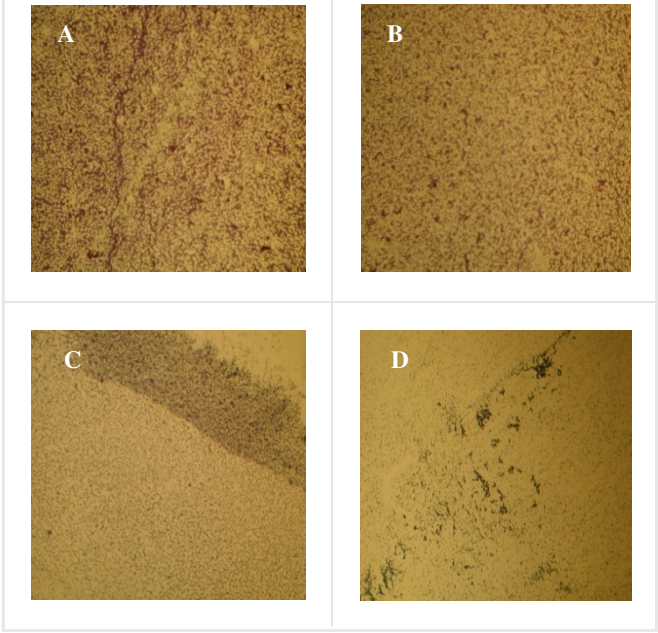
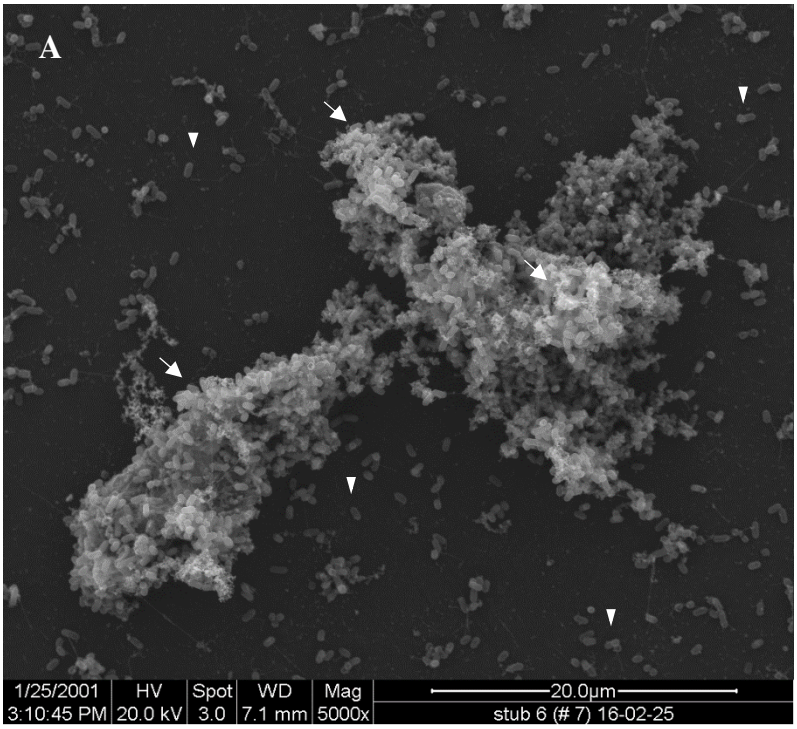

A
B
C
D

Fig 6-1: Inverted Light microscopy images of representative isolates. Images taken at a magnification of ×20 show a correlation between crystal violet staining of cells and biofilm OD_{595} values. (A) strain 148 (OD_{595} – 0.491 ± 0.041); (B) strain 140 (OD_{595} - 0.472±0.029); (C) strain 127 (OD_{595} - 0.304±0.090); (D) strain 107 (OD_{595} – 0.076±0.035).


A
1/25/2001 | HV | Spot | WD | Mag | 20.0µm
3:10:45 PM | 20.0 kV | 3.0 | 7.1 mm | 5000x | stub 6 (# 7) 16-02-25

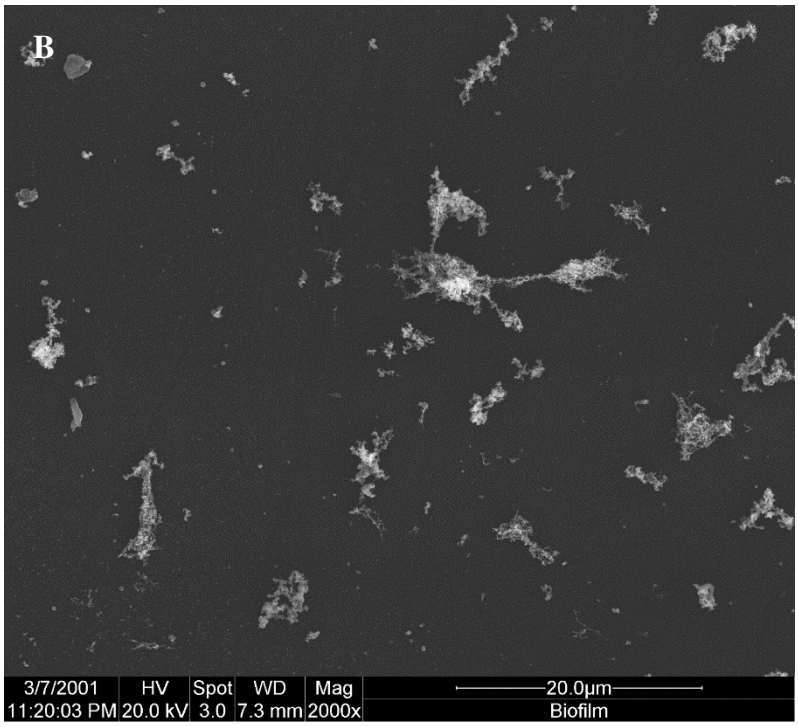

B
3/7/2001 | HV | Spot | WD | Mag | 20.0µm
11:20:03 PM | 20.0 kV | 3.0 | 7.3 mm | 2000x | Biofilm

Fig 6-2: Scanning Electron Microscopy image of strain 148: Images taken at a magnification of ×5000 and ×2000, respectively. Arrows indicate cluster of cells and arrow heads indicate adherent cells. Biofilm formation correlated with increase in biofilm OD_{595} values. A: strain 148 (OD_{595} – 0.491 ± 0.041); B: Negative control without bacterial inoculation showing debris from beef broth. Scale bars, 20 μm.

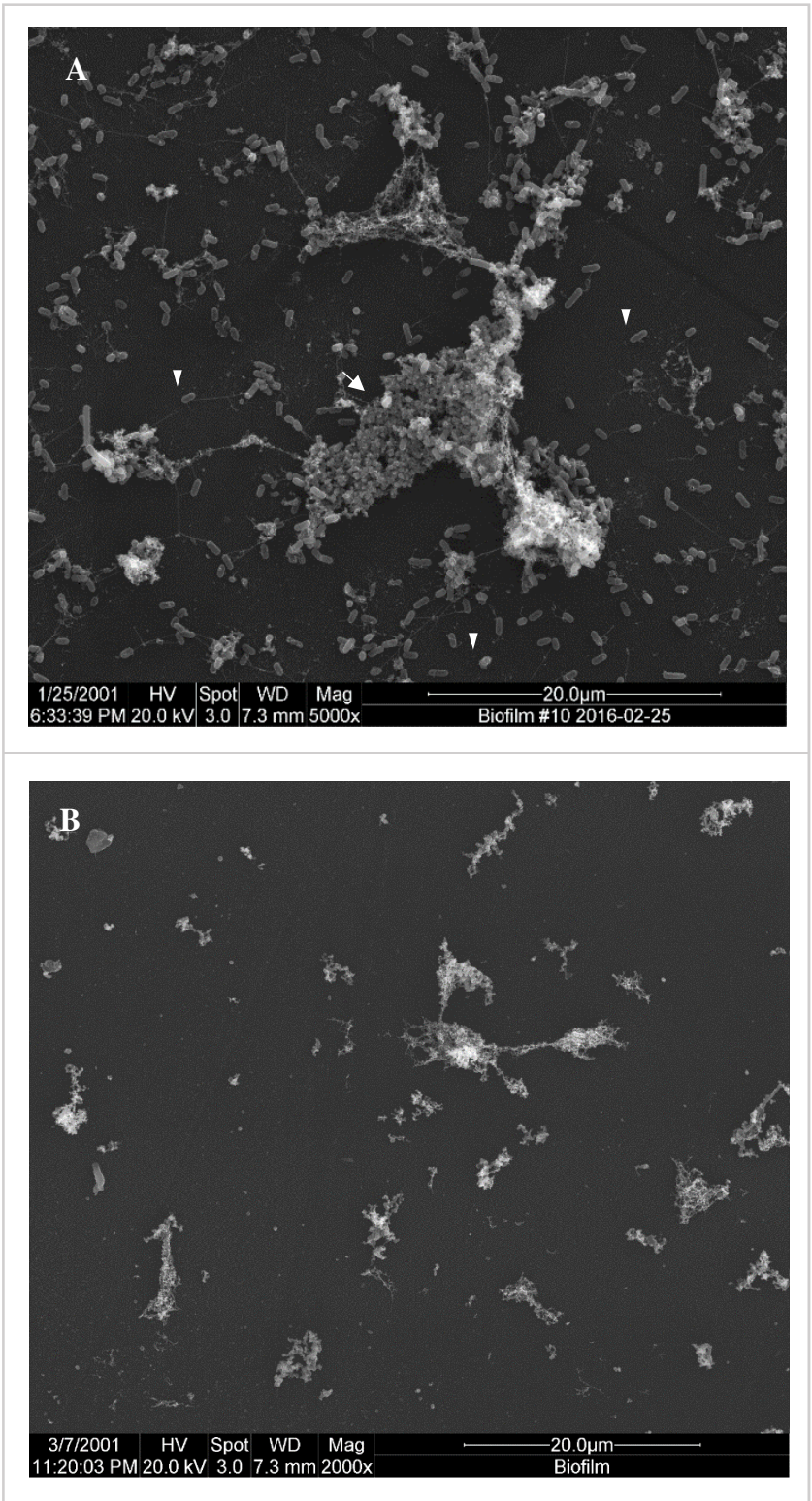
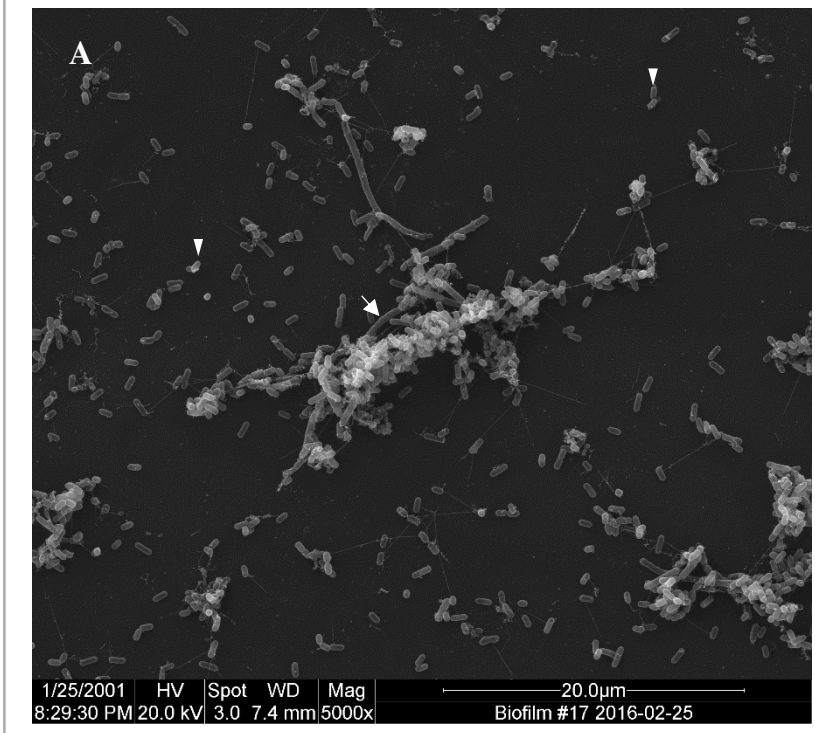

A
1/25/2001 HV Spot WD Mag
6:33:39 PM 20.0 kV 3.0 7.3 mm 5000x
20.0µm
Biofilm #10 2016-02-25
B
3/7/2001 HV Spot WD Mag
11:20:03 PM 20.0 kV 3.0 7.3 mm 2000x
20.0µm
Biofilm

Fig 6-3: Scanning Electron Microscopy image of strain 140: Images taken at a magnification of ×5000 and ×2000, respectively. Arrows indicate cluster of cells and arrow heads indicate adherent cells. Biofilm formation correlated with increase in biofilm OD_{595} values. A: strain 140 (OD_{595} - 0.472±0.029); B: Negative control without bacterial inoculation showing debris from beef broth. Scale bars, 20 μm.


A
1/25/2001 HV Spot WD Mag
8:29:30 PM 20.0 kV 3.0 7.4 mm 5000x
20.0µm
Biofilm #17 2016-02-25

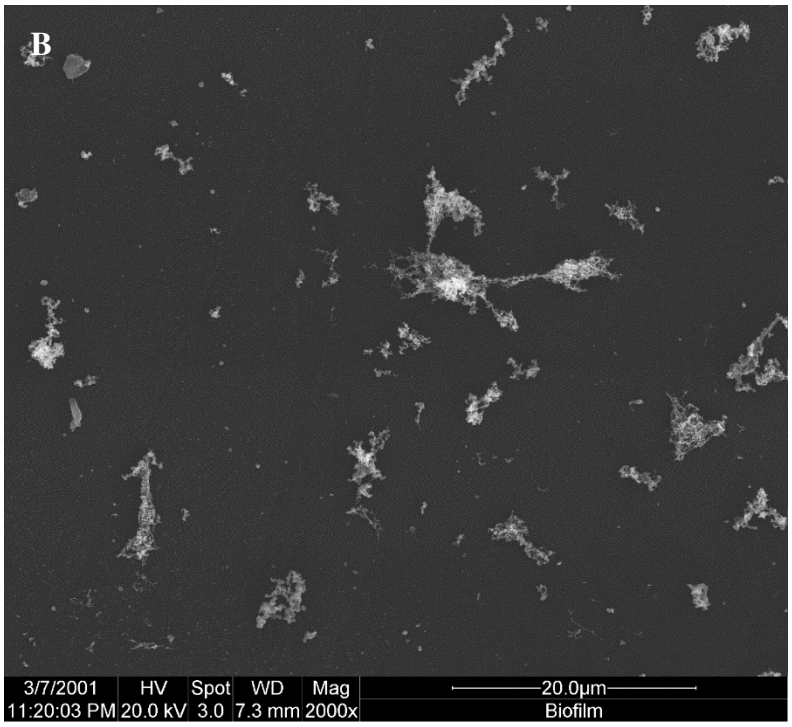

B
3/7/2001 HV Spot WD Mag
11:20:03 PM 20.0 kV 3.0 7.3 mm 2000x
20.0µm
Biofilm

Fig 6-4: Scanning Electron Microscopy image of strain 127: Images taken at a magnification of ×5000 and ×2000, respectively. Arrows indicate cluster of cells and arrow heads indicate adherent cells. Biofilm formation correlated with increase in biofilm OD_{595} values. A: strain 127 (OD_{595} - 0.304±0.090); B: Negative control without bacterial inoculation showing debris from beef broth. Scale bars, 20 μm.

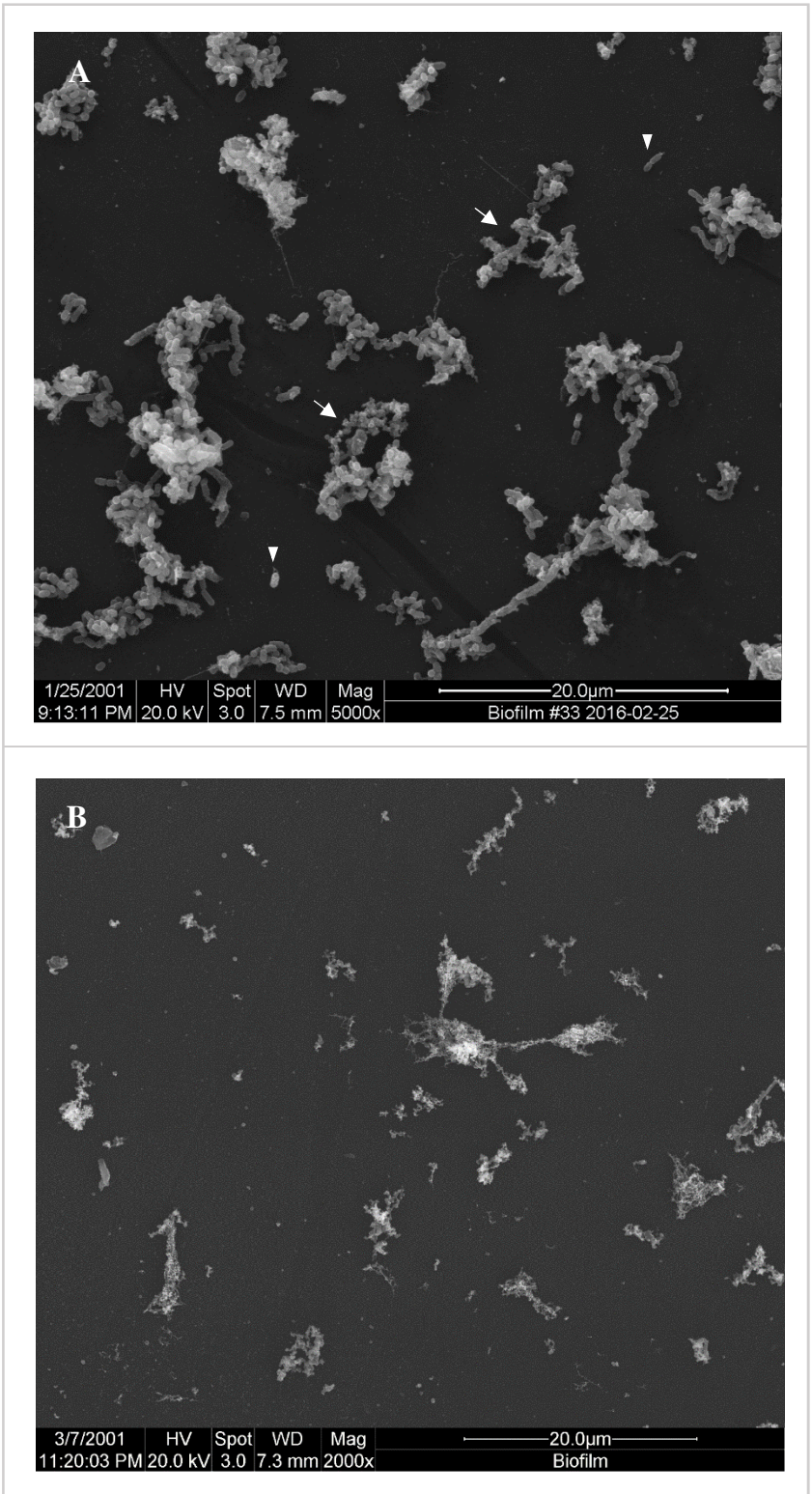

A
1/25/2001 HV Spot WD Mag
9:13:11 PM 20.0 kV 3.0 7.5 mm 5000x
20.0µm
Biofilm #33 2016-02-25
B
3/7/2001 HV Spot WD Mag
11:20:03 PM 20.0 kV 3.0 7.3 mm 2000x
20.0µm
Biofilm

Fig 6-5: Scanning Electron Microscopy image of strain 107: Images taken at a magnification of ×5000 and ×2000, respectively. Arrows indicate cluster of cells and arrow heads indicate adherent cells. Biofilm formation correlated with increase in biofilm OD_{595} values. A: strain 107 (OD_{595} – 0.076±0.035); B: Negative control without bacterial inoculation showing debris from beef broth. Scale bars, 20 μm.

4.3 Analysis of biofilm associated genes using PCR

Twenty seven isolates (including the isolate with weak biofilm forming ability, four isolates with moderate biofilm forming ability and twenty two isolates with strong biofilm forming ability) were tested using a combination of singleplex and multiplex PCR with 22 sets of primers targeting 20 genes associated with biofilm formation. The isolates within the strong biofilm forming group were selected in such a way to represent all the three ranges of OD_{595} values observed within the phenotype (low, mid-range and high). Among the 20 biofilm associated genes tested, all the isolates (Table 4) showed the presence of 16 genes including *agrA, agrB, agrC, gad2, hlyA, inlB, iap, mpl, plcA, plcB, rpoB, rrn, sigB, inlJ, luxS*, and *prfA*. The PCR products of these genes migrated to the expected positions when analyzed by agarose gel electrophoresis.

The gene *actA*, which was also detected in all the isolates, however exhibited polymorphism among the isolates. While 17 of the test isolates showed the presence of the amplicon *actA1* at ~ 950 bp instead of the expected 839 bp, 10 isolates showed the presence of the amplicon *actA2* at 268 bp as opposed to the 385 bp (polymorphism indicated with ▲ and color coded blue). Isolate 7 alone did not test positive for the PCR product of actA1 (absence indicated with **-** and color coded pink). However with the help of the second set of primers actA2, *actA* gene was also detected in this isolate. PCR products for the gene *agrD, bapL* and *inlA* were detected in 11, 4 and 24 isolates respectively with the expected sizes (absence indicated with **-** and color coded pink). The positive control 115 showed the presence of all the genes at the expected positions except for the gene *actA* for which amplicon *actA1* was detected at ~ 950 bp.

Overall, only 14.8% (4 out of 27 isolates - including 3 strong and 1 moderate isolate) of the isolates showed the presence of all the 20 biofilm associated genes. 22.2% of the isolates (6 out of 27 – including 1 isolate in the high range, 1 isolate in the mid-range and 4 isolates in lower range of

strong biofilm forming spectrum) showed the presence of 19 genes as the gene *bapL* could not be detected in these isolates. Eighteen genes were detected in 55.5% of the isolates (15 out of 27 – including 11 isolates with OD values from high, mid and low ranges of the strong biofilm forming phenotype, 3 isolates from the moderate phenotype and the weak biofilm forming isolate). Out of these 15 isolates, 14 of them showed the absence of genes *agrD* and *bapL* and one of them showed the absence of genes *bapL* and *inlA*. Finally, 7.4% (2 out of 26 isolates – including one isolate from the mid –range and one isolate from the lower range of strong biofilm forming phenotype) of the isolates showed the absence of genes *agrD*, *bapL* and *inlA*. Examples for the absence of amplicons for *agrD*, *bapL* and *actA* (detected using primers actA2) genes are shown in Fig. 7 (A-C).

There was no difference in biofilm formation between isolates in which all the 20 biofilm associated genes were detected and isolates in which one or few genes were not detected. Moreover none of the genes showed exclusivity to only one phenotype. Therefore there was no correlation between the genes tested and the biofilm forming phenotype of the tested isolates. Results from the analysis are listed in Table 4. (Please refer *Appendix A1* for the representative gels images).

Table 4. PCR results for biofilm associated genes of *L. monocytogenes*

Strains	Biofilm Phenotype	No of genes detected (20)	Biofilm associated genes																				
			*actA**		*agrA*	*agrB*	*agrC*	*agrD*	*bapL*	*gad2*	*hlyA*	*inlA*	*inlB*	*iap**		*intJ*	*luxS*	*mpl*	*plcA*	*plcB*	*prfA*	*rpoB*	*rrn*	*sigB*
			actA1	actA2										iap1	list1									
Lab ID			839 or ~950 bp	268 or 385 bp	500 bp	500 bp	500 bp	100 bp	343 bp	268 bp	456 bp	760 bp	148 bp	131 bp	660 bp	611 bp	486 bp	502 bp	129 bp	1150 bp	274 bp	520 bp	938 bp	310 bp
4	Strong (Mid – range)	20	▲	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	Strong (Mid – range)	20	-	▲	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
108	Strong (low)	20	▲	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
122	Moderate	20	▲	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
115	Moderate	20	▲	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
161	Strong (High)	19	▲	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
101	Strong (Mid – range)	19	▲	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
127	Strong (low)	19	▲	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
102	Strong (low)	19	▲	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	Strong (low)	19	▲	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
123	Strong (low)	19	▲	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
162	Strong (High)	18	▲	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
158	Strong (High)	18	+	▲	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
148	Strong (High)	18	+	▲	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
132	Strong (High)	18	▲	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
140	Strong (High)	18	▲	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
133	Strong (Mid – range)	18	+	▲	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	Strong (Mid – range)	18	+	▲	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
137	Strong (Mid – range)	18	▲	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	Strong (Mid – range)	18	+	▲	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
103	Strong (low)	18	▲	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
111	Strong (low)	18	▲	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
121	Moderate	18	+	▲	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
126	Moderate	18	+	▲	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
106	Moderate	18	+	▲	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
107	Weak	18	+	▲	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
128	Strong (Mid – range)	17	▲	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
104	Strong (low)	17	▲	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+

* - Genes *actA* and *iap* were detected using two sets of primers

\+ Presence

– Absence

▲ Polymorphism

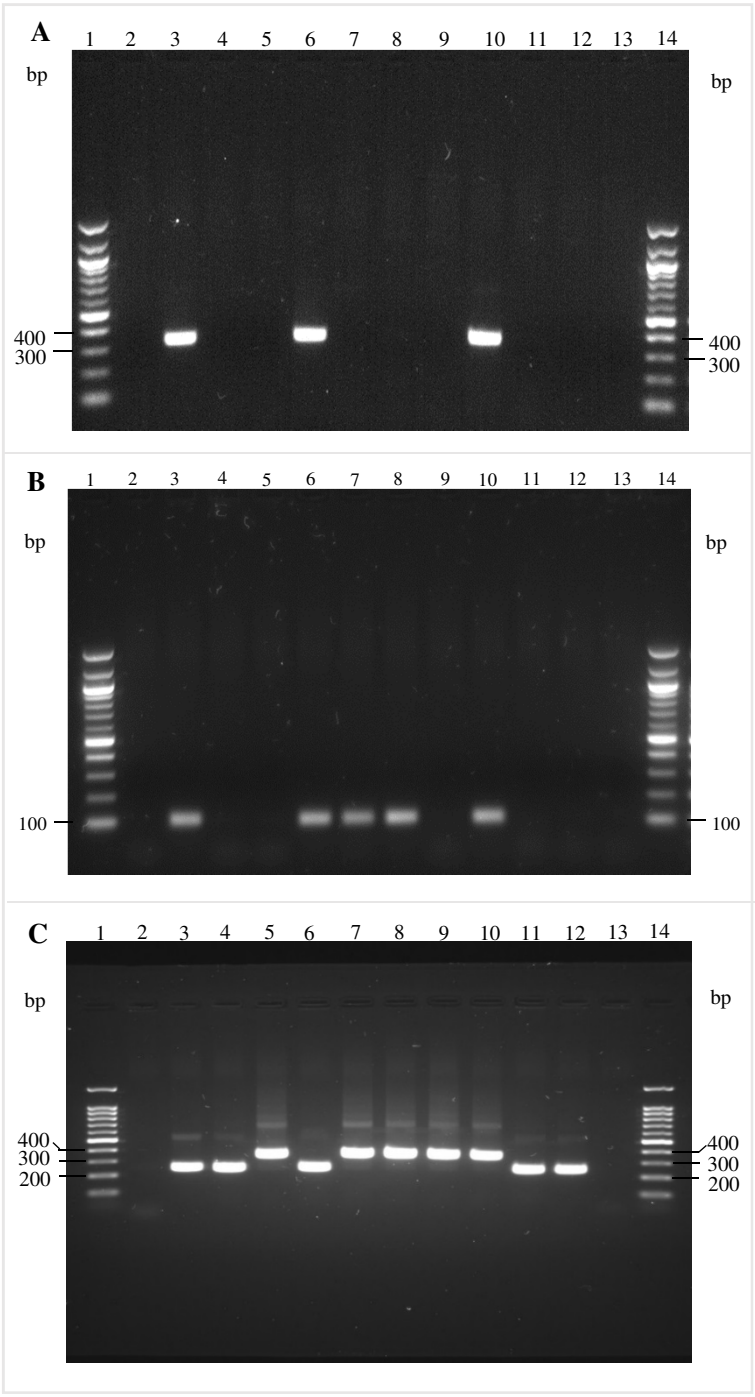

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
bp
bp
400
300
400
300
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
bp
bp
100
100
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14
bp
bp
400
300
200
400
300
200

Fig 7: Gel electrophoresis analysis of the genes *bapL*, *agrD* and *actA* amplified by PCR. Electrophoresis of the PCR products obtained from singleplex PCR reactions when run on 1.5% agarose gel. In all gels represented: Molecular weight marker (lane 1 and 14); Negative control (lane 2 and lane 13); strain 4 (lane 3); strain 148 (lane 4); strain 140 (lane 5); strain 7 (lane 6); strain 103 (lane 7); strain 127 (lane 8); strain 137 (lane 9); strain 122 (lane 10); strain 106 (lane 11); strain 107 (lane 12). (A) Presence of *bapL at* 343 bp; (B) Presence of *agrD* at 100 bp; (C) Presence of *actA* at 268 or 385 bp (primers actA2).

4.4. Analysis of biofilm associated genes using BLAST

BLAST similarity analysis of the genomes of ~ 700 *L. monocytogenes* isolates was performed against 106 biofilm associated genes obtained from six reference strains (EGD-e, 10403S, F2365, LL195, L312 and M7). Out of these 106 genes, isolates showed an absence of either one or more than one of 18 genes encoding for the following proteins: biofilm associated protein *(bapL or lmo0435 in the table)*, Lac 1 family transcriptional protein *(lmo0734*), cell division suppressor protein (*yneA*), transcriptional regulator (*lmo1262*), hypothetical protein (*lmrg_02457*), peptidoglycan linked protein *(lmo1666),* merR family transcriptional regulator (*LMOf2365_1497*), transcriptional anti-terminator gene (*bglG* or *lmo0501*), hypothetical protein (*lmrg_00049*), isocitrate dehydrogenase (*citC* or *lmo1566*), flagellin (*flaA or lmo0690*), ferritin like protein (*flp* or *lmo0943*), hexose phosphate transport protein (*hpt*), two-component sensor histidine kinase (*lmo1378* or *lisk*), branched chain fatty acid kinase (*lmo1370*), hypothetical protein (*lmo2056*), *N*-acetylglucosaminyl-phosphatidylinositol biosynthetic protein (*lmo2555*) and xanthine ribosyltransferase (*lmrg_01032*). Results from the analysis is presented in Table 5.

Out of 66 isolates tested, 12.12% of them (8 out of 66 – including 6 isolates from mid- range and 1 isolate from low range of the strong biofilm forming spectrum and 1 isolate from the moderate biofilm forming phenotype) showed the presence of all the 106 genes. In addition, the positive control 115 that displayed a moderate biofilm forming ability also carried all the 106 tested genes. However in almost 51.51% of the isolates (34 out of 66 – including 6, 14 and 10 isolates from high, mid, and low ranges of the strong biofilm forming group, 3 isolates from the moderate biofilm forming phenotype and the 1 weak biofilm forming isolate) only 104 genes were present. Among these isolates, 31 of them showed the absence of the genes, *lmo0435* and *lmo0734* and the remaining three isolates showed the absence of genes *yneA, lmo1262* and *lmrg_02457* respectively

in addition to the absence of the gene *lmo0435*. The next common pattern observed, was the presence of 105 genes among 30.3% of the isolates (20 out of 66 – 2, 15 and 13 isolates from high, mid and low ranges of the strong biofilm forming group) as they all showed an absence of the gene *lmo0435*. A small percentage (3%) of the sample population showed the absence of genes *lmo0435*, *lmo0734* and *lmo1666*. Both the isolates that showed an absence of the above three genes were found to be from the lower range of the strong biofilm forming spectrum. Finally, out of the 66 test isolates two isolates were found to have a completely different profile with one isolate (Strong (low-range) showing the absence of 6 genes (*LMOf2365_1497, lmo0501, lmo0435, lmo0734, lmo1666, lmrg_00049*) and one isolate (moderate) showing the absence of 14 genes (*lmo1566, lmo0690, lmo0943, hpt, lmo1378, lmo0435, lmo0734, lmo1262, lmo1370, lmo2056, lmo2555, lmrg_02457, yneA* and *lmrg_01032*).

However, even when a certain number of isolates exhibited the same genotype, (where all the genes were detected or one or more genes were found to be absent), the isolates within that genotype varied considerably in their OD values/phenotype. Hence there was no solid correlation observed between the presence/absence of the detected genes and the biofilm forming phenotype of the tested *L. monocytogenes* strains.

Table 5. BLAST results for biofilm associated genes of *L. monocytogenes*

Strains / Lab ID	Genes Absent	No. of genes detected (106)	Phenotype
130	-	106	Strong (mid-range)
5	-	106	Strong (mid-range)
7	-	106	Strong (mid-range)
8	-	106	Strong (mid-range)
4	-	106	Strong (mid-range)
110	-	106	Strong (mid-range)
108	-	106	Strong (low)
122	-	106	Moderate
115	-	106	Moderate
140	*lmo0435*	105	Strong (high)
157	*lmo0435*	105	Strong (high)
125	*lmo0435*	105	Strong (mid-range)
143	*lmo0435*	105	Strong (mid-range)
124	*lmo0435*	105	Strong (mid-range)
144	*lmo0435*	105	Strong (mid-range)
160	*lmo0435*	105	Strong (mid-range)
1	*lmo0435*	105	Strong (low)
123	*lmo0435*	105	Strong (low)
127	*lmo0435*	105	Strong (low)
102	*lmo0435*	105	Strong (low)
109	*lmo0435*	105	Strong (low)
139	*lmo0435*	105	Strong (low)
141	*lmo0435*	105	Strong (low)
149	*lmo0435*	105	Strong (low)
150	*lmo0435*	105	Strong (low)
151	*lmo0435*	105	Strong (low)
152	*lmo0435*	105	Strong (low)
155	*lmo0435*	105	Strong (low)
159	*lmo0435*	105	Strong (low)
161	*lmo0435, yneA*	104	Strong (high)
132	*lmo0435,lmo0734*	104	Strong (high)
148	*lmo0435,lmo0734*	104	Strong (high)
114	*lmo0435,lmo0734*	104	Strong (high)
158	*lmo0435,lmo0734*	104	Strong (high)
162	*lmo0435,lmo0734*	104	Strong (high)
130	*lmo0435,lmo0734*	104	Strong (mid-range)
131	*lmo0435,lmo0734*	104	Strong (mid-range)
135	*lmo0435,lmo0734*	104	Strong (mid-range)

Strains Lab ID	Genes Absent	No. of genes detected (106)	Phenotype
133	*lmo0435,lmo0734*	104	Strong (mid-range)
128	*lmo0435,lmo0734*	104	Strong (mid-range)
137	*lmo0435,lmo0734*	104	Strong (mid-range)
134	*lmo0435,lmo0734*	104	Strong (mid-range)
9	*lmo0435,lmo0734*	104	Strong (mid-range)
2	*lmo0435,lmo0734*	104	Strong (mid-range)
101	*lmo0435,lmo0734*	104	Strong (mid-range)
138	*lmo0435,lmo0734*	104	Strong (mid-range)
146	*lmo0435,lmo0734*	104	Strong (mid-range)
147	*lmo0435,lmo0734*	104	Strong (mid-range)
154	*lmo0435,lmo0734*	104	Strong (mid-range)
136	*lmo0435,lmo0734*	104	Strong (low)
6	*lmo0435,lmo0734*	104	Strong (low)
104	*lmo0435,lmo0734*	104	Strong (low)
111	*lmo0435,lmo0734*	104	Strong (low)
112	*lmo0435,lmo0734*	104	Strong (low)
113	*lmo0435,lmo0734*	104	Strong (low)
142	*lmo0435,lmo0734*	104	Strong (low)
145	*lmo0435,lmo0734*	104	Strong (low)
156	*lmo0435,lmo0734*	104	Strong (low)
3	*lmo0435,lmo1262*	104	Strong (low)
121	*lmo0435,lmo0734*	104	Moderate
106	*lmo0435,lmo0734*	104	Moderate
S1	*lmo0734, lmrg_02457*	104	Moderate
107	*lmo0435,lmo0734*	104	Weak
103	*lmo0435,lmo0734,lmo1666*	103	Strong (low)
105	*lmo0435,lmo0734,lmo1666*	103	Strong (low)
153	*LMOf2365_1497,lmo0501, lmo0435,lmo0734, lmo1666, lmrg_00049*	100	Strong (low)
126	*lmo1566, lmo0690, lmo0943, hpt, lmo1378, lmo0435, lmo0734, lmo1262, lmo1370, lmo2056, lmo2555, lmrg_02457,yneA lmrg_01032,*	92	Moderate

biofilm associated protein *(bapL or lmo0435)*; Lac 1 family transcriptional protein *(lmo0734)*; cell division suppressor protein (*yneA*); transcriptional regulator (*lmo1262*); hypothetical protein (*lmrg_02457*); peptidoglycan linked protein *(lmo1666);* merR family transcriptional regulator (*LMOf2365_1497*); transcriptional anti-terminator gene (*bglG* or *lmo0501*); hypothetical protein (*lmrg_00049*); isoitrate dehydrogenase (*citC* or *lmo1566*); flagellin (*flaA or lmo0690*); ferritin like protein (*flp* or *lmo0943*); hexose phosphate transport protein (*hpt*); two-component sensor histidine kinase (*lmo1378* or *lisk*); branched chain fatty acid kinase (*lmo1370*); hypothetical protein (*lmo2056*); *N*-acetylglucosaminyl-phosphatidylinositol biosynthetic protein (*lmo2555*); xanthine ribosyltransferase (*lmrg_01032*)

Chapter 5: Discussion

In 2010, the World Health Organization (WHO) estimated the global burden of listeriosis to be 23,150 illnesses, 5,463 deaths and 1, 72,823 DAILYs (disability-adjusted life-years) (Maertens De Noordhout et al., 2014). To date, food recalls due to contamination with *L. monocytogenes* are reported on a regular basis, despite the implementation of various surveillance and control strategies (CFIA, 2016). Thus the prevention of contamination by this pathogen at the food processing level remains a serious challenge to the industry and the regulatory agencies like the CFIA.

Biofilm formation is considered to be one of the major causes of persistence of *L. monocytogenes* within food processing environments (Kadam et al., 2013; Nilsson et al., 2011; Tresse et al., 2006; Valderrama & Cutter, 2013). Consequently the main focus of ongoing research efforts has always been to identify factors that facilitate *Listeria* to form biofilms in an environment where routine cleaning and disinfection occur. Analysis of biofilm formation among the strains of *L. monocytogenes* and correlation of biofilm formation with lineage, serotype, strain origin and test surfaces have been the key aspects of phenotype studies in literature (Djordjevic et al., 2002; Folsom et al., 2006; Milanov et al., 2009; Nilsson et al., 2011). Nevertheless, the outcomes from these studies have been fairly inconclusive, as a result of which, the properties that facilitate persistence of certain subtypes of *L. monocytogenes* have not been clearly identified.

The disparity in the outcome of these studies stems from the fact that every study makes use of different growth conditions for biofilm formation. In addition, the use of optimal conditions for biofilm formation makes the outcomes from these studies less relatable to *in-situ* conditions (Doijad et al., 2015; Kadam et al., 2013; Moltz and Martin, 2005).

As it is known that different growth conditions can greatly modify biofilm forming capabilities, it was therefore important to demonstrate that this was true for *Listeria* biofilms. To that end biofilm formation was assessed using different growth conditions including one that closely relates to the food processing environment (12 ºC, BB). This section in the study was aimed at highlighting the need for growth conditions that truly reflected the conditions experienced by this pathogen in the food processing environment in order to assess biofilm forming capabilities in an unbiased manner.

Multiple factors had to be considered when the biofilm model was being designed. It was important to bear in mind that in a food processing environment pathogens have limited access to nutrients due to routine cleaning and disinfection, however sufficient growth needed to be achieved to produce quantifiable biofilms. It was found that 1% BB was able to provide a growth environment that was limited in resources yet sufficient enough to produce quantifiable biofilms, as a result it was used as the growth medium for this model. The CFIA recommends 10 ºC as the ambient temperature for food processing plants. (CFIA, 2014). However it is common for temperatures to be set between 10 ºC – 20 ºC within these environments (Møretrø et al., 2013; Piercey et al., 2016). Furthermore few studies, in which 12 ºC was used as the incubation temperature, showed long term survival of biofilms at this temperature and have also demonstrated an increased resistance to sanitizers when compared to biofilms grown at 37 ºC (Hassan et al., 2004; Lourenço et al., 2011). Hence 12 ºC was picked to imitate ambient food processing temperature in our study. As previously described, MWB at 37 ºC was selected to represent optimal biofilm forming conditions for *L. monocytogenes* as it is commonly used in other studies (Djordjevic et al., 2002; Moltz and Martin, 2005). Two other growth conditions used were 12 ºC, MWB and 37 ºC, BB to assess the differences in biofilm formation at varying temperatures when compared to the optimal and food processing conditions. Another factor that was considered is the abiotic surface on which the

biofilms were allowed to adhere to. Studies have used materials like polystyrene, stainless steel and glass to test biofilm formation (Choi et al., 2013; Herald & Zottola, 1988; Min et al., 2006). There are reports that claim a lower bacterial retention on stainless steel (more resistant to abrasion) compared to other materials like polycarbonate, mineral resin and enameled steel (Holah & Thorpe, 1990; Wirtanen et al., 1996). Consequently, polystyrene was chosen as the abiotic surface to be used to facilitate biofilm formation in our study.

Biofilm formation of two strains, S1 and 115 (positive control) was analyzed in the four growth conditions over a period of 14 days to obtain a clear picture of the biofilm growth pattern over an extended period of time. The effects of media on biofilm formation was not evident at 12 ºC but a media dependent biofilm formation was observed when incubated at 37 ºC. A similar case, where the combination of both media and temperature influenced biofilm formation has been reported previously (Kadam et al., 2013). In the studies published by Harvey et al. (2007), high biofilm formation was observed in less nutrient rich conditions (MWB) compared to nutrient limiting conditions (dTSB) irrespective of the incubation period. However in our study there was no significant difference between biofilm formation in nutrient limiting conditions (BB) and less nutrient rich conditions (MWB) throughout the period of investigation when incubated at 12 ºC. On the contrary biofilm formation at 37 ºC in MWB, was much higher than biofilm formation obtained in both the growth media at 12 ºC. Many studies have demonstrated that biofilm formation increased with increase in temperature (Kadam et al., 2013; Moltz and Martin, 2005; Nilsson et al., 2011). However in our study, the lowest amount of biofilm formation was observed in BB when incubated at 37 ºC and the reason for this observation is unknown. Strain dependent biofilm formation has been observed in *L. monocytogenes* in response to different growth conditions (Di Bonaventura et al., 2008; Folsom et al., 2006; Kadam et al., 2013). This was

evidenced by the trends observed for biofilm formation by both strains assessed in the conditions tested (Fig 1A and 1B). Incubation periods in assays to investigate biofilm formation of *L. monocytogenes* in the literature varied between 24 hours to 14 days (Di Bonaventura et al., 2008; Harvey et al., 2007; Milanov et al., 2009). However, measuring biofilm formation every day over a period of 14 days proved valuable, as it enabled us to select the best time point at which highest OD values were observed for our testing conditions (12 ºC, BB). Even though there were no statistical differences in biofilm growth obtained at days 6, 7, 9, 10 and 11, day 9 gave the most consistent results and therefore it was picked as the time point for screening large number of *L. monocytogenes* isolates in our study.

Since the conditions used in this study were different from most of the published studies (including new broth, incubation temperature and time) morphological observations by light microscopy and SEM were conducted in order to correlate the results obtained using crystal violet assay. The ability of the established growth condition to support biofilm formation was visually confirmed using the positive control isolate 115 (data not shown) before screening the test isolates.

Subsequent to establishing the above described model, biofilm assays were carried out for 66 randomly picked isolates from food and clinical origin at 12 ºC in BB for 9 days. Though a range of OD values were displayed by the isolates, 60 of the 66 isolates were found to be strong biofilm formers, while 5 were found to be moderate and 1 was found to be weak. It is noteworthy that OD values obtained for the isolates in this study were low, however the values obtained correlate with values reported in other studies that used optimal biofilm forming conditions (Doijad et al., 2015; Harvey et al., 2007). Our findings are further supported by multiple studies that have suggested that *L. monocytogenes* have lower biofilm forming capacities when compared to other Gram positive and Gram negative species (Hood and Zottola, 1997a, 1997b; Jeong and Frank, 1994a,

1994b). Upon comparison of biofilm forming potential based on the source of isolation, clinical isolates appeared to have slightly better biofilm forming abilities than food isolates. These findings are in direct contrast to studies that observed no significant correlation between biofilm forming potential and origin of isolates (Harvey et al., 2007; Kalmokoff et al., 2001). The model used in our study might have been able to detect this potential of clinical isolates which may not have been discernable under optimal conditions. In addition, we also found a good correlation between the results obtained using biofilm assay and microscopical observations using light microscopy and SEM (Fig 5-1 and 5-2).

The next part of the study focused on identifying genetic determinants responsible for varying capacities of biofilm formation within *L. monocytogenes*. Previous mutagenesis studies in literature have identified numerous genes that influence biofilm formation including those required for virulence, stress response, gene regulation, quorum sensing, metabolism and motility (Alonso et al., 2014; Chang et al., 2012; Ouyang et al., 2012) . However which of these genes contribute towards different capacities of biofilm formation observed among *L. monocytogenes* isolates is not well understood as the phenotypic studies performed to elucidate the function of these genes were performed under different conditions. Furthermore, the use of optimal conditions for biofilm formation may have hampered our understanding of true biofilm forming abilities of *L. monocytogenes* from a food processing perspective.

In our study we sought to identify biofilm forming phenotypes in conditions relatable to the food processing environment and to correlate these phenotypes to their corresponding genetic determinants. We tried to identify genetic determinants by analyzing the gene profiles of the test isolates. Gene profiles have been used in earlier studies to discriminate between phenotypes such as antibiotic resistance and susceptibility (Fazeli & Momtaz, 2014; Slanec et al., 2009). Virulence

gene profiles of *L. monocytogenes* generated in a study using PCR revealed, that there was a positive correlation between the number of genes detected and the capacity to form biofilms (Meloni et al., 2012). In this study we hoped to analyze the gene profiles of selected isolates by assessing the presence/absence of biofilm associated genes using PCR and BLAST.

To obtain a comprehensive understanding of the genes that contribute to different biofilm forming capabilities, representatives of each type of biofilm former were chosen for further analysis using PCR. However since most of the isolates were found to be strong biofilm formers and were found to exhibit varying OD values, isolates were also chosen within this group to represent the varying OD values observed (lower range OD values: 0.230 ± 0.105 and 0.336 ± 0.03 OD, mid-range OD values: 0.338 ± 0.207 and 0.437 ± 0.15, upper range OD values: 0.456 ± 0.034 and 0.517 ± 0.027). A total of 27 representative isolates were selected and a combination of singleplex and multiplex PCRs were performed to detect 20 biofilm associated genes (including genes related to virulence, stress response and quorum sensing).

Meloni et al. (2012) reported that isolates with a near complete profile of 9 out of 10 virulence genes (lack of *inl B*) showed stronger biofilm formation than isolates in which two or more genes were not detected. In our study, the most prevalent profile was the presence of 18 genes (lack of *agrD* and *bapL*) in 55.5% of the isolates followed by the presence of 19 genes (absence of *bapL*) in 22.2% of the isolates. Only a very small percentage (14.8%) of the test isolates showed the presence of all the 20 genes. However, variation in biofilm formation could not be attributed to difference in gene profiles as every profile consisted of isolates from all the three phenotypes. Therefore we found no correlation between the number of genes that were determined as being present by PCR and the capacity to form biofilms.

A high rate of polymorphism has been reported in the gene *actA* and *iap* (Bubert et al., 1999; Conter et al., 2010; Jiang et al., 2006; Suárez et al., 2001). We therefore used two sets of primers to detect the presence of these genes. This in fact proved useful in the case of isolate 7 as we were able to detect *actA* only with the primer set actA2 and not actA1. Surprisingly none of the tested isolates showed a polymorphism for the gene *iap*. On the contrary all the isolates showed a polymorphism for the gene *actA*. As observed previously, detection using primers actA2 yielded a polymorphic 268 bp band in 10 isolates instead of the expected 385 bp (Conter et al., 2010; Meloni et al., 2012). Interestingly we also observed that *actA* gene was detected in 18 isolates including the positive control (115) at ~950 bp when they were supposed to be detected at 839 bp using the primer set actA1. This has not been reported previously. We also found a good correlation between the results obtained using PCR and BLAST for this gene. Polymorphism in *actA* has been reported to correlate with enhanced virulence of *Listeria* strains (Wiedmann et al., 1997). Nevertheless in our study polymorphism in the *actA* gene did not seem to contribute towards varying biofilm forming capacities among the tested *L. monocytogenes* isolates.

The gene *bapL*, was detected only in 4 isolates. BLAST searches also confirmed the results obtained by PCR for this gene. Moreover, our results are also in line with the studies published by Jordan et al. (2008). While the protein Bap is known to play a major role in biofilm formation of *S. aureus*, the gene that encodes for the orthologue in *L. monocytogenes* was found to be absent in a number of isolates (Jordan et al., 2008). Furthermore, Jordan and colleagues reported that there was no difference in biofilm formation between isolates that either showed the presence or absence of the gene. This was true in our case as well, as there were many isolates that did not test positive for the gene but showed a similar biofilm forming capacity as the 4 isolates that carried the gene.

Therefore as suggested by Jordan et al. (2008), BapL may not have a crucial role to play in biofilm formation of *L. monocytogenes.*

Many food isolates have been reported to carry a premature stop codon (PMSC) that encodes for a truncated InlA (Nightingale et al., 2005). In our study *inlA* was not detected in 3 isolates that were of food origin. However these isolates were found to show the presence of the gene in BLAST analysis. The primers targeting *inlA* were directed towards the B repeats region of the gene. Therefore it is possible that a PMSC may have occurred before this region as reported earlier, disrupting the primer binding sequence, as a result of which the gene could not be detected by PCR (Nightingale et al., 2005; Van Stelten et al., 2010).

A high number of isolates (16 out of 27) tested negative for the gene *agrD.* This was suspicious given that the gene played an important role in biofilm formation (Kumar et al., 2009; Riedel et al., 2009; Rieu et al., 2007). However all the isolates were shown to contain the gene when analyzed using BLAST. Therefore there may have been single nucleotide polymorphisms (SNPs), insertions or deletions at the primer annealing site that prevented the primers from binding and amplifying the target gene (Hayashi et al., 2001).

Since we were unable to identify specific genetic determinants responsible for varying capacities of biofilm formation using PCR, we decided to expand our BLAST similarity search to 106 biofilm associated genes (including those related to gene regulation, virulence, general and adaptive stress response, motility, quorum sensing, metabolism and biosynthesis) for all the isolates including the positive control. These 106 genes included 16 genes that were tested using PCR as a means of confirming the results obtained with PCR.

The results obtained using BLAST mirrored the same trend as the results obtained using PCR. Even when a certain number of isolates seemed to share the same gene profile they all differed notably in their phenotypes. For e.g. the most prevalent gene profile among isolates was the presence of 104 genes (lack of *lmo0435* and *lmo0734*). However this profile was found not to be exclusive, as isolates from all the three biofilm forming phenotypes (weak, moderate and strong) were found to share this gene profile.

There was also no correlation between the number of genes that were found to be present/absent in isolates and their ability to form biofilms. For instance, out of 66 test isolates only 8 of them showed the presence of all the 106 tested genes. While the majority of these isolates were from the strong (mid-range) biofilm forming phenotype, there was however one isolate in this group that belonged to the moderate biofilm forming phenotype. Moreover, there were 14 other isolates that showed the same biofilm forming ability as these 8 isolates but were found to carry only 104 genes (lack of *lmo0435* and *lmo0734*). Similarly, there was no difference in biofilm formation between the isolates in the strong (low-range) biofilm forming phenotype that showed the absence of either one gene (lack of *lmo0435*) or three genes (lack of *lmo0435*, *lmo0734* and *lmo1666*).

We hypothesized that certain genetic determinants or a combination of them might be responsible for the varying capacities of biofilm formation. Among the 106 genes tested, the gene *lmo0435* (*bapL*) was found to be absent from 87.87% (58 out of the 66) isolates, gene *lmo0734* (LacI family transcriptional regulator) was absent from 53% (35 out of 66) of the isolates and the combination of just these two genes was absent from 46.96% (31 out of 66) of the isolates. As mentioned previously the results obtained for the gene *lmo0435* or *bapL* using BLAST correlated well with those obtained using PCR. Mutation in the gene *lmo0734* that encodes for Lac I family transcriptional regulator, was found to cause a reduction in biofilm formation when compared to

the wild type strain (Ouyang et al., 2012). However in our study the gene *lmo0734* or the lack of it did not seem to affect biofilm formation. Therefore the absence of the gene *lmo0435* alone or the combination of genes *lmo435* and *lm0734* may not play a crucial role for varying capacities of biofilm formation.

On further examination of the results, we found that isolates 161 (Strong biofilm forming phenotype), 3 (Strong biofilm forming phenotype) and S1 (moderate biofilm forming phenotype), in addition to showing an absence of the gene *lmo0435* or *bapL* showed the absence of genes *yneA*, *lmo1262* and *lmrg_02457*. The gene *yneA* encodes for cell division suppressor protein YneA, gene *lmo1262* encodes for a transcriptional regulator and gene *lmrg_02457* encodes for a hypothetical protein. Mutation in all the three genes have been shown to have a negative effect on biofilm formation (Alonso et al., 2014; Ouyang et al., 2012; Van Der Veen and Abee, 2010). However if the absence of only these three genes are considered in these isolates and not the gene *lmo0435* (as it does not seem to be a crucial contributor to biofilm formation), these isolates showed no difference in biofilm formation from those isolates containing all the three genes.

Furthermore there were 2 isolates (153 and 126), for which it was difficult to obtain a correlation between combinations of the genes that were absent from the isolates and their corresponding biofilm forming phenotypes. The reason for this was because no other isolate in the sample population showed an absence of the same combination of genes as these 2 isolates. Isolate 153, within the low-range of a strong biofilm forming group showed the absence of 6 genes including *LMOf2365_1497, lmo0501, lmo0435, lmo0734, lmo1666* and *lmrg_00049*. Gene *LMOf2365_1497* encodes for a merR family transcriptional regulator; *lmo0501* encodes for transcriptional antiterminator BglG; *lmo1666* encodes for peptidoglycan linked protein; and *lmrg_00049* encodes for a hypothetical protein (Alonso et al., 2014; Chang et al., 2012; Liu et al., 2002). It is noteworthy

that this isolate happens to have the lowest absorbance value out of all the tested clinical isolates. While it might be speculated that the absence of these 6 genes resulted in reduction of the biofilm forming capacity of this isolate, it cannot be confirmed unless more isolates belonging to the same genotype have been investigated.

The most striking of all the profiles was that of the moderate biofilm forming isolate 126 as it showed the presence of only 92 genes and the lack of 14 genes including *lmo1566, lmo0690, lmo0943, hpt, lmo1378, lmo0435, lmo0734, lmo1262, lmo1370, lmo2056, lmo2555, lmrg_02457, yneA* and *lmrg_01032*. Gene *lmo1566* encodes for isocitrate dehydrogenase; *lmo0690* encodes for flagellin; *lmo0943* encodes for ferritin like protein; *hpt* encodes for hexose phosphate transport protein; *lmo1378* encodes for a two-component sensor histidine kinase; *lmo1370* encodes for branched chain fatty acid kinase; *lmo2056* encodes for a hypothetical protein*;* and *lmo2555* encodes for *N*-acetylglucosaminyl- phosphatidylinositol biosynthetic protein (Chang et al., 2012; Liu et al., 2002; Ouyang et al., 2012; Taylor et al., 2002). Genes *LMOf2365_149*, lmo1666, *lmrg_00049*, *lmo0690*, *lmo0943*, *lmo1378*, *lmo1370*, *lmo2056* and *lmo2555* have all been implicated to play a role in biofilm formation as mutation in these genes reduces the biofilm forming ability of *L. monocytogenes* (Alonso et al., 2014; Chang et al., 2012; Huang et al., 2012; Liu et al., 2002; Ouyang et al., 2012). The genes *lmo1566*, *lmo0943* and *lmo0501* were reported to be expressed in response to lower temperatures (10 ºC) (Liu et al., 2002). Therefore in this study along with these three genes we evaluated 17 other genes that were reported to be expressed at a lower temperature to see if they played a role in biofilm formation under our testing conditions.

Whether or not, the absence of 14 genes in isolate 126, contributes to the moderate biofilm forming phenotype would require further investigation. The correlation between the presence/absence of genes and biofilm formation becomes especially difficult to be established within moderate biofilm

forming phenotype as there were only 5 isolates in this group and they belonged to 4 different gene profiles. Besides the positive control isolate 115 showed the presence of all the 106 genes even though it displayed a moderate biofilm forming ability under the tested condition. Based on our results for the 110 genes tested altogether using PCR and BLAST, we were unable to identify if any genetic determinants or a combination of them contributed to varying capacities of biofilm formation of *L. monocytogenes.*

Biofilm formation is a multifactorial process and different pathways can be triggered under different growth environments (Kadam et al., 2013; Lemon et al., 2010). The findings from the current study (Section 4.1) also substantiates the fact as the same strain showed different patterns of biofilm formation when exposed to different growth conditions. While the model established in the study relates more closely to the food processing environment, validation using more isolates would be required before it can be used in regulatory agencies like the CFIA. The genes uncovered so far have been identified in earlier studies under optimal conditions. In addition, molecular mechanisms employed by *L. monocytogenes* is yet to be fully understood. Thus the genes tested in the study may not reflect the entire scope of biofilm formation in this pathogen. However if a large number of isolates from food and clinical origin are screened for their biofilm forming potential using our model, a pattern might emerge between the combinations of genes present/absent and the biofilm forming phenotypes.

Concluding remarks and future work

In this study we were able to successfully establish a model that is relatable to the food processing environment. Since a model has been created it can now be used to screen a large number of *L. monocytogenes* isolates from the food processing environment and create a repertoire of their

biofilm forming capacities. This in turn could be used to provide risk information for the food processing industries, based on which they can make changes to their cleaning and disinfection practices whenever necessary.

In a natural setting biofilms are often found as mixed microbial communities (Davey and O'Toole, 2000; Donlan, 2002). However in our study, we only looked at *L. monocytogenes* as we wanted to first understand the biofilm forming abilities of this pathogen when introduced into a food processing setting. Once the genetic determinants for their varying capacities of biofilm formation have been identified, future work should however focus on studying biofilm formation of *L. monocytogenes* in the presence of microorganisms commonly encountered in food processing plants (Carpentier and Chassaing, 2004; Sasahara and Zottola, 1993). It is also worthwhile for future studies to test more isolates with various ways of determining the biofilm formation phenotypes (Habimanaa et al., 2011; Merritt et al., 2005).

Among the 110 genes tested together using PCR and BLAST, we were not able to single out any gene profiles as being specific to one particular phenotype. Even though there were a range of OD values within the strong phenotype, there was no correlation between the tested biomarkers and the OD values. The main reason for this could be due to the fact that there were unequal number of isolates in each phenotype (Strong: 60 isolates, Moderate: 5 isolates, Weak: 1 isolate). As a result we were limited in our ability to draw comparisons between these isolates.

From our study it is evident that the results obtained by using PCR must be treated with caution, unless the primers are designed for conserved regions of a gene. Also if the primers are not directed to target conserved sequences then confirmation of results using a second method would be absolutely necessary (Park et al., 2013; Rawool et al., 2007; Somer et al., 2005). While a 30%

cut-off in BLAST analysis is considered to be significant, the possibility of false negatives cannot be overlooked. Therefore the cut-off percentage can be lowered further to see if the genes that were shown to be absent at this threshold (30%) were indeed absent (Pearson, 2013). If the genes become detectable at < 20% then it could be an indication that the proteins coded by these genes have changed drastically in their function (Chothia and Lesk, 1986). Further investigation could be done to see if these isolates (< 20% homology) occur frequently within one particular phenotype.

With gene based analysis such as PCR and BLAST, there could be a bias as genes are pre-selected for analysis (Read and Massey, 2014). Therefore, work is under progress in our lab to compare all the isolates at the genome level using Genome Wide Association Studies (GWAS) to determine if there are any SNPs that can be associated with a particular phenotype. Any SNPs identified as being specific to a particular phenotype, can be validated for their role in biofilm forming capacity using our model. These SNPs can then be used as biomarkers for identifying the risk of persistence of *L. monocytogenes* in food processing plants.

A study by Fox et al. (2011) reported that 92 genes (including genes *purE*, *purL*, *purF*, *purN*, *purH* and *inlA* used in this study) were differently regulated between a persistent and a non-persistent isolate of *L. monocytogenes* in the presence of BZT. The proposed model in this study can be used for transcriptional and translational analysis of all the genes in question to see even if they are present in all the isolates, if they are regulated differently across different phenotypes of biofilm formation. This could shed some light on the key players that are involved in strong biofilm formation and serve as targets for designing strategies to eliminate *L. monocytogenes* from food processing plants.

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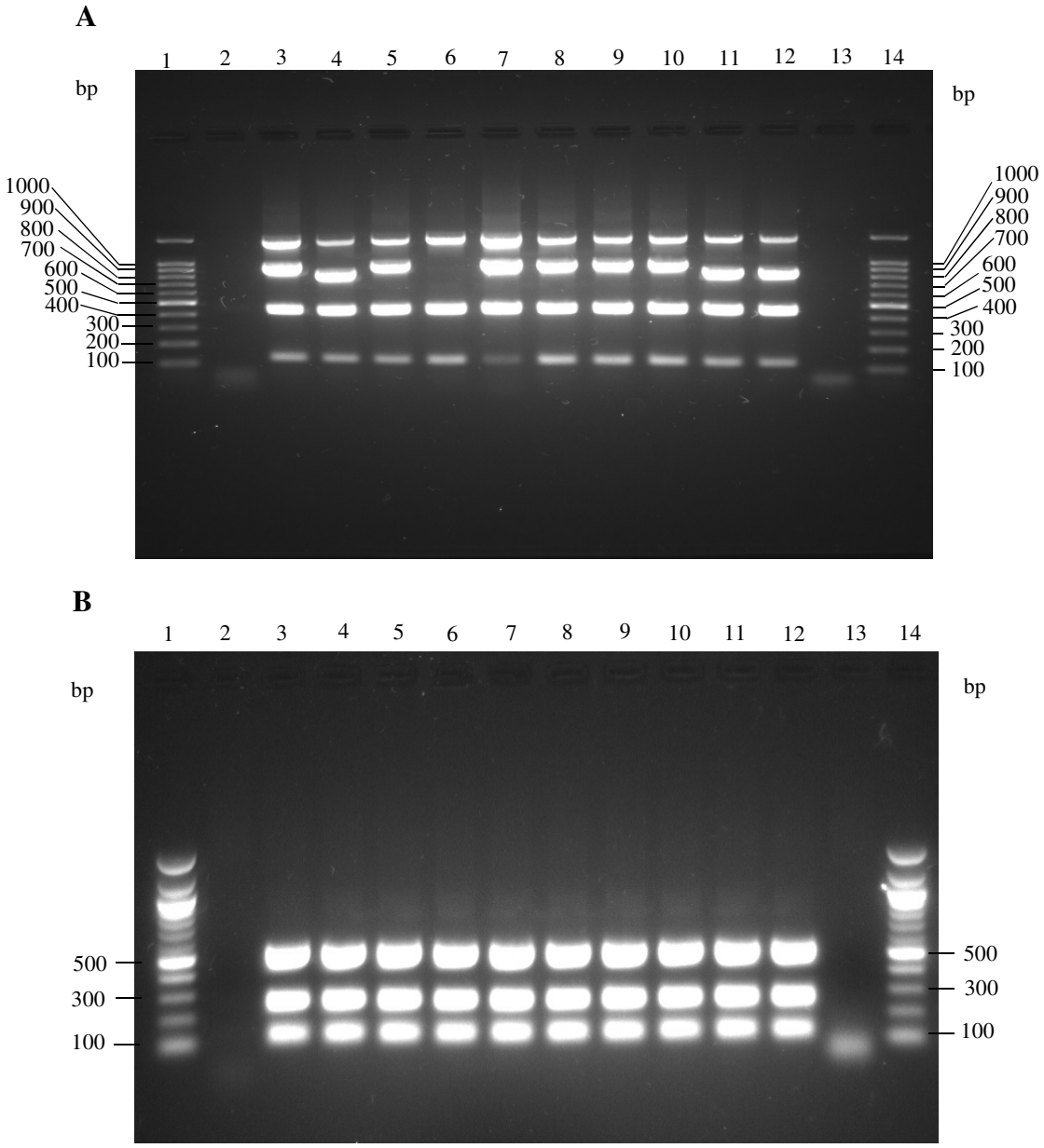

Fig A1-1: Gel electrophoresis analysis of the genes *plcB*, *actA*, *hly*, *iap*, *agrC*, *gad2* and *plcA* amplified by PCR. Electrophoresis of the PCR products obtained from multiplex PCR reactions when run on 1.5% agarose gel. In all gels represented: (lane 1 and 14) Molecular weight marker; (lane 2 and lane 13) Negative control; (lane 3) strain 4; (lane 4) strain 148; (lane 5) strain 140; (lane 6) strain 7; (lane 7) strain 103; (lane 8) strain 127; (lane 9) strain 137; (lane 10) strain 122; (lane 11) strain 106; (lane 12) strain 107. **(A)** Presence of genes *plcB at* 1150 bp, actA (primers actA1) at 839 bp and ~ 950 bp, *hly* at 456 bp and *iap* (primers iap1) at 131 bp; **(B)** Presence of gene *agrC* at 500 bp, *gad2* at 268 bp and *plcA* at 129 bp.

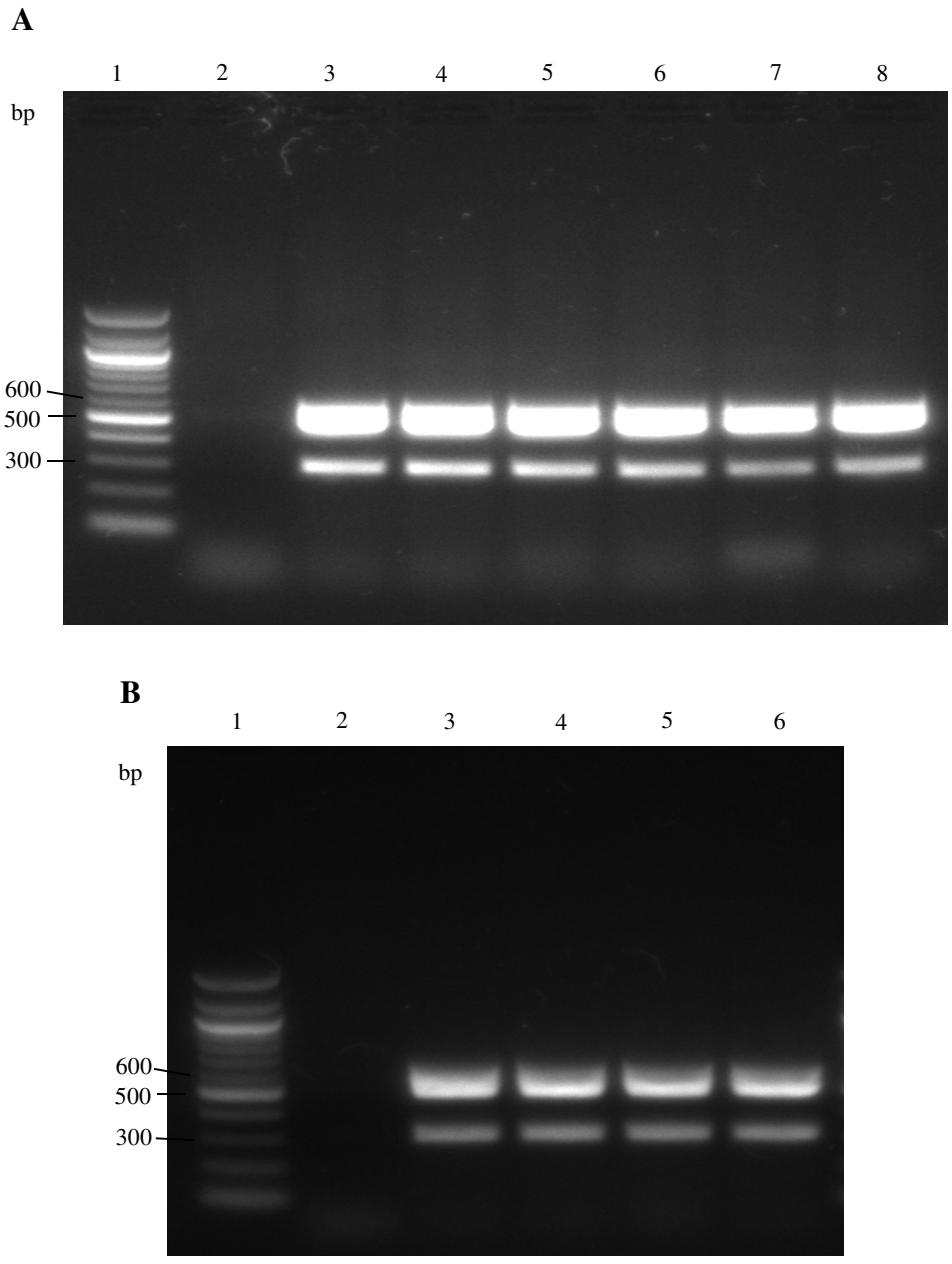

Fig A1-2: Gel electrophoresis analysis of the gene *sigB* amplified by PCR. Electrophoresis of the PCR products obtained from multiplex PCR reactions when run on 1.5% agarose gel. In gel A: (lane 1) Molecular weight marker; (lane 2) Negative control; (lane 3) strain 4; (lane 4) strain 148; (lane 5) strain 140; (lane 6) strain 122; (lane 7) strain 106; (lane 8) strain 107**.** In gel B: (lane 1) Molecular weight marker; (lane 2) Negative control; (lane 3) strain 7; (lane 4) strain 103; (lane 5) strain 127; (lane 6) strain 137. While the gels A and B clearly show the presence of *sigB at* 310 bp*,* the bands for *inlJ* (600 bp) and *rpoB* (520 bp) are not clearly distinguishable.

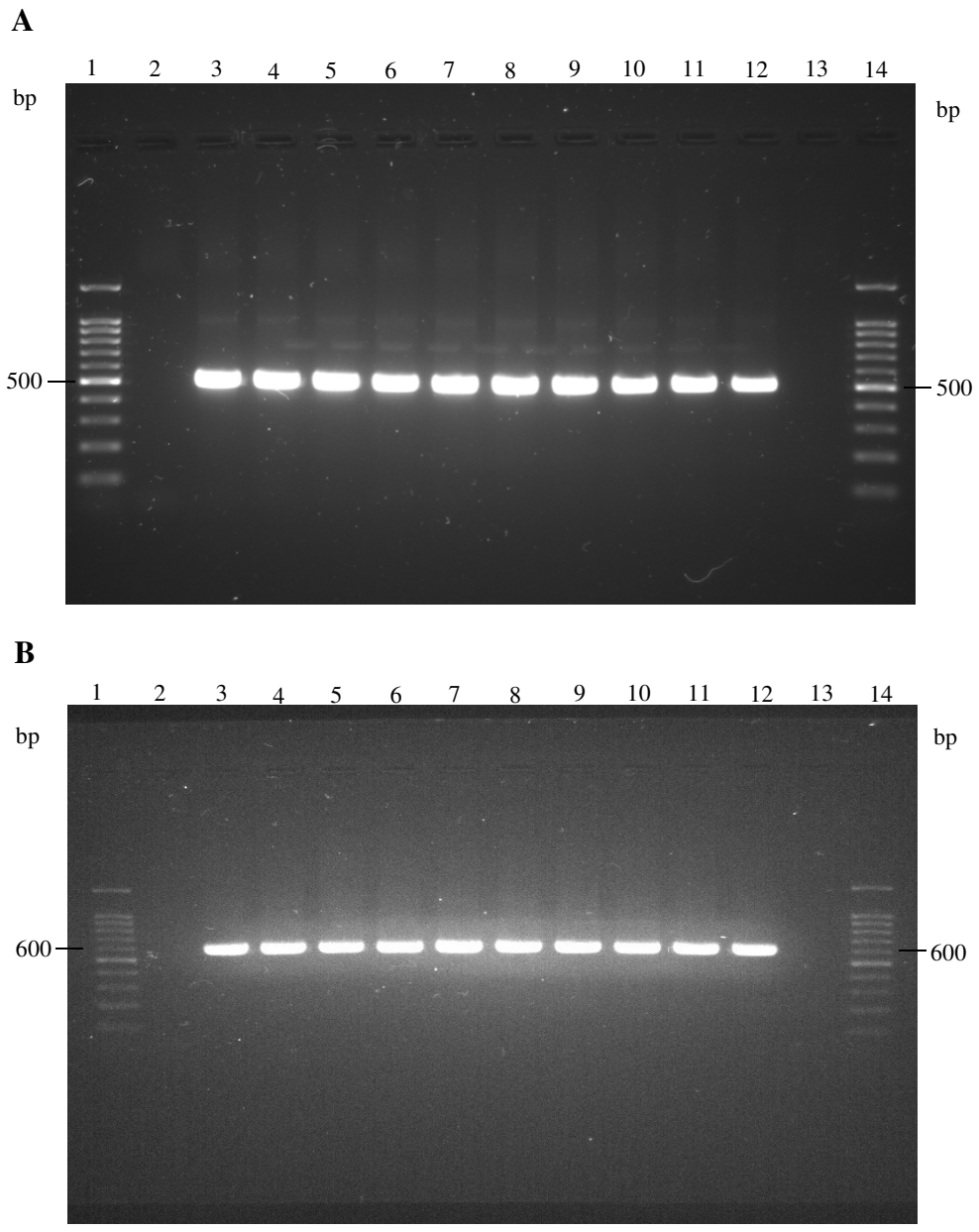

Fig A1-3. Gel electrophoresis analysis of the genes *rpoB* and *inlJ* amplified by PCR. Electrophoresis of the PCR products obtained from singleplex PCR reactions when run on 1.5% agarose gel. In all gels represented: (lane 1 and 14) Molecular weight marker; (lane 2 and lane 13) Negative control; (lane 3) strain 4; (lane 4) strain 148; (lane 5) strain 140; (lane 6) strain 7; (lane 7) strain 103; (lane 8) strain 127; (lane 9) strain 137; (lane 10) strain 122; (lane 11) strain 106; (lane 12) strain 107. **(A)** Presence of gene *rpoB* at 520 bp; **(B)** Presence of gene *inlJ* at 600 bp. Singleplex PCR reactions were performed in order to confirm the presence of genes *rpoB* and *inlJ* as it could not be determined in Multiplex PCR (Fig A1-2).

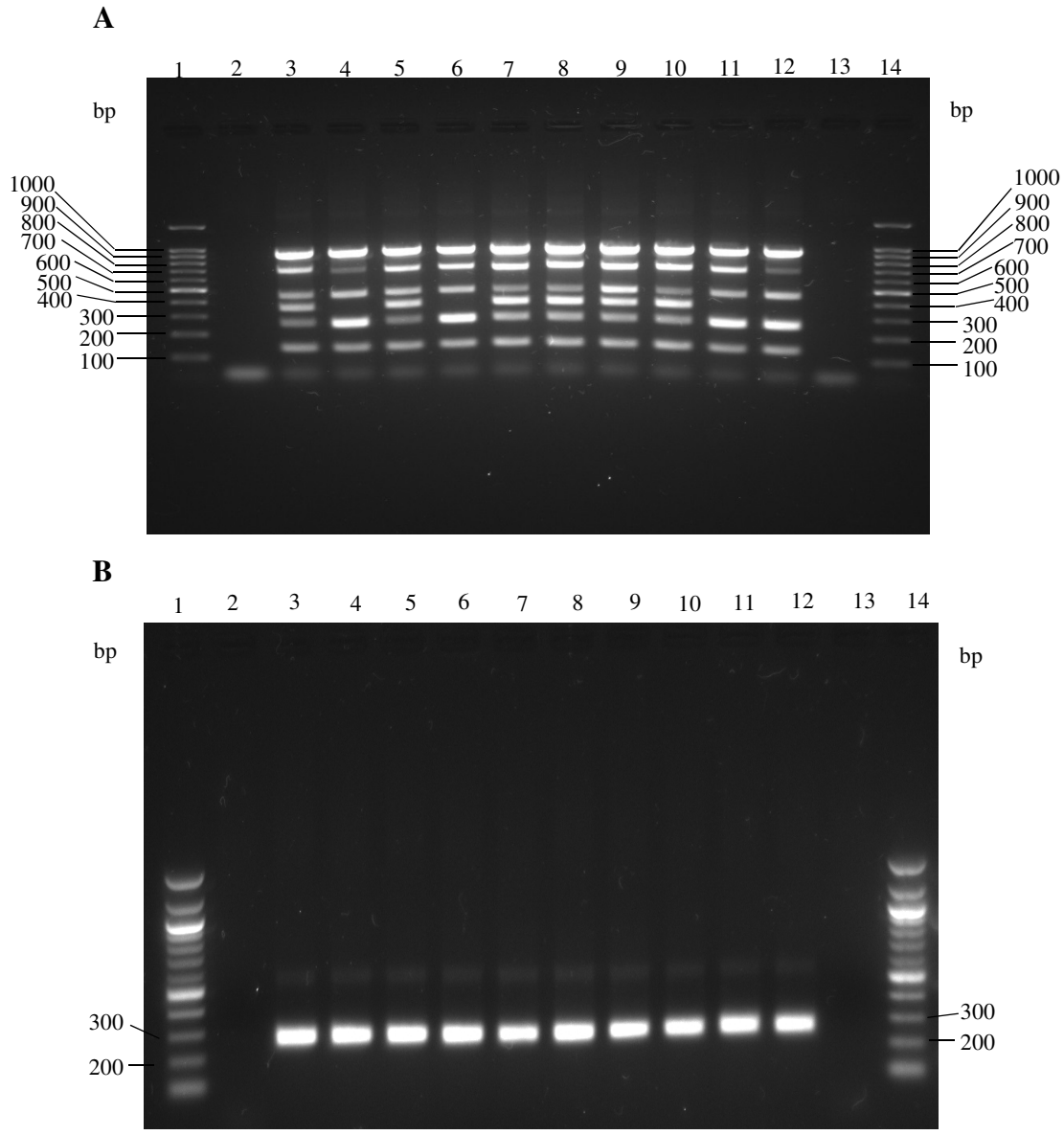

Fig A1-4. Gel electrophoresis analysis of the genes *rrn*, *iap*, *luxS*, *actA*, *prfA* and *inlB* amplified by PCR. Electrophoresis of the PCR products obtained from multiplex and singleplex PCR reactions when run on 1.5% agarose gel. In all gels represented: (lane 1 and 14) Molecular weight marker; (lane 2 and lane 13) Negative control; (lane 3) strain 4; (lane 4) strain 148; (lane 5) strain 140; (lane 6) strain 7; (lane 7) strain 103; (lane 8) strain 127; (lane 9) strain 137; (lane 10) strain 122; (lane 11) strain 106; (lane 12) strain 107. **(A)** Presence of genes *rrn* at 938 bp, *iap* (primers list1) at 660 bp, *luxS* at ~500 bp, *actA* (primers actA2) at 268 or 385 bp, *prfA* at 280 bp and *inlB* at 148 bp; **(B)** Presence of gene *prfA* at 148 bp.

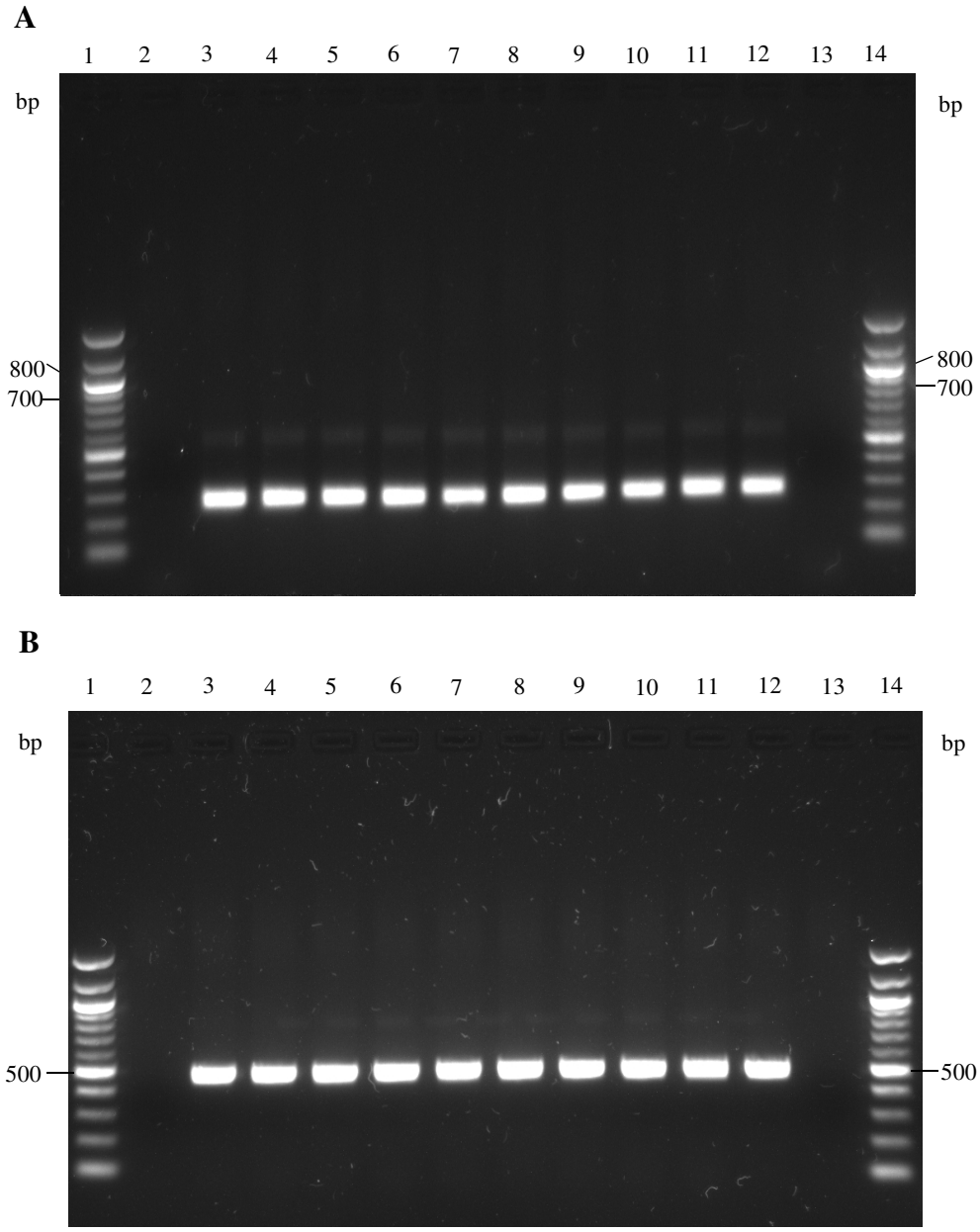

Fig A1-5 Gel electrophoresis analysis of the genes *inlA* and *mpl* amplified by PCR. Electrophoresis of the PCR products obtained from singleplex PCR reactions when run on 1.5% agarose gel. In all gels represented: (lane 1 and 14) Molecular weight marker; (lane 2 and lane 13) Negative control; (lane 3) strain 4; (lane 4) strain 148; (lane 5) strain 140; (lane 6) strain 7; (lane 7) strain 103; (lane 8) strain 127; (lane 9) strain 137; (lane 10) strain 122; (lane 11) strain 106; (lane 12) strain 107. **(A)** Presence of gene *inlA* at 760 bp; **(B)** presence of gene *mpl* at 502 bp.

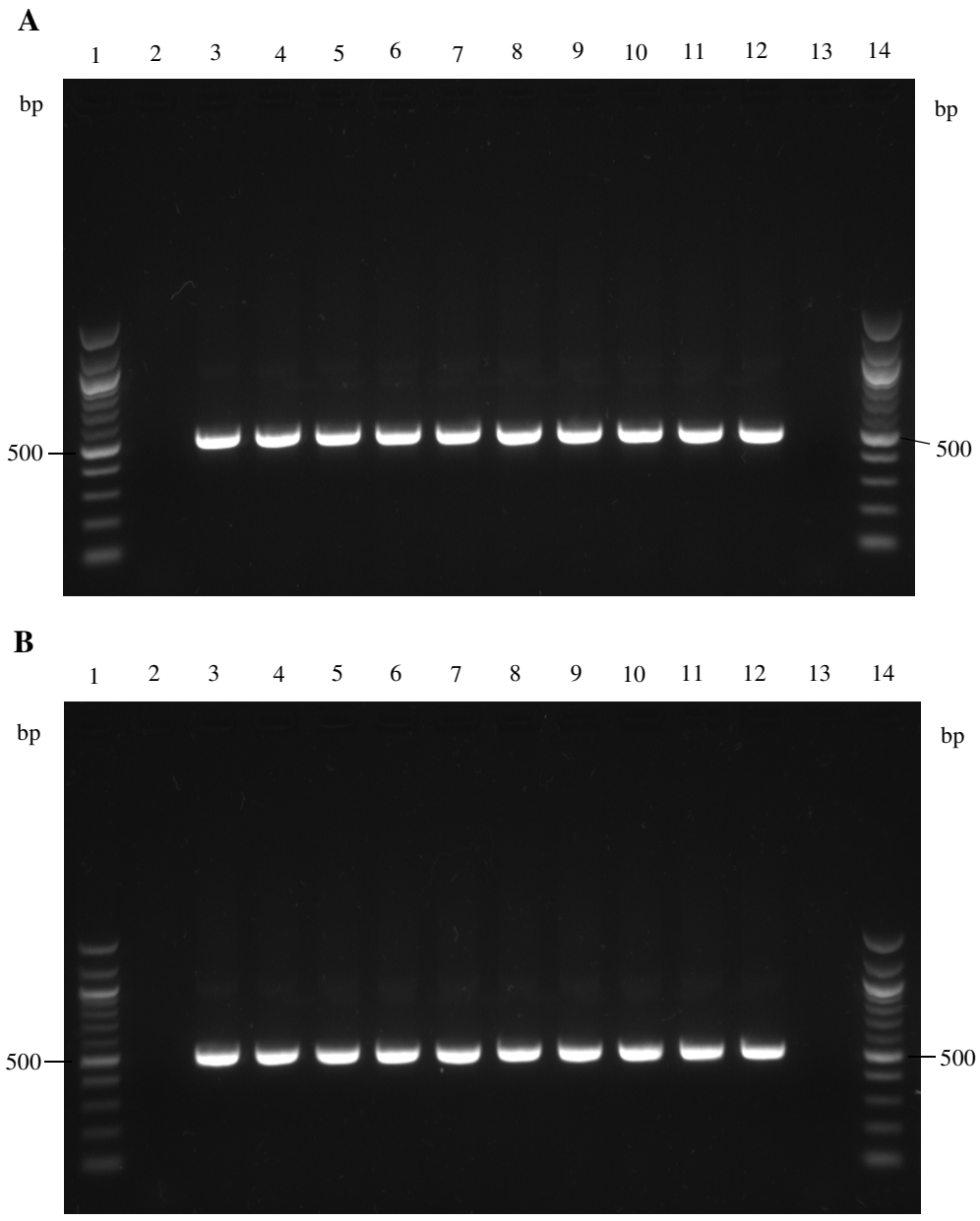

Fig A1-6.Gel electrophoresis analysis of the genes *agrA* and *agrB* amplified by PCR. Electrophoresis of the PCR products obtained from singleplex PCR reactions when run on 1.5% agarose gel. In all gels represented: (lane 1 and 14) Molecular weight marker; (lane 2 and lane 13) Negative control; (lane 3) strain 4; (lane 4) strain 148; (lane 5) strain 140; (lane 6) strain 7; (lane 7) strain 103; (lane 8) strain 127; (lane 9) strain 137; (lane 10) strain 122; (lane 11) strain 106; (lane 12) strain 107. **(A)** presence of gene *agrA* at 500 bp; **(B)** presence of gene *agrB* at 500 bp.

Appendix – A2

Table A1. List of genes used in BLAST study

General functional group	Genes			Reference strain	Reference
	Locus Tag	symbol	Names		
Biosynthesis	*lmo1641*	*citB*	aconitate Hydratase	EGD-e	Alonso et al., 2014
	lmo1566	*citC*	isocitrate dehydrogenase	EGD-e	
	lmo0974	*dltA*	D - alanine polyligase subunit 1	EGD-e	
	lmo0973	*dltB*	DltB protein for D-alanine esterification of lipoteichoic and wall teichoic acids	EGD-e	
	lmo0972	*dltC*	D-alanine polyligase subunit 2	EGD-e	
	lmo0971	*dltD*	DltD protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid	EGD-e	
	lmrg_00107	-	peptidoglycan N - acetylglucosamine deacetylase	10403S	
	lmrg_00823	-	2-oxoisovalerate dehydrogenase E1 component	10403S	
	lmrg_01032	-	xanthine ribosyltransferase	10403S	
	lmrg_01304	-	asparagine synthase	10403S	
	lmrg_01700	-	homoserine dehydrogenase	10403S	
	lmrg_01771	-	UDP-glucose 4-epimerase	10403S	
	lmrg_02497	*purE*	phosphoribosylaminoimidazole carboxylase	10403S	
	lmrg_02502	*purL*	phophoribosylformylglycinamide synthase II	10403S	
	lmrg_02503	*purF*	amidophosphoribosyltransferase	10403S	
	lmrg_02505	*purN*	phosphoribosylglycinamide formyltransferase	10403S	
	lmrg_02506	*purH*	phosphoribosylaminoimidazolecarboxamide formyltransferase	10403S	
	lmo0644	-	membrane sulfatase family protein	EGD-e	Chang et al., 2012
	lmo1370	-	branched chain fatty acid kinase	EGD-e	Ouyang et al., 2012
	lmo2229	-	penicillin binding protein	EGD-e	
	lmo2554	-	galactosyltransferase	EGD-e	
	lmo2555	-	N-acetylglucosaminyl-phosphatidylinositol biosynthesis protein		

General functional group	Genes			Reference strain	Reference
	Locus Tag	Symbol	Name		
Gene Regulation	*lmo2501*	*phoP*	two component response phosphate regulator	EGD-e	Alonso et al., 2014
	lmo2500	*phoR*	two component sensor histidine kinase	EGD-e	
	lmrg_00721	-	signal peptidase I	10403S	
	lmrg_01251	-	GntR family regulator	10403S	
	lmrg_01305	-	putative rRNA methylase	10403S	
	lmrg_01402	-	DNA polymerase	10403S	
	lmrg_01481	-	putative Rrf2 family regulator	10403S	
	lmo1386	-	DNA translocase	EGD-e	Chang et al., 2012
	lmo1378	*lisK*	two component system histidine kinase	EGD-e	
	lmo1878	-	manganese transport transcriptional regulator	EGD-e	
	lmo0246	*nusG*	transcription antitermination protein NusG	EGD-e	
	lmo0734	-	LacI family trancriptional regulator	EGD-e	Ouyang et al., 2012
	lmo1262	-	transcriptional regulator	EGD-e	
	LMOf2365_1497	-	MerR family transcriptional regulator	F2365	Huang et al., 2012
	lmo0258	*rpoB*	DNA directed RNA polymerase subunit beta	EGD-e	van der Veen & Abee, 2011
	lmo2515	*degU*	two component response regulator	EGD-e	Kumar et al., 2009

General functional group	Genes			Reference strain	Reference
	Locus tag	Symbol	Name		
Metabolism	*lmrg_00956*	*plsX*	phospholipid synthesis protein PlsX	10403S	Alonso et al., 2014
	lmo0372	-	beta-glucosidase	EGD-e	Chang et al., 2012
	lmo1600	*aroA*	bifunctional 3-deoxy-7phosphoheptulose synthase/ chorismate mutase	EGD-e	Liu et al., 2002
	lmo0239	*cysS*	cysteinyl-tRNA synthetase	EGD-e	
	lmo0570	*hisJ*	histidinol phosphatase	EGD-e	
	lmo1719	*celD*	endoglucanase D	EGD-e	
	lmo2749	*trpG*	anthranilate synthase	EGD-e	
	lmo1175	*eutB*	ethanolamine ammonia lyase large subunit	EGD-e	
	lmo1915	*mleA*	malolactic enzyme	EGD-e	
	lmo2205	-	phophoglyceromutase I	EGD-e	Ouyang et al., 2012
	lmo2535	*atpB*	ATP synthase F0F1 subunit A	EGD-e	
	lmo2529	*atpD*	ATP synthase F0F1 subunit beta	EGD-e	Ouyang et al., 2012
	lmo2534	*atpE*	ATP synthase F0F1 subunit c	EGD-e	
Motility	*lmo0685*	*motA*	flagellar motor protein	EGD-e	Alonso et al., 2014
	lmrg_00365	*fliQ*	flagellar biosynthetic protein FliQ	10403S	
	lmrg_00405	*fliI*	flagellar protein export ATPase FliI	10403S	
	lmo0697	*flgE*	flagellar hook protein FlgE	EGD-e	Chang et al., 2012
	lmo0680	*flhA*	flagellar biosynthesis protein	EGD-e	
	lmo0707	*fliD*	flagellar capping protein	EGD-e	
	lmo0714	*fliG*	flagellar motor switch protein	EGD-e	
	lmo0688	-	Thermosensing antirepressor protein GmaR	EGD-e	

General functional group	Genes			Reference strain	Reference
	Locus Tag	Symbol	Name		
Virulence	*Lmo0203*	*mpl*	zinc metalloproteinase protein	EGD-e	Meloni et al., 2012
	lmo0201	*plcA*	phosphatidylinositol specific phospholipase - C	EGD-e	
	lmo0205	*plcB*	phosphatidylcholine phospholipase – C	EGD-e	
	lmo0433	*inlA*	internalin A	EGD-e	Franciosa et al., 2009
	lmo2558	*ami*	autolytic amidase	EGD-e	Kumar et al., 2009
	lmrg_02624	*hly*	listeriolysin	10403S	Lemon et al., 2010
	lmo0200	*prfA*	listeriolysin positive regulatory protein	EGD-e	
	lmo0434	*inlB*	internalin B	EGD-e	
	lmo0394	*iap*	invasion associated protein	EGD-e	Monk et al., 2004
	lmo0204	*actA*	actin polymerization inducing protein	EGD-e	Travier et al., 2013
Stress response	*lmrg_01912*	-	catalase	10403S	Alonso et al., 2014
	lmo0690	*flaA*	flagellin	EGD-e	Liu et al., 2002
	lmo0943	*flp*	ferritin like protein	EGD-e	
	lmo2206	*clpB*	Clp protease subunit B	EGD-e	
	lmo2068	*groEL*	major heat shock protein	EGD-e	
	lmo2478	*trxB*	thioredoxin reductase	EGD-e	
	lmo2461	*sigL*	RNA polymerase factor sigma54	EGD-e	
	lmo0501	*bglG*	transcriptional anti-terminator BglG	EGD-e	
	lmo0291	*yycj*	two component signal transduction system	EGD-e	Liu et al., 2002
	lmo0571	*adaB*	methyltransferase	EGD-e	
	lmo1508	*lkhA*	histidine kinase sensor	EGD-e	
	BN389_13280	*yneA*	cell division suppressor protein YneA	LL195	van der Veen and Abee., 2010b
	lmo0895	*sigB*	RNA polymerase sigma factor SigB	EGD-e	van der Veen and Abee., 2010a
	lmo0292	*hrtA*	serine protease	EGD-e	Wilson et al., 2006
	lmo1523	*relA*	(p)ppGpp synthetase	EGD-e	Taylor et al., 2002
	LMOL312_0838	*hpt*	hexose phosphate transport protein	L312	
	lmo1439	*sod*	superoxide dismutase	EGD-e	Suo et al., 2012

General functional group	Genes			Reference strain	Reference
	Locus Tag	Symbol	Name		
Quorum Sensing	*lmo0048*	*agrB*	sensor histidine kinase AgrB	EGD-e	Chang et al., 2012
	lmo0051	*agrA*	response regulator AgrA	EGD-e	
	lmrg_02479	*agrC*	sensor histidine kinase AgrC	10403S	Rieu et al., 2007
	LMM7_0043	*agrD*	putative auto-inducing peptide AgrD	M7	
Other Functions					
LPXTG surface proteins and anchoring of LPXTG proteins	*lmo0929*	*srtA*	sortase	EGD-e	Chang et al., 2012
	lmo1666	-	peptidoglycan linked protein	EGD-e	
cell wall associated protein	*lmo0435*	*bapL*	peptidoglycan binding protein	EGD-e	Jordan et al., 2008
protein translocation	*lmo2612*	*secY*	SecY preprotein translocase subunit	EGD-e	Durack et al., 2015
cell surface alterations	*lmo0443*	*psr*	PBP5 synthesis repressor	EGD-e	Liu et al., 2002
	lmo0721	*fbp*	fibronectin binding protein	EGD-e	
unknown	*lmo2504*	-	cell wall binding protein	EGD-e	Lourenço et al., 2013
	lmrg_00049	-	hypothetical protein	10403S	Alonso et al., 2014
	lmrg_01872	-	efflux protein	10403S	
	lmrg_02457	-	hypothetical protein	10403S	
	lmrg_02487	-	adenyl synthase	10403S	
	lmo1918	-	hypothetical protein	EGD-e	Chang et al., 2012
	lmo2056	-	hypothetical protein	EGD-e	
	lmo2566	-	hypothetical protein	EGD-e	
	lmo2402	-	similar to B. subtilis YutD protein	EGD-e	
	lmo2553	-	hypothetical protein	EGD-e	Ouyang et al., 2012

Contribution of Collaborators

The experiments to determine the influence of different growth conditions on biofilm formation were performed with the assistance of Jenny Han Zhang, Jeffrey Zhang and Elizabeth Johnston.

SEM and optimization of the conditions for singleplex and multiplex PCR was done by Beverly Phipps-Todd. PCR analysis for all the test isolates was performed by Krishna Gelda and PCR for isolate 115 was performed by Alyssa Lee.

Whole genome sequencing of the isolates were done by Dr. Catherine Carrillo and others at the Carling lab. The script Gene Seeker used for BLAST search was provided by Adam Koziol. BLAST similarity searches were performed by Adam Koziol and Jackson Eyres at the Carling lab.