Identification and understanding the roles of biofilm formation-related genes in *Listeria monocytogenes* isolated from seafood

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This work is dedicated to my mother and father

Ihr gebt mir Wurzeln in die eine, und Flügel in die andere Hand und einen Kuss auf meine Stirn, der sagt mir: „Ich bin nicht alleine."
Dann legt ihr zwischen uns ein Band, sodass wir uns nicht verlieren, sagt ihr. Und dass ich gehen kann wenn ich will.
Und irgendwann geh ich raus. Aber hier draußen ist es so still, so ohne euch. Ihr seid nicht da wenn ich aufstehe, seid nicht da wenn ich schlafen gehe. Also schon, aber woanders und das ist nicht leicht.
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Julia Engelmann

Abstract

Listeria monocytogenes is a foodborne pathogen that can lead to severe bacterial infections in immunocompromised people, the elderly and pregnant women and their unborn. Seafood is one of several contamination sources and as the seafood industry is of high economic value to New Zealand, this pathogen needs to be controlled. The main route for contamination is thought to be from biofilms in the seafood-processing environment and their persistence through cleaning and sanitation.

Persistent and sporadic strains of *L. monocytogenes* isolated from mussel-processing facilities were compared using phenotypic assays. Biofilm formation was greater for persistent strains compared to sporadic strains (30°C, 48h) using cell counts and crystal violet staining (CV). The persistent isolate 15G01 exhibited greatest biofilm formation and was therefore chosen to be studied for biofilm formation using transposon mutagenesis.

A screen of the transposon library for biofilm-forming ability using the crystal violet assay identified 27 genes to be associated with biofilm formation. Three low biofilm formers (33E11, 39G5, 44D3) and one high biofilm formers (34F11) were analysed with the fluorescent LIVE/DEAD stain and the scanning electron microscope revealing coccoid-shaped cells and long chains for 33E11 and 44D3, respectively.

The four mutants and a previously identified fifth (6B4) were investigated for their biofilm-forming ability, the surface characteristics of the cells and the influence of cations on biofilm formation. Three different biofilm formation assays were used to assess the composition of the biofilm. The CV assay was used to determine the whole biofilm mass, cell enumeration was applied to calculate viable cells in the biofilm and a formazan based assay (XTT) measured metabolic activity. All three assays showed a significant correlation, however, no correlation with cell surface characteristics was observed. Confocal laser scanning microscopy revealed a unique sandwich structure for the biofilm formed by 44D3, which has not been reported before, and was reversed at higher magnesium concentrations.

Magnesium influenced biofilm formation at a concentration of 5 mM resulting in enhanced biofilm formation for the wild-type and the mutant 44D3 and in reduced biofilm formation in 39G5 whereas calcium showed no gene-specific effect on biofilm formation.

The research presented in this study provides useful data for the prevention and control of biofilm formation by *L. monocytogenes* in seafood-processing plants.

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Declaration

The presented thesis is comprised of seven chapters. Chapters 3, 4, 5 and 6 are structured as manuscripts that have either been submitted and published or are to be submitted to peer-reviewed journals. Therefore, sections in the materials and methods are repeated in some chapters, however, results and discussion are unique to each chapter.

Contents

List of Publications

Nowak J., Cruz C., Visnovsky S., Palmer J., Fletcher G., Pitman A., Flint S. (in advanced preparation for submission). The *mgtB* mutant of the persistent *Listeria monocytogenes* 15G01 strain produces a unique biofilm sandwich structure which changes its phenotype upon addition of magnesium.

Nowak J., Cruz C., Visnovsky S., Palmer J., Fletcher G., Pitman A., Flint S. (in advanced preparation for submission). Biofilm formation of *Listeria monocytogenes* 15G01, a persistent isolate from a seafood-processing plant in New Zealand, is influenced by inactivation of multiple genes belonging to different functional groups.

Nowak J., Cruz, C., Tempelaars, M., Abee, T., van Vliet, A., Fletcher G., Hedderley, D., Palmer J., Flint S. (2017). Persistent *Listeria monocytogenes* isolates from mussel production facilities form more biofilm but are not linked to specific genetic markers. *International Journal of Food Microbiology*, Volume 256, 45-53

Nowak J., Visnovsky S., Pitman A., Cruz C., Fletcher G., Palmer J., Flint S. (Nov 2016). Magnesium and stress response play a vital role in biofilm formation of *Listeria monocytogenes.* NZMS Conference 2016, Christchurch, New Zealand (oral presentation).

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Chapter 1

General Introduction

L. monocytogenes is a foodborne human pathogen which is commonly found in the environment. This Gram-positive microorganism is a threat, especially for the immunocompromised, the elderly and pregnant women as well as their unborn babies and newborns (Rocourt et al. 2000). *L. monocytogenes* can cause a disease named listeriosis. This infection is relatively uncommon, however, if an invasive infection occurs, the fatality rate can be as high as 30% (Rocourt et al. 2000). Most of the *L. monocytogenes* infections derive from consuming food like dairy products, vegetables and fruit, meat and seafood, that have become contaminated. The major source of contamination is thought to be due to biofilm formation and/or persistence of *L. monocytogene*s strains in food-processing plants. A biofilm is a community of cells that has become sessile by attaching to a surface and producing extracellular polymeric substances (EPS) (Lasa 2006). The EPS can consist of polysaccharides, lipids, DNA and other components and varies between species and strain. By establishing as a biofilm, cells can become more resistant to environmental threats, such as desiccation, high temperatures, and sanitisers. Bacterial cells tend to settle down in niches and harbourage sites where they are hard to eradicate. This along with the increased resistance to environmental threats through formation of biofilms and persistence cause a problem for the food-processing industry.

New Zealand's waters are a great breeding ground for an abundance of fish species. The purity of the water and the distance from major pollution hubs produce seafood of high quality that is highly sought after internationally. New Zealand exports large amounts of seafood (approximately 290,000 tonnes of seafood were exported in 2015) to China (including Hong Kong), the USA, Australia and Japan (Seafood New Zealand n.d.). The New Zealand aquaculture industry generated around $500m in revenue in 2015 (Aquaculture New Zealand n.d.) of which $338.1m were generated through exports. Seafood is important to New Zealand's economy – in 2015 exports were valued at NZ$1.71B and growth in aquaculture is expected to reach NZ$1B by 2025. The amount of food traded internationally increases every year and in order to be able to compete and maintain a position on the international market, the high food safety standards of New Zealand's industries need to be maintained.

Infections with *L. monocytogenes* derived from contaminated food will not only cause financial damage to the producing company but will also result in damage to New Zealand's excellent reputation for food safety, as demonstrated by Fonterra's botulism scare in 2013. The true financial loss to Fonterra is unclear but an estimate of NZ$60 million was announced shortly

after the incident (Hussain and Dawson 2013, Government inquiry into the whey protein concentrate contamination 2014). The recovery after such an incident takes time and money. If contamination can be avoided in the first place by tackling biofilms, ongoing sampling can be reduced and money can be saved.

An example that demonstrates the major implications contamination of food with *L. monocytogenes* can have, is a recall of mussel meat through a New Zealand mussel-processing company (Dominion Post 2009, NZ Herald 2007). This company voluntarily recalled 280 tonnes of green-lipped mussel meat which was possibly contaminated with *L. monocytogenes* in low levels in 2007. The majority of the produce was exported, but some of the meat was sold in Nelson. After an investigation, a contaminated roller and conveyor belt were removed from the processing plant. Luckily the financial loss was not "too bad" and the food safety reputation was not majorly affected, but this case shows that a little contamination can have severe effects. By preventing contamination through prevention of biofilm formation, incidents like those can be avoided.

Effort has been made to clarify the mechanism of biofilm formation, yet the exact mechanism remains elusive. Several genes have been identified to be associated with biofilm formation in *L. monocytogenes*, however, the ability of *L. monocytogenes* to adapt quickly to changes in environmental conditions makes it hard to control and study. Biofilm formation is no doubt a highly complex process which needs to be further investigated in order to develop strategies to prevent contamination in food-processing plants. This is particularly important in seafood-processing plants, as seafood is often consumed raw or as a chilled ready-to-eat (RTE) processed product. Those products represent a serious food safety hazard if contaminated with *L. monocytogenes* and consumed by sensitive consumers.

Therefore, the aim of the present study was to 1) identify differences between sporadic and persistent *L. monocytogenes* strains isolated from mussel-processing plants, 2) identify optimum conditions for biofilm formation of one persistent strain, 3) identify genes that are associated with biofilm formation by screening a mutant library of the persistent strain *L. monocytogenes* 15G01 and 4) identify the involvement of the selected genes in biofilm formation.

The research hypotheses of this study are

- Biofilm formation of *L. monocytogenes* is linked to persistence.
- Biofilm formation of *L. monocytogenes* is influenced by a number of unidentified genes belonging to different functional groups.
- Biofilm formation of *L. monocytogenes* is strongly dependent on temperature and nutrient availability.
- Biofilm formation of *L. monocytogenes* is influenced by presence of cations.

Chapter 2

Literature Review

Summary

Listeria monocytogenes is a foodborne pathogen that can cause a disease named listeriosis which is an infection that occurs predominantly in immunocompromised people, producing serious symptoms and possibly leading to death. This bacterium, like many others, has the ability to form biofilms not only on biotic but also on abiotic surfaces. Once biofilms are established on surfaces they are very hard to eradicate. After the initial attachment, bacteria change their properties and become irreversibly attached and form a mature biofilm with characteristics somewhat different to their planktonic counterpart.

To induce the changes in characteristics bacteria need to communicate with each other – they use quorum sensing, a communication system correlated to population density. The LuxS-system and the *agr*-system are both part thereof and are proven to be involved in biofilm formation. Flagella, which are appendages responsible for the cell's motility, are involved in biofilm formation. Flagella may function as adhesins, but it has also been proposed that flagella mediated motility is critical for biofilm formation rather than the flagella themselves. However, it has been found that the absence of flagella results in the formation of hyperbiofilms in flow conditions, which suggests that flagella are not necessary for biofilm formation. Flagella are synthesised by a complex mechanism which includes at least five regulators, but the exact mechanism and the role in biofilm formation remain unclear. During biofilm formation, bacteria excrete and form substances that stabilise the biofilm structure. Recent studies, with a focus on the extracellular matrix, showed the importance of extracellular DNA in initial attachment, however the mechanism is unknown, and the origin of eDNA and its release remain unclear. While comparing exoproteomes of biofilms and planktonic bacteria, Lmo2504, a putative cell wall binding protein, it was found to positively influence biofilm formation. Another protein associated with biofilm formation is BapL, a cell wall anchored protein. However, the importance of extracellular proteins in biofilm formation is not fully understood.

Other molecular determinants which play an important role in biofilm formation are DegU (degradation enzyme regulator), (p)ppGpp, whose synthesis is influenced by *relA* and *hpt*, and *prfA*, a gene encoding for a virulence factor that also regulates genes involved in biofilm formation. Internalin A and B, both part of *L. monocytogenes'* pathogenicity, influence biofilm growth to a greater extent when present together compared to only one being present. A putative DNA translocase (Lmo1386), a putative Crp/Fnr family transcription factor (Lmo0753)

and the GntR-family response regulator LbrA were also linked to biofilm formation. Other genes involved in biofilm formation and stress response are *yneA*, *recA*, *sod*, *sigB*, *gltB* and *gltC*, *hrcA* and *dnaK*.

The activation of the oxidative stress and SOS responses during biofilm formation is an interesting finding. The SOS response is usually activated when damage occurs to the DNA. Cells experience stress, not only when they are exposed to heat or oxidative agents, but also during biofilm formation which results in upregulation of antioxidative genes and a change in cell structure. Cells grown in flow systems experience elongation and form biofilms consisting of microcolonies with knitted chains.

In food-processing plants *L. monocytogenes* is exposed to different environmental conditions such as pH and temperature which change, usually due to cleaning and sanitising treatments. These conditions plus nutrient availability and the presence of other microorganisms also influence biofilm growth. The presence of *Flavobacterium* spp and *Pseudomonas* spp was found to enhance biofilm formation of *L. monocytogenes* whereas the presence of lactic acid bacteria and *Listeria innocua* decreased the ability to form biofilm. Although there are some contradictory findings, it is clearly stated that co-cultures influence biofilm formation although the extent and fundamental understanding of these interactions is unclear.

Most of the *L. monocytogenes* infections derive from consuming food like dairy products, vegetables and fruit, meat and also seafood, that have become contaminated. New Zealand exports large amounts of seafood to, in particular, European and Asian markets. Contamination of these products is believed to be due to persistence and biofilm formation either on the food or in the processing plant. To ensure New Zealand's food safety and to sustain its international reputation, it is important to minimise the contamination risk. To enable appropriate strategies to minimise contamination, an understanding of the genes and the molecular mechanism of biofilm formation of *L. monocytogenes* originating from seafood is required.

2.1 Introduction

Listeria monocytogenes is a pathogen which can cause severe infections in humans, especially immunocompromised people, and is usually derived from food contamination. Foods like ready-to-eat meat and seafood as well as vegetables and fruit have been identified as vectors. *L. monocytogenes* is not the only bacterium responsible for foodborne outbreaks, but it has one of the highest fatality rates of all foodborne pathogens. *L. monocytogenes'* ability to persist in food-processing plants and to produce biofilms has been thoroughly investigated. Biofilms are

a sessile living form of bacteria that provides many ecological benefits to the bacterium, including protection from environmental damage. Through the formation of an extracellular matrix, bacteria are able to withstand environmental threats such as UV light, nutrient deficiency, acids, osmotic pressure, antibiotics and sanitisers, and can therefore secure their survival. As *L. monocytogenes* is commonly found on food surfaces it can also be found regularly in food-processing plants. *L. monocytogenes* tends to settle down as biofilms in niches where they are protected from cleaning and sanitising treatments and in addition, once a biofilm has formed, this enhances the bacterium's resistance to chemical treatments. Many genes are known to be involved in biofilm formation. However, the exact mechanism of biofilm formation is still unclear. This review summarises the known mechanisms and genes involved in *L. monocytogenes'* biofilm formation and outlines the importance of controlling biofilms of *L. monocytogenes* for New Zealand's seafood industry.

2.2 *Listeria monocytogenes*

L. monocytogenes is a foodborne human pathogen (or saprophyte depending on the environment) which is commonly found in the environment. This Gram-positive microorganism is a threat especially for the immunocompromised, the elderly and pregnant women as well as their unborn babies and new-borns (Rocourt et al. 2000). *L. monocytogenes* can cause a disease named listeriosis. This infection is relatively uncommon, however, if an infection occurs, the fatality rate can be as high as 30 % (Rocourt et al. 2000). *L. monocytogenes* enters the human body mainly through contaminated food. It survives the acidity of the stomach and enters the small intestine where it passes through the mucosal layer and adheres to the epithelial cells of the intestinal wall with the cell wall proteins Internalin A and Internalin B and enters it via phagocytosis (Vazquez-Boland et al. 2001). Listeriolysin O and phospholipases C are expressed and release the bacterium through lysis of the vacuolar membrane. Then it spreads from cell to cell via an actin-based motility process (de las Heras et al. 2011). Most of the genes encoding for virulence factors are regulated by the transcription factor PrfA. *L. monocytogenes* can cross the epithelial barrier and spread to the liver, spleen and the central nervous system and in pregnant women to the foetus. Listeriosis can manifest itself in two different phenotypes in adults, the invasive and the non-invasive disease. The symptoms of the non-invasive form equal a bacterial gastroenteritis whereas the invasive form shows severe symptoms of meningitis, meningoencephalitis and sepsis. The invasive form occurs usually in immunocompromised people such as the elderly and people with an underlying disease. Humans can be asymptomatic carriers of *L. monocytogenes,* making it even more difficult to control. Infections in pregnant

women can result in fetal loss, stillbirth, premature delivery or neonatal infection (meningitis). The neonatal infection is separated into early onset (infection in utero and symptomatology during first weeks of life) and late onset (symptomatology from first weeks to several weeks after birth) (John and Laurence 2007).

L. monocytogenes is a non-spore-forming rod which belongs to the genus *Listeria* along with *L. ivanovii, L. innocua, L. welshimeri, L. selligeri, L. grayi*, *L. rocourtiae* and *L. marthii* (Graves et al. 2010, Leclercq et al. 2010, Rocourt and Buchrieser 2007). They belong to the class Bacilli and the order Bacillales with a minimum of four evolutionary lineages with 13 serovars. The most common serotypes derive from lineage I and II (Orsi et al. 2011). The majority of human listeriosis cases are caused by three different serovars: 1/2a (lineage II), 1/2b and 4b (lineage I) (Schuchat et al. 1991); in fact more than 95% of the listeriosis cases are caused by the three serovars (Kathariou 2002). *L. monocytogene*s is acid tolerant, able to grow in temperatures from 0°C to 45°C, in a broad pH range and can survive at freezing temperatures which makes it a bacterium that is hard to eradicate and control (Yousef 1999). The optimal growth temperature ranges from 30°C to 37°C. *L. monocytogenes* is a facultative anaerobic microorganism with 4 to 6 peritrichous flagella which are responsible for its motility at room temperature and below. Comparative genomic studies between *L. monocytogenes* EGDe *and L. innocua* showed great similarity with *Bacillus subtilis*, suggesting that regulation or molecular mechanisms might be very similar (Glaser et al. 2001).

2.3 *L. monocytogenes* in seafood in New Zealand

New Zealand's coast line is 15,134 km long and marine fisheries waters cover 4.4 million km^2 with 130 different species being commercially fished in these waters. A total of 659,552 tonnes of seafood is harvested each year and in 2015 289,911 tonnes valued at NZ$1.71B were exported (Seafood New Zealand n.d.). This makes the seafood industry an important part of the New Zealand economy. Aquaculture, the cultivation of seafood is also important. Products from aquaculture are not only sold in New Zealand but also exported into 79 countries worldwide thereby generating $500 million in revenue in 2015 (Aquaculture New Zealand n.d.).

While *L. monocytogenes* is able to attach to and contaminate raw products, contamination usually occurs through processing rather than on raw products (Autio et al. 1999, Fonnesbech-Vogel et al. 2001).

As seafood can be contaminated with *L. monocytogenes*, it is important to ensure quality standards are met. Standard 1.6.1 Microbiological limits for foods of the Australia New Zealand

Food Standards Code (FSC) regulates the limit for all types of food that is produced in New Zealand and Australia (Food Standards Australia New Zealand 2016a). The updated version became effective on 1st March 2016 to meet the latest standards in the international market in order to support an efficient and internationally competitive food industry. The revised microbial limits for *L. monocytogenes* can be found in the Food Standards Code 1.6.1. – Microbial Limits for Food (Ministry for Primary Industries n.d.). The updated version distinguishes between ready-to-eat foods where growth can occur and where growth cannot occur. According to Standard 1.6.1. "no growth can occur (1) if the food has a pH less than 4.4 regardless of water activity; or (2) the food has a water activity less than 0.92 regardless of pH; or (3) the food has a pH less than 5.0 in combination with a water activity of less than 0.94; or (4) the food has a refrigerated shelf life no greater than 5 days; or (5) the food is frozen (including foods consumed frozen and those intended to be thawed immediately before consumption); or (6) it can be validated that the level of *L. monocytogenes* will not increase by greater than 0.5 log CFU/g over the food's stated shelf life." Moreover "a ready-to-eat food that does not receive a listericidal process during manufacture is taken to be a food in which growth of *L. monocytogenes* will not occur if the level of *L. monocytogenes* will not exceed 100 CFU/g within the food's expected shelf life (Food Standards Australia New Zealand 2016b, Food Standards Australia New Zealand 2016c)." In ready-to-eat food where growth can occur, *L. monocytogenes* needs to be absent in 25g (<0.04 CFU/g) and the limit for *L. monocytogenes* in ready-to-eat-food where no growth can occur is 100 CFU/g. This update is based on Codex 2007 and has been adopted by many countries so far, including Canada and the European Commission (Food Standards Australia New Zealand n.d.), however, the USA has got a zero-tolerance policy and requires absence of *L. monocytogenes* in all ready-to-eat foods (US Food and Drug Administration 2015).

In 2014 and 2015, 25 and 26 cases of listeriosis were reported in New Zealand, respectively (The Institute of Environmental Science and Research Ltd. 2016). From 1997 to 2015, the cases of listeriosis have fluctuated between 17 and 35 reported notifications per year. Those numbers include all cases reported in New Zealand regardless of the contamination source. The highest incidence of listeriosis occurred in the elderly. In 2009, 2010 and 2011, 21 out of 28 cases, 18 out of 23 cases and 21 out of 26 cases respectively were food related (The Institute of Environmental Science and Research Ltd. 2013, The Institute of Environmental Science and Research Ltd. 2014). Of the 25 and 26 cases reported in 2014 and 2015, 22 and 21 are estimated to be food related (Horn et al. 2015, Lopez et al. 2016). From 2002 to 2013 two *Listeria* outbreaks were reported in (in 2012 and 2009). The outbreak in 2012 could be linked to RTE

meat products (beef) sold in the Hawke's Bay and Tauranga regions whereas the outbreak in 2009 was food associated, but the vehicle is unknown. An outbreak is defined as two or more listeriosis cases by the same serotype from a common food source. The only documented outbreak of listeriosis associated with seafood in New Zealand happened in 1992, where contaminated smoked mussels were consumed by two people in Auckland. Analysis of 15 other clinical isolates revealed two other cases that were linked to the same identified serotype 1/2a (Brett et al. 1998). Two deaths occurred out of those four cases stated above. Other than that, there have been outbreaks in earlier years, but they could not be linked to specific food (Faoagali and Schousboe 1985, Flight 1971). Contamination of seafood, or food in general, with *L. monocytogenes* in New Zealand, as well as in other countries, is more likely to occur during processing rather than in the raw product (Fletcher et al. 1994, Gudbjörnsdóttir et al. 2004). This underlines the importance of the identification of *L. monocytogenes* genes involved in biofilm formation in seafood-processing plants.

2.4 Biofilm formation in *Listeria monocytogenes*

2.4.1 Biofilm

Forming biofilms enables bacteria to gain resistance against environmental factors, such as pH, temperature changes, and cleaning agents. Bacteria tend to attach to surfaces and form biofilm not only in the natural environment but also, and probably more importantly to us humans, in food-processing plants where they are then hard to eradicate and capable of spreading and hence a risk for food (re)contamination.

Lasa defines the five stages of biofilm development as follows: (1) initial attachment of planktonic bacteria to a surface, (2) production of extracellular matrix consisting of exopolysaccharides, DNA and lipids (3) building of multilayer construction, (4) maturation of biofilm structure and (5) detachment of single cells from the biofilm to spread and colonize other surfaces (Lasa 2006).

According to the Oxford Dictionary, adhesion is defined as the action or process of adhering to a surface or object. In the case of biofilm formation adhesion is defined as the attachment of a cell to a substrate and cell to cell attachment is defined as cohesion (Garrett et al. 2008). Opinions differ in regard to the meanings of each term amongst scientists. In order to guard against misunderstandings, the terms attachment and adhesion will be used as synonyms in this review.

The biofilm structure of *L. monocytogenes* depends on the environment. Biofilms grown in static conditions show a homogenous layer of cells, whereas biofilms which develop in a continuous flow system build ball-shaped microcolonies, which are surrounded by a network of knitted chains and increase in thickness over time (Rieu et al. 2008a). It has been found that the elongation of cells which is responsible for forming the latter is dependent on activation of YneA, a SOS response factor (van der Veen and Abee 2010a, van der Veen et al. 2010). Generally, biofilms developed under static conditions are thinner than those generated in flow conditions (Rieu et al. 2008a). *L. monocytogenes* can not only form ball-shaped microcolonies surrounded by a network of knitted chains but also honeycomb-like structures (Pilchova et al. 2014).

Hence, the industrial environment has a big influence on the biofilm structure and needs to be considered when analysing biofilms.

2.4.2 Cell-to-cell communication (Quorum sensing)

Quorum sensing is a communication system which is induced when bacteria reach a high cell density. Two systems are known for *L. monocytogenes,* the LuxS- and the *agr*-system.

2.4.2.1 LuxS-System

L. monocytogenes produces an autoinducer-2 (AI-2) like molecule whose formation is catalysed by LuxS (S-ribosylhomocysteinase). LuxS is involved in a two-step conversion of S-adenosyl homocysteine (SAH) to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) with S-ribosyl-L-homocysteine (SRH) as a key precursor. DPD is very unstable and degrades to furanosyl borate diester and an autoinducer-2 like molecule. It was observed that a *L. monocytogenes* strain with a mutation in *luxS* gene formed a biofilm with higher density than the control, and also demonstrated greater attachment to glass and polystyrene surfaces (Sela et al. 2006). Adding synthesised AI-2 to the mutant did not restore the wild-type function, which indicated that another substance associated with *luxS* must have an influence on biofilm formation. SRH as well as SAH were present in higher amounts in the cell supernatant of the *luxS* mutant, but further tests showed that only SRH was capable of increasing biofilm formation. The hypothesis was that SRH enhances the ability to form biofilms and AI-2 might only be present in *L. monocytogenes* to detoxify the cell from the toxic compound SAH (Challan Belval et al. 2006, Sela et al. 2006). The mechanisms on how SRH and SAH can accumulate outside the cell and how SRH increases biofilm formation remain unclear. Although LuxS is involved in biofilm formation, it is not essential as it was observed that the *luxS* mutant is still capable of becoming sessile (Bonsaglia et al. 2014).

2.4.2.2 *agr*-System

The *agr*-system (accessory gene regulator) is another cell-to-cell communication system used by *L. monocytogenes*. Four genes (*agrB*, *agrD*, *agrC* and *agrA*) are organised as an operon. Those genes encode histidine kinase AgrC, response regulator AgrA, precursor peptide AgrD and AgrB, which is involved in processing AgrD into a mature autoinducer peptide. Mutants of *agrA* and *agrD* were created to examine the effect on biofilm formation (Rieu et al. 2007, Garmyn et al). Both mutants showed the same phenotype: initial attachment was reduced, less biofilm was produced in the first 24 h, but no difference to the wild-type strain was observed in the later stages of biofilm formation, which indicates that the Agr-system plays an important role in the early stages of biofilm development.

Gene expression studies compared the expression level of *agr* in planktonic versus sessile cells (Rieu et al. 2007). Expression levels were growth dependent for sessile but not planktonic cells, which indicates the involvement in biofilm formation. Expression of *agr* was decreased in sessile cells, which was surprising but might indicate a complex mechanism with a possible regulation of other proteins involved in biofilm maturation by the *agr* operon.

Gene expression studies of planktonic growth showed lower expression for *agrB*, *agrC* and *agrD* in the mutants compared to the wild-type (Rieu et al. 2007), but no difference for *agrA* levels, which indicates that there is constitutive expression of *agrA* and a possible autoregulation of *agrB*, *agrC* and *agrD*. The basic level of AgrA might be present in order to reply or react to certain environmental conditions faster. Chemical half-life and 5'-RACE experiments showed posttranscriptional cleavage of the full length transcript with degradation of *agrC* and *agrA*. This finding and results from mRNA quantification support the theory of a complex autoregulation of the *agr*-system.

In another study, low expression of *agr* was only observed under static conditions, but when grown in a flow system, a progressive increase in expression could be detected (Rieu et al. 2008a). Cells expressing *agr* were mostly found outside the microcolonies, in the network of knitted chains. It is assumed that there is a possible connection between *agr* expression and the structure of biofilm as there were differences in biofilm organisation under static or dynamic conditions.

Another study showed that a mutation of the *agrD* gene led to a larger decrease in biofilm formation than previously observed (Riedel et al. 2009). The use of a different medium (10-fold diluted Brain-Heart-Infusion versus Tryptic Soy Broth) showed greater influence on biofilm

formation than previously expected, suggesting that nutrient availability can play a very important role in biofilm formation.

Garmyn et al. (2011) suggested that the primary function of the Agr system is not only to sense population density, but possibly participating in generating heterogeneity. However, this needs further investigation.

Quorum sensing is a communication system that is not only used by *L. monocytogenes* but also by other species and might be therefore a target for eradicating bacteria from food processing premises. The exact involvement of the LuxS-system and the regulatory process of *agr* in biofilm formation remain unclear.

2.4.3 Flagella

L. monocytogenes can not only move around passively but also actively using flagella. A flagellum is composed of thousands of flagellin monomers encoded by the *flaA* gene. *L. monocytogenes* possesses four to six peritrichous flagella whose expression is temperature dependent. It is generally agreed that flagella are produced at 20°C to 25°C (Peel et al. 1988) and are not produced at 37°C, but it has been found that some laboratory-adapted strains and about 20% of clinical isolates have the ability to express flagella at 37°C (Way et al. 2004).

Although most of the *L. monocytogenes* strains do not express flagella at temperatures above 30°C *L. monocytogenes* is still able to attach to surfaces in temperatures greater than 30°C through passive attachment (Tresse et al. 2009), indicating that flagella are not necessary for biofilm formation. Although they are not necessary, they can still play a vital role in food-processing environments as adhesion at room temperature compared to 37°C is enhanced due to the presence of flagella (Vatanyoopaisarn et al. 2000).

Flagella synthesis is complex and regulated by at least five regulators including PrfA (positive regulatory factor A), which down regulates its motility at temperatures above 30°C (Lemon et al. 2007), FlaR (Flagellin regulator), MogR (motility gene repressor), which regulates temperature dependent expression of flagella (Gründling et al. 2004) and represses gene transcription of *flaA* at 37°C (Shen and Higgins 2006), DegU (degradation enzyme regulator) and GmaR (glycosyltransferase and motility antirepressor). DegU is a positive indirect activator of flagellum biosynthesis. It has been shown that the deletion of the *degU* gene led to decreased biofilm formation (Gueriri et al. 2008) and that the absence of flagella and motility (Knudsen et al. 2004). DegU activates *gmaR* expression and GmaR then activates *flaA* transcription and flagella synthesis through binding to MogR.

Vatanyoopaisarn et al. (2000) showed that initial attachment was greater for the wild-type strain lacking motility (using PBS as a low nutrient medium) but expressing flagella compared to a flagella mutant at 22°C, indicating that flagella function as adhesins. However, another study by Lemon et al. (2007) found that there was no difference in initial attachment for paralysed flagellated and non-flagellated strains in biofilm formation which suggests that flagella do not act as an adhesin in biofilm formation (Lemon et al. 2007). These results could be strengthened by a motile mutant lacking flagella glycosylation that formed the same amount of biofilm as the wild-type strain. Glycosylation is thought to change surface characteristics and therefore possibly attachment to surfaces. Consequently, it is claimed that motility is critical for initial surface attachment and biofilm formation, rather than the flagella itself (Lemon et al. 2007). Mutants with the loss of motility or flagella showed the same biofilm phenotype as the wild-type strain when motility was generated through centrifugation. These results support the theory that flagella act rather as a motility factor than an adhesin (Lemon et al. 2007).

Differences in the outcome of the studies might be due to the use of different media and strains and also different surfaces. Vatanyoopaisarn et al. (2000) used PBS and BHI as media and observed attachment on stainless steel coupons whereas Lemon et al. (2007) performed biofilm formation studies in Hsiang-Ning Tsai medium (HTM) and on microtitre polystyrene plates.

Another interesting finding is the formation of hyperbiofilms in flow conditions (Todhanakasem and Young 2008). Todhanakasem and Young (2008) defined hyperbiofilms as “an accumulation of surface attached cells that impede the flow of medium to the flow cell apparatus”. The attachment ability of mutants that lack motility and the wild-type strain in static as well as in flow systems were examined at ambient temperature. The initial attachment of the strains with a mutation in motility related genes was lower compared to the wild-type but the mutants colonised the surface faster than the wild-type strain and formed a microscopically visible hyperbiofilm, whereas in static conditions a decrease in biofilm formation was observed. These results suggest that flagellum based motility is not necessary for biofilm formation in flow systems, so might therefore not be an ideal target for the prevention of biofilm formation (Todhanakasem and Young 2008).

The DNA glycosylase AdlP (alkylbase DNA glycosylase-like protein) was recently found to be involved in biofilm formation. A deletion mutant showed reduced biofilm formation after three days compared with the wild-type. AdlP is furthermore involved in regulation of expression of flagellar motility genes and in stress resistance shown through treatment with H_2O_2 and methyl methanesulfonate (Zhang et al. 2016).

All in all, flagella have been shown to be beneficial for attachment in static conditions, but not in flow systems, so therefore consideration needs to be given to the validity of static biofilm assays in an industrial environment where the manufacturing or processing system requires flow.

2.4.4 Extracellular matrix

The biofilm matrix consists of exopolysaccharides, lipids, glycolipid, DNA and proteins, surfactants, membrane vesicles and ions (Karatan and Watnick 2009) and has a multifunctional role. It acts as a stabiliser, a nutrient deliverer and as a barrier against environmental damage.

Harmsen et al. (2010) discovered that extracellular DNA (eDNA) found in the extrapolymeric substance plays an important role in initial surface attachment and subsequent biofilm growth, which can be shown by the addition of DNase I: biofilm formation and growth in the early stage (0–24h) was inhibited to a far greater extent than in the later stages of biofilm formation (24–48h) (Harmsen et al. 2010). Purification and analysis of eDNA showed its chromosomal origin. eDNA is not alone responsible for initial attachment, only combined addition of culture supernatant and genomic DNA to an eDNA free culture could restore the attachment ability. Further investigation showed that adding high molecular weight DNA and supernatant to eDNA free cells resulted in adhesion and formation of biofilms, whereas the addition of low molecular weight DNA or high molecular weight DNA on its own could not restore adhesion ability. These findings indicate that another substance must be involved in biofilm formation. When N-acetylglucosamine (NAG) was added to eDNA free cells with genomic DNA, adhesion was observed to the same extent as the wild-type strain (Harmsen et al. 2010).

Another protein found to be involved in biofilm formation is Lmo1386, which encodes a putative DNA-translocase (Chang et al. 2013). A lmo1386 mutant showed reduced biofilm formation in static and flow systems. Not only was there less biofilm compared to the wild-type but the biofilm was also described as a flatter biofilm structure. A possible relationship between the extracellular DNA and the putative DNA-translocase could be rejected through studies showing no difference in eDNA production and biofilm reduction when DNAse I was applied to both the wild-type strain and the mutant. eDNA could be found in both strains. In addition, the mutant showed less adhesion ability than the wild-type, suggesting that Lmo1386 is involved in initial attachment (Chang et al. 2013).

Other components of the extracellular polymeric substance have been shown to be involved in biofilm formation. Lmo2504, a putative cell wall binding protein, was found in high numbers in

biofilm exoproteomes when the composition of exoproteomes in planktonic and biofilm cells were compared (Lourenco et al. 2013). The *lmo2504* mutant showed reduced biofilm-forming ability.

BapL is a putative cell wall anchored protein with the LPXTG cell wall anchor domain. It has been found to be involved in surface attachment but it is not necessary for biofilm formation (Jordan et al. 2008). Strains were found that can form biofilms without having the gene encoding for BapL. A *bapL* mutant showed reduced attachment to surfaces compared to the wild-type. Consequently, there are two different methods of attachment to surfaces: one BapL dependent and one BapL independent (Jordan et al. 2008).

Extracellular polymeric substances seem to be involved in initial attachment rather than in maturation of biofilms and the production of extracellular polymeric carbohydrates may increase the ability to form a biofilm (Chae et al. 2006). The exact mechanism of eDNA release remains unclear and needs to be further investigated. It is suggested that eDNA is derived from autolysis or active secretion as both mechanisms have been shown for other bacteria species (Qin et al. 2007, Whitchurch et al. 2002).

2.4.5 Virulence factors

prfA is a transcriptional virulence factor which not only regulates genes responsible for *L. monocytogenes'* virulence but it also seems to be positively involved in biofilm formation. A mutation in the *prfA* gene led to decreased biofilm formation (Salazar et al. 2013). Recent studies focused on the regulatory influence of PrfA in biofilm formation (Lemon et al. 2010, Luo et al. 2013). Lemon et al. (2010) showed that the *prfA* mutant could attach to the surface as well as the wild-type, but exhibited decreased biofilm formation, indicating that *prfA* is involved in biofilm formation and not in initial attachment (Lemon et al. 2010). Furthermore, double mutants of genes regulated by PrfA lacking *inlA* and *inlB* (Internalin A and Internalin B) or *plcA* and *plcB* (phosphatidylinositol-specific phospholipase C and phosphatidylcholine-specific phospholipase C) did not reveal the same defect in biofilm formation as the *prfA* mutant, suggesting that none of them alone is the *prfA* dependent effector of biofilm formation (Lemon et al. 2010).

Luo et al. (2013) compared the *prfA* mutant with the parent strain through whole genome microarray sequencing and they observed a total of 185 genes that are associated with PrfA and biofilm formation (Luo et al. 2013). The *prfA* gene was not differentially expressed in the biofilm

cells compared to planktonic cells, but the gene knockout resulted in decreased biofilm formation, which suggests that PrfA regulates biofilm formation indirectly (Luo et al. 2013).

Further studies revealed that PrfA plays a vital role in biofilm formation by *L. monocytogenes* but not by *L. innocua* (Zhou et al. 2011). An insertion of the *prfA* gene into *L. innocua* showed no difference in biofilm formation (Zhou et al. 2011). This supports the theory that *prfA* is not directly involved in biofilm formation.

Other virulence factors that were investigated regarding biofilm formation are InlA and B (Chen et al. 2008). Mutants were created to prove that Internalin A and B are involved in initial attachment for biofilm formation. The double mutant (mutation in the *inlA* and *inlB* genes) showed the lowest amount of biofilm compared to the wild-type strain on a glass surface and inhibited initial attachment to a greater extent than the single mutants, which suggests that both InlA and B are participating in initial attachment (Chen et al. 2008).

Those findings suggest that PrfA and InlA and B, like DegU, are not only acting as virulence factors but are also important for biofilm formation, indicating that there is a possible connection between biofilm-forming ability and virulence, which might therefore be a target of further investigations on the prevention of biofilm formation.

2.4.6 Stress response

When cells are exposed to oxidative stress, a cascade of mechanisms is activated to prevent the cell from damage. In *L. monocytogenes,* there are two systems involved in heat-shock response (van der Veen et al. 2007). The class I heat-shock system is regulated by the autoregulatory repressor HrcA, which regulates genes encoding chaperones. The class III heat-shock response is responsible for encoding chaperones and ATP-dependent Clp proteases.

Van der Veen et al. (2007) investigated the heat-shock response of *L. monocytogenes* through whole-genome expression profiles of cells and showed that the heat-shock response and the SOS-response are activated after exposure to high temperature. The *hrcA* gene encodes a transcriptional regulator of the class I heat-shock response, an autoregulatory repressor, which regulates 56 genes, among those its regulon member *dnaK*. The *dnaK* gene encodes a class I heat-shock response chaperone protein, which is important for correct protein folding.

Promoter reporter studies showed that *dnaK* and *hrcA* were activated in continuous flow biofilms (van der Veen and Abee 2010b). In-frame deletion mutant studies showed no difference in growth for the mutants in a planktonic state, but increased biofilm formation for

the *hrcA* mutant (through increased expression of *dnaK*) and a decreased ability to form biofilms for the *dnaK* mutant in static conditions. Moreover, it was found that biofilm formation was reduced in a continuous flow environment for both mutants. Those results showed that *dnaK* as well as *hrcA* are important for biofilm formation. Surprisingly, biofilm formation was reduced for the *hrcA* mutant in continuous flow conditions. Van der Veen and Abee (2010b) suggest a possible connection to the SOS-response. The *hrcA* mutant might prevent the activation of the SOS-response through an unknown mechanism (van der Veen and Abee 2010b).

Single-stranded DNA which usually occurs as a result of DNA damage through stress exposure, like heat, and increased presence of ROS (reactive oxygen species), activates RecA which in turn activates the SOS-response (van der Veen et al. 2010). The SOS-response is a system which induces DNA repair and mutagenesis and is regulated by RecA (recombinase A), a DNA-repair protein, and LexA, a repressor. RecA activates YneA, a cell division factor, which triggers cell elongation in continuous flow biofilms to form a network of knitted chains (van der Veen and Abee 2010a, van der Veen et al. 2010). Comparison of the wild-type strain and the *yneA* mutant revealed that the *yneA* mutant could not change cell morphology (elongation of cells) after exposure to stress, confirming the involvement of YneA for cell elongation in biofilms formed in continuous flow systems. Biofilms grown in continuous flow systems exhibited enhanced radical formation and an increase in RecA, but when grown in the presence of radical inhibitors, RecA was not activated which in turn did not activate YneA and hence the formation of knitted-chains composed of elongated cells was not observed (van der Veen and Abee 2011a). Deletion mutant studies could prove the necessity of the presence of radicals and activation of RecA for the induced generation of genetic variants in continuous flow systems (van der Veen and Abee 2011a). Moreover, generation of variants is associated with the formation of persistent strains. Strains that are repeatedly found in a specific environment over a specified period of time are often referred to as persistent strains (Schmitz-Esser et al. 2015).

Suo et al. (2012) found a correlation between a putative ABC transporter permease (lm.G_1771) and SOD (superoxide dismutase) expression. SOD is an oxidative stress response gene which scavenges reactive oxygen species to prevent damage to the cell. lm.G_1771 negatively regulates biofilm formation through the expression of SOD (Zhu et al. 2008, Zhu et al. 2011). The lm.G_1771 mutant exhibits an upregulation of *sod* expression. Proteomic and genomic studies revealed differential expression of genes encoding for cell surface proteins for the mutant which might indicate that lm.G_1771 is involved in a novel signal transduction pathway regulating expression of cell wall surface proteins. It is hypothesised that biofilm formation or

increased oxygen supply causes oxidative stress to cells and therefore induces expression of *sod* (Suo et al. 2012). SOD may be involved in biofilm formation but the exact mechanism is unknown.

The class II general stress response is regulated by SigB (RNA polymerase sigma factor SigB) and the genes of this response encode general stress proteins. The SigB is a major transcriptional regulator of stress response genes in both, static and continuous flow biofilms. SigB is activated in static biofilms as well as in continuous flow biofilms, but more than double in the latter (van der Veen and Abee 2010c). The mutation of *sigB* results in poor biofilm formation. The theory is that *L. monocytogenes* experiences stress during biofilm formation and therefore activates its stress response and *sigB* expression. SigB is highly present in biofilms, but to a greater extent in continuous flow systems, which supports the hypothesis that *L. monocytogenes*, being facultatively anaerobic, is exposed to higher stress in a continuous flow biofilm than in a static biofilm, through exposure to higher shear stress and increased oxygen availability. SigB is clearly involved in biofilm formation but is not essential for initial attachment of *L. monocytogenes* (Schwab et al. 2005).

Another study examined the involvement of *gltB* and *gltC* (Huang et al. 2013). The mutants with insertions in the *gltB* and *gltC* genes show a reduced ability to form biofilms and reduced oxidative stress tolerance. *gltC*, known as a LysR family member, encodes a positive transcriptional regulator of *gltB* (involved in encoding a glutamate synthase). The mutation in either gene led to different transcription after stress induction of the other gene, indicating that a positive mutual regulation might be present (Huang et al. 2013). Initial tests showed a decreased attachment ability of the mutants in high flow systems and to a lesser extent in low flow systems, indicating that the deletion of those genes led to altered surface characteristics of the mutants. No difference in cell motility or growth between the mutants and the wild-type could be observed, suggesting that altered surface characteristics are unrelated to motility. The mechanism by which *gltC* and *gltB* are involved in the attachment of *L. monocytogenes* to surfaces is unknown.

Through screening a library of generated mutants, two strains were found with an altered ability to form biofilm (Taylor et al. 2002). Insertions were identified in the *relA* and *hpt* genes. *relA* encodes guanosine pentaphosphate synthetase which catalyses the formation of (p)ppGpp, and *hpt* encodes a hypoxanthin-guanine phosphoribosyl-transferase (Hgprt) which converts the purine base (guanine) into its corresponding nucleotide (GMP) and is involved in the purine salvage pathway (Taylor et al. 2002). Both generated mutants could attach to surfaces but could

not grow and mature as a biofilm in comparison to the wild-type, however, planktonic cultures showed no differences in growth compared to the wild-type. In contrast to the wild-type strain, the mutants could not accumulate (p)ppGpp after nutritional starvation nor activate the stringent response. The stringent response is a stress response activated through different stress factors such as heat and amino acid starvation. Therefore, (p)ppGpp might be important for biofilm formation. Interestingly the *hpt* mutant could not accumulate (p)ppGpp and not grow after adhesion, which indicates that a functional Hgprt is important for the synthesis of (p)ppGpp. A possible explanation is that the RelA protein has a high K_m and Hgprt is present to maintain that K_m to ensure a functional RelA activity. This theory is supported by the fact that Hgprt is negatively regulated by (p)ppGpp. The role of (p)ppGpp in biofilm formation has yet to be determined.

In another study, a mutation in *lmG1497*, resulted in decreased biofilm formation (Huang et al. 2012). This gene has been identified as a MerR family transcriptional regulator. MerR family regulator genes are expressed after activation through exposure to oxidative stress, heavy metals or antibiotics. The biggest difference in biofilm formation could be observed on day 3. The differences of biofilm growth on days 1 and 2 between the wild-type and the mutants were minimal, suggesting that the MerR family regulator is involved in maturation rather than initial attachment. The hypothesis is that in the later stages of biofilm formation, oxidative stress increases and therefore the decrease in biofilm formation might be a result of the mutants' inability to activate stress response genes due to the absence of the MerR family regulator (Huang et al. 2012).

Lmo0753, a putative Crp (Cyclic AMP receptor protein)/Fnr (fumarate nitrate reductase regulator) family transcription factor was found to be involved in biofilm formation (Salazar et al. 2013). Those transcription factors are associated with environmental stress (oxygen, nutrient-availability and extreme temperatures). A mutation in that gene led to decreased biofilm formation.

The activation of the oxidative stress response and SOS-response during biofilm formation is an interesting finding. The SOS-response is usually activated when damage occurs to the DNA. Cells experience stress not only when they are exposed to heat or oxidative agents, but also during biofilm formation which results in the upregulation of antioxidative genes and change in cell structure. At this stage, it cannot be clarified whether biofilm formation is a result of enhanced stress or if biofilm formation is causing stress or whether it is a combination of both. However,

it is clear that genes involved in *L. monocytogenes'* stress response are involved in biofilm formation.

2.4.7 Other molecular determinants

LbrA (*Listeria* biofilm regulator A) mutations disrupt biofilm formation (Wassinger et al. 2013). Less biofilm with collapsed secondary structure was observed for the mutant, whereas the complemented strain could restore a honeycomb structure. LbrA is thought to act as a regulator rather than being a direct factor for biofilm formation as the microarray comparison between mutant and parent strain revealed 304 differentially expressed genes.

Other determinants found to be involved in biofilm formation are the *dltABCD* operon, which is responsible for D-alanylation of lipoteichoic acids) and the *phoPR* regulon (phosphate-sensing PhoPR two-component system). Both mutants exhibited a thinner biofilm phenotype than the wild-type (Alonso et al. 2014).

All in all, there are several genes involved in biofilm formation and there may be more genes involved in biofilm formation than those already described. There is still lack on clarity of the mechanism of biofilm formation.

2.4.8 Co-cultures

Biofilms occur in the environment in mono-cultural, bi-cultural and most commonly multi-cultural forms. Several studies have investigated the influence of other microorganisms on the *L. monocytogenes'* capability to form biofilms.

Rieu et al. (2008b) showed that the population of *L. monocytogenes* was not influenced by *Staphylococcus aureus* strains, except for one. When grown together on stainless steel with that specific strain, this enhanced the settlement of *L. monocytogenes*. In seafood-processing plants in Iceland the predominant Gram-negative genus that attached to steel coupons placed near food contact surfaces was *Pseudomonas* spp for shrimp-processing plants and various genera of Enterobacteriaceae for fish-processing plants (Gudbjörnsdóttir et al. 2005). While examining the microflora found in those seafood-processing plants, it has been found that a mixture of *Pseudomonas* spp increased colonisation of the surface by *L. monocytogenes* on stainless steel compared to other bacteria species. This finding is supported by results of other studies which showed that *Pseudomonas fragi* and *Flavobacterium* spp increased colonisation of surfaces by *L. monocytogenes* on glass and stainless steel, respectively (Bremer et al. 2001, Sashara and Zottola 1993,). But on the contrary, it has been found that *L. monocytogenes* forms less biofilm

on stainless steel when *Pseudomonas fragi* and *Staphylococcus* spp are present (Norwood and Gilmour 2001). The contrary results might be due to the difference in serotype of the *L. monocytogenes* strain (3a versus 1/2a and 4b). Other studies revealed the influence of *Staphylococcus sciuri* and *L. innocua* on biofilm formation. *Staphylococcus sciuri* decreased biofilm formation of *L. monocytogenes* in three tested media on stainless steel (Leriche and Carpentier 2000) and *L. innocua* decreased initial attachment of *L. monocytogenes* on stainless steel as well as aluminium surfaces (Koo et al. 2014). Lactic acid bacteria were also found to influence biofilm formation (Metselaar et al. 2015). A *Lactococcus lactis* strain was found to decrease the adhesion of *L. monocytogenes* (Habimana et al. 2009). In another study, three lactic acid bacteria (LAB) strains were investigated for their ability to interfere with *L. monocyotgenes* adhesion on stainless steel surfaces (Ndahetuye et al. 2012). The influence of a multi-strain mixture was examined in three different scenarios: (1) Mixture of the LAB strains was added to the coupons 24h before the addition of *L. monocytogenes*; (2) both, the LAB-mixture and *L. monocytogenes* were added at the same time and (3) *L. monocytogenes* was added 24h before the LAB cocktail. The results showed that LAB reduces the biofilm formation of *L. monocytogenes* in all three scenarios. This indicates that the presence alone of LAB regardless of addition time is able to influence biofilm formation. The authors suggest that the reduction in biofilm formation might be due to bacteriocins which are known to be produced by LAB, surface coating through LAB, direct competition between *L. monocytogenes* and LAB or even a combination of all of them.

The influence of 29 different strains of bacteria from food processing plants on *L. monocytogenes'* ability to form biofilm has been examined and 16 strains decreased biofilm formation of *L. monocytogenes*, while four strains led to an increase (Carpentier and Chassaing 2004). No correlation between the quantity of exopolysaccharides produced by the examined strains and biofilm formation of *L. monocytogenes* could be found. The presence of other strains also influenced the biofilm structure of *L. monocytogenes*. The background flora can have a strong influence on the biofilm-forming ability of *L. monocytogenes*. However, only mono-culture and binary culture biofilms were examined and it is more likely to find more than two species in many food-processing environments (Carpentier and Chassaing 2004).

So far most of the studies regarding biofilm formation have focused on particular species, but only a few publications are available on the microflora of food-processing plants and the influence on biofilm growth and the interaction between different species. It is highly unlikely that *L. monocytogenes* occurs as a single species culture in food-processing plants. It is therefore

important to look closer at other microorganisms found in those plants and gain a better understanding of interactions between different species. A study performed in 2003 looked closer at the microflora in processing equipment in different fish industries (Bagge-Ravn et al. 2003). This study might be useful for further studies regarding the interaction between *L. monocytogenes* and other microorganisms. Interestingly the number of detected *Listeria* spp*/Kurthia* spp (no differentiation between those two) increased after cleaning and disinfection, which could be an indication of *L. monocytogenes'* poor ability to compete with other microorganisms for nutrients. This is line with the findings of Carlin et al. (1996) and Cosentino and Palmas (1997) who found that the population of *L. monocytogenes* increased when disinfectant reduced the background microflora and that no *Listeria* and *Salmonella* could be isolated in small dairies with poor hygienic conditions.

Although there are some contradictory findings, it is clearly stated that co-cultures influence biofilm formation. Possible reasons for the influence of other microorganisms on *L. monocytogenes* biofilm formation might be competition for nutrients, bacteriocin production or a difference in electronegativity and therefore stronger surface-bacteria interaction.

2.4.9 Influence of surface properties and nutrient availability

Bacterial attachment to a surface depends not only on the bacteria's motility but also on the interaction between the surface and bacteria, thus it is obvious that the surface properties influence attachment. The impact of hydrophobicity (of surface and bacteria), roughness of surface, nutrient availability, shear stress and also origin and serovars to biofilm formation have been investigated in recent years, showing clear but sometimes also contradictory findings.

Many different surfaces can be found in food-processing plants, which can act as possible attachment sites for *L. monocytogenes.* These include stainless steel and less frequently glass (hydrophilic) and also polystyrene (hydrophobic), PVC, PTFE, PP, aluminium, Teflon, and rubber (Møretrø and Langsrud 2004). The influence of those surfaces on biofilm formation was investigated and it was found that *L. monocytogenes* generally tends to adhere best to glass and stainless steel (Bonsaglia et al. 2014, Di Bonaventura et al. 2008, Takahashi et al. 2010), but is also capable of adhering to other surfaces. Some studies report a link between hydrophobicity and adhesion but other studies show no correlation between surface hydrophobicity and initial adhesion (Silva et al. 2008, Szlavik et al. 2012).

Nutrient-availability was investigated in order to show its influence on biofilm formation. Kadam et al. (2013) performed studies to examine biofilm growth in nutrient-rich and nutrient-

deficient media at different temperatures. Better growth was observed in a nutrient-poor medium and at increasing temperature (temperatures tested ranged from 12°C–37°C) (Kadam et al. 2013), which is in line with findings of other studies: Todhanakasem and Young (2008), Pilchova et al. (2014) and Djordjevic et al. (2002) found that *L. monocytogenes* grown in modified Welshimer's broth (MWB) formed good biofilms (Djordjevic et al. 2002, Pilchova et al. 2014, Todhanakasem and Young 2008) and Luo et al. (2013) observed that greater biofilm mass was produced in minimal essential medium (MEM) compared to BHI (Luo et al. 2013). But Kadam et al. (2013) found that the influence of temperature was more significant than the medium. Biofilm formation decreased with decreasing temperature: the least amount of biofilm was formed at 12°C and the highest at 37°C.

Although biofilms are preferably formed in nutrient-deficient medium (Combrouse et al. 2013, Djordjevic et al. 2002, Ochiai et al. 2014, Pilchova et al. 2014, Todhanakasem and Young 2008), some strains perform better in nutrient-rich conditions (Folsom et al. 2006). By dividing *L. monocytogenes* into serotypes, the former study observed that serotype 4b (lineage I) forms better biofilms than serotype 1/2a in high-nutrient medium (TSB), but in low nutrient medium (1:10 dilution of TSB) serovar 1/2a strains are better biofilm formers (Folsom et al. 2006). In another study it was shown that serovars 1/2a and 1/2b formed more biofilm than 4b in nutrient-rich medium, but in nutrient-deficient medium 1/2b formed more than 1/2a and 4b (Kadam et al. 2013). Regardless of the contradictory finding, other studies showed that strains of serovar 1/2a form better biofilms with higher density than 4b strains (Pan et al. 2010) and strong biofilm formation was observed for serovar 1/2a and serovar 1/2b isolates but not for 4b (Doijad et al. 2015). It is suggested that this might be the reason why strains of serovar 1/2a are isolated from food-processing plants more often than other strains (Orsi et al. 2011, Pan 2009, Pan et al. 2009, Pan et al. 2010), whereas serovar 4b (lineage I) was found to be more associated with clinical cases (Pan 2009, Pan et al. 2009, Pan et al. 2010).

2.4.10 Influence of strain characteristics and persistence

Some studies have focused on the correlation between lineage and biofilm formation. Djordjevic et al. (2002) found that strains belonging to lineage I form better biofilms than lineage II, whereas other studies showed enhanced biofilm formation for lineage II strains (Borucki et al. 2003, Combrouse et al. 2013, Djordjevic et al. 2002, Nilsson et al. 2011).

Persistence has also been considered in reference to biofilm formation. Persistent strains are strains that are repeatedly found in a food-processing plant over a defined timeframe, whereas

sporadic strains are only occasionally found. Borucki et al. (2003), who not only found enhanced ability to form biofilms for lineage II strains, showed that persistent strains form better biofilms than sporadic strains (Borucki et al. 2003). It was shown that persistent strains attach in higher numbers to food contact surfaces than non-persistent strains in the first few hours of biofilm formation, but no difference could be observed after 72 h (Lunden et al. 2000). On the other hand, it has also been shown that known persisting strains did not adhere better than non-persistent ones (Szlavik et al. 2012). It has been suggested that persistent strains in one plant might be sporadic in another because no specific lineage for persistent strains could be found through PFGE (pulsed-field gel electrophoresis) and AFLP (amplified fragment length polymorphism) and are therefore independent of genotype but rather dependent on environmental factors and adaptation (Autio et al. 2003). This is line with findings of Ochiai et al. (2014) who showed that there was no difference in biofilm growth for persistent and non-persistent strains at 30°C (Ochiai et al. 2014), however, the persistent strain formed significantly more biofilm at 37°C which is possibly due to its greater ability to adapt to environmental conditions. Another study showed higher resistance for persistent strains to benzalkonium chloride in a fish-processing plant compared to sporadic strains which supports the hypothesis of higher adaptability of persistent strains (Nakamura et al. 2013). It has even been stated that *L. monocytogenes* does not have any special ability to persist in the food industry environment, and its occurrence and "persistence" in the food industry is simply because of the inability to clean and sanitise the niche and harbourage sites properly and the bacteria's ability to grow at low temperatures (Carpentier and Cerf 2011).

Epidemic strains (frequently involved in outbreaks) attach in higher numbers to surfaces than sporadic strains (occasionally associated with listeriosis) and cell numbers in biofilms after 24 h are different for epidemic strains and sporadic strains, suggesting that there are differences between sporadic and epidemic strains (Chae et al. 2006).

Some researchers suggest a correlation between strain origin and variability in biofilm formation (Nilsson et al. 2011), whereas others found no correlation between the origin of the strain and biofilm formation (Di Bonaventura et al. 2008, Kadam et al. 2013). Strains used for studies originate from several sources, including environmental, clinical, human and bovine sources.

However, all these different findings about correlation between origin of strain, lineage, serovars or persistent or non-persistent strains of *L. monocytogenes* and biofilm formation, might be due to the fact that different methods and systems were used in these studies and *L.*

monocytogenes was cultivated in different media at different temperatures. What is clear is that *L. monocytogenes*' initial adhesion and biofilm formation strongly depend on environmental factors. Although there are some contradictory findings they all concluded that biofilm formation is a complex process and *L. monocytogenes* a highly adaptable bacterium. These adaptions enable *L. monocytogenes* to form biofilms that protect it from damage which in turn makes it harder to eradicate. *L. monocytogenes* can persist in food-processing plants and (re)contaminate food through detachment from the biofilm colonising the surface. To examine the behaviour of *L. monocytogenes* in the food industry, study conditions need to be carefully selected to simulate conditions from food-processing plants.

2.4.11 Biofilm control

Biofilms are a community of irreversibly attached cells producing extracellular matrix which protects the cells from damage and removal. In food-processing plants biofilms pose a risk for (re)contamination of food and therefore their formation needs to be prevented or/and removal needs to be ensured by regular cleaning and disinfecting. Cleaning is a procedure to remove food residues and/or other substances from surfaces in food-processing plants to avoid possible interference with disinfectant treatment. High temperature and physical force are used to enhance cleaning. Ideally, the cleaning process should break up EPS so that disinfectants can reach the bacteria. Cleaning may also remove bacterial cells from the surface, which can result in recontamination of other surfaces, therefore disinfectants need to be applied in order to kill bacterial cells. Moreover, the removal of biofilms could leave cell surface fragments and extracellular substances behind, which might attract bacteria of the same species or another species to settle down and mature as a biofilm.

Disinfection treatments can either be carried out to prevent attachment or to alter the biofilm that has already established (Meyer 2003). Prevention of attachment can be achieved through regular disinfecting or through surface alterations. Sub-lethal doses of disinfectants and/or sanitisers might encourage biofilm formation by creating resistance/tolerance (Møretrø et al. 2017). Sources for *L. monocytogenes* contamination into food-processing plants can be raw product, water as well as the general environment, due to its ubiquitous nature (Farber and Peterkin 1991).

Disinfectants used in industrial environments are sodium hypochlorite, peracetic acid, hydrogen peroxide, lactic acid, quaternary ammonium compounds, ozone and also so called biological controls such as enzymes and phages (da Silva and De Martinis 2013, Srey et al. 2013).

A variety of enzymes are used to degrade the EPS of biofilms. These enzymes can be divided into three classes: proteases, deoxyribonucleases and glycoside hydrolases (Fleming and Rumbaugh 2017). Proteases such as pronase and proteinase K have been shown to influence biofilm formation of *L. monocytogenes* (Rodriguez-Lopez et al. 2017, Nguyen and Burrows 2014). DnaseI, a deoxyribonuclease, influenced biofilm formation in *L. monocytogenes* (Rodriguez-Lopez et al. 2017, Nguyen and Burrows 2014). Cellulase, a glycoside hydrolase, degraded biofilms formed by *L. monocytogenes* (Rodriguez-Lopez et al. 2017). Although different species and serovars produce different amounts of EPS, the use of enzymes has potential to remove biofilm from all of these (Rodriguez-Lopez et al. 2017).

Phages seem to be an effective tool to control biofilm of *L. monocytogenes* (Guenther et al. 2009). They have been shown to be effective in the decontamination of fish coated coupons contaminated with *L. monocytogenes*, as would likely occur in seafood-processing plants (Ganegama Arachchi et al. 2013).

Another option to control biofilm formation of *L. monocytogenes* might be the use of plant-derived essential oils as an antilisterial effect has been shown for thyme essential oil (Abdollahzadeh et al. 2014) and other plant extracts (Nostro et al. 2016).

Another possibility which has been investigated is controlling biofilm formation by using competitive exclusion (Gomez et al. 2016, Guerrieri et al. 2009, Koo et al. 2014, Ndahetuye et al. 2012). Non-pathogenic bacteria could either be used as surface coating agent or applied with the water to actively compete with *L. monocytogenes*, but these need to be carefully selected as some strains could enhance resistance to a subsequent disinfection procedure (van der Veen and Abee 2011b). Bacteriocin-producing bacteria can be used to prevent biofilm formation or to remove existing biofilms (Bolocan et al. 2017, Perez-Ibarreche et al. 2016) as they have been shown to remove biofilms of *L. monocytogenes*. Subtilosin, an antimicrobial peptide, could successfully remove biofilm formed by *L. monocytogenes*, *Escherichia coli* and other microorganisms (Algburi et al. 2016).

As stated previously, quorum sensing is a communication system between bacteria based on chemical signal molecules (Waters and Bassler 2005) involved in biofilm formation. By inhibiting communication between bacterial cells, biofilm formation can be hindered (Algburi et al. 2016). As biofilms in food-processing plants may consist of more than one species, quorum sensing should be considered as a possible target for multi-species biofilm eradication.

Using the right sanitiser in the right concentration and varying between different sanitisers prevents the development of populations of bacteria resistant to a particular sanitiser. The efficacy of a sanitising system not only depends on the sanitising agent itself but on the influence of environmental conditions, such as the surface, presence of other microorganisms, organic material and the properties of the biofilm, like thickness and age. As biofilm formation is time dependent, disinfection of processing plant should be carried out as soon as possible after production on a regular basis.

2.4.12 Biofilm measurement

A variety of methods are used to measure biofilm formation of bacterial cells. Microplate assays are the most popular to assess the biofilm forming ability of large numbers of strains (O'Toole 2011). Commonly used stains to visualize the biofilm formed in the plates are crystal violet, congo red and 2,3,5-Triphenyltetrazolium chloride (TTC) (Brown et al. 2014) , which bind to the whole biofilm mass present. The biofilm produced is then quantified by destaining and subsequent optical density measurements. Microplate assays are a fast and economic way of measuring biofilm mass and therefore the first choice in most research labs.

Another method of measuring biofilm formation is the enumeration of viable cells by detaching biofilms from their surface and plating onto agar. This method only measures living cells and cannot measure dead cells or extracellular polymeric substances, which are both known to be major/important components of biofilms. However, used in combination with other methods it serves as a valuable tool providing additional information.

Advances in microscopy now allow the visualisation of biofilms and their individual components in three dimensions using confocal laser scanning microscopy (CLSM) after staining with fluorescent dyes (Franklin et al. 2015). A commonly used dye is the LIVE/DEAD stain which distinguishes between living (stained green by SYTO9) and dead cells (coloured in red by propidium iodide). Fluorescent probes can also visualize extracellular DNA (propidium iodide, DDAO) or polysaccharide components (Calcofluor). Mathematical programmes, such as COMSTAT (Heydorn et al. 2000, Vorregaard 2008), which uses the ImageJ platform, can then be used to calculate biofilm mass from the confocal images. The main advantage of confocal analysis is the ability to measure the thickness of the biofilm and to produce three dimensional images of the biofilm structure.

Other biofilm imaging techniques include scanning electron microscopy (SEM) and atomic force microscopy (AFM) which enable high resolution imaging (da Silva and De Martinis 2013).

There is a variety of methods available to measure biofilm formation with both, advantages and disadvantages. It is therefore important to choose the best method depending on the purpose. In this thesis, a variety of biofilm formation assays were used: the crystal violet assay to test large sets of strains for their phenotypic diversity and to screen a large transposon library, cell enumeration to identify correlations between viable cells and whole biofilm mass and SEM and CLSM to evaluate the three dimensional structure.

2.5 Conclusion

As outlined in this review, biofilm formation depends on environmental factors and differs between strains. Some strains express genes that are involved in biofilm formation, but are not essential, since strains without those genes are still able to grow in a sessile form.

In order to identify traits that enable *L. monocytogenes* to persist in food-processing plants, genomic and phenotyping analyses (including biofilm assays, growth studies, motility and autolysis assays, DNA sequencing and bioinformatic analyses) are needed.

It is important to identify genes that are associated with biofilm formation and persistence of *L. monocytogenes* and then understand the environmental factors that influence gene expression in order to develop new ways to prevent biofilm formation in food-processing plants. The continued success of the seafood industry to New Zealand's economy depends upon ensuring food safety through effective control of biofilms of pathogenic bacteria such as *L. monocytogenes.*

2.6 References

Abdollahzadeh, E., M. Rezaei and H. Hosseini (2014). Antibacterial activity of plant essential oils and extracts: The role of thyme essential oil, nisin, and their combination to control Listeria monocytogenes inoculated in minced fish meat. Food Control **35**(1): 177-183.

Aquaculture New Zealand (n.d.). Overview. Retrieved 16 January 2017 from http://www.aquaculture.org.nz/industry/overview/.

Algburi, A., S. Zehm, V. Netrebov, A. B. Bren, V. Chistyakov and M. L. Chikindas (2016). Subtilosin prevents biofilm formation by inhibiting bacterial quorum sensing. Probiotics Antimicrob Proteins.

Alonso, A. N., K. J. Perry, J. M. Regeimbal, P. M. Regan and D. E. Higgins (2014). Identification of *Listeria monocytogenes* determinants required for biofilm formation. PLoS One **9**(12): e113696.

Autio, T., S. Hielm, M. Miettinen, A. M. Sjoberg, K. Aarnisalo, J. Bjorkroth, T. Mattila-Sandholm and H. Korkeala (1999). Sources of *Listeria monocytogenes* contamination in a cold-smoked rainbow trout processing plant detected by pulsed-field gel electrophoresis typing. Appl Environ Microbiol **65**(1): 150-155.

Autio, T., R. Keto-Timonen, J. Lunden, J. Bjorkroth and H. Korkeala (2003). Characterisation of persistent and sporadic *Listeria monocytogenes* strains by pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). Syst Appl Microbiol **26**(4): 539-545.

Bagge-Ravn, D., Y. Ng, M. Hjelm, J. N. Christiansen, C. Johansen and L. Gram (2003). The microbial ecology of processing equipment in different fish industries-analysis of the microflora during processing and following cleaning and disinfection. Int J Food Microbiol **87**(3): 239-250.

Bolocan, A. S., V. Pennone, P. M. O'Connor, A. Coffey, A. I. Nicolau, O. McAuliffe and K. Jordan (2017). Inhibition of *Listeria monocytogenes* biofilms by bacteriocin-producing bacteria isolated from mushroom substrate. J Appl Microbiol **122**(1): 279-293.

Bonsaglia, E. C. R., N. C. C. Silva, A. Fernades, J. P. Araujo, M. H. Tsunemi and V. L. M. Rall (2014). Production of biofilm by *Listeria monocytogenes* in different materials and temperatures. Food Control **35**(1): 386-391.

Borucki, M. K., J. D. Peppin, D. White, F. Loge and D. R. Call (2003). Variation in biofilm formation among strains of *Listeria monocytogenes*. Appl Environ Microbiol **69**(12): 7336-7342.

Bremer, P. J., I. Monk and C. M. Osborne (2001). Survival of *Listeria monocytogenes* attached to stainless steel surfaces in the presence or absence of Flavobacterium spp. J Food Prot **64**(9): 1369-1376.

Brett, M. S., P. Short and J. McLauchlin (1998). A small outbreak of listeriosis associated with smoked mussels. Int J Food Microbiol **43**(3): 223-229.

Brown, H. L., M. Reuter, L. J. Salt, K. L. Cross, R. P. Betts, A. H. van Vliet (2014). Chicken juice enhances surface attachment and biofilm formation of *Campylobacter jejuni*. Appl Environ Microbiol **80**(22), 7053-7060.

Carlin, F., C. NguyenThe and C. E. Morris (1996). Influence of background microflora on *Listeria monocytogenes* on minimally processed fresh broad-leaved endive (Cichorium endivia var latifolia). J Food Prot **59**(7): 698-703.

Carpentier, B. and O. Cerf (2011). Review-Persistence of *Listeria monocytogenes* in food industry equipment and premises. Int J Food Microbiol **145**(1): 1-8.

Carpentier, B. and D. Chassaing (2004). Interactions in biofilms between *Listeria monocytogenes* and resident microorganisms from food industry premises. Int J Food Microbiol **97**(2): 111-122.

Chae, M. S., H. Schraft, L. Truelstrup Hansen and R. Mackereth (2006). Effects of physicochemical surface characteristics of *Listeria monocytogenes* strains on attachment to glass. Food Microbiol **23**(3): 250-259.

Challan Belval, S., L. Gal, S. Margiewes, D. Garmyn, P. Piveteau and J. Guzzo (2006). Assessment of the roles of LuxS, S-ribosyl homocysteine, and autoinducer 2 in cell attachment during biofilm formation by *Listeria monocytogenes* EGD-e. Appl Environ Microbiol **72**(4): 2644-2650.

Chang, Y., W. Gu, F. Zhang and L. McLandsborough (2013). Disruption of lmo1386, a putative DNA translocase gene, affects biofilm formation of *Listeria monocytogenes* on abiotic surfaces. Int J Food Microbiol **161**(3): 158-163.

Chen, B. Y., T. J. Kim, Y. S. Jung and J. L. Silva (2008). Attachment strength of *Listeria monocytogenes* and its internalin-negative mutants. Food Biophysics **3**(3): 329-332.

Combrouse, T., I. Sadovskaya, C. Faille, O. Kol, Y. Guerardel and G. Midelet-Bourdin (2013). Quantification of the extracellular matrix of the *Listeria monocytogenes* biofilms of different phylogenic lineages with optimization of culture conditions. J Appl Microbiol **114**(4): 1120-1131.

Cosentino, S. and F. Palmas (1997). Hygienic conditions and microbial contamination in six ewe's-milk-processing plants in Sardinia, Italy. J Food Prot **60**(3): 283-287.

da Silva, E. P. and E. C. De Martinis (2013). Current knowledge and perspectives on biofilm formation: the case of *Listeria monocytogenes*. Appl Microbiol Biotechnol **97**(3): 957-968.

de las Heras, A., R. J. Cain, M. K. Bielecka and J. A. Vazquez-Boland (2011). Regulation of *Listeria* virulence: PrfA master and commander. Curr Opin Microbiol **14**(2): 118-127.

Di Bonaventura, G., R. Piccolomini, D. Paludi, V. D'Orio, A. Vergara, M. Conter and A. Ianieri (2008). Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: relationship with motility and cell surface hydrophobicity. J Appl Microbiol **104**(6): 1552-1561.

Djordjevic, D., M. Wiedmann and L. A. McLandsborough (2002). Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl Environ Microbiol **68**(6): 2950-2958.

Doijad, S. P., S. B. Barbuddhe, S. Garg, K. V. Poharkar, D. R. Kalorey, N. V. Kurkure, D. B. Rawool and T. Chakraborty (2015). Biofilm-forming abilities of *Listeria monocytogenes* serotypes isolated from different sources. PLoS One **10**(9): e0137046.

Dominion Post (2009). *Listeria* found in tonnes of export Kiwi mussel meat. Retrieved from http://www.stuff.co.nz/dominion-post/archive/national-news/140211/Listeria-found-in-tonnes-of-export-Kiwi-mussel-meat.

Faoagali, J. L. and M. Schousboe (1985). Listeriosis in Christchurch 1967-1984. N Z Med J **98**(772): 64-66.

Farber, J. M. and P. I. Peterkin (1991). *Listeria monocytogenes*, a food-borne pathogen. Microbiol Rev **55**(3): 476-511.

Fleming, D. and K. P. Rumbaugh (2017). Approaches to dispersing medical biofilms. Microorganisms **5**(2): 15.

Food Standards Australia New Zealand (2016a). Food Standards Code. Retrieved from http://www.foodstandards.govt.nz/code/Pages/default.aspx

Food Standards Australia New Zealand (2016b). Australia New Zealand Food Standards Code – Standard 1.6.1 – Microbiological limits in food. Retrieved from https://www.legislation.gov.au/Details/F2016C00172

Food Standards Australia New Zealand (2016c). Australia New Zealand Food Standards Code Schedule 27 Microbial Limits in Food. Retrieved from http://www.foodstandards.gov.au/code/Documents/Sched%2027%20Micro%20limits%20v151.pdf

Fletcher, G., M. Rogers and R. Wong (1994). Survey of *Listeria monocytogenes* in New Zealand seafood. J Aquat Food Prod T **3**(2): 13-24.

Flight, R. J. (1971). Listeriosis in Auckland. N Z Med J **73**(469): 349-351.

Folsom, J. P., G. R. Siragusa and J. F. Frank (2006). Formation of biofilm at different nutrient levels by various genotypes of *Listeria monocytogenes*. J Food Protect **69**(4): 826-834.

Fonnesbech Vogel, B., H. H. Huss, B. Ojeniyi, P. Ahrens and L. Gram (2001). Elucidation of *Listeria monocytogenes* contamination routes in cold-smoked salmon processing plants detected by DNA-based typing methods. Appl Environ Microbiol **67**(6): 2586-2595.

Franklin, M. J., C. Chang, T. Akiyama and B. Bothner (2015). New technologies for studying biofilms. Microbiol Spectr **3**(4).

Ganegama Arachchi, G. J., A. G. Cridge, B. M. Dias-Wanigasekera, C. D. Cruz, L. McIntyre, R. Liu, S. H. Flint and A. N. Mutukumira (2013). Effectiveness of phages in the decontamination of *Listeria monocytogenes* adhered to clean stainless steel, stainless steel coated with fish protein, and as a biofilm. J Ind Microbiol Biotechnol **40**(10): 1105-1116.

Garmyn, D., L. Gal, J.P. Lemaitre, A. Hartmann and P. Piveteau (2009). Communication and autoinduction in the species *Listeria monocytogenes*: A central role for the agr system. Commun Integr Biol **2**(4): 371-374.

Garmyn, D., L. Gal, R. Briandet, M. Guilbaud,J. P. Lemaître, A. Hartmann and P. Piveteau (2011). Evidence of autoinduction heterogeneity via expression of the Agr system of *Listeria monocytogenes* at the single-cell level. Appl Environ Microbiol **77**(17): 6286-6289.

Garrett, T. R., M. Bhakoo and Z. Zhang (2008). Bacterial adhesion and biofilms on surfaces. Prog Nat Sci **18**(9): 1049-1056.

Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, P. Berche, H. Bloecker, P. Brandt, T. Chakraborty, A. Charbit, F. Chetouani, E. Couve, A. de Daruvar, P. Dehoux, E. Domann, G. Dominguez-Bernal, E. Duchaud, L. Durant, O. Dussurget, K. D. Entian, H. Fsihi, F. Garcia-del Portillo, P. Garrido, L. Gautier, W. Goebel, N. Gomez-Lopez, T. Hain, J. Hauf, D. Jackson, L. M. Jones, U. Kaerst, J. Kreft, M. Kuhn, F. Kunst, G. Kurapkat, E. Madueno, A. Maitournam, J. M. Vicente, E. Ng, H. Nedjari, G. Nordsiek, S. Novella, B. de Pablos, J. C. Perez-Diaz, R. Purcell, B. Remmel, M. Rose, T. Schlueter, N. Simoes, A. Tierrez, J. A. Vazquez-Boland, H. Voss, J. Wehland and P. Cossart (2001). Comparative genomics of Listeria species. Science **294**(5543): 849-852.

Gomez, N. C., J. M. Ramiro, B. X. Quecan and B. D. de Melo Franco (2016). Use of potential probiotic lactic acid bacteria (LAB) biofilms for the control of *Listeria monocytogenes*, *Salmonella typhimurium*, and *Escherichia coli* O157:H7 biofilms formation. Front Microbiol **7**: 863.

Government inquiry into the whey protein concentrate contamination (2014). The WPC80 incident: causes & responses. Retrieved from https://www.dia.govt.nz/vwluResources/Government-Whey-Inquiry-Report-November-2014/$file/Government-Whey-Inquiry-Report-November-2014.PDF

Graves, L. M., L. O. Helsel, A. G. Steigerwalt, R. E. Morey, M. I. Daneshvar, S. E. Roof, R. H. Orsi, E. D. Fortes, S. R. Milillo, H. C. den Bakker, M. Wiedmann, B. Swaminathan and B. D. Sauders (2010). *Listeria marthii* sp. nov., isolated from the natural environment, Finger Lakes National Forest. Int J Syst Evol Microbiol **60**(Pt 6): 1280-1288.

Gründling, A., L. S. Burrack, H. A. Bouwer and D. E. Higgins (2004). *Listeria monocytogenes* regulates flagellar motility gene expression through MogR, a transcriptional repressor required for virulence. Proc Natl Acad Sci U S A **101**(33): 12318-12323.

Gudbjörnsdóttir, B., M.L. Suihko, P. Gustavsson, G. Thorkelsson, S. Salo, A.M. Sjöberg, O. Niclasen and S. Bredholt (2004). The incidence of *Listeria monocytogenes* in meat, poultry and seafood plants in the Nordic countries. Food Microbiol **21**(2): 217-225.

Gudbjörnsdóttir, B., H. Einarsson and G. Thorkelsson (2005). Microbial adhesion to processing lines for fish fillets and cooked shrimp: influence of stainless steel surface finish and presence of gram-negative bacteria on the attachment of *Listeria monocytogenes*. Food Technol Biotechnol **43**(1): 55-61.

Guenther, S., D. Huwyler, S. Richard and M. J. Loessner (2009). Virulent bacteriophage for efficient biocontrol of *Listeria monocytogenes* in ready-to-eat foods. Appl Environ Microbiol **75**(1): 93-100.

Gueriri, I., C. Cyncynatus, S. Dubrac, A. T. Arana, O. Dussurget and T. Msadek (2008). The DegU orphan response regulator of *Listeria monocytogenes* autorepresses its own synthesis and is required for bacterial motility, virulence and biofilm formation. Microbiology **154**(Pt 8): 2251-2264.

Guerrieri, E., S. de Niederhausern, P. Messi, C. Sabia, R. Iseppi, I. Anacarso and M. Bondi (2009). Use of lactic acid bacteria (LAB) biofilms for the control of Listeria monocytogenes in a small-scale model. Food Control **20**(9): 861-865.

Habimana, O., M. Meyrand, T. Meylheuc, S. Kulakauskas and R. Briandet (2009). Genetic features of resident biofilms determine attachment of *Listeria monocytogenes*. Appl Environ Microbiol **75**(24): 7814-7821.

Harmsen, M., M. Lappann, S. Knochel and S. Molin (2010). Role of extracellular DNA during biofilm formation by *Listeria monocytogenes*. Appl Environ Microbiol **76**(7): 2271-2279.

Heydorn, A., A. T. Nielsen, M. Hentzer, C. Sternberg, M. Givskov, B. K. Ersboll and S. Molin (2000). Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology **146**(Pt 10): 2395-2407.

Horn, B., L. Lopez, P. a. Cressey and R. Roos (2015). Annual Report Concerning Foodborne Disease in New Zealand 2014. ESR Client Report FW15016, Christchurch, New Zealand.

Huang, Y., C. Shi, S. Yu, K. Li and X. Shi (2012). A putative MerR family regulator involved in biofilm formation in *Listeria monocytogenes* 4b G. Foodborne Pathog Dis **9**(8): 767-772.

Huang, Y., Y. Suo, C. Shi, J. Szlavik, X. M. Shi and S. Knochel (2013). Mutations in *gltB* and *gltC* reduce oxidative stress tolerance and biofilm formation in *Listeria monocytogenes* 4b G. Int J Food Microbiol **163**(2-3): 223-230.

Hussain, M. A. and C. O. Dawson (2013). Economic impact of food safety outbreaks on food businesses. Foods **2**(4): 585-589.

John, P. and S. Laurence (2007). Listeriosis in humans. Listeria, Listeriosis, and Food Safety, Third Edition, CRC Press**:** 85-109.

Jordan, S. J., S. Perni, S. Glenn, I. Fernandes, M. Barbosa, M. Sol, R. P. Tenreiro, L. Chambel, B. Barata, I. Zilhao, T. G. Aldsworth, A. Adriao, M. L. Faleiro, G. Shama and P. W. Andrew (2008). *Listeria monocytogenes* biofilm-associated protein (BapL) may contribute to surface attachment of *L. monocytogenes* but is absent from many field isolates. Appl Environ Microbiol **74**(17): 5451-5456.

Kadam, S. R., H. M. den Besten, S. van der Veen, M. H. Zwietering, R. Moezelaar and T. Abee (2013). Diversity assessment of *Listeria monocytogenes* biofilm formation: impact of growth condition, serotype and strain origin. Int J Food Microbiol **165**(3): 259-264.

Karatan, E. and P. Watnick (2009). Signals, regulatory networks, and materials that build and break bacterial biofilms. Microbiol Mol Biol Rev **73**(2): 310-347.

Kathariou, S. (2002). *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. J Food Prot **65**(11): 1811-1829.

Knudsen, G. M., J. E. Olsen and L. Dons (2004). Characterization of DegU, a response regulator in *Listeria monocytogenes*, involved in regulation of motility and contributes to virulence. FEMS Microbiol Lett **240**(2): 171-179.

Koo, O. K., J. B. Ndahetuye, C. A. O'Bryan, S. C. Ricke and P. G. Crandall (2014). Influence of *Listeria innocua* on the attachment of *Listeria monocytogenes* to stainless steel and aluminum surfaces. Food Control **39**(0): 135-138.

Lasa, I. (2006). Towards the identification of the common features of bacterial biofilm development. Int Microbiol **9**(1): 21-28.

Leclercq, A., D. Clermont, C. Bizet, P. A. Grimont, A. Le Fleche-Mateos, S. M. Roche, C. Buchrieser, V. Cadet-Daniel, A. Le Monnier, M. Lecuit and F. Allerberger (2010). *Listeria rocourtiae* sp. nov. Int J Syst Evol Microbiol **60**(Pt 9): 2210-2214.

Lemon, K. P., N. E. Freitag and R. Kolter (2010). The virulence regulator PrfA promotes biofilm formation by *Listeria monocytogenes*. J Bacteriol **192**(15): 3969-3976.

Lemon, K. P., D. E. Higgins and R. Kolter (2007). Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. J Bacteriol **189**(12): 4418-4424.

Leriche, V. and B. Carpentier (2000). Limitation of adhesion and growth of *Listeria monocytogenes* on stainless steel surfaces by *Staphylococcus sciuri* biofilms. J Appl Microbiol **88**(4): 594-605.

Lopez, L., R. Roos, P. a. Cressey and B. Horn (2016). Annual report concerning foodborne disease in New Zealand 2015. ESR Client Report FW16020, Christchurch, New Zealand.

Lourenco, A., A. de Las Heras, M. Scortti, J. Vazquez-Boland, J. F. Frank and L. Brito (2013). Comparison of *Listeria monocytogenes* Exoproteomes from biofilm and planktonic state: Lmo2504, a protein associated with biofilms. Appl Environ Microbiol **79**(19): 6075-6082.

Lunden, J. M., M. K. Miettinen, T. J. Autio and H. J. Korkeala (2000). Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. J Food Prot **63**(9): 1204-1207.

Luo, Q., J. Shang, X. Feng, X. Guo, L. Zhang and Q. Zhou (2013). PrfA led to reduced biofilm formation and contributed to altered gene expression patterns in biofilm-forming *Listeria monocytogenes*. Curr Microbiol **67**(3): 372-378.

Metselaar, K. I., P. Saa Ibusquiza, A. R. Ortiz Camargo, M. Krieg, M. H. Zwietering, H. M. den Besten and T. Abee (2015). Performance of stress resistant variants of *Listeria monocytogenes* in mixed species biofilms with *Lactobacillus plantarum*. Int J Food Microbiol **213**: 24-30.

Meyer, B. (2003). Approaches to prevention, removal and killing of biofilms. Int Biodeter Biodegr **51**(4): 249-253.

Ministry for Primary Industries (n.d.). *Listeria* Resources and Documents. Retrieved 20 February 2017 from http://www.foodsafety.govt.nz/science-risk/programmes/hazard-risk-management/listeria.htm.

Møretrø, T. and S. Langsrud (2004). *Listeria monocytogenes*: biofilm formation and persistence in food-processing environments. Biofilms **1**(2): 107-121.

Møretrø, T., B. C. Schirmer, E. Heir, A. Fagerlund, P. Hjemli and S. Langsrud (2017). Tolerance to quaternary ammonium compound disinfectants may enhance growth of *Listeria monocytogenes* in the food industry. Int J Food Microbiol **241**: 215-224.

Nakamura, H., K. Takakura, Y. Sone, Y. Itano and Y. Nishikawa (2013). Biofilm formation and resistance to benzalkonium chloride in *Listeria monocytogenes* isolated from a fish processing plant. J Food Prot **76**(7): 1179-1186.

Ndahetuye, J. B., O. K. Koo, C. A. O'Bryan, S. C. Ricke and P. G. Crandall (2012). Role of lactic acid bacteria as a biosanitizer to prevent attachment of *Listeria monocytogenes* F6900 on deli slicer contact surfaces. J Food Prot **75**(8): 1429-1436.

Nilsson, R. E., T. Ross and J. P. Bowman (2011). Variability in biofilm production by *Listeria monocytogenes* correlated to strain origin and growth conditions. Int J Food Microbiol **150**(1): 14-24.

Nguyen, U. T. and L.L. Burrows (2014). DNase I and proteinase K impair *Listeria monocytogenes* biofilm formation and induce dispersal of pre-existing biofilms. Int J Food Microbiol **187**: 26-32.

Norwood, D. E. and A. Gilmour (2001). The differential adherence capabilities of two *Listeria monocytogenes* strains in monoculture and multispecies biofilms as a function of temperature. Lett Appl Microbiol **33**(4): 320-324.

Nostro, A., A. Guerrini, A. Marino, M. Tacchini, M. Di Giulio, A. Grandini, M. Akin, L. Cellini, G. Bisignano and H. T. Saracoglu (2016). In vitro activity of plant extracts against biofilm-producing food-related bacteria. Int J Food Microbiol **238**: 33-39.

NZ Herald (2007). *Listeria* sparks mussel recall. Retrieved from http://www.nzherald.co.nz/nz/news/article.cfm?c_id=1&objectid=10478766

Ochiai, Y., F. Yamada, M. Mochizuki, T. Takano, R. Hondo and F. Ueda (2014). Biofilm formation under different temperature conditions by a single genotype of persistent *Listeria monocytogenes* strains. J Food Prot **77**(1): 133-140.

Orsi, R. H., H. C. den Bakker and M. Wiedmann (2011). *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. Int J Med Microbiol **301**(2): 79-96.

O'Toole G. A. (2011). Microtiter dish biofilm formation assay. J Vis Exp (47):2437.

Pan, Y. (2009). A comparative study of serotype 1/2a and serotype 4b strains of *Listeria monocytogenes* in biofilms using a simulated food processing system.

Pan, Y., F. Breidt, Jr. and L. Gorski (2010). Synergistic effects of sodium chloride, glucose, and temperature on biofilm formation by *Listeria monocytogenes* serotype 1/2a and 4b strains. Appl Environ Microbiol **76**(5): 1433-1441.

Pan, Y., F. Breidt, Jr. and S. Kathariou (2009). Competition of *Listeria monocytogenes* serotype 1/2a and 4b strains in mixed-culture biofilms. Appl Environ Microbiol **75**(18): 5846-5852.

Peel, M., W. Donachie and A. Shaw (1988). Temperature-dependent expression of flagella of *Listeria manocytogenes* studied by electron microscopy, SDS-PAGE and western blotting. J Gen Microbiol **134**(8): 2171-2178.

Perez-Ibarreche, M., P. Castellano, A. Leclercq and G. Vignolo (2016). Control of *Listeria monocytogenes* biofilms on industrial surfaces by the bacteriocin-producing *Lactobacillus sakei* CRL1862. FEMS Microbiol Lett **363**(12).

Pilchova, T., M. Hernould, H. Prevost, K. Demnerova, J. Pazlarova and O. Tresse (2014). Influence of food processing environments on structure initiation of static biofilm of *Listeria monocytogenes.* Food Control **35**(1): 366-372.

Qin, Z., Y. Ou, L. Yang, Y. Zhu, T. Tolker-Nielsen, S. Molin and D. Qu (2007). Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. Microbiology **153**(7): 2083-2092.

Riedel, C. U., I. R. Monk, P. G. Casey, M. S. Waidmann, C. G. Gahan and C. Hill (2009). AgrD-dependent quorum sensing affects biofilm formation, invasion, virulence and global gene expression profiles in *Listeria monocytogenes*. Mol Microbiol **71**(5): 1177-1189.

Rieu, A., R. Briandet, O. Habimana, D. Garmyn, J. Guzzo and P. Piveteau (2008a). *Listeria monocytogenes* EGD-e biofilms: no mushrooms but a network of knitted chains. Appl Environ Microbiol **74**(14): 4491-4497.

Rieu, A., J. P. Lemaitre, J. Guzzo and P. Piveteau (2008b). Interactions in dual species biofilms between *Listeria monocytogenes* EGD-e and several strains of *Staphylococcus aureus*. Int J Food Microbiol **126**(1-2): 76-82.

Rieu, A., S. Weidmann, D. Garmyn, P. Piveteau and J. Guzzo (2007). Agr system of *Listeria monocytogenes* EGD-e: role in adherence and differential expression pattern. Appl Environ Microbiol **73**(19): 6125-6133.

Rocourt, J. and C. Buchrieser (2007). 1 The genus *Listeria* and *Listeria monocytogenes*: phylogenetic position, taxonomy, and identification. Listeria, listeriosis, and food safety: 1.

Rocourt, J., C. Jacquet and A. Reilly (2000). Epidemiology of human listeriosis and seafoods. Int J Food Microbiol **62**(3): 197-209.

Rodriguez-Lopez, P., A. Carballo-Justo, L. A. Draper and M. L. Cabo (2017). Removal of *Listeria monocytogenes* dual-species biofilms using combined enzyme-benzalkonium chloride treatments. Biofouling **33**(1): 45-58.

Salazar, J. K., Z. Wu, W. Yang, N. E. Freitag, M. L. Tortorello, H. Wang and W. Zhang (2013). Roles of a novel Crp/Fnr family transcription factor Lmo0753 in soil survival, biofilm production and surface attachment to fresh produce of *Listeria monocytogenes*. PLoS One **8**(9): e75736.

Sashara, K. and E. A. Zottola (1993). Biofilm formation by *Listeria monocytogenes* utilizes a primary colonizing microorganism in flowing systems. J Food Prot **56**(12): 1022-1028

Schmitz-Esser, S., A. Muller, B. Stessl and M. Wagner (2015). Genomes of sequence type 121 *Listeria monocytogenes* strains harbor highly conserved plasmids and prophages. Front Microbiol **6**: 380.

Schuchat, A., B. Swaminathan and C. V. Broome (1991). Epidemiology of human listeriosis. Clin Microbiol Rev **4**(2): 169-183.

Schwab, U., Y. Hu, M. Wiedmann and K. J. Boor (2005). Alternative sigma factor σ B is not essential for *Listeria monocytogenes* surface attachment. J Food Prot **68**(2): 311-317.

Seafood New Zealand (n.d.). Key Facts. Retrieved 16 January 2017 from http://www.seafood.org.nz/industry/key-facts/

Sela, S., S. Frank, E. Belausov and R. Pinto (2006). A mutation in the luxS gene influences *Listeria monocytogenes* biofilm formation. Appl Environ Microbiol **72**(8): 5653-5658.

Shen, A. and D. E. Higgins (2006). The MogR transcriptional repressor regulates nonhierarchal expression of flagellar motility genes and virulence in *Listeria monocytogenes*. PLoS Pathog **2**(4): e30.

Silva, S., P. Teixeira, R. Oliveira and J. Azeredo (2008). Adhesion to and viability of *Listeria monocytogenes* on food contact surfaces. J Food Prot **71**(7): 1379-1385.

Srey, S., I. K. Jahid and S. D. Ha (2013). Biofilm formation in food industries: A food safety concern. Food Control **31**(2): 572-585.

Suo, Y., Y. Huang, Y. Liu, C. Shi and X. Shi (2012). The expression of superoxide dismutase (SOD) and a putative ABC transporter permease is inversely correlated during biofilm formation in *Listeria monocytogenes* 4b G. PLoS One **7**(10): e48467.

Szlavik, J., D. S. Paiva, N. Mork, F. van den Berg, J. Verran, K. Whitehead, S. Knochel and D. S. Nielsen (2012). Initial adhesion of *Listeria monocytogenes* to solid surfaces under liquid flow. Int J Food Microbiol **152**(3): 181-188.

Takahashi, H., T. Suda, Y. Tanaka and B. Kimura (2010). Cellular hydrophobicity of *Listeria monocytogenes* involves initial attachment and biofilm formation on the surface of polyvinyl chloride. Lett Appl Microbiol **50**(6): 618-625.

Taylor, C. M., M. Beresford, H. A. Epton, D. C. Sigee, G. Shama, P. W. Andrew and I. S. Roberts (2002). *Listeria monocytogenes* relA and hpt mutants are impaired in surface-attached growth and virulence. J Bacteriol **184**(3): 621-628.

The Institute of Environmental Science and Research Ltd. (2013). Annual summary of outbreaks in New Zealand 2012 Porirua, New Zealand. Retrieved from https://surv.esr.cri.nz/PDF_surveillance/AnnualRpt/AnnualOutbreak/2012/2012OutbreakRpt.pdf

The Institute of Environmental Science and Research Ltd. (2014). Annual summary of outbreaks in New Zealand 2013 Wallaceville, New Zealand. Retrieved from https://surv.esr.cri.nz/PDF_surveillance/AnnualRpt/AnnualOutbreak/2013/2013OutbreakRpt.pdf

The Institute of Environmental Science and Research Ltd. (2016). Notifiable diseases in New Zealand: Annual report 2015 Porirua, New Zealand. Retrieved from https://surv.esr.cri.nz/PDF_surveillance/AnnualRpt/AnnualSurv/2015/2015AnnualReportFinal.pdf

Todhanakasem, T. and G. M. Young (2008). Loss of flagellum-based motility by *Listeria monocytogenes* results in formation of hyperbiofilms. J Bacteriol **190**(17): 6030-6034.

Tresse, O., V. Lebret, D. Garmyn and O. Dussurget (2009). The impact of growth history and flagellation on the adhesion of various *Listeria monocytogenes* strains to polystyrene. Can J Microbiol **55**(2): 189-196.

US Food and Drug Administration (2015). Quantitative assessment of relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods, retrieved 16 January 2017 from http://www.fda.gov/Food/FoodScienceResearch/RiskSafetyAssessment/ucm183966.htm.

van der Veen, S. and T. Abee (2010a). Dependence of continuous-flow biofilm formation by *Listeria monocytogenes* EGD-e on SOS response factor YneA. Appl Environ Microbiol **76**(6): 1992-1995.

van der Veen, S. and T. Abee (2010b). HrcA and DnaK are important for static and continuous-flow biofilm formation and disinfectant resistance in *Listeria monocytogenes*. Microbiology **156**(Pt 12): 3782-3790.

van der Veen, S. and T. Abee (2010c). Importance of SigB for *Listeria monocytogenes* static and continuous-flow biofilm formation and disinfectant resistance. Appl Environ Microbiol **76**(23): 7854-7860.

van der Veen, S. and T. Abee (2011a). Generation of variants in *Listeria monocytogenes* continuous-flow biofilms is dependent on radical-induced DNA damage and RecA-mediated repair. PLoS One **6**(12): e28590.

van der Veen, S. and T. Abee (2011b). Mixed species biofilms of *Listeria monocytogenes* and *Lactobacillus plantarum* show enhanced resistance to benzalkonium chloride and peracetic acid. Int J Food Microbiol **144**(3): 421-431.

van der Veen, S., T. Hain, J. A. Wouters, H. Hossain, W. M. de Vos, T. Abee, T. Chakraborty and M. H. Wells-Bennik (2007). The heat-shock response of *Listeria monocytogenes* comprises genes involved in heat shock, cell division, cell wall synthesis, and the SOS response. Microbiology **153**(Pt 10): 3593-3607.

van der Veen, S., S. van Schalkwijk, D. Molenaar, W. M. de Vos, T. Abee and M. H. Wells-Bennik (2010). The SOS response of *Listeria monocytogenes* is involved in stress resistance and mutagenesis. Microbiology **156**(Pt 2): 374-384.

Vatanyoopaisarn, S., A. Nazli, C. E. Dodd, C. E. Rees and W. M. Waites (2000). Effect of flagella on initial attachment of *Listeria monocytogenes* to stainless steel. Appl Environ Microbiol **66**(2): 860-863.

Vazquez-Boland, J.A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, J. Kreft (2001). *Listeria* pathogenesis and molecular virulence determinants. Clin Microbiol Rev **14**(3): 584-640.

Vorregaard, M. (2008). Comstat2-a modern 3D image analysis environment for biofilms, Technical University of Denmark, DTU, DK-2800 Kgs. Lyngby, Denmark.

Wassinger, A., L. Zhang, E. Tracy, R. S. Munson, Jr., S. Kathariou and H. H. Wang (2013). Role of a GntR-family response regulator LbrA in *Listeria monocytogenes* biofilm formation. PLoS One **8**(7): e70448.

Waters, C. M. and B. L. Bassler (2005). Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol **21**: 319-346.

Way, S. S., L. J. Thompson, J. E. Lopes, A. M. Hajjar, T. R. Kollmann, N. E. Freitag and C. B. Wilson (2004). Characterization of flagellin expression and its role in *Listeria monocytogenes* infection and immunity. Cell Microbiol **6**(3): 235-242.

Whitchurch, C. B., T. Tolker-Nielsen, P. C. Ragas and J. S. Mattick (2002). Extracellular DNA required for bacterial biofilm formation. Science **295**(5559): 1487.

Yousef, A. E. (1999). Characteristics of *Listeria monocytogenes* important to food processors. Listeria: Listeriosis, and Food Safety: 131.

Zhang, T., D. Bae and C. Wang (2016). The DNA glycosylase, Adlp affects flagellar motility, biofilm formation, virulence, and stress response in *Listeria monocytogenes*. Appl Environ Microbiol **82**(17): 5144-52

Zhou, Q., F. Feng, L. Wang, X. Feng, X. Yin and Q. Luo (2011). Virulence regulator PrfA is essential for biofilm formation in *Listeria monocytogenes* but not in *Listeria innocua*. Curr Microbiol **63**(2): 186-192.

Zhu, X., W. Liu, R. Lametsch, F. Aarestrup, C. Shi, Q. She, X. Shi and S. Knochel (2011). Phenotypic, proteomic, and genomic characterization of a putative ABC-transporter permease involved in *Listeria monocytogenes* biofilm formation. Foodborne Pathog Dis **8**(4): 495-501.

Zhu, X., F. Long, Y. Chen, S. Knochel, Q. She and X. Shi (2008). A putative ABC transporter is involved in negative regulation of biofilm formation by *Listeria monocytogenes*. Appl Environ Microbiol **74**(24): 7675-7683.

Chapter 3

Persistent *Listeria monocytogenes* isolates from mussel production facilities form more biofilm but are not linked to specific genetic markers

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Abstract

Contamination of mussels with the human pathogen *Listeria monocytogenes* can occur during processing in the factory environment. In this study, a selection of persistent (n=8) and sporadic (n=8) *Listeria monocytogenes* isolates associated with mussel-processing premises in New Zealand were investigated for their phenotypic and genomic characteristics. To identify traits that favour or contribute to bacterial persistence, biofilm formation, heat resistance, motility and recovery from dry surfaces were compared between persistent and sporadic isolates. All isolates exhibited low biofilm formation at 20°C, however, at 30°C persistent isolates showed significantly greater biofilm formation after 48 h when analysed using cell enumeration. All 16 isolates were motile at 20°C and 30°C, but no significant difference was observed. We found that biofilm cells of the persistent isolates survived better than the planktonic cells. Two of the three most heat-resistant isolates were persistent, while four of five isolates lacking heat resistance were sporadic isolates. Comparison of genome sequences of persistent and sporadic isolates showed that there was no overall clustering of persistent or sporadic isolates, and that differences in prophages and plasmids were not associated with persistence. Our phenotypic results suggest a link between persistence and biofilm formation, which is most likely multifactorial, however, there were no genotypic differences that could be correlated to persistence in these isolates.

3.1 Introduction

Listeria monocytogenes is a Gram-positive motile rod-shaped bacterium that is commonly encountered in the environment. It is capable of surviving and even growing in harsh environmental conditions, over a wide temperature range, in acidic environments and it also tolerates high osmolarity (Yousef 1999).

L. monocytogenes is capable of infecting animals as well as humans, causing the bacterial infection listeriosis, which leads to death in up to 30% of the cases (Rocourt et al. 2000). High-risk groups include pregnant women, immunocompromised people and the elderly. *L. monocytogenes* can cross the placental barrier, thereby leading to the abortion of the foetus. It can also cross the blood-brain barrier and/or the intestinal barrier, leading to life-threatening bacterial infections. Common sources of infection are processed food, including raw milk products and ready-to-eat (RTE) chilled products (Allerberger and Wagner 2010). However, unprocessed foods such as fruit and vegetables have also been identified as vectors (Garner and Kathariou 2016, Martinez et al. 2016).

Studies have shown that contamination of seafood with *L. monocytogenes* usually occurs during processing rather than from raw products in the seafood-processing environment (Autio et al. 1999, Fletcher et al. 1994). The source of contamination therefore consists of either persistent strains harboured within a processing facility or from transient sporadic strains passing through the facility. Bacterial persistence is generally defined as the long-term occurrence of genetically indistinguishable strains isolated from the same environment (Schmitz-Esser et al. 2015).

The ability of strains to persist in a food-processing environment has been the topic of two reviews (Carpentier and Cerf 2011, Ferreira et al. 2014), however the reason for persistence remains poorly understood. Persistence of strains in food-processing plants can either be due to genotypic and phenotypic features that facilitate the expression of specific genes and lead to enhanced tolerance towards environmental threats, or to the attachment of bacteria to niches and harbourage sites by chance and subsequent survival due to insufficient cleaning and sanitation procedures (Abee et al. 2016).

Bacteria persisting in a food-processing plant not only pose a health concern/threat but also present a risk factor for the economy (Ferreira et al. 2014, Ryser and Marth 2007). The ongoing sampling and testing for *L. monocytogenes* in food-processing plants is a huge financial burden. To reduce the risk for consumers and the financial stress for the producer, it is important to identify genetic traits and/or phenotypic behaviour that favour persistence of *L. monocytogenes*

within or around a processing facility. Interventions that reduce attachment to surfaces and persistence of bacteria can assist in preventing contamination.

During a two-year weekly sampling programme in the New Zealand mussel industry, sporadic and persistent *L. monocytogenes* isolates were collected and characterised (Cruz and Fletcher 2011). Strains were considered persistent if they had been isolated over a time period of 6 months or more from the same processing facility and were genetically indistinguishable by pulsed field gel electrophoresis (PFGE) (Cruz and Fletcher 2011). The current study was designed to compare the phenotypes of such persistent isolates with those of presumed non-persistent isolates isolated from the mussel-processing environment.

During processing in a typical New Zealand mussel-processing plant, bacterial strains encounter different environmental conditions (Figure 3.1). After arrival of the mussels in the processing factory from aquaculture sites, they are kept in large bags under chilled storage in the bale store (7-8°C). The mussels are transferred to the cook room where they undergo heat treatment (which differs between different plants) to facilitate opening and to inactivate microorganisms. A debyssing process removes the mussel beards before the mussels are shucked manually or by an automated system. The product is then cooled, individually quick frozen and packed for export. There are several steps in the processing chain that may induce persistence of bacteria.

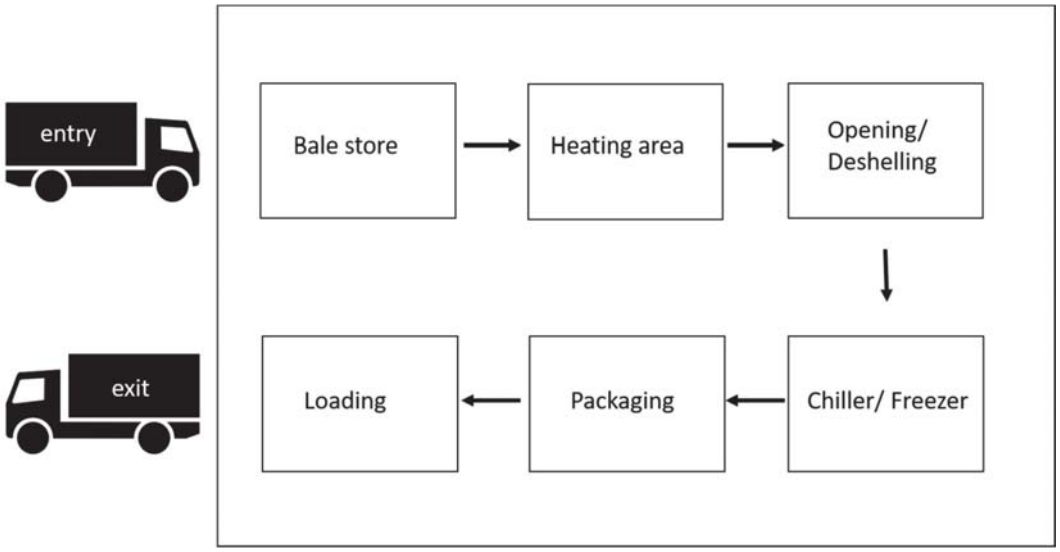

Figure 3.1. Schematic process line for mussel-processing. Mussels are stored in bales for up to 6 days, but ideally are processed after 3-4 days. The processing from leaving the bale store (7-8°C) to final packaging takes about two hours. The heat treatment varies in different plants: One stage heat treatments with temperatures of 76-78°C are applied for around 150 s or a 2-stage heat-shock process with higher temperatures at first (93-96°C for 22 s) and a subsequent second step with lower temperatures. The packaged product is stored at -18°C after being individually quick frozen ready for export.

The relationship between biofilm formation and persistence has been investigated previously (Abdallah et al. 2014, Bonsaglia et al. 2014, Møretrø and Langsrud 2004). The sessile living form has been shown to be the preferred form of existence for many bacteria and it is known that microbial biofilms exhibit higher resistance to cleaning and sanitising (Pan et al. 2006), which may result in bacterial persistence in food-processing plants. Biofilm formation is a response to nutrient deprivation and is seen as a stress response (Blaschek et al. 2015). Motility has been linked to biofilm formation: some researchers found that flagella function as an adhesin (Vatanyoopaisarn et al. 2000), whereas others suggest flagella are used as a motility factor (Lemon et al. 2007). Regardless of the different findings, flagella seem to play an important role in attachment to surfaces, which is the first step of biofilm formation. Lemon et al. (2007) found that non-motile mutants including flagella-deficient mutants and mutants with non-functional (paralyzed) flagella, exhibited impaired biofilm formation at 30°C. Most of the *L. monocytogenes* strains do not express flagella above 30°C (Peel et al. 1988), but are still able to form biofilms, suggesting that there are different mechanisms of attachment for biofilm formation. As most of the food-processing plants and much of a mussel-processing facility operates at temperatures below 30°C, bacterial flagella and motility may influence attachment.

Previous research focused on the influence of pH, salinity and resistance to sanitisers to identify features that may lead to bacterial persistence in food-processing plants (Nilsson et al. 2011). The focus of the current study was to identify links between persistent isolates and biofilm formation, motility, survival under dry conditions and heat sensitivity and to identify genetic factors contributing to persistence.

3.2. Materials and Methods

3.2.1. Strains and growth conditions

To assess the phenotype of the persistent and sporadic isolates, two representative isolates for four persistent pulsotypes (three identified in a previous study (Cruz and Fletcher 2011) and a fourth pulsotype identified from a fourth factory) were selected. Seven sporadic isolates with different pulsotypes (isolated only once over the sampling period of 2 years) and a strain isolated from a food-poisoning outbreak in New Zealand due to contaminated smoked mussels (Brett et al. 1998) were used for this study (Table 3.1). These strains were chosen from different locations at five different factories at different times in an attempt to capture diversity from those sourced. The selected strains had all been isolated by environmental swabbing either within processing plants or on their factory grounds except for the outbreak strain that had been isolated from the final product. The outbreak strain could be linked to isolates from the

environment of the mussel-processing facility by several typing methods (Brett et al. 1998), but is considered sporadic for the present study as the environmental swabs were collected with a big time gap (November 1990 and July 1993) suggesting reintroduction into the processing facility. It is likely that environmental swabbing was continued during this time without detection of the outbreak isolate, however, Brett et al. (1998) did not state this. All 16 isolates belonging to the 12 pulsotypes were recovered from -80°C stock and grown on Tryptic Soy Agar enriched with 0.6% Yeast Extract (TSAYE) (Difco, BD, USA) plates. A colony was freshly picked for each experiment. Overnight cultures were usually prepared in Tryptic Soy Broth enriched with 0.6% Yeast Extract (TSBYE) (Difco, BD, USA) and then used for subsequent experiments.

Table 3.1. Strains of *Listeria monocytogenes* used in the experiments

Strain	Pulsotype	Persistence	Plant[1]	Isolation site	Isolation date
15A04	3814	+	A	heating	29/08/07
27A05	3814	+	B	heating	07/09/10
15G01	5132	+	C	external area[2]	07/02/08
16J10	5132	+	B	opening	01/05/08
32C06	5588	+	D	opening	03/06/11
33H04	5588	+	D	heating	01/12/11
15A07	6502	+	A	bale store	06/09/07
31H06	6502	+	A	heating	07/02/11
16A01	3860		outbreak	smoked mussels	??/12/92
15B09	8779		B	opening	07/12/07
15D07	5176		C	heating	19/12/07
15G10	3832		A	packaging	08/02/08
16H02	0101		E	opening	23/04/08
16J08	8981		C	bale store	30/04/08
17A02	7002		A	external area[2]	28/04/08
19B07	3880		C	heating	07/04/09

[1] Plants A, B and C are coded as published in Cruz and Fletcher (2011).

[2] Strains obtained from areas surrounding the factory (shell, truck park, waste belt roof, entrance doors and floors).

3.2.2. Biofilm formation

Biofilm formation was measured according to a method described previously with modifications (Djordjevic et al. 2002). An overnight culture (2 µL) grown in TSBYE (Difco, BD, USA) at 37°C was used to inoculate 96-well plates (polystyrene, Greiner, F-Bottom, 655161) filled with 198 µL fresh Brain-Heart-Infusion broth (BHI) (Difco, BD, USA) and incubated statically at 20°C and 30°C for 24 and 48 h, respectively. Even though these temperatures are probably not the most common ones in the food processing environment, they still may be encountered. This study aimed to identify differences in the biofilm formation of persistent and sporadic isolates after 24 and 48 h incubation. Temperatures lower than 20°C do not support biofilm formation therefore 30°C was chosen for this study. After incubation, media was removed by inverting plates and wells were washed three times with double-distilled water (ddH_2O). To determine biofilm production the biofilm mass was stained with 200 µL of an aqueous 0.1% crystal violet (BDH) solution and incubated for 45 min at 30°C. After incubation the wells were washed three times with ddH_2O to remove excess dye and then air dried. Bound crystal violet was then destained using 200 µL 96% ethanol (EtOH) per well. The optical density was measured at 595nm with a microplate reader (VersaMax, Molecular Devices). Obtained values were blank corrected and averaged for each isolate. The experiment was performed twice with two replicates.

For cell enumeration performed in parallel, the bacteria were grown as above and the wells washed three times with ddH_20 to remove planktonic cells. The sessile cells were detached and resuspended by rigorous pipetting using 500 µL phosphate buffered saline (PBS – NaCl 8.0 g/L, KCl 0.2 g/L, Na_2HPO_4 1.42 g/L, KH_2PO_4 0.24 g/L, pH 7.4). Tenfold dilutions were prepared in PBS and plated on Brain Heart Infusion (BHI) agar plates using the drop plate method (Chen et al. 2003). Colony forming units (CFU) were enumerated after 24 h of incubation at 37°C. The experiment was performed twice with five replicates.

3.2.3. Motility Assay

Motility was tested on semi-solid agar plates composed of BHI broth and 0.3% agar. The plates were inoculated from single colonies using a sterile pick and incubated at 20, 30 and 37°C for 24 h according to a method described previously (Knudsen et al. 2004). Motility was measured using the visible halo around the inoculum. The experiment was performed once with three technical replicates. Results were analysed using analysis of variance (ANOVA) and the variation between the three replicates was used as the residual error.

3.2.4. Survival of planktonic and biofilm cells on dry surfaces

Survival on dry surfaces was tested as described previously with minor modifications (Castelijn et al. 2013). To grow the biofilms, colonies were picked from TSAYE agar plates and used to inoculate 10 mL TSBYE and then incubated at 37°C overnight. An overnight culture (2 μL) was used to inoculate 96-well plates containing 198 μL BHI per well. The 96-well plates were incubated at 30°C for 48 h and then washed three times with ddH_2O to remove planktonic and loosely attached cells. The plates were air dried under a laminar air flow until fully dried. For the planktonic cultures, 10 mL of TSBYE was inoculated with colonies picked from TSAYE plates and incubated overnight at 37°C. This overnight culture (100 μL) was then used to inoculate 10 mL of BHI and incubated for 24 h at 25°C. Each culture (10 μL, ~10^7CFU) was then pipetted onto 96-well plates and left to air dry under the laminar airflow. Dilutions of planktonic cultures were prepared and plated to determine the cell concentration (Day 0). The 96-well plates were stored at 25°C and viable cells were determined on days 0, 1, 2, 5, 7 and 14. PBS (500 μL, pH 7.4) was used to detach and resuspend the biofilms and planktonic cells from the 96-well plates by rigorous pipetting. Tenfold dilutions were prepared and cell concentration was determined using the drop plate method (Chen et al. 2003). Cultures were plated on BHI agar plates and incubated at 37°C for 24 h. The experiment was performed twice with five replicates.

The student's t-test was used to compare persistent with sporadic isolates and a significant difference was concluded for $p \leq 0.05$. Analysis of variance (ANOVA) was used to identify differences between the means for days, persistent versus sporadic isolates and biofilm vs planktonic cultures, allowing for the difference between isolates within those groups. An antedependence model (which allows the variability of the data to be different at different time points, and estimates the correlation between each time point and the preceding one) was used because observations on a culture/well may have been correlated over time.

3.2.5. Heat resistance

Overnight cultures grown in TSBYE at 37°C (100 μL) were used to inoculate 9.9 mL BHI and grown for 24 h at 30°C. The cell concentration before heat treatment ranged from 8.5 to 9.5 $\log_{10}$ CFU/mL. A temperature of 58°C was chosen for heat treatment after preliminary trials showed a 10% reduction in cell number for the representative strain 15G01 after 5 min at this temperature. The 10% reduction was used as the target reduction to evaluate differences between the strains. Heat resistance of all strains was therefore determined after treatment at 58°C for 5 min. Overnight culture (4 x 1.5 mL) was pipetted in 4 x 2 mL Eppendorf tubes and heat treated. After 5 min cultures were allowed to cool down at room temperature for 5 min or

2 h, respectively. Decimal dilutions of untreated (t0) and treated cultures (t5min and t2h) in PBS were plated on BHI agar plates. Agar plates were incubated at 37°C and colonies were enumerated after 24 h of incubation. CFU counts at each time were compared using ANOVA, with experiment as a block term (random effect) and strain as the treatment factor; the change from time 0 to 5 min, time 0 to 2 h and 5 min to 2 h was also analysed using ANOVA. Furthermore, the results before and 5 min after heat treatment were analysed using a one-tailed t-test.

Furthermore, 1.5 mL culture of untreated or heat treated cells (cooled down for 5 min or 2 h) was centrifuged and resuspended in the same volume of PBS containing the red fluorescent stain propidium iodide (PI – 20 µM). Non-fluorescent PI is excluded from cells with intact membranes and can only enter and bind to DNA in cells with damaged (permeabilised) membranes. The fractions of unstained cells with intact membranes and that of PI-stained cells with (transiently) permeabilised membranes were determined using a BD FACS Aria III flow cytometer (50000 events per measurement).

Combining flow cytometry (FCM) analysis and plate count methods allows analysis of short-term membrane-damaging effects (after 5 min and 2 h recovery) and the impact on culturability of cells assayed after 24 h incubation on BHI agar medium.

CFU/mL at each time were compared using ANOVA, with experiment as a block term (random effect) and strain as the treatment factor; the change from time 0 to 5 min, time 0 to 2 h and 5 min to 2 h was also analysed using ANOVA. CFU values were $\log_{10}$ transformed before analysis. The results were also analysed using a one-tailed t-test before and 5 min after heat treatment.

3.2.6. Genome sequencing and analysis

Genomic DNA was isolated from *L. monocytogenes* isolates using the DNeasy Blood & Tissue Kit (Qiagen), and used for genome sequencing at the Earlham Centre, Norwich, UK. Libraries had an average insert size of ~600 bp and were generated using the Illumina NexteraXT kit, according to the manufacturer's instructions (Illumina UK, Cambridge). The libraries were used for high-throughput DNA sequencing using the Illumina MiSeq with 250 nt paired end reads. Reads were QC-ed, trimmed and adaptors removed using standard protocols. Genome sequences were assembled using SPAdes 3.6.2 (Bankevich et al. 2012) with assembly statistics generated by Quast v. 2.0 (Gurevich et al. 2013). Genomes were annotated using Prokka v. 1.11b (Seemann 2014). Core genome single nucleotide polymorphisms (SNPs) were identified using the parSNP program v. 1.2 from the Harvest suite (Treangen et al. 2014) with the "-a 13 -

c -x" switches, and used for the generation of phylogenetic trees using the default settings of parSNP. Multilocus sequence typing (MLST) was performed *in silico* using the scheme provided by the Institut Pasteur (http://bigsdb.pasteur.fr/listeria/listeria.html), with Figtree (http://tree.bio.ed.ac.uk/software/figtree/) used for annotation of phylogenetic trees. After clustering, genome sequences were compared using Roary (Page et al. 2015) for pangenome analysis with Scoary (Brynildsrud et al. 2016) for statistical evaluation, and Mauve (Darling et al. 2004) for direct comparison. PHASTER (Arndt et al. 2016) was used for assessment on whether prophages in the *L. monocytogenes* genomes were intact, questionable or incomplete, using the default settings. The FASTQ reads, isolate information and genome assemblies have been uploaded to the European Nucleotide Archive (ENA) at the EMBL-EBI (http://www.ebi.ac.uk/ena), as project PRJEB19211. Accession numbers for ENA (reads and assemblies) are provided in Supplementary Table S3.4.

3.3 Results

3.3.1 Persistent isolates form more biofilm at 30°C

We evaluated the ability of the *L. monocytogenes* isolates to produce biofilm by the crystal violet assay and determined the number of viable biofilm cells by plating. Generally, biofilms are composed of living cells, dead cells and extracellular polymeric substance including carbohydrates, DNA and lipids. Crystal violet attaches to negatively charged surface molecules and measures the whole biofilm mass including all of the above mentioned. Viable biofilm cells are of special risk as they are the ones capable of reproducing and contaminating other surfaces and foods. Combining both methods gives a good indication of *L. monocytogenes* persistent and sporadic isolates biofilm formation capability and associated contamination risk.

Biofilm formation was studied at two different temperatures (20°C and 30°C) after 24h and 48h incubation. At 20°C biofilm mass was greater after 48 h compared to 24h incubation for all isolates (Figure 3.2 a). However, biofilm mass was relatively low with all OD_{595nm} values ≤ 0.066 after 24 h. Viable cell counts ranged between 3.83 and 4.94 log_{10} CFU/well after 24 h incubation and between 3.31 and 5.82 log CFU/well for 48 h incubation (Figure 3.2. a). Prolonged incubation times resulted in a marginal increase in the number of viable cells but greater increases in biomass (Figure 3.2). The average for all measurements after 48 h was 0.28 log CFU/well higher than after 24 h incubation. Log_{10} counts were higher after 24 h incubation compared to 48 h incubation for some isolates, which could be due to increased cell death by 48 h.

At 30°C, biofilm mass and cell counts were greater than at 20°C for all isolates tested. The average mean for the cell counts was 0.94 $\log_{10}$ CFU/well higher after 48 h incubation compared with 24 h (Figure 3.2 b). All 16 isolates had more viable cells present in their biofilm after 48 h incubation. Strain 15G01 exhibited an interesting phenotype with $\log_{10}$-counts not changing significantly between 24 and 48 h incubation but crystal violet staining showing a major increase after 48 h incubation compared with 24 h (94.08% increase). This might be due to increased death of part of the population as cells move into late stationary/death phase (death rate equalling growth rate) or increased production of extracellular polymeric substance with increasing incubation time or a combination of both.

Comparing the dataset for the persistent isolates with the sporadic isolates (Supplementary Table S3.1), ANOVA revealed a significant difference between persistent and non-persistent isolates; the number of cells in the biofilm was higher after 48 h incubation at 30°C for the persistent isolates (average of 6.68 $\log_{10}$ CFU/well for the persistent isolates compared with 6.33 $\log_{10}$ CFU/well for the sporadic isolates; $p = 0.031$). Overall, biofilm formation increased for both the sporadic and persistent isolates with increasing incubation time and temperature.

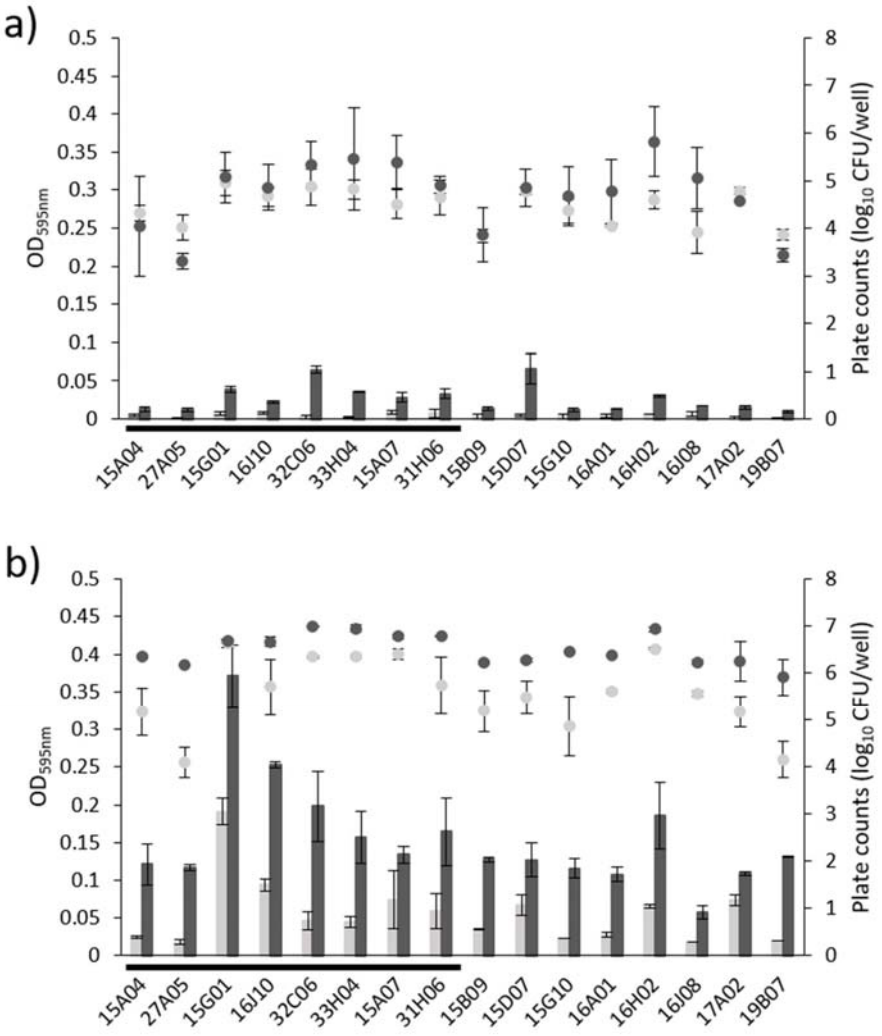

Figure 3.2. Biofilm formation of sporadic and persistent *L. monocytogenes* isolates measured through crystal violet staining (bars) and plate count cell enumeration (dots) at 20°C (a) and at 30°C (b) after 24 h (light grey bars and dots) and 48 h (dark grey bars and dots) incubation in Brain Heart Infusion Broth. Underlined strains are persistent. Isolates of the same pulsotype are found next to each other. Error bars represent standard deviation of 2 independent experiments with n=2 for the crystal violet staining and n=5 for the plate count cell enumeration. No visible error bars are due to low standard deviation.

3.3.2 Persistence is not correlated to motility

The involvement of flagella and motility in *L. monocytogenes'* attachment to surfaces has been shown in previous research (Lemon et al. 2007, Todhanakasem and Young 2008, Vatanyoopaisarn et al. 2000). Therefore motility of the 16 strains was measured. All tested isolates were motile and showed higher motility at 30°C compared with 20°C, which could be due to higher growth rate at 30°C. Six out of 16 isolates were motile at 37°C, with two of the six

isolates being persistent. The difference in motility between 20°C and 30°C and the interaction (temperature/strain) was significant ($p<0.05$), however, the proportion of variability was comparatively small, indicating the differences were not very large (the 20°C and 30°C results had a correlation of 0.68). The mean motility was higher for sporadic isolates compared to persistent isolates at 20°C and 30°C (Supplementary Table S3.2). Testing whether there was a difference between persistent and sporadic isolates, against the variation between isolates, indicated no significant main effect ($F = 1.2$ on 1 and 14 df, $p = 0.294$) and no significant persistent x temperature interaction ($F = 1.6$ on 1 and 14 df, $p = 0.224$) (Supplementary Figure S3.1).

3.3.3 Heat resistance might contribute to persistence

Heat resistance was examined by exposing all 16 isolates to 58°C heat for 5 min. The short-term effect of heat exposure on the (non)persistent isolates was investigated by determining the fraction of membrane-damaged cells with propidium iodide (PI) using flow cytometry and the long-term effect was measured through cell plating.

Notably, for seven out of eight persistent isolates (all except 27A05), the fraction of non-stained cells indicating intact (non-damaged) membranes assayed 5 min after heat treatment, was higher than 90%, whereas only three out of eight sporadic isolates showed the same effect (Figure 3.3 a). After 2 h only four out of eight persistent isolates and the same three out of eight sporadic isolates showed the same effect.

Plate counts were determined before heat treatment and 5 min and 2 h after heat treatment, to determine whether recovery occurs. Heat treatment for 5 min at 58°C led to a decrease in cell numbers for all isolates. Three isolates (15G01, 15A07, 17A02) showed highest heat resistance with the surviving fraction after 5 min being approximately 43.7 %, 56.5 %, and 50.3 %, respectively (Figure 3.3 b). The first two of the three belong to the persistent group. The sporadic isolate 16H02 and the persistent isolate 16J10 showed a heat resistance of 26.78% and 28.02%, respectively. All the remaining isolates exhibited survival below 10% of the original population after heat treatment (Figure 3.3 b).

The difference between 5 min after heat treatment and 2 hours after heat treatment was examined to determine whether any strains are capable of recovering in that timeframe after initial heat damage. The results showed that there was no significant difference (Figure 3.3 b) ($p=0.232$, ANOVA), between 5 min after heat treatment and 2 h post treatment. However, the difference between 0 and 5 min was significant for 12 of the 16 isolates examined ($p=0.005$,

one-tailed t-test). Four isolates showed no significant decrease in cell numbers 5 min after heat treatment, hence are more heat-resistant. Three (15G01, 16J10, 15A07) of the four isolates were persistent.

The three most heat-resistant isolates (15A07, 15G01 and 17A02) were isolated from the environment of the bale storage area or from the external areas of the factory, so had not been exposed to any heat prior. However, they could represent contamination of these areas from strains present within the plant. The other two isolates that exhibited higher heat resistance (16H02 and 16J10) were isolated in the opening area just after the heating step so may have gone through heat treatment if introduced before the opening step, but they also might have been introduced from outside sources.

Flow cytometry assesses recovery based on staining damaged cells, whereas cell plating measures viable culturable cells. The high percentage of intact cells after heat treatment measured through flow cytometry and the lower levels of recovered cells measured through plating suggest that significant irreversible damage occurs.

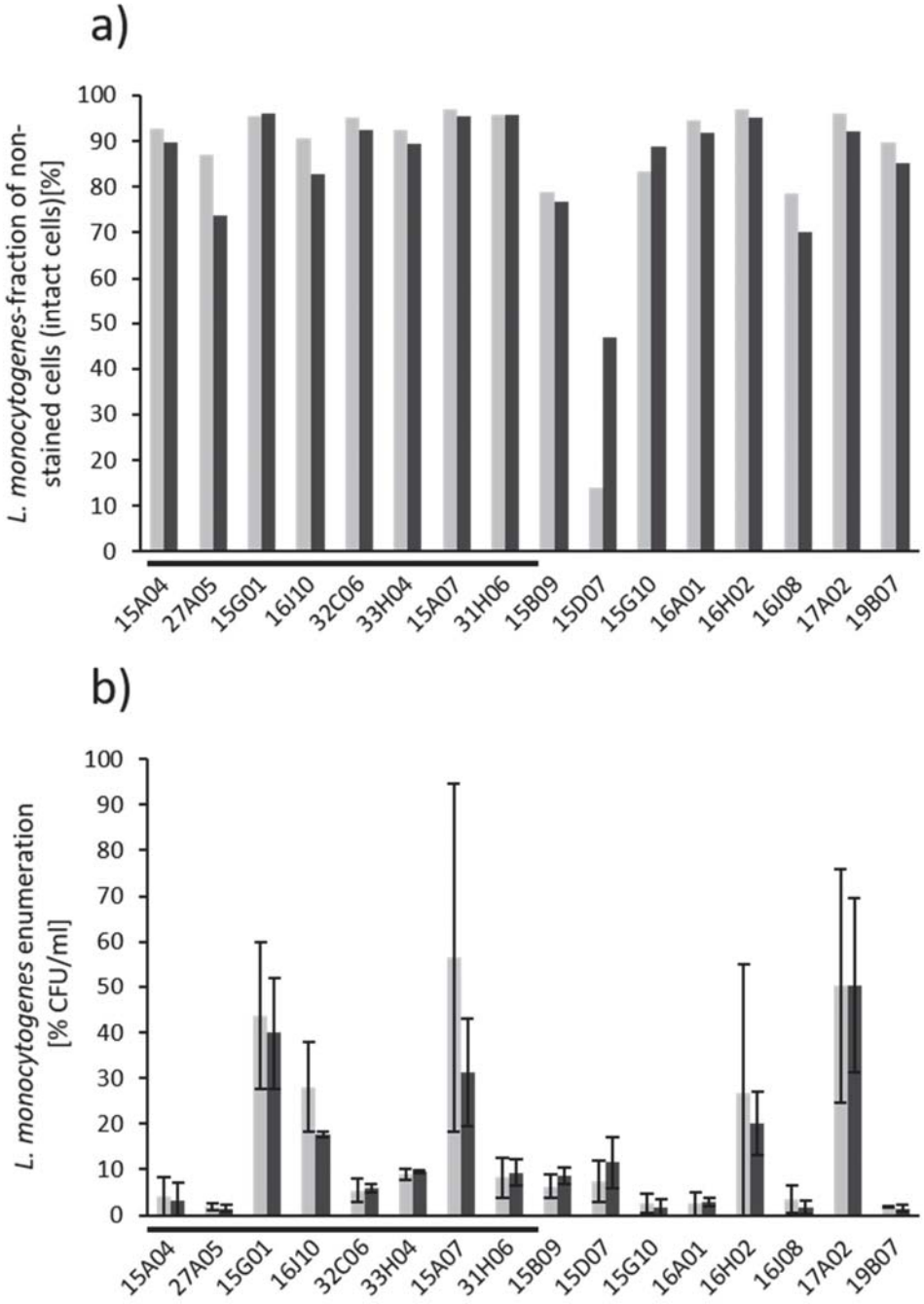

Figure 3.3. *Listeria monocytogenes* enumerated 5 min and 2 h after heat treatment at 58°C for 5 min expressed as fraction of non-damaged cells assessed by flow cytometry (a) (grey bars showing results for 5 min and black bars for 2 h after heat treatment) and expressed as percentage of those present before the heat treatment for cell plating (b) (CFU/mL, grey bars 5 min after heat treatment and black bars 2 h after heat treatment). Persistent strains are grouped together under the black bars shown on the left side of the axes.

3.3.4 Persistent biofilm cells survive better than persistent planktonic under dry conditions

Two experiments were run, testing 16 isolates comparing numbers of biofilm cells versus planktonic cells on dry surfaces over a period of 14 days. Colony forming units (CFU) were counted at days 0, 1, 2, 5, 7 and 14.

Biofilm and planktonic cell numbers decreased sharply on day 1 compared with the initial concentration (Table 3.2) for all isolates. The mean reduction for biofilm and planktonic cells were 1.83 $\log_{10}$ CFU/well and 1.93 $\log_{10}$ CFU/well respectively after 1 day. Cell numbers decreased continuously over subsequent time periods.

The cell numbers were higher for planktonic cells (Table 3.2) due to methodology so the changes from day 0 were analysed for all groups. Biofilm cells of the persistent isolates survived better than the planktonic cells after 7 d and 14 d ($p \leq 0.05$). However, there was no difference for the biofilm and planktonic cells of the sporadic isolates. When comparing the sporadic with the persistent isolates only one significant difference was observed; biofilm cells of the persistent cells survived better at day 2 compared to biofilm cells of the sporadic isolates. No significant difference was found at any other sampling date. Planktonic cells of all isolates tested showed reductions between 3.01-5.29 $\log_{10}$ CFU/well after 14 d incubation on dry surfaces, whereas biofilm cells exhibited reductions ranging between 2.57-5.05 $\log_{10}$ CFU/well after 14 d. Comparing persistent versus sporadic isolates, the planktonic cultures of the sporadic isolates showed the highest reductions (3.76-5.29 $\log_{10}$ CFU/well after 14 d) and biofilm cells of the persistent isolates showed the lowest reductions (2.57-4.12 $\log_{10}$ CFU/well) (Table 3.2 and Supplementary Table S3.3).

Table 3.2. Enumeration of planktonic and biofilm cells obtained from persistent and sporadic *L. monocytogenes* isolates (see materials and methods) after incubation of cells on dry surfaces (96-well plates) for 1, 2, 5, 7 and 14 d at 25°C. After incubation the cells were resuspended in PBS and counted at the times indicated. Mean counts for each isolate (n=5 per isolate for two independent experiments) are shown as Log_{10} values with the standard deviation shown in brackets.

	persistent		**sporadic**	
Days on dry surface	planktonic	biofilm	planktonic	biofilm
0	7.364 (0.142)	5.996 (0.838)	7.263 (0.148)	6.140 (0.640)
1	5.587 (0.703)	4.457 (0.808)	5.185 (0.489)	4.014 (0.767)
2	5.350 (0.540)	4.078 (0.573)	4.949 (0.429)	3.299 (0.824)
5	5.005 (0.499)	3.512 (0.597)	4.633 (0.454)	3.103 (0.770)
7	3.950 (0.466)	3.161 (0.618)	3.636 (0.439)	2.746 (0.519)
14	3.459 (0.676)	2.805 (0.678)	2.836 (0.697)	2.274 (0.977)

3.3.5 The two persistent isolates of each pulsotype behaved similarly

Two representatives for each persistent pulsotype (Table 3.1) were subjected to phenotype analyses. The representatives of each pulsotype behaved similar (Figures 3.2 and 3.3). This supports the use of PFGE typing as a method of selecting persistent strains. The exception was 15A07 which had higher heat resistance by the plate count method than 31H06. This was surprising in that 31H06 was isolated from the heating area whereas 15A07 was from the bale store.

The selected representatives of pulsotype 3814 were isolated from two different processing plants, and this pulsotype was also isolated from a third factory (Cruz and Fletcher 2011), suggesting that the strain has been introduced from a common source, e.g., raw material. Both selected strains had been isolated from the heating areas (outlined in Figure 3.1) as had most (13) isolates of this pulsotype (Cruz and Fletcher 2011). However, both tested isolates were rather less heat-resistant compared to the other persistent strains (Figure 3.3). This pulsotype was widely distributed in factories with three isolated from an external area, two from bale stores, one from an opening area and two from a packing area (Cruz and Fletcher 2011).

The selected representatives of pulsotype 5132 were isolated in the opening area and the external area of the same plant. Strains of this pulsotype had been regularly isolated in these areas (four isolates from the external area and 15 from the opening area) but was also found in the packing area of the plant (five isolates) and the opening area (two isolates) and in product (one isolate) from another nearby plant (Cruz and Fletcher 2011). This strain might have been introduced from the outside environment possibly through the raw product or bags for mussel storage. It is likely that this strain made it through the processing line into the opening and packing areas (Figure 3.1) surviving the heat treatment, suggesting high heat resistance. Both isolates belonging to pulsotype 5132 exhibited high viable cell numbers after heat treatment (two of the three highest heat-resistant strains determined by plating).

The two selected isolates belonging to pulsotype 6502 were isolated from the bale storage and the opening area of the same plant and eight other isolates with the same pulsotype had been obtained in these locations but none from the other plants (Cruz and Fletcher 2011). This suggests introduction from an outside source from the environment.

The two representatives of the pulsotype 5588 were isolated from the same factory in the opening area and heating area and the pulsotype has been found in numerous other locations

in that factory including product, suggesting survival of the isolates through heat treatment and contaminating the opening area.

3.3.6 Comparative genomics of persistent and sporadic *L. monocytogenes* isolates

The draft genome sequences of the eight persistent and eight sporadic isolates were determined using Illumina sequencing technology with 250 nt paired end reads. The genome sizes varied between 2.9-3.3 Mbp, consistent with previously released *L. monocytogenes* genomes. The phylogenetic relationship between the 16 genomes was analysed using phylogenomic clustering based on core genome single nucleotide polymorphisms (SNPs) (Figure 3.4). The 16 isolates were all lineage II genomes, and except of isolate 16J08 which clustered on its own, aligned into four distinct clusters of five, two, three and five genomes (labelled cluster 1-4 in Figure 3.4). Clusters 1, 3 and 4 contained both persistent and sporadic isolates, with cluster 2 only containing persistent isolates. The genomes within the clusters showed virtually no differences in the core genome SNPs, shown by the lack of further sub-branching within the clusters. The clusters each consisted of genomes with a separate MLST sequence type, with cluster 1 consisting of ST-321, cluster 2 of ST-204, cluster 3 of ST-120 and cluster 4 of ST-399, with 16J08 being ST-31.

All genomes were provisionally annotated using Prokka (Seemann 2014), and pangenome analysis using Roary (Page et al. 2015) was used to search for genes or markers associated with persistence or non-persistence within the 16 genomes, or markers associated with specific factories from which the isolates were obtained. We were unable to detect such markers for either group, consistent with previous reports where persistence and virulence of *L. monocytogenes* could not be linked to specific genetic markers (Fagerlund et al. 2016, Fang et al. 2016, Stasiewicz et al. 2015). As there were differences in pulsotypes within the four clusters, we used a combination of comparative genomics utilities to identify the genomic changes that may account for the differences in pulsotypes between the genomes within each cluster, using the multiple genome aligner Mauve (Darling et al. 2004). Within the ST-321 genomes in cluster 1 were two persistent isolates and three sporadic isolates isolated from factories B, C and E.

The primary differences were in mobile genetic elements, with isolates 16H02 and 15G01 lacking a ϕtRNA-Ser prophage, 16H02 lacking a ϕtRNA-Leu prophage, and 16H02 having a transposon with a restriction-modification system lacking in the other four genomes (Fagerlund et al. 2016) (not shown in Figure 3.4). The two ST-204 isolates from Cluster 2 were virtually identical, both persistent and from the same factory (D), but isolate 33H04 carries a ϕ*tufA* prophage, while isolate 32C06 contains plasmid contigs also found in cluster 1. The ST-120

isolates from cluster 3 all came from factory A, with two out of three isolates persistent (15A07 and 31H08). These differed from sporadic isolate 17A02 by lacking ϕtRNA-Ser and ϕtRNA-Arg prophages, as well as differences in the specificity subunits of a *hsd* Type I restriction modification system (not shown), and having an inactivated gene encoding a putative surface-located protein (not shown). Finally, the ST-399 genomes in cluster 4 included isolate 16A01 from an outbreak in 1992, and this genome lacked a ϕRNA-methyltransferase prophage, and the ϕtRNA-Asn prophage in 16H02 was scored as questionable (i.e. possibly incomplete) while it scored as intact in the four other isolates. When compared to the pulsotypes of the isolates, the differences in these 30-50 kb prophages could in part explain differences in pulsotypes, for instance in cluster 3, but not in clusters 1, 2 and 4.

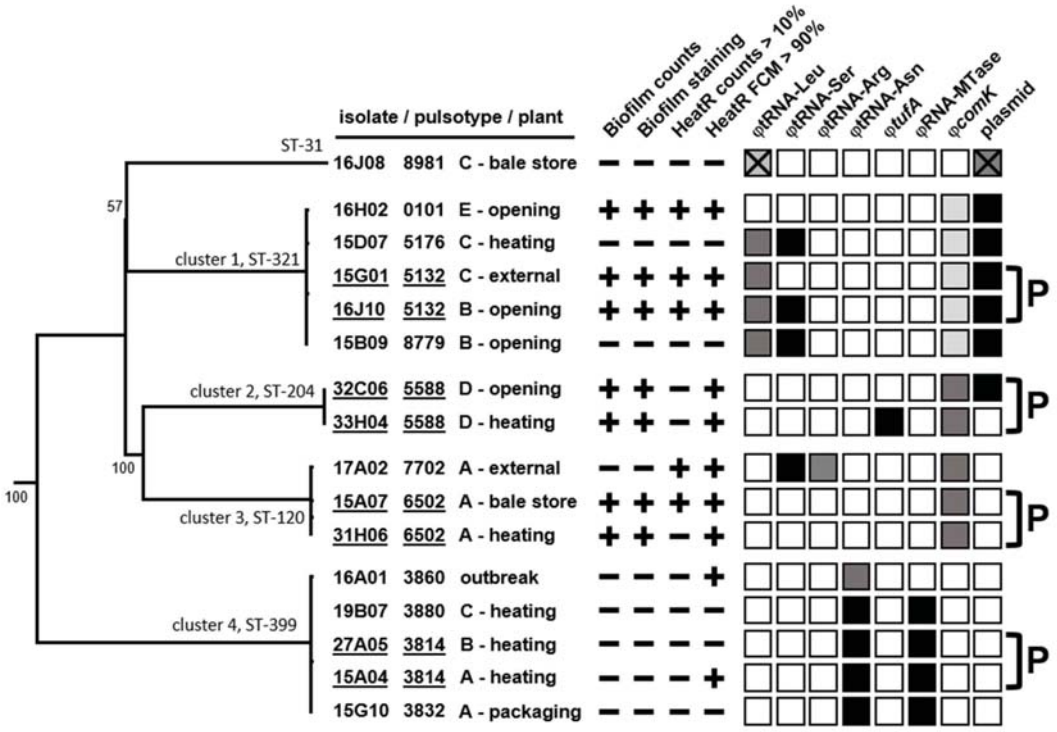

Figure 3.4. Whole-genome comparison of persistent and non-persistent *L. monocytogenes* isolates demonstrates persistence is not associated with a specific genetic lineage, and does not allow identification of genetic markers contributing to persistence in the isolates investigated. The phylogenetic tree on the left is based on core genome single nucleotide polymorphisms identified with the ParSNP program, with bootstrap values indicated at the branching points. The code of the isolates, pulsotypes and the plant and location of isolation are indicated, with underlined isolate codes showing persistent isolates. The clusters and the MLST sequence type are indicated in the tree, demonstrating that persistence is not associated with a specific genetic lineage. The plus and minus symbols indicate level of biofilm formation based on viable counts and crystal violet staining, whereas HeatR represents heat resistance, and FCM flow cytometry. Isolates that produced higher biofilm counts and biofilm staining than the median at 30°C are labelled with + (plus) and isolates that produced lower biofilm counts and staining are labelled with – (minus). Six of the eight persistent isolates showed high results in at least three of the four shown tests whereas the sporadic isolates exhibited lower biofilm formation and heat resistance. The sporadic isolate 16H02 was an outlier and might be in the process of becoming persistent. The boxes on the right indicate differentially present mobile genetic elements (prophages and putative plasmids) in these isolates. The φ code indicates the genomic locus where the prophage is inserted. The shading of the prophage boxes indicates whether it is an intact (black), questionable (dark grey) or incomplete prophage (light grey), based on the analysis performed using PHASTER. The crossed boxes for isolate 16J08 highlight that although it does have a prophage at the tRNA-Leu locus, it differs from the prophages of cluster 1 in the same location. Similarly, the putative plasmid-contigs are show significant sequence difference with those of clusters 1 and isolate 32C06 of cluster 2. There were no prophages or plasmids specifically associated with persistence. Minor genetic changes were observed (not shown), but again did not correlate with persistence, but only with genetic background/clusters.

3.4 Discussion

Persistence of pathogenic bacteria in a food-processing environment causes a health risk for the consumer of the food products and a high financial risk for the producer, thus constituting both a public health and economic concern. Identification of phenotypic and/or genotypic features of pathogenic bacteria that favour persistence has been the focus of several research groups across the world. In this study, we analysed phenotypic and genotypic features of *L. monocytogenes* to identify differences between sporadic and persistent strains isolated from a mussel-processing environment to determine key factors that might contribute to persistence in food-processing plants.

In this study eight persistent strains isolated from four different factories and 8 sporadic strains from four different sites (three factories identical with the persistent isolation) including one outbreak isolate were subject to phenotypic assays and genomic analysis.

We observed that biofilm formation was significantly greater at 30°C compared with 20°C for persistent isolates. In a previous study by Cruz and Fletcher (2011), where some of the same isolates used in this study were investigated, persistence could not be linked to biofilm formation using TSBYE medium (Cruz and Fletcher 2011). However,in the current study BHI medium was used, as it is a commonly used medium for *L. monocytogenes* studies and a nutrient-rich medium that was previously shown to promote *L. monocytogenes* biofilm formation (Nowak et al. 2015). Differences in biofilm formation might be due to nutrient availability and composition as discussed in our previous work (Nowak et al. 2015) and others (Kadam et al. 2013).

A recent study focused on growth behaviour of 31 persistent (repetitive isolation over 4-5 years for small-scale industrial cheese producer and 8 – 15 months artisanal raw ewes' milk cheese producer) and 10 sporadic *L. monocytogenes* strains at different temperatures (4°C, 22°C, 37°C) (Magalhaes et al. 2016). Average growth rates for persistent strains were found to be higher at 22°C compared to sporadic strains, which highlights the fact that certain temperatures (22°C) may favour bacterial persistence.

In the present study persistent isolates were found to form significantly greater amounts of biofilm at 30°C after 48 h incubation determined by plate counts. Nakamura et al. (2013) analysed biofilm formation of persistent and transient strains at 32°C with the crystal violet assay and viable cell counts using Modified Welshimer's Broth (MWB) as the medium (Nakamura et al. 2013). They found that persistent strains formed significantly more biofilm

than transient strains after 48 h incubation and that there was no significant difference after 24h incubation. Viable cell counts were not significantly different for persistent and transient strains in Nakamura's study, however, we noted significant higher viable cell counts after 48h incubation for persistent strains. Furthermore, Borucki et al. (2003) found that persistent strains (n=11) form more biofilm than sporadic strains (n=15) (p=0.027) after 40 h incubation at 30°C determined by the crystal violet assay in MWB (Borucki et al. 2003). Ochiai et al. (2014) investigated biofilm-forming ability of persistent strains at 30°C and 37°C using the crystal violet assay and showed that persistent strains did not form more biofilm at 30°C, but at 37°C (Ochiai et al. 2014).

However, some other research groups found no difference between biofilm-forming ability of persistent and sporadic strains which might be due to differences in media and growth conditions as discussed, next to strain variability (Djordjevic et al. 2002, Nilsson et al. 2011)

In the present study we evaluated the recovery/survival of persistent and sporadic isolates from dry surfaces. Not surprisingly, we observed a reduction in cell numbers with increasing incubation for persistent and sporadic isolates. Planktonic cells showed a reduction of 3.01 – 5.29 $\log_{10}$ CFU/ well after 14 d. Survival of planktonic cells of *L. monocytogenes* on dry surfaces (coated and uncoated stainless steel coupons) has been tested previously (Møretrø et al. 2013, Takahashi et al. 2011). Møretrø et al. (2013) found that there was a 1.5-2 $\log_{10}$ reduction per coupon for bacteria after 7 and 14 days, when incubated at 12°C. Takahashi et al. (2011) coated coupons with a sterile food filtrate (minced tuna, cabbage or ground pork) and tested survival of *L. monocytogenes* Scott A at 25°C compared to survival on uncoated stainless steel coupons. After 15 d, cell numbers reduced from an initial concentration of 10^7 CFU/coupon to ~3 $\log_{10}$ CFU/coupon on uncoated coupons, which is consistent with our findings. On coated coupons cell numbers reduced to ~3.5-5.5 $\log_{10}$ CFU/coupon after 15 d and after 30 d to 2.32-3.7 $\log_{10}$ CFU/coupon. Castelijn et al. (2013) examined survival of *Salmonella* spp. on dry surfaces and observed that survival of biofilm cells is higher than survival of the planktonic counterpart. We observed a difference between planktonic and biofilm cells of persistent isolates, but no difference between sporadic and persistent isolates. Survival on dry surfaces has also been tested for *Acinetobacter baumannii* where biofilm-forming and non-biofilm-forming strains were compared (Espinal et al. 2012). Biofilm-forming strains survived longer than the non-biofilm formers in dry conditions (36 days versus 15 days). We observed a reduction in cell numbers over time for all isolates tested, but no significant differences in survival between sporadic and persistent isolates, except for day 2. Biofilm cells showed a steady decline in cell

numbers up until day 14 whereas planktonic cells had a sharp decline from day five to seven. It could be considered useful to test survival after longer incubation time (up to 30 d), however, previous studies could not observe a further reduction from 15 d to 30 d (Takahashi et al. 2011).

In the present study we also investigated heat resistance but there was no consistent difference between persistent and sporadic isolates observed after heat treatment at 58°C for 5 min although two of the three most heat-resistant isolates were classified as persistent. Lunden et al. (2008) analysed 17 persistent and 23 non-persistent strains for heat resistance and found no significant difference in survival of persistent and sporadic strains (55°C, 40 min), which is consistent with our findings. In the present study heat resistance was investigated at 58°C in a model setting to evaluate overall robustness of the sporadic versus the persistent isolates. The majority of the isolates had been sampled after the heating stage of the processing line. Heat treatment at a sub-lethal level might encourage cross-protection, which could result in higher resistance towards other environmental stresses, including sanitisers and cleaning reagents. Cross-protection has been reported for several bacterial species, where bacterial cells become more resistant to different environmental stresses after being exposed to another stress, e.g. higher heat resistance after alkali stress exposure for *Salmonella enteritidi*s (Inagaki et al. 2009) and *Enterococcus faecalis*. *L. monocytogenes* also showed increased H_2O_2 resistance after exposure to salt stress (Bergholz et al. 2012) and exhibited higher heat resistance after exposure to other environmental stresses (Lou and Yousef 1996).

We also used genome sequencing to assess whether there were any genetic markers that could distinguish persistent and sporadic isolates used in this study. The 16 isolates used here were from five different MLST sequence types, with four of the five sequence types containing both persistent and sporadic isolates. While the isolates within each sequence type contained only a few core genome SNPs which did not lead to major differences in genes predicted, there were differences in the accessory genome, especially mobile genetic elements such as prophages and plasmids (Figure 3.4). As the prophages and plasmids are relatively large fragments (30-50 kb of sequence each), they could be large enough to affect the PFGE banding pattern and hence result in a different pulsotype. However, there was no clear association between the prophages/plasmids with persistence or pulsotype observed (Figure 3.4). This is consistent with several recent studies on genomics of *L. monocytogenes*, where significant variations in prophages have been observed, but were not linked to specific phenotypes (Fagerlund et al. 2016, Fang et al. 2016, Stasiewicz et al. 2015). This strongly suggests that the phenotypic differences are not caused by single mutations, phages or other genetic differences, but could

be unique to distinct *L. monocytogenes* lineages, or multifactorial combining phenotypic stimuli and genetic background.

In food production plants such as mussel-processing facilities, products undergo several treatment steps including washing and cooling as well as heating, before being packed for export. Persistent isolates produced more biofilm and showed higher heat resistance in some cases. Additionally, biofilm cultures of persistent isolates survived better than the planktonic cultures after prolonged incubation time emphasising the need to critically evaluate and implement adequate cleaning procedures to eliminate the risk for product contamination by persistent *L. monocytogenes.*

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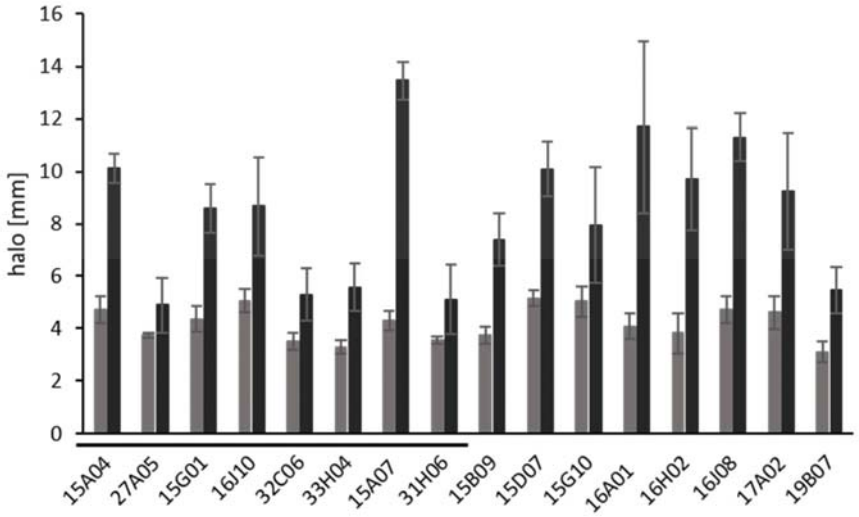
3.6 Supplementary Information



Supplementary Figure S3.1. Motility of the eight persistent and eight sporadic *Listeria monocytogenes* isolates after 24 h incubation at 20°C (light grey bars) and at 30°C (dark grey bars) measured in semi-solid agar plates. Error bars represent the mean of three technical replicates. Underlined strains are persistent.

Supplementary Table S3.1. Means and standard deviations (in brackets) of biofilm formation of the persistent and sporadic *L. monocytogenes* strains measured by the crystal-violet assay and cell enumeration at 20°C and 30°C after 24 and 48 h.

Biofilm formation			**Crystal violet assay**	**Cell enumeration**
	Temperature (°C)	incubation (h)	OD_{595nm}	Log_{10} CFU/well
persistent n=8	20	24	0.005 (0.003)	4.597 (0.311)
		48	0.031 (0.017)	4.798 (0.749)
	30	24	0.069 (0.055)	5.809 (0.839)
		48	0.190 (0.086)	6.676 (0.284)
sporadic n=8	20	24	0.004 (0.002)	4.273 (0.407)
		48	0.022 (0.019)	4.628 (0.727)
	30	24	0.041 (0.024)	5.323 (0.676)
		48	0.120 (0.036)	6.332 (0.288)

Supplementary Table S3.2. Motility of persistent (n=8) and sporadic (n=8) *L. monocytogenes* isolates after 24h incubation at 20°C and 30°C.

Motility	**20°C**		**30°C**	
	halo [mm]	SD	halo [mm]	SD
Persistent	4.05	0.63	7.70	3.07
Sporadic	4.27	0.71	9.10	2.09

Supplementary Table S3.3. Survival on dry surfaces of persistent and sporadic *L. monocytogene*s isolates before and after 1, 2, 5, 7 and 14 d incubation at 25°C. The experiment was performed in duplicate with n=5. Persistent strains are labelled by an asterisk.

Survival on dry surfaces [log_{10} CFU/well]												
Day / Strain	Planktonic cells						Biofilm cells					
	0	1	2	5	7	14	0	1	2	5	7	14
15A04*	7.25	4.40	4.59	4.48	3.54	2.49	5.24	3.69	3.22	2.71	2.52	2.19
SD	0.04	0.41	0.37	0.29	0.60	1.12	0.51	0.22	0.74	0.04	0.58	0.34
27A05*	7.14	4.78	4.41	3.98	3.01	3.16	4.50	2.87	3.28	2.60	2.39	1.72
SD	0.09	0.74	0.89	0.76	0.52	0.29	0.35	0.38	0.06	0.26	0.22	0.34
15G01*	7.37	5.89	5.70	5.35	4.31	3.25	6.66	5.42	4.87	4.10	4.34	3.25
SD	0.20	0.18	0.43	0.64	0.64	1.26	0.29	0.20	0.06	0.37	0.06	0.15
16J10*	7.46	5.57	5.55	5.26	4.09	2.97	6.98	5.06	4.29	4.03	3.34	3.87
SD	0.07	0.59	0.72	0.55	0.94	1.38	0.04	0.17	0.34	0.47	0.06	0.16
32C06*	7.28	5.92	5.63	5.16	3.98	4.27	5.85	4.69	4.51	3.76	3.54	3.08
SD	0.19	0.59	0.88	0.87	0.42	0.36	0.64	0.04	0.15	0.34	0.21	0.11
33H04*	7.35	5.70	5.77	5.18	3.98	4.33	5.75	4.58	4.27	4.05	3.24	3.18
SD	0.19	0.59	0.69	0.80	0.57	0.18	0.32	0.23	0.35	0.33	0.02	0.04
15A07*	7.49	5.79	5.41	5.34	4.43	3.13	6.75	4.75	4.23	3.57	2.98	2.62
SD	0.08	0.07	0.54	0.91	0.34	0.93	0.20	0.19	0.37	0.07	0.32	0.17
31H06*	7.57	6.66	5.74	5.29	4.25	4.07	6.23	4.60	3.96	3.28	2.93	2.53
SD	0.18	1.39	1.16	1.13	0.50	0.64	0.81	0.64	0.57	0.14	0.38	0.21
15B09	7.07	4.56	4.29	4.11	3.13	1.78	5.81	3.12	2.04	1.63	2.07	0.75
SD	0.25	0.01	0.56	0.58	0.70	1.81	1.27	0.12	1.04	0.21	0.16	0.35
15D07	7.22	4.95	5.01	4.92	3.60	2.85	6.09	4.63	3.27	2.99	2.90	1.50
SD	0.06	0.08	0.64	0.72	1.15	1.08	0.30	0.09	0.90	0.77	0.54	0.71
15G10	7.33	5.36	4.93	4.37	3.58	2.42	6.11	3.65	2.68	3.18	2.36	1.78
SD	0.13	0.08	0.68	0.94	0.53	1.58	0.65	0.15	0.45	0.10	0.03	1.11
16A01	7.05	4.65	4.70	4.10	3.07	2.09	6.52	3.33	3.12	3.20	2.27	2.33
SD	0.10	0.18	0.67	0.14	1.12	1.12	0.03	0.18	0.39	0.57	0.05	0.10
16H02	7.33	5.96	5.70	5.23	4.36	3.35	7.23	4.96	4.51	4.00	3.48	3.42
SD	0.04	0.38	0.88	0.80	0.61	1.14	0.11	0.45	0.21	0.36	0.03	0.47
16J08	7.26	5.05	4.74	4.41	3.65	2.94	6.50	4.00	3.06	3.16	3.20	3.43
SD	0.03	0.33	0.88	0.46	1.14	1.22	0.14	0.43	0.65	0.36	0.85	0.16
17A02	7.49	5.71	5.37	5.20	4.13	3.73	5.80	5.03	4.41	4.05	3.20	3.11
SD	0.19	0.33	0.94	0.89	0.86	0.21	0.17	0.24	0.10	0.79	0.33	0.41
19B07	7.36	5.25	4.85	4.73	3.57	3.53	5.06	3.38	3.30	2.61	2.50	1.87
SD	0.27	0.75	0.84	0.55	1.01	0.39	0.50	0.05	0.03	0.21	0.34	0.12

Supplementary Table S3.4. Accession numbers for genome assemblies and Illumina paired end sequencing reads. The sequencing reads and genome assemblies have been deposited in the European Nucleotide Archive (ENA) as part of BioProject PRJEB19211.

BioProject	Isolate	Sample code	Sequencing reads	Assembly	Contigs	Persistence
PRJEB19211	15A04	LDI10680	ERR1816990	GCA_900162275	FUIK01000001-FUIK01000018	Persistent
PRJEB19211	27A05	LDI10675	ERR1816985	GCA_900162135	FUJP01000001-FUJP01000018	Persistent
PRJEB19211	15G01	LDI10683	ERR1816993	GCA_900162555	FUIO01000001-FUIO01000023	Persistent
PRJEB19211	16J10	LDI14780	ERR1817086	GCA_900162285	FUJD01000001-FUJD01000027	Persistent
PRJEB19211	32C06	LDI10677	ERR1816987	GCA_900162495	FUKF01000001-FUKF01000018	Persistent
PRJEB19211	33H04	LDI10679	ERR1816989	GCA_900162345	FUKA01000001-FUKA01000023	Persistent
PRJEB19211	15A07	LDI14788	ERR1817093	GCA_900162245	FUIL01000001-FUIL01000028	Persistent
PRJEB19211	31H06	LDI14777	ERR1817095	GCA_900162415	FUKE01000001-FUKE01000028	Persistent
PRJEB19211	16A01	LDI10686	ERR1816996	GCA_900162565	FUIW01000001-FUIW01000017	Non-persistent
PRJEB19211	15B09	LDI10681	ERR1816991	GCA_900162295	FUIJ01000001-FUIJ01000027	Non-persistent
PRJEB19211	15D07	LDI10682	ERR1816992	GCA_900162155	FUIN01000001-FUIN01000026	Non-persistent
PRJEB19211	15G10	LDI10684	ERR1816994	GCA_900162115	FUIU01000001-FUIU01000018	Non-persistent
PRJEB19211	16H02	LDI10687	ERR1816997	GCA_900162165	FUIV01000001-FUIV01000024	Non-persistent

PRJEB19211	16J08	LDI14782	ERR1817088	GCA_900162445	FUIR01000001-FUIR01000048	Non-persistent
PRJEB19211	17A02	LDI10689	ERR1816999	GCA_900162215	FUJA01000001-FUJA01000040	Non-persistent
PRJEB19211	19B07	LDI14766	ERR1817005	GCA_900162375	FUJI01000001-FUJI01000016	Non-persistent

3.7 References

Abdallah, M., C. Benoliel, D. Drider, P. Dhulster and N. E. Chihib (2014). Biofilm formation and persistence on abiotic surfaces in the context of food and medical environments. Arch Microbiol **196**(7): 453-472.

Abee, T., J. Koomen, K. I. Metselaar, M. H. Zwietering and H. M. den Besten (2016). Impact of pathogen population heterogeneity and stress-resistant variants on food safety. Annu Rev Food Sci Technol **7**: 439-456.

Allerberger, F. and M. Wagner (2010). Listeriosis: a resurgent foodborne infection. Clin Microbiol Infect **16**(1): 16-23.

Arndt, D., J. R. Grant, A. Marcu, T. Sajed, A. Pon, Y. Liang and D. S. Wishart (2016). PHASTER: a better, faster version of the PHAST phage search tool. Nucleic Acids Res **44**(W1): W16-21.

Autio, T., S. Hielm, M. Miettinen, A. M. Sjoberg, K. Aarnisalo, J. Bjorkroth, T. Mattila-Sandholm and H. Korkeala (1999). Sources of *Listeria monocytogenes* contamination in a cold-smoked rainbow trout processing plant detected by pulsed-field gel electrophoresis typing. Appl Environ Microbiol **65**(1): 150-155.

Bankevich, A., S. Nurk, D. Antipov, A. A. Gurevich, M. Dvorkin, A. S. Kulikov, V. M. Lesin, S. I. Nikolenko, S. Pham, A. D. Prjibelski, A. V. Pyshkin, A. V. Sirotkin, N. Vyahhi, G. Tesler, M. A. Alekseyev and P. A. Pevzner (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol **19**(5): 455-477.

Bergholz, T. M., B. Bowen, M. Wiedmann and K. J. Boor (2012). *Listeria monocytogenes* shows temperature-dependent and -independent responses to salt stress, including responses that induce cross-protection against other stresses. Appl Environ Microbiol **78**(8): 2602-2612.

Blaschek, H. P., H. H. Wang, A. L. Pometto III, A. Demirci and M. E. Agle (2015). Biofilms in the food environment, John Wiley & Sons.

Bonsaglia, E. C. R., N. C. C. Silva, A. Fernades, J. P. Araujo, M. H. Tsunemi and V. L. M. Rall (2014). Production of biofilm by *Listeria monocytogenes* in different materials and temperatures. Food Control **35**(1): 386-391.

Borucki, M. K., J. D. Peppin, D. White, F. Loge and D. R. Call (2003). Variation in biofilm formation among strains of *Listeria monocytogenes*. Appl Environ Microbiol **69**(12): 7336-7342.

Brett, M. S., P. Short and J. McLauchlin (1998). A small outbreak of listeriosis associated with smoked mussels. Int J Food Microbiol **43**(3): 223-229.

Brynildsrud, O., J. Bohlin, L. Scheffer and V. Eldholm (2016). Rapid scoring of genes in microbial pan-genome-wide association studies with Scoary. Genome Biol **17**(1): 238.

Carpentier, B. and O. Cerf (2011). Review--Persistence of *Listeria monocytogenes* in food industry equipment and premises. Int J Food Microbiol **145**(1): 1-8.

Castelijn, G. A., J. A. Parabirsing, M. H. Zwietering, R. Moezelaar and T. Abee (2013). Surface behaviour of *S. typhimurium, S. derby*, *S. brandenburg* and *S. infantis*. Vet Microbiol **161**(3-4): 305-314.

Chen, C. Y., G. W. Nace and P. L. Irwin (2003). A 6 x 6 drop plate method for simultaneous colony counting and MPN enumeration of *Campylobacter jejuni*, *Listeria monocytogenes*, and *Escherichia coli*. J Microbiol Methods **55**(2): 475-479.

Cruz, C. D. and G. C. Fletcher (2011). Prevalence and biofilm-forming ability of *Listeria monocytogenes* in New Zealand mussel (*Perna canaliculus*) processing plants. Food Microbiol **28**(7): 1387-1393.

Darling, A. C., B. Mau, F. R. Blattner and N. T. Perna (2004). Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res **14**(7): 1394-1403.

Djordjevic, D., M. Wiedmann and L. A. McLandsborough (2002). Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl Environ Microbiol **68**(6): 2950-2958.

Espinal, P., S. Marti and J. Vila (2012). Effect of biofilm formation on the survival of *Acinetobacter baumannii* on dry surfaces. J Hosp Infect **80**(1): 56-60.

Fagerlund, A., S. Langsrud, B. C. Schirmer, T. Moretro and E. Heir (2016). Genome analysis of *Listeria monocytogenes* sequence type 8 strains persisting in salmon and Ppultry processing environments and comparison with related strains. PLoS One **11**(3): e0151117.

Fang, C., T. Cao, Y. Shan, Y. Xia, Y. Xin, C. Cheng, H. Song, J. Bowman, X. Li, X. Zhou and W. Fang (2016). Comparative genomic analysis reveals that the 20K and 38K prophages in *Listeria monocytogenes* serovar 4a strains Lm850658 and M7 contribute to genetic diversity but not to virulence. J Microbiol Biotechnol **26**(1): 197-206.

Ferreira, V., M. Wiedmann, P. Teixeira and M. J. Stasiewicz (2014). *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. J Food Prot **77**(1): 150-170.

Fletcher, G., M. Rogers and R. Wong (1994). Survey of *Listeria monocytogenes* in New Zealand seafood. J Aquat Food Prod T **3**(2): 13-24.

Garner, D. and S. Kathariou (2016). Fresh produce-associated listeriosis outbreaks, sources of concern, teachable moments, and insights. J Food Prot **79**(2): 337-344.

Gurevich, A., V. Saveliev, N. Vyahhi and G. Tesler (2013). QUAST: quality assessment tool for genome assemblies. Bioinformatics **29**(8): 1072-1075.

Inagaki, S., M. Matsumoto-Nakano, K. Fujita, K. Nagayama, J. Funao and T. Ooshima (2009). Effects of recombinase A deficiency on biofilm formation by *Streptococcus mutans*. Oral Microbiol Immunol **24**(2): 104-108.

Kadam, S. R., H. M. den Besten, S. van der Veen, M. H. Zwietering, R. Moezelaar and T. Abee (2013). Diversity assessment of *Listeria monocytogenes* biofilm formation: impact of growth condition, serotype and strain origin. Int J Food Microbiol **165**(3): 259-264.

Knudsen, G. M., J. E. Olsen and L. Dons (2004). Characterization of DegU, a response regulator in *Listeria monocytogenes*, involved in regulation of motility and contributes to virulence. FEMS Microbiol Lett **240**(2): 171-179.

Lemon, K. P., D. E. Higgins and R. Kolter (2007). Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. J Bacteriol **189**(12): 4418-4424.

Lou, Y. and A. E. Yousef (1996). Resistance of *Listeria monocytogenes* to heat after adaptation to environmental stresses. J Food Prot **59**(5): 465-471.

Lunden, J., R. Tolvanen and H. Korkeala (2008). Acid and heat tolerance of persistent and nonpersistent *Listeria monocytogenes* food plant strains. Lett Appl Microbiol **46**(2): 276-280.

Magalhaes, R., V. Ferreira, T. R. Brandao, R. C. Palencia, G. Almeida and P. Teixeira (2016). Persistent and non-persistent strains of *Listeria monocytogenes*: A focus on growth kinetics under different temperature, salt, and pH conditions and their sensitivity to sanitizers. Food Microbiol **57**: 103-108.

Martinez, M. R., J. Osborne, V. O. Jayeola, V. Katic and S. Kathariou (2016). Capacity of *Listeria monocytogenes* strains from the 2011 cantaloupe outbreak to adhere, survive, and grow on cantaloupe. J Food Prot **79**(5): 757-763.

Møretrø, T. and S. Langsrud (2004). *Listeria monocytogenes*: biofilm formation and persistence in food-processing environments. Biofilms **1**(2): 107-121.

Møretrø, T., S. Langsrud and E. Heir (2013). Bacteria on meat abattoir process surfaces after sanitation: characterisation of survival properties of *Listeria monocytogenes* and the commensal bacterial flora. Adv Microbiol **3**(03): 255.

Nakamura, H., K. Takakura, Y. Sone, Y. Itano and Y. Nishikawa (2013). Biofilm formation and resistance to benzalkonium chloride in *Listeria monocytogenes* isolated from a fish processing plant. J Food Prot **76**(7): 1179-1186.

Nilsson, R. E., T. Ross and J. P. Bowman (2011). Variability in biofilm production by *Listeria monocytogenes* correlated to strain origin and growth conditions. Int J Food Microbiol **150**(1): 14-24.

Nowak, J., C. D. Cruz, J. Palmer, G. C. Fletcher and S. Flint (2015). Biofilm formation of the *L. monocytogenes* strain 15G01 is influenced by changes in environmental conditions. J Microbiol Methods **119**: 189-195.

Ochiai, Y., F. Yamada, M. Mochizuki, T. Takano, R. Hondo and F. Ueda (2014). Biofilm formation under different temperature conditions by a single genotype of persistent *Listeria monocytogenes* strains. J Food Prot **77**(1): 133-140.

Page, A. J., C. A. Cummins, M. Hunt, V. K. Wong, S. Reuter, M. T. Holden, M. Fookes, D. Falush, J. A. Keane and J. Parkhill (2015). Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics **31**(22): 3691-3693.

Pan, Y., F. Breidt, Jr. and S. Kathariou (2006). Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. Appl Environ Microbiol **72**(12): 7711-7717.

Peel, M., W. Donachie and A. Shaw (1988). Temperature-dependent expression of flagella of *Listeria manocytogenes* studied by electron microscopy, SDS-PAGE and western blotting. J Gen Microbiol **134**(8): 2171-2178.

Rocourt, J., C. Jacquet and A. Reilly (2000). Epidemiology of human listeriosis and seafoods. Int J Food Microbiol **62**(3): 197-209.

Ryser, E. T. and E. H. Marth (2007). Listeria, listeriosis, and food safety, CRC Press.

Schmitz-Esser, S., A. Muller, B. Stessl and M. Wagner (2015). Genomes of sequence type 121 *Listeria monocytogenes* strains harbor highly conserved plasmids and prophages. Front Microbiol **6**: 380.

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. Bioinformatics **30**(14): 2068-2069.

Stasiewicz, M. J., H. F. Oliver, M. Wiedmann and H. C. den Bakker (2015). Whole-genome sequencing allows for improved identification of persistent *Listeria monocytogenes* in food-associated environments. Appl Environ Microbiol **81**(17): 6024-6037.

Takahashi, H., S. Kuramoto, S. Miya and B. Kimura (2011). Desiccation survival of *Listeria monocytogenes* and other potential foodborne pathogens on stainless steel surfaces is affected by different food soils. Food Control **22**(3-4): 633-637.

Todhanakasem, T. and G. M. Young (2008). Loss of flagellum-based motility by *Listeria monocytogenes* results in formation of hyperbiofilms. J Bacteriol **190**(17): 6030-6034.

Treangen, T. J., B. D. Ondov, S. Koren and A. M. Phillippy (2014). The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol **15**(11): 524.

Vatanyoopaisarn, S., A. Nazli, C. E. Dodd, C. E. Rees and W. M. Waites (2000). Effect of flagella on initial attachment of *Listeria monocytogenes* to stainless steel. Appl Environ Microbiol **66**(2): 860-863.

Yousef, A. E. (1999). Characteristics of *Listeria monocytogenes* important to food processors. Listeria: Listeriosis, and Food Safety: 131.

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Jessika Nowak

Name/Title of Principal Supervisor: Steve Flint

Name of Published Research Output and full reference:

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In which Chapter is the Published Work: Chapter 3

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

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  The candidate carried out the laboratory work and the analysis (apart from the genetic analysis and parts of the statistical analysis, which was conducted by A. van Vliet and D. Hedderley, respectively) and prepared the manuscript with input in guidance of direction and editorial help from the co-authors and supervisors.

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Chapter 4

Biofilm formation of the *L. monocytogenes* strain 15G01 is influenced by changes in environmental conditions

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Abstract

Listeria monocytogenes 15G01, a strain belonging to the persistent pulsotype 5132, was isolated from a seafood-processing plant in New Zealand. Simple monoculture assays using crystal violet staining showed good biofilm formation for this strain and it was therefore chosen to be further investigated in regard to its biofilm-forming ability. To evaluate its behaviour in different conditions, biofilm assays and growth studies were performed using common laboratory media under a range of temperatures (20°C, 30°C and 37°C). Furthermore, the effects of incubation time and different environmental conditions including static, dynamic and anaerobic incubation on biofilm formation were investigated. Changes in the environmental conditions resulted in different biofilm phenotype of *L. monocytogenes* 15G01. We demonstrated that increasing temperature and incubation time led to a greater biofilm mass and that dynamic incubation has little effect on biofilm formation at 37°C but encourages biofilm formation at 30°C. Biofilm production at 20°C was minimal regardless of the medium used. We furthermore observed that anaerobic environment led to reduced biofilm mass at 30°C for all tested media but not at 37°C. Biofilm formation could not be narrowed down to one factor but was rather dependent on multiple factors with temperature and medium having the biggest effects.

4.1 Introduction

Listeria monocytogenes is a foodborne human pathogen which is ubiquitious in our environment. This versatile facultative anaerobic microorganism has the ability to adapt quickly to environmental changes, which in turn makes it difficult to control. Not only is *L. monocytogenes* able to grow at temperatures ranging from 0 to 45°C, it can survive in freezing temperatures (-18°C and below) (Yousef 1999) and tolerate acidic conditions and low water activity (Nolan et al. 1992, O'Driscoll et al. 1996). *L. monocytogenes* expresses four to six peritrichous flagella which are responsible for its motility at room temperature and below. Motility is one of many key factors known to influence biofilm formation of bacteria including *L. monocytogenes* (Lemon et al. 2007, Tresse et al. 2009, Vatanyoopaisarn et al. 2000). A biofilm is a community of cells, consisting of single or multiple species that are attached to each other and to a surface through a self-produced matrix, known as the extracellular polymeric substance (EPS) (Lasa 2006). The EPS is composed of proteins, DNA, polysaccharides and other extracellular polymeric substances and helps protect the cells from stress encountered in the environment. The conversion from a planktonic to a sessile lifestyle enables bacteria to gain resistance against environmental factors such as pH and temperature change, and therefore improves chances of survival. Biofilm development occurs in many environments, one of the most important being food-processing plants, where they provide a source of cross-contamination to food products (Chmielewski and Frank 2003). These biofilms are difficult to eradicate as they are often resistant to the cleaners and sanitisers used in food-processing facilities (Pan et al. 2006). *L. monocytogenes* has been found to contaminate a variety of raw food such as vegetables and meat but it is mostly found in processed food products including seafood, cheese and meat products (Srey et al. 2013).

To control and/or prevent biofilm formation an understanding of the factors involved in biofilm formation is needed. One approach to gain this understanding is the identification of genes and the molecular mechanisms involved in biofilm development. Biofilm formation of *L. monocytogenes* has been thoroughly investigated; however, the molecular mechanisms remain unclear. Studies performed on biofilm formation so far used different strains from different origins. Serotypes 1/2a and 4b are the most common ones isolated from clinical cases and/or the food environment (Pan et al. 2009). Biofilm formation of those has been investigated in different commonly used laboratory media, such as Tryptic Soy Broth (TSB), Tryptic Soy Broth enriched with 0.6% Yeast Extract (TSBYE), Brain-Heart-Infusion (BHI), Luria-Bertani-Broth (LB), Modified Welshimer's Broth (MWB) and Hsiang-Tsai-Medium (HTM) under a range of incubation times (Bonsaglia et al. 2014, Borucki et al. 2003, Challan Belval et al. 2006, Djordjevic

et al. 2002, Harmsen et al. 2010, Kadam et al. 2013, Nilsson et al. 2011, Ochiai et al. 2014, Ouyang et al. 2012). Moreover, biofilm formation has been investigated in different environmental conditions, including static (Bonsaglia et al. 2014, Djordjevic et al. 2002, Lemon et al. 2007, Nilsson et al. 2011, Ochiai et al. 2014, Pilchova et al. 2014), dynamic (Habimana et al. 2009, van der Veen and Abee 2010, van der Veen and Abee 2011), aerobic and anaerobic environments. Those studies resulted in some controversial findings, which is not surprising considering that *L. monocytogenes* is extremely versatile and adaptable.

This study was conducted to reveal the influence of different environmental conditions on biofilm formation of *L. monocytogenes* 15G01, thus providing useful information for downstream experiments to help to identify factors and genes contributing to biofilm formation in food-processing premises.

4.2 Materials and Methods

4.2.1 Bacterial strain and growth

Listeria monocytogenes 15G01 was isolated during a seafood industry sampling programme. During this programme four persistent pulsotypes (persisted in factories for at least 6 months) were found and the pulsotype 5132 was the only one where individual isolates showed greater biofilm formation ability (Cruz and Fletcher 2011). *L. monocytogenes* 15G01 was one of the strains belonging to pulsotype 5132 that showed greater biofilm formation in a monoculture assay. Planktonic growth was determined in a 96-well plate in BHI (Difco, BD, USA), TSB (Difco, BD, USA), TSBYE (Difco, BD, USA) and MWB (Himedia, India) at 20°C, 30°C and 37°C under static conditions. Measurements were taken at a wavelength of 595 nm using a microplate reader (Multiskan EX, ThermoFisher).

4.2.2 Biofilm formation assay

The biofilm formation assay was performed according to a method described previously by Djordjevic et al. (2002) with some modifications. Briefly, a single *L. monocytogenes* colony was picked from Columbia sheep agar plate (Fort Richard, NZ) with a sterile pick and used to inoculate a 96-well plate by dipping that pick in the wells, each of which was filled with 200 µL TSBYE. This 96-well plate was then incubated at 37°C overnight used to inoculate a new 96-well plate (sterile, polystyrene, Interlab, NZ) with each well containing 200 µL media (BHI, TSB, TSBYE or MWB) using a 96-well replicator, which was first dipped into the overnight culture and then into the new plate. The cultures were incubated for 48 h at 20°C, 30°C or 37°C and then washed

three times with 200 µL sterile water using a microplate strip washer (ELx50, Biotek). The plates were air dried at 30°C for 30 min and then 150 µL of 1% aqueous crystal violet solution (Acros Organics, USA) was added. After 45 min of incubation at 30°C the crystal violet solution was removed and the cultures were washed six times with 150 µL sterile water. After drying for 30 min at 30°C, 96% ethanol was added and the crystal violet was destained for 1 h. The OD was measured at 595 nm with a microplate reader (Multiskan EX, ThermoFisher). The OD of a negative control (microtitre wells containing uninoculated media) was subtracted from the OD from the wells inoculated with culture. Biofilm formation assays were performed under several conditions: static, on an orbital shaker set on 100 rpm and in an anaerobic environment (BD GasPak).

4.2.3 Motility Assay

Semi-solid agar plates (TSB+0.25% Agar) were inoculated with *L. monocytogenes* 15G01 using a sterile pick and incubated at 20°C, 30°C and 37°C for 24 h according to a method described previously (Knudsen et al. 2004). Motility was expressed as a visible halo. This experiment was conducted in triplicate and the results of one experiment are shown in Figure 4.3.

4.2.4 Mussel juice for the biofilm formation assay

Greenshell™ mussels obtained from the local supermarket were stored for 24 h at 10°C and the next day they were evenly spread in a wok, closed with a lid and boiled without addition of water. After 1 min, when all mussels opened and released the intervalvular juice, the liquid was collected and stored in a sterile plastic tube to be used immediately (cooked mussel juice=CMJ). Before biofilm assays were performed, CMJ was filtered with a membrane filter with 0.45 µm pore size. Biofilm assays were then performed according to the method described above. Incubation time was 48 h and temperatures tested were 20°C, 30°C and 37°C. Biofilm formation in MWB was tested on the same plate to compare the results.

4.2.5 Statistical analysis

Statistical analysis was performed using Microsoft Excel software. Significant differences were concluded when P was ≤0.05 for two tailed t-test and ANOVA, respectively.

4.3 Results and Discussion

4.3.1 Planktonic growth of *L. monocytogenes* 15G01

Commonly used laboratory media chosen for this study of *L. monocytogenes* were BHI, TSB, TSBYE and MWB. The first three are undefined rich media, whereas the latter is a defined synthetic medium with minimal nutrients for *Listeria* growth (Premaratne et al. 1991). To assess the influence of nutrients on growth, growth curves were obtained in undiluted rich (BHI, TSB, TSBYE) and defined media (MWB) and 1:10 dilutions of rich media with sterile H_2O (dBHI, dTSB and dTSBYE).

The results in Figure 4.1 show that planktonic growth was slower at 20°C than 30°C and 37°C. Growth in diluted undefined media was minimal and independent of temperature, whereas growth in MWB showed a different pattern in all three temperatures tested: the exponential phase was prolonged and the stationary phase was reached later. MWB is known to influence a sessile living form of *L. monocytogenes* positively (Djordjevic et al. 2002). Therefore it can be presumed that planktonic cells in the wells of the 96-well plates change to a sessile lifestyle and it is known that sessile cells sacrifice their maximum growth rate to benefit the biofilm community (Kreft 2004), which explains slower growth. Undefined media are rich in nutrients which stimulate growth of the bacteria. Diluted media offer only limited nutrient availability to *L. monocytogenes* and therefore only minimal growth with minimal cell numbers was observed independent of temperature.

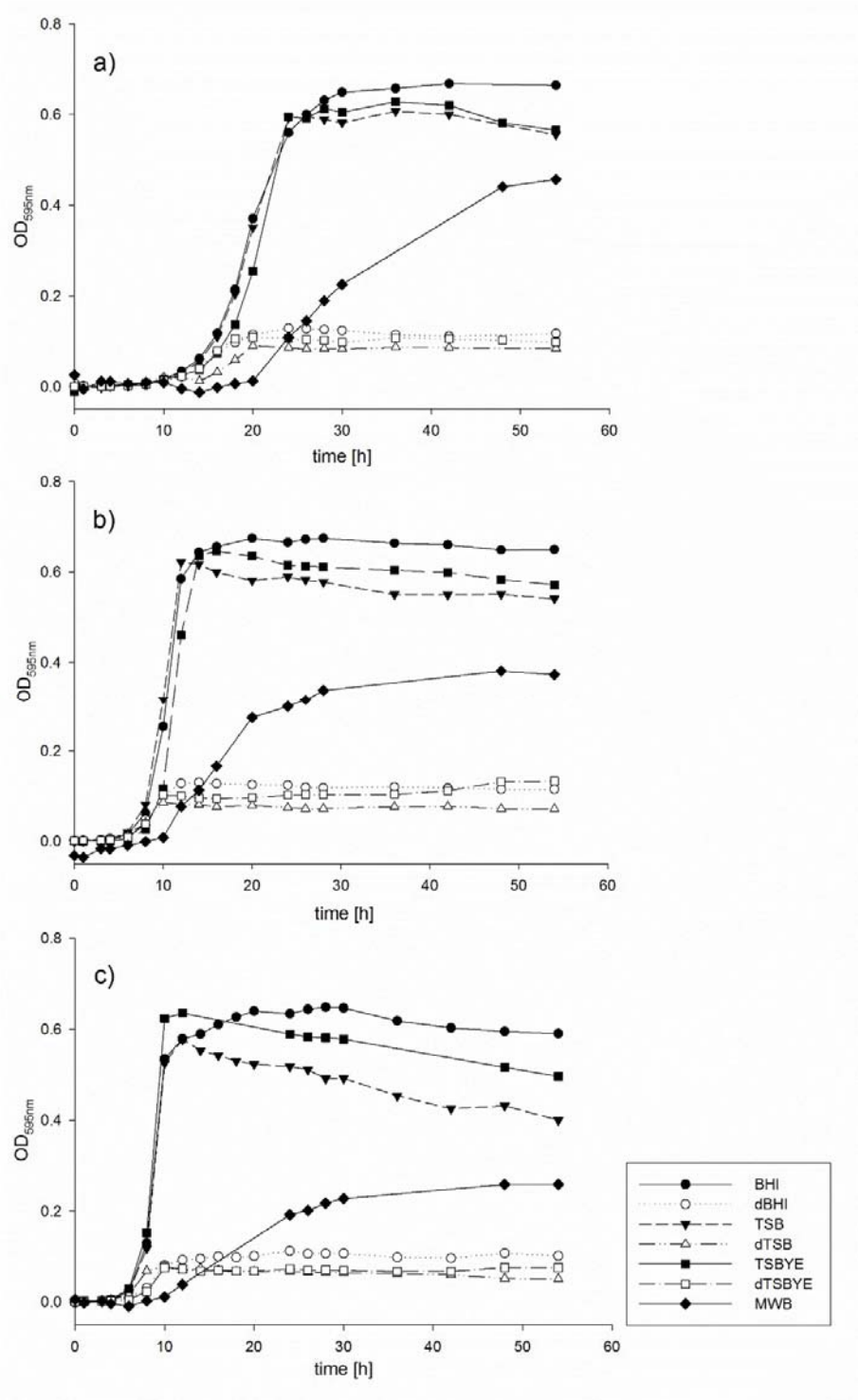

Figure 4.1. Optical density of *L. monocytogenes* 15G01 grown at a) 20°C, b) 30°C and c) 37°C in seven different media analysed in one experiment with n=12.

4.3.2 Biofilm formation of *L. monocytogenes* 15G01

4.3.2.1 Effect of nutrient availability

Biofilm formation was tested in the media stated previously to identify the effect of nutrient availability on biofilm formation.

Biofilm formation in undiluted nutrient rich media increased with increasing temperature, and in diluted media was independent of temperature and minimal at all temperatures tested, which might be due to attachment only rather than biofilm formation. Biofilm formation in MWB showed a different pattern: greatest biofilm formation was observed at 30°C and lowest at 20°C (Figure 4.2).

Nutrient availability influences biofilm formation. Kadam et al. (2013) observed that biofilm formation was enhanced in nutrient-poor media compared with nutrient-rich media, which is in line with findings of our studies and other studies where *L. monocytogenes* formed good biofilms when grown in MWB (Djordjevic et al. 2002, Pilchova et al. 2014, Todhanakasem and Young 2008) and greater biofilm mass was produced in minimum essential media (MEM) than BHI (Luo et al. 2013). Deprived accessibility to nutrients in minimum defined media is likely to cause stress in *Listeria* cells resulting in enhanced biofilm formation. Biofilm formation is believed to be induced by stress in the form of increased heat, limited nutrient availability or enhanced oxygen presence.

Other studies showed that defined nutrient-poor media enhance initial attachment and subsequently promote biofilm formation. Pilchova et al. (2014) found that three out of four strains showed greater biofilm formation at 20°C in MWB than TSBYE and one strain showed similar biofilm formation in MWB and TSBYE. Moltz and Martin (2005) observed greater biofilm formation in MWB for six out of eight strains and greater biofilm formation in TSB for the remaining two strains. Although in general biofilms formed preferentially in nutrient-deficient medium (Combrouse et al. 2013, Djordjevic et al. 2002, Ochiai et al. 2014, Pilchova et al. 2014, Todhanakasem and Young 2008), some strains performed better in nutrient-rich conditions (Folsom et al. 2006). This shows that biofilm formation of *L. monocytogenes* cannot be generalised and highlights the importance of performing preliminary tests for *L. monocytogenes* strains before continuing with further studies.

Moreover, the use of different media might also trigger different phenotypes. Chang et al. (2013) observed that cells grown in TSBYE showed elongation whereas when grown in MWB

that phenomenon could not be observed. This should be taken into account when choosing a medium for further experiments.

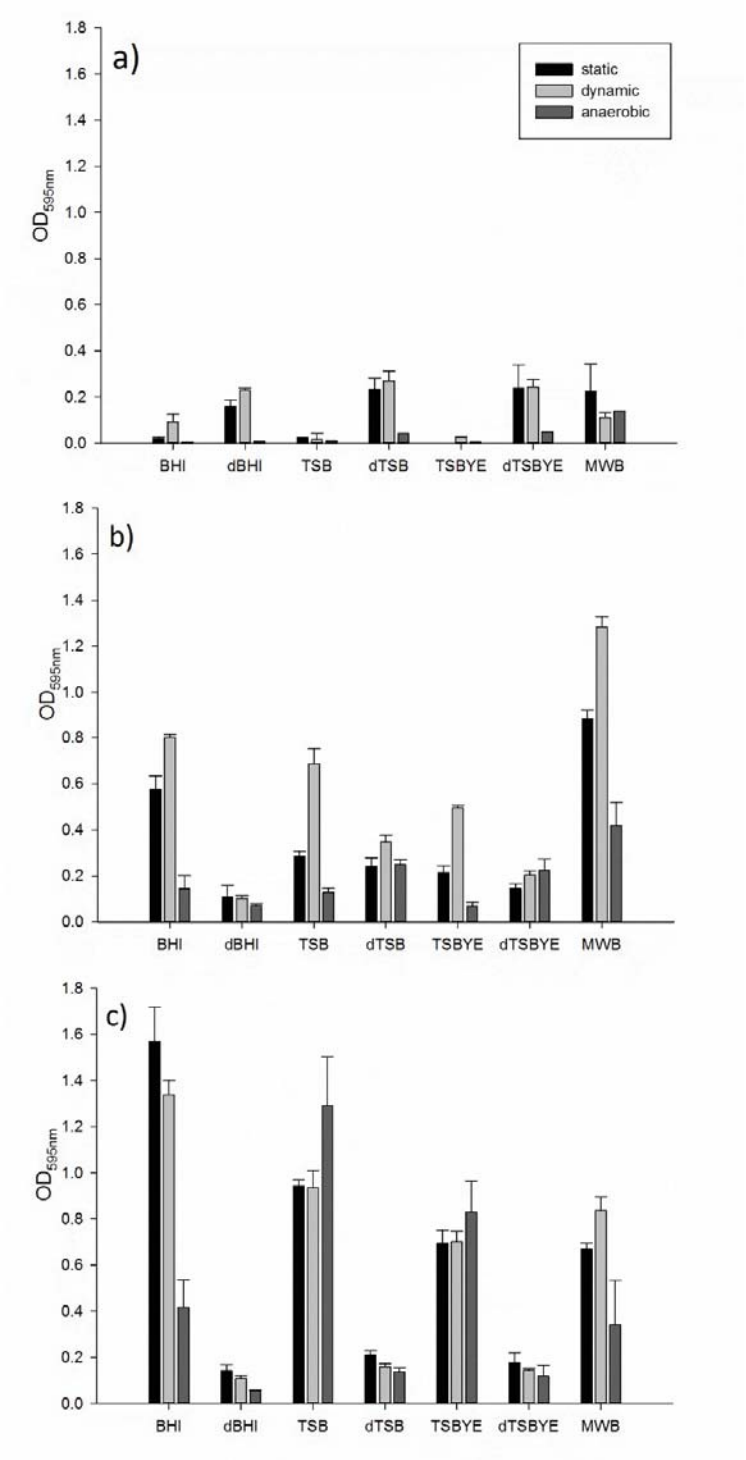

Figure 4.2. Biofilm formation of *L. monocytogenes* 15G01 at a) 20°C, b) 30°C and c) 37°C after 48 h (error bars represent standard deviation for means of at least 2 experiments with n = 40). The results of the statistical analysis are shown in the Supplementary Table 4.1.

4.3.2.2 Effect of static, dynamic and anaerobic incubation

Biofilm formation of *L. monocytogenes* was tested in static and dynamic conditions, since it is a facultative anaerobic microorganism but prefers an anaerobic environment (Buchrieser et al. 2003). We postulated that increased presence of oxygen, through shaking of the 96-well plates, resulted in enhanced stress for the bacteria and subsequently more biofilm formation. To verify that this putative effect was strictly related to the presence of oxygen, biofilm formation was also tested under anaerobic conditions.

The differences in the measurements of the OD_{595nm} at 20°C between static, dynamic and anaerobic conditions were minimal and the OD_{595nm} values were generally very low (Figure 4.2). As stated before, this might be due to attachment of the cells to the surface rather than biofilm formation itself. Enhanced biofilm formation was observed at 30°C when grown under dynamic conditions and lowest biofilm formation when grown in anaerobic conditions. Oxygen present in the media is able to cross cell membranes and inside the cells superoxide and hydroxyl radicals can be formed through a series of chemical reactions (van der Veen and Abee 2011). The biofilm mass measured when grown under anaerobic conditions at 20°C and 30°C was lower than under aerobic conditions, which supports the hypothesis that less oxygen presence resulted in decreased stress. Statistical analysis (ANOVA) revealed that differences in biofilm formation between static, dynamic and anaerobic incubation were highly significant ($p \leq 0.001$) at 30°C for BHI, TSB, TSBYE and MWB but not for diluted media and that medium and temperature had the biggest effect on biofilm formation.

Our hypothesis was accepted for MWB at 37°C, but not for BHI, TSB and TSBYE. Biofilm formation was greatest when grown under static conditions in BHI (OD_{595nm} = 1.57); and dynamic incubation did not enhance biofilm formation, but differences were highly significant ($p=0.001$). All media used provide glucose as the main carbon source. BHI consists of animal-derived brain and heart infusions whereas TSB and TSBYE use a soybean and casein digest as a protein source, which might also influence biofilm formation of *L. monocytogenes* 15G01. Biofilm formation in static and dynamic conditions was similar for TSB and TSBYE (Figure 4.2). We observed that *L. monocytogenes* 15G01 formed better biofilms at 37°C in TSB and TSBYE when grown anaerobically (OD_{595nm}= 1.29 and 0.82, respectively) rather than aerobically (OD_{595nm} = 0.94 and 0.69 or 0.94 and 0.70, respectively). ANOVA showed no significant difference between static, dynamic and anaerobic incubation for TSBYE ($p=0.154$), but for static and dynamic vs. anaerobic incubation for TSB ($p=0.021$). It seemed that oxygen presence had a relatively minor effect on biofilm formation in TSB and TSBYE at 37°C and that temperature was

the main influencing factor of biofilm formation in these media. *Listeria monocytogenes* 15G01 might be able to use certain compounds and/or degradation products in TSB and TSBYE to promote anaerobic growth.

Premaratne et al. (1991) found that glucose and glutamine are required as primary carbon and nitrogen source for *L. monocytogenes* Scott A. Several other sugars were able to replace glucose, but the best growth was observed in glucose. The same research group found that essential amino acids for *Listeria* growth are leucine, isoleucine, valine, methionine, arginine, cysteine, glutamine, histidine and tryptophane. Moreover iron, riboflavin, thiamine, biotin and thioctic acid are required for *Listeria* growth. However, Tsai and Hodgson (2003) found that *L. monocytogenes* 10403S requires only two essential amino acids (methionine and cysteine) and only glucose, fructose, glycerol and mannose were able to act as carbon sources. That shows again that behaviour and growth differ between different serovars and strains (*L. monocytogenes* Scott A and *L. monocytogenes* 10403S are serovars 4b and 1/2a respectively) and must not be generalised.

4.3.3 Effect of temperature

Biofilm formation was tested at 20°C, 30°C and 37°C to cover common temperatures used in previous studies.

The motility assay was performed to analyse the ability of *L. monocytogenes* 15G01 to express flagella at different temperatures. Flagella, which are expressed in a temperature dependent process, are responsible for *L. monocytogenes'* motility and motility is believed to be important in biofilm formation (Lemon et al. 2007). It is generally agreed that flagella are produced at 20°C to 25°C (Peel et al. 1988) and are not produced at 37°C, but it has been found that some laboratory-adapted strains and about 20% of clinical isolates have the ability to express flagella at 37°C (Way et al. 2004).

In this study a halo was visible on the motility plates at 20°C and 30°C which showed that *L. monocytogenes* is motile at those temperatures, whereas at 37°C only growth was visible but no halo revealing that flagella were not expressed by *L. monocytogenes* 15G01 at 37°C (Figure 4.3).

As mentioned before, biofilm formation increased with increasing temperature, except in MWB (Figure 4.2). Biofilm formation was greatest at 30°C and not at 37°C, which could be due to the lack of flagella. However, the lack of flagella did not decrease *L. monocytogenes'* ability to form biofilms in rich undefined media at 37°C compared with 30°C showing that flagella are not

always required for biofilm formation and suggest that bacteria can induce biofilm formation by several mechanisms. Another study showed that the lack of flagella resulted in decreased adhesion but subsequently led to a "hyperbiofilm" phenotype showing that flagella are not crucial for biofilm formation (Todhanakasem and Young 2008). Moreover, Tresse et al. (2009) found that strains lacking flagella could attach to surfaces through passive attachment, suggesting that attachment can occur in a temperature independent manner and through different mechanisms ensuring *L. monocytogenes*' survival.

Kadam et al. (2013) found that the influence of temperature was more significant than the medium on biofilm formation with biofilm formation decreasing with decreasing temperature. The least amount of biofilm was formed at 12°C and the greatest at 37°C, which is in line with our findings for undefined rich media: lowest biofilm formation was observed at 20°C and greatest biofilm formation at 37°C.

Greater biofilm formation with increasing temperature does not necessarily mean that more cells are present (due to enhanced growth) in the biofilm but it could also be due to increased production of extracellular matrix and/or presence of non-viable cells. Mai et al. (2007) showed that cell numbers attached to a surface were lower at 42°C than at 37°C and 30°C when grown in rich medium and lower at 42°C than all other temperatures tested in nutrient-deficient medium (Mai and Conner 2007). Kadam et al. (2013) found that extracellular polymeric substance and /or numbers of non-viable cells of a biofilm were higher when grown in nutrient deficient medium (NB) than BHI through LIVE/DEAD *Bac*Light staining. This should be taken into consideration when studying biofilm mass.

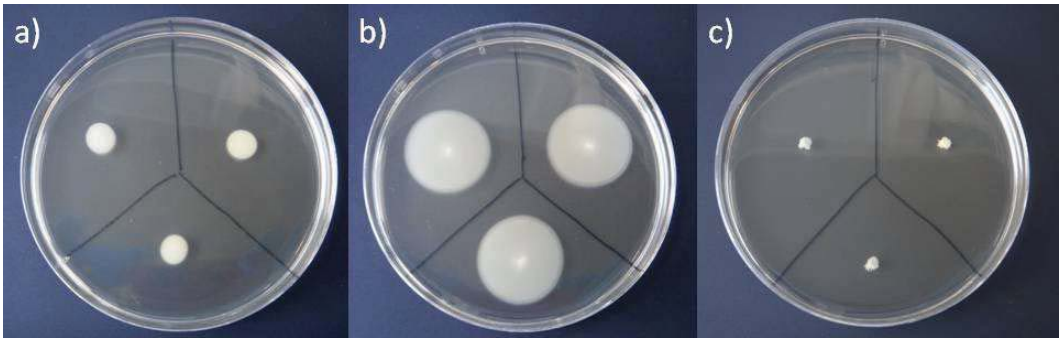

Figure 4.3. Motility assay of *Listeria monocytogenes* 15G01 after 24 h incubation at a) 20°C, b) 30°C and c) 37°C

4.3.4 Effect of incubation time

Biofilm formation in BHI, MWB, TSBYE and TSB at 30°C and 37°C was measured after incubation ranging from 12 to 48 h (Figure 4.4). Biofilm formation at 20°C was only measured after 48 h of incubation. As biofilm mass was low after 48 h incubation (Figure 4.2) it was concluded that decreasing incubation time will not have a positive effect on biofilm formation. Previous tests at 20°C with a prolonged incubation time up to 120 h did not result in an increase of biofilm formation at 20°C (data not shown) and therefore it was concluded that this temperature does not favour biofilm formation.

Figure 4.4 shows the effect of incubation time on biofilm formation at 30°C and 37°C.

At 30°C, longer incubation times resulted in greater biofilm formation in BHI, TSB and TSBYE. Biofilm formation in MWB at 30°C reached a maximum OD_{595nm} 1.24 after 24 h. At 37°C, enhanced biofilm formation could be observed with increasing temperature in BHI, whereas biofilm mass decreased in TSB and TSBYE after reaching a maximum OD_{595nm} of 1.19 and 1.23 after 36 and 30 h, respectively. Biofilm formation decreased at 37°C after reaching a maximum OD_{595nm} of 0.99 in MWB after 30 h to an OD_{595nm} of 0.56 after 48 h of incubation.

Maximum biofilm formation in MWB at 30°C and 37°C could be observed after 24 h and 30 h, respectively, whereas in nutrient-rich media biofilm formation increased with increasing temperature.

Incubation time is another critical determinant for biofilm formation. Biofilm formation can be divided into five steps: reversible attachment, irreversible attachment, growth, maturation and dispersal (Lasa 2006). The start of one stage or transition to another is dependent on certain environmental factors. Depending on what stage of biofilm formation is to be studied, incubation time needs to be chosen carefully as expressions of genes can vary between stages (Fiorini et al. 2008, O'Driscoll et al. 1996). Depending on the environmental conditions, bacteria might reach stages faster than originally expected, so once optimum medium and temperature are identified it is advisable to study the kinetics of biofilm formation. Growth studies showed that stationary phase was reached after 10 h in nutrient-rich media and after about 30 h in MWB. After 24 h of incubation cells/biofilm grown in MWB were still in exponential phase whereas cells/biofilm grown in nutrient-rich media were already in stationary phase (Figure 4.4). This might also account for differences in the amount of biofilm formed as gene expression is dependent on the stage of growth based on the growth curve of the cells.

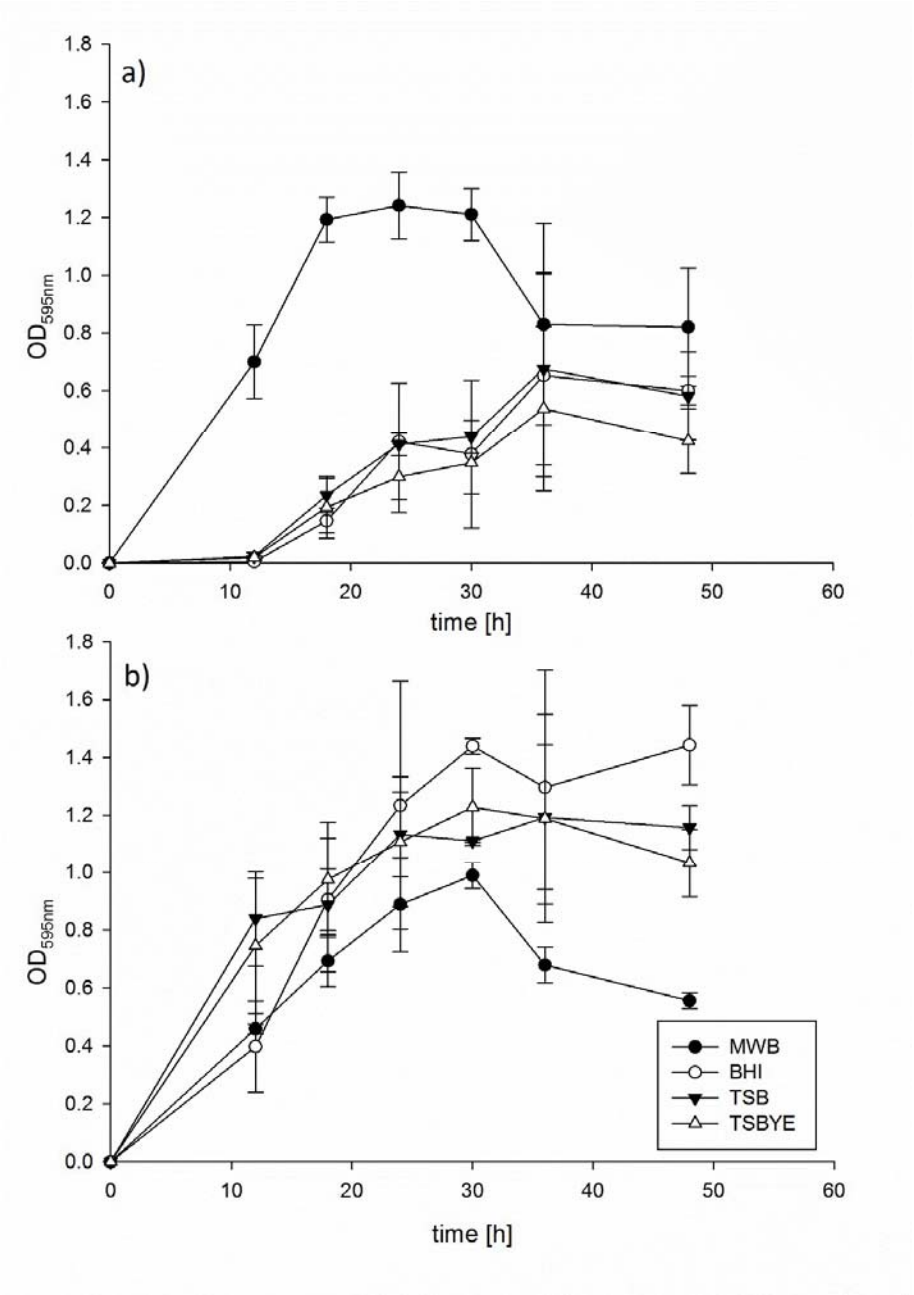

Figure 4.4. Biofilm formation at a) 30°C and b) 37°C in BHI, MWB, TSB and TSBYE after 12, 18, 24, 30, 36 and 48 h incubation (error bars represent standard deviation for 3 experiments; n=16)

4.3.5 Biofilm formation in mussel juice

To mimic conditions in a mussel-processing facility biofilm formation was also tested in cooked mussel juice (CMJ), which was obtained through heat-shucking (Saa et al. 2009). Biofilm assays were performed at 20°C, 30°C and 37°C in static aerobic conditions in CMJ and MWB and turbidity was measured before staining with crystal violet to determine growth. Growth

decreased with increasing temperature for MWB, whereas growth in CMJ was minimal at all temperatures tested. Biofilm formation was low in CMJ ranging from OD_{595nm} 0.0259 to 0.0850 indicating that *Listeria* cells attach rather than form biofilms (Figure 4.5).

To identify whether initial concentration of *Listeria* cells was the issue for reduced growth and biofilm formation, growth was measured in a 2 mL microtube inoculated with a single colony picked from Columbian sheep blood agar. OD_{595nm} was determined after 24 h. The OD_{595nm} value was 0.642 revealing that CMJ induces bacterial growth, but is obviously depending on inoculation size. For biofilm formation assays and growth studies 96-well plates were inoculated by using a 96-well replicator which only transferred little amount of cells.

pH and salinity of CMJ were 7.6 and 53 g/L, respectively. Xu et al. (2010) observed that NaCl stabilizes biofilm formation of *L. monocytogenes* by hindering detachment phase (incubation up to 10 days) with increasing salt concentrations (6–10%). After 48 h of incubation greatest biofilm formation could be observed in medium containing 0% NaCl and second greatest in medium containing 2% NaCl. Previous growth studies revealed that exponential phase extends with increasing NaCl concentration (1–9%) (Zarei et al. 2012). Therefore, in our case salt concentration was adjusted to 2% and biofilm formation was measured in CMJ-2% NaCl, but no difference to undiluted CMJ could be detected (data not shown).

A change from a planktonic to a sessile lifestyle is induced by certain environmental triggers including pH change, temperature change or nutrient availability. Mimicking a complex environment as present in food-processing plants is not an easy task considering that *L. monocytogenes* is a bacterium that can easily adapt to environmental changes (Gray et al. 2006). Cleaning and sanitising change the environment on a regular basis can therefore alter bacteria's characteristics. *Listeria monocytogenes* 15G01 was isolated in a mussel-processing facility and classified as a persistent high biofilm former in exact defined conditions. It showed greatest biofilm formation capacity in MWB but not in CMJ in a laboratory environment, which may simply be due to the inability of mimicking a complex always evolving environment. Previous research showed that *L. monocytogenes* preferably attaches to porous hydrophilic surfaces (Bonsaglia et al. 2014, Habimana et al. 2009) — despite thorough and regular cleaning organic material of processed foods might remain on equipment in food-processing plants and function as an attachment site for *L. monocytogenes*. In our studies sterile polystyrene plates were used to test attachment and biofilm formation with no additional surface conditioning which might be another reason for low biofilm formation of *L. monocytogenes* 15G01 in CMJ.

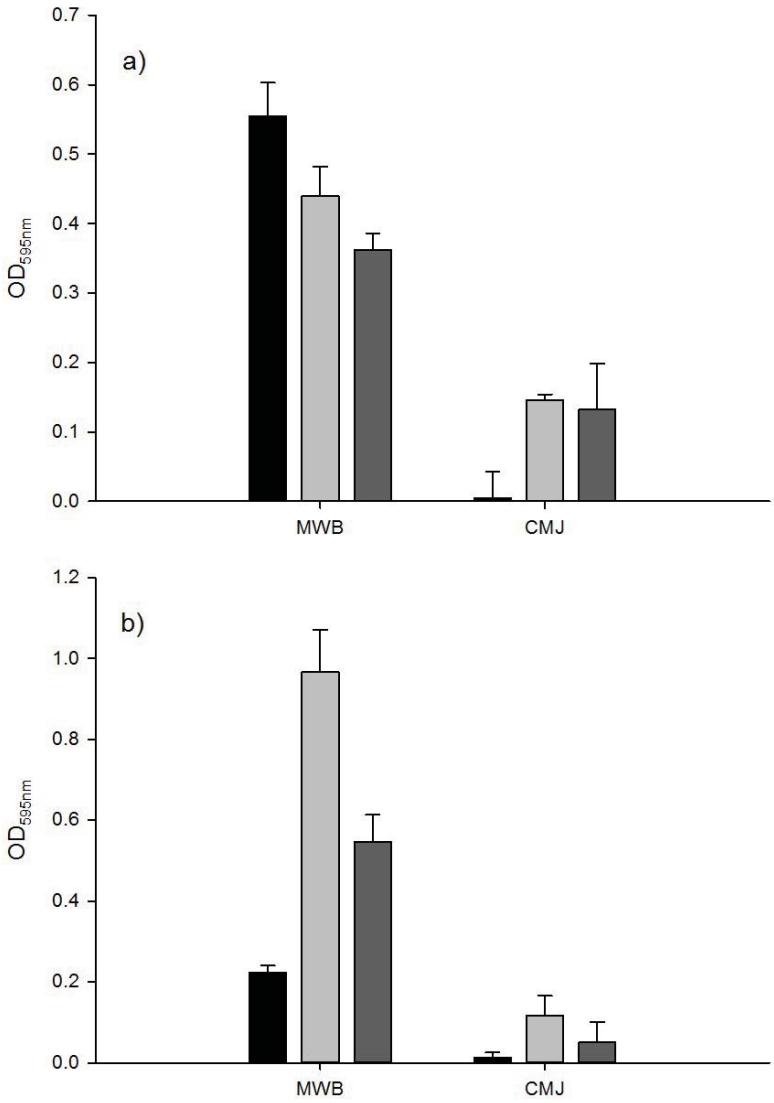

Figure 4.5. Comparison of a) growth and b) biofilm formation of *Listeria monocytogenes* 15G01 after 48 h in MWB and CMJ at 20°C (black bar), 30°C (grey bar) and 37°C (dark grey bar) (error bars represent standard deviation for 3 experiments with n=16)

4.4 Conclusion

In our studies we examined the influence of different factors on biofilm formation of the persistent *L. monocytogenes* strain 15G01.

Although growth studies revealed that cell numbers were higher in nutrient-rich media (shown through higher OD-measurements), biofilm formation was lower in nutrient-rich media than the minimal medium (MWB), indicating that biofilm formation was not due to high cell numbers but rather triggered by the medium itself. Greatest biofilm formation was observed under

dynamic conditions, which supports the hypothesis of stress in the form of oxygen being a biofilm-inducing factor.

To conclude, biofilm formation of *L. monocytogenes* 15G01 strongly depends on multiple environmental factors, but medium and temperature have the biggest effects on biofilm formation. Our results emphasize *L. monocytogenes'* ability to adapt to changing environmental conditions, which contribute to its persistence in the food-processing environment and can help to understand the underlying mechanisms.

4.5 Acknowledgements

The authors would like to thank Duncan Hedderley for his help with the statistical analysis. This research was funded through The New Zealand Ministry of Business, Innovation and Employment (MBIE) funding: contract CAWX0301.

4.6 Supplementary information

Supplementary Table S4.1. Statistical analysis of biofilms formed in seven different media at three different temperatures after static, dynamic and anaerobic incubation for 48 hours.

20°C						**medium**			
shaking	**anaerobic**	n	BHI	dBHI	TSB	dTSB	TSBYE	dTSBYE	MWB
no	**no**	3	0.02	0.16	0.02	0.24	0.00	0.24	0.22
no	**yes**	1	0.01	0.01	0.01	0.04	0.01	0.05	0.14
yes	**no**	2	0.09	0.23	0.02	0.27	0.03	0.24	0.11
ANOVA	**F (2,3 df)**		9.4	33.1	0.2	9.0	1.4	2.0	0.9
	p		0.051	0.009	0.847	0.054	0.372	0.279	0.516
	LSD		0.07	0.08	0.06	0.17	0.07	0.32	0.45
30°C						**medium**			
shaking	**anaerobic**	n	BHI	dBHI	TSB	dTSB	TSBYE	dTSBYE	MWB
no	**no**	3	0.57	0.11	0.29	0.24	0.21	0.14	0.89
no	**yes**	2	0.15	0.07	0.13	0.25	0.07	0.22	0.42
yes	**no**	2	0.80	0.10	0.69	0.35	0.50	0.20	1.28
ANOVA	**F (2,4 df)**		85.9	0.7	122.2	7.0	168.1	4.3	98.3
	p		<.001	0.552	<.001	0.049	<.001	0.101	<.001
	LSD		0.13	0.09	0.09	0.08	0.06	0.08	0.16
37°C						**medium**			
shaking	**anaerobic**	n	BHI	dBHI	TSB	dTSB	TSBYE	dTSBYE	MWB (2,1,1)
no	**no**	3	1.57	0.14	0.94	0.21	0.70	0.18	0.67
no	**yes**	2	0.42	0.06	1.38	0.14	0.88	0.12	0.48
yes	**no**	2	1.34	0.11	0.94	0.16	0.70	0.15	0.79
ANOVA	**F (2,4 df)**		53.3	12.2	11.8	11.9	3.1	1.5	46.0
	p		0.001	0.02	0.021	0.021	0.154	0.33	0.104
	LSD		0.32	0.05	0.27	0.04	0.22	0.10	0.37

4.7 References

Bonsaglia, E. C. R., N. C. C. Silva, A. Fernades, J. P. Araujo, M. H. Tsunemi and V. L. M. Rall (2014). Production of biofilm by *Listeria monocytogenes* in different materials and temperatures. Food Control **35**(1): 386-391.

Borucki, M. K., J. D. Peppin, D. White, F. Loge and D. R. Call (2003). Variation in biofilm formation among strains of *Listeria monocytogenes*. Appl Environ Microbiol **69**(12): 7336-7342.

Buchrieser, C., C. Rusniok, F. Kunst, P. Cossart and P. Glaser (2003). Comparison of the genome of *Listeria monocytogenes* and *Listeria innocua*: clues for evolution and pathogenicity. FEMS Immunol Med Microbiol **35**(3): 207-213.

Challan Belval, S., L. Gal, S. Margiewes, D. Garmyn, P. Piveteau and J. Guzzo (2006). Assessment of the roles of LuxS, S-ribosyl homocysteine, and autoinducer 2 in cell attachment during biofilm formation by *Listeria monocytogenes* EGD-e. Appl Environ Microbiol **72**(4): 2644-2650.

Chang, Y., W. Gu, F. Zhang and L. McLandsborough (2013). Disruption of lmo1386, a putative DNA translocase gene, affects biofilm formation of *Listeria monocytogenes* on abiotic surfaces. Int J Food Microbiol **161**(3): 158-163.

Chmielewski, R. A. N. and J. F. Frank (2003). Biofilm formation and control in food processing facilities. Compr Rev Food Sci Food Saf **2**(1): 22-32.

Combrouse, T., I. Sadovskaya, C. Faille, O. Kol, Y. Guerardel and G. Midelet-Bourdin (2013). Quantification of the extracellular matrix of the *Listeria monocytogenes* biofilms of different phylogenic lineages with optimization of culture conditions. J Appl Microbiol **114**(4): 1120-1131.

Cruz, C. D. and G. C. Fletcher (2011). Prevalence and biofilm-forming ability of *Listeria monocytogenes* in New Zealand mussel (*Perna canaliculus*) processing plants. Food Microbiol **28**(7): 1387-1393.

Djordjevic, D., M. Wiedmann and L. A. McLandsborough (2002). Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl Environ Microbiol **68**(6): 2950-2958.

Fiorini, F., S. Stefanini, P. Valenti, E. Chiancone and D. De Biase (2008). Transcription of the *Listeria monocytogenes* fri gene is growth-phase dependent and is repressed directly by Fur, the ferric uptake regulator. Gene **410**(1): 113-121.

Folsom, J. P., G. R. Siragusa and J. F. Frank (2006). Formation of biofilm at different nutrient levels by various genotypes of *Listeria monocytogenes*. J Food Protect **69**(4): 826-834.

Gray, M. J., N. E. Freitag and K. J. Boor (2006). How the bacterial pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. Infection and Immunity **74**(5): 2505-2512.

Habimana, O., M. Meyrand, T. Meylheuc, S. Kulakauskas and R. Briandet (2009). Genetic features of resident biofilms determine attachment of *Listeria monocytogenes*. Appl Environ Microbiol **75**(24): 7814-7821.

Harmsen, M., M. Lappann, S. Knochel and S. Molin (2010). Role of extracellular DNA during biofilm formation by *Listeria monocytogenes*. Appl Environ Microbiol **76**(7): 2271-2279.

Kadam, S. R., H. M. den Besten, S. van der Veen, M. H. Zwietering, R. Moezelaar and T. Abee (2013). Diversity assessment of *Listeria monocytogenes* biofilm formation: impact of growth condition, serotype and strain origin. Int J Food Microbiol **165**(3): 259-264.

Knudsen, G. M., J. E. Olsen and L. Dons (2004). Characterization of DegU, a response regulator in *Listeria monocytogenes*, involved in regulation of motility and contributes to virulence. FEMS Microbiol Lett **240**(2): 171-179.

Kreft, J. U. (2004). Biofilms promote altruism. Microbiology **150**(Pt 8): 2751-2760.

Lasa, I. (2006). Towards the identification of the common features of bacterial biofilm development. Int Microbiol **9**(1): 21-28.

Lemon, K. P., D. E. Higgins and R. Kolter (2007). Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. J Bacteriol **189**(12): 4418-4424.

Luo, Q., J. Shang, X. Feng, X. Guo, L. Zhang and Q. Zhou (2013). PrfA led to reduced biofilm formation and contributed to altered gene expression patterns in biofilm-forming *Listeria monocytogenes*. Curr Microbiol **67**(3): 372-378.

Mai, T. L. and D. E. Conner (2007). Effect of temperature and growth media on the attachment of *Listeria monocytogenes* to stainless steel. Int J Food Microbiol **120**(3): 282-286.

Moltz, A. G. and S. E. Martin (2005). Formation of biofilms by *Listeria monocytogenes* under various growth conditions. J Food Prot **68**(1): 92-97.

Nilsson, R. E., T. Ross and J. P. Bowman (2011). Variability in biofilm production by *Listeria monocytogenes* correlated to strain origin and growth conditions. Int J Food Microbiol **150**(1): 14-24.

Nolan, D. A., D. C. Chamblin and J. A. Troller (1992). Minimal water activity levels for growth and survival of *Listeria monocytogenes* and *Listeria innocua*. Int J Food Microbiol **16**(4): 323-335.

O'Driscoll, B., C. G. Gahan and C. Hill (1996). Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased virulence. Appl Environ Microbiol **62**(5): 1693-1698.

Ochiai, Y., F. Yamada, M. Mochizuki, T. Takano, R. Hondo and F. Ueda (2014). Biofilm formation under different temperature conditions by a single genotype of persistent *Listeria monocytogenes* strains. J Food Prot **77**(1): 133-140.

Ouyang, Y., J. Li, Y. Dong, L. V. Blakely and M. Cao (2012). Genome-wide screening of genes required for *Listeria monocytogenes* biofilm formation. J Biotech Res **4**: 13-25.

Pan, Y., F. Breidt, Jr. and S. Kathariou (2006). Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. Appl Environ Microbiol **72**(12): 7711-7717.

Pan, Y., F. Breidt, Jr. and S. Kathariou (2009). Competition of *Listeria monocytogenes* serotype 1/2a and 4b strains in mixed-culture biofilms. Appl Environ Microbiol **75**(18): 5846-5852.

Peel, M., W. Donachie and A. Shaw (1988). Temperature-dependent expression of flagella of *Listeria manocytogenes* studied by electron microscopy, SDS-PAGE and western blotting. J Gen Microbiol **134**(8): 2171-2178.

Pilchova, T., M. Hernould, H. Prevost, K. Demnerova, J. Pazlarova and O. Tresse (2014). Influence of food processing environments on structure initiation of static biofilm of *Listeria monocytogenes*. Food Control **35**(1): 366-372.

Premaratne, R. J., W. J. Lin and E. A. Johnson (1991). Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. Appl Environ Microbiol **57**(10): 3046-3048.

Saa, P., M. L. Cabo and J. J. Rodriguez (2009). Effects of mussel processing soils on the adherence of *Listeria monocytogenes* to polypropylene and stainless steel. J Food Prot **72**(9): 1885-1890.

Srey, S., I. K. Jahid and S. D. Ha (2013). Biofilm formation in food industries: A food safety concern. Food Control **31**(2): 572-585.

Todhanakasem, T. and G. M. Young (2008). Loss of flagellum-based motility by *Listeria monocytogenes* results in formation of hyperbiofilms. J Bacteriol **190**(17): 6030-6034.

Tresse, O., V. Lebret, D. Garmyn and O. Dussurget (2009). The impact of growth history and flagellation on the adhesion of various *Listeria monocytogenes* strains to polystyrene. Can J Microbiol **55**(2): 189-196.

Tsai, H. N. and D. A. Hodgson (2003). Development of a synthetic minimal medium for *Listeria monocytogenes.* Appl Environ Microbiol **69**(11): 6943-6945.

van der Veen, S. and T. Abee (2010). Dependence of continuous-flow biofilm formation by *Listeria monocytogenes* EGD-e on SOS response factor YneA. Appl Environ Microbiol **76**(6): 1992-1995.

van der Veen, S. and T. Abee (2011). Generation of variants in *Listeria monocytogenes* continuous-flow biofilms is dependent on radical-induced DNA damage and RecA-mediated repair. PLoS One **6**(12): e28590.

Vatanyoopaisarn, S., A. Nazli, C. E. Dodd, C. E. Rees and W. M. Waites (2000). Effect of flagella on initial attachment of *Listeria monocytogenes* to stainless steel. Appl Environ Microbiol **66**(2): 860-863.

Way, S. S., L. J. Thompson, J. E. Lopes, A. M. Hajjar, T. R. Kollmann, N. E. Freitag and C. B. Wilson (2004). Characterization of flagellin expression and its role in *Listeria monocytogenes* infection and immunity. Cell Microbiol **6**(3): 235-242.

Xu, H., Y. Zou, H. Y. Lee and J. Ahn (2010). Effect of NaCl on the biofilm formation by foodborne pathogens. J Food Sci **75**(9): M580-585.

Yousef, A. E. (1999). Characteristics of *Listeria monocytogenes* important to food processors. Listeria: Listeriosis, and Food Safety: 131.

Zarei, M., M. Khezizadeh, S. Kazemipour, G. Hesami and E. Bemani (2012). Growth and cell morphology of *Listeria monocytogenes* as affected by various concentrations of NaCl and KCl. J Appl Biol Sci **6**(1): 55-58.

DRC 16

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

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We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Jessika Nowak

Name/Title of Principal Supervisor: Steve Flint

Name of Published Research Output and full reference:

Nowak, J., Cruz, C., Palmer, J., Fletcher, G. & Flint, S. (2015). Biofilm formation of the L. monocytogenes strain 15G01 is influenced by changes in environmental conditions. Journal of Microbiological Methods.

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Chapter 5

Biofilm formation of *Listeria monocytogenes* 15G01, a persistent isolate from a seafood-processing plant in New Zealand, is influenced by inactivation of multiple genes belonging to different functional groups

Prepared for submission to PLoS ONE

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Abstract

Listeria monocytogenes is a ubiquitous foodborne pathogen that results in a high rate of mortality in sensitive and immunocompromised people. Contamination of food with *L. monocytogenes* is thought to occur during food-processing, when the pathogen produces a biofilm that persists in the environment and acts as the source for subsequent dispersal of cells onto food. A survey of seafood-processing plants in New Zealand identified the persistent strain, 15G01, which has a high capacity to form biofilms. In this study, a transposon library of *L. monocytogenes* 15G01 was screened for mutants with altered biofilm formation using a crystal violet assay to identify genes that are associated with biofilm formation in this strain. This screen identified 36 transposants that showed a significant increase or decrease of biofilm formation compared to the wild-type. Nested semi-arbitrary PCR and subsequent sequencing of the amplified products identified the transposon insertion sites. The insertion sites were in 27 genes, of which 20 led to decreased biofilm formation and seven to an increase. Two insertions were in intergenic regions. Annotation of the genes suggested that they were involved in diverse cellular processes, including stress response, autolysis, transporter systems and cell wall/membrane synthesis. Subsequent analysis of the biofilms produced by the transposants using scanning electron microscopy and fluorescence microscopy showed notable differences in the structure of the biofilms produced by several mutants when compared with the wild-type. In particular, inactivation of *uvrB* and *mltD* produced coccoid-shaped cells and elongated cells in long chains, respectively. The disruption of *clsA* resulted in greater aggregation/clumping of the cells on stainless steel coated with mussel juice and a cloudy structure observed for the *mgtB* mutant using fluorescence suggested an increased level of extracellular DNA in the biofilm. The identification of these changes in biofilm structure corroborated the altered biofilm formation detected using the *in vitro* assay and confirmed the involvement of four genes with different cellular activity in biofilm formation in *L. monocytogenes* 15G01.

5.1 Introduction

The foodborne pathogen *Listeria monocytogenes* is a serious health threat to immunocompromised people, the elderly and pregnant women (Rocourt et al. 2000). Its capacity to cross the main protective barriers in the body, including the blood-brain barrier, the intestinal barrier and the placental barrier leads to a high mortality rate (up to 30%) in susceptible groups, which can include unborn and newborn babies (Lecuit 2005). *L. monocytogenes* is ubiquitous in the environment, with contamination of food usually occurring during processing rather than in raw food (Fletcher et al. 1994). The breadth of foods contaminated with *L. monocytogenes* is extensive, and includes ready-to-eat meat and seafood as well as vegetables and fruit (Srey et al. 2013).

L. monocytogenes is ubiquitous in the environment because it has the capacity to adapt quickly to environmental changes to secure its survival. It has the capacity to grow in harsh conditions such as acidic environments as well as over a broad temperature range (Yousef 1999). It is also capable of surviving in high salt concentrations (up to 11.5 %) and at freezing temperatures (Yousef 1999). *L. monocytogenes* is motile across a broad range of temperatures too, possessing four to six peritrichous flagella that are responsible for its movement at room temperature and below. All these attributes contribute to the adaptability of this pathogen and impede its control when encountered in food-processing environments.

Surface attachment and biofilm formation are important to the environmental persistence of *L. monocytogenes* (Carpentier and Cerf 2011). A biofilm is a community of cells that exists in a sessile lifestyle rather than a planktonic one in order to use resources more efficiently and to resist environmental threats. Cells in a biofilm are attached to each other and to a surface. They are held together by an extracellular polymeric matrix that consists of DNA, proteins, lipopolysaccharides and other substances that contribute to its stability and act as a protective barrier. For *L. monocytogenes*, the capacity to form biofilms in so-called 'harbourage' sites, enables the pathogen to establish itself and to act as a source for subsequent dispersal of single cells. Management of *L. monocytogenes* is made more difficult because biofilm cells are more resistant to cleaning agents and sanitisers as well as antibiotics (Van Houdt and Michiels 2010). As a result, *L. monocytogenes* is able to contaminate surfaces and food products more persistently.

Transposon mutagenesis has proven to be a successful tool for identification of genes involved in biofilm formation in *L. monocytogenes* (Huang et al. 2012, Huang et al. 2013). Cao et al.

(2007), however, developed a Himar1 mariner-based transposition system that increased the efficiency of mutagenesis in this pathogen compared to the Tn917 transposition system. As a result, to date, four research groups have successfully identified genes involved in biofilm formation of *L. monocytogenes* using the Himar1-based transposition system (Alonso et al. 2014, Chang et al. 2012, Ouyang et al. 2012, Piercey et al. 2016). The majority of the identified genes were associated with biosynthesis or motility. This transposition system has also been used to identify the genetic factors underlying other phenotypic changes, such as desiccation survival or nisin-sensitivity (Collins et al. 2010b, Hingston et al. 2015).

A survey of seafood-processing plants in New Zealand identified four persistent strains (persisted in factories for at least 6 months), classified by their unique pulsotypes (Cruz and Fletcher 2011). One of these pulsotypes (5132), represented by *L. monocytogenes* 15G01, was shown to have a high capacity to form biofilms in *in vitro* assays (Cruz and Fletcher 2011). As the capacity to form biofilms is believed to be a major contributing factor in persistence of *L. monocytogenes* and subsequent contamination of food in food-processing premises, a library of mutants of *L. monocytogenes* 15G01, previously generated using the Himar1 mariner-based transposition system (unpublished), was screened for mutants with altered biofilm formation using the crystal violet assay. By undertaking this screen, this study aimed to identify additional genes in *L. monocytogenes* 15G01 that are associated with biofilm formation and to further characterise the role of these genes in this process. Selected mutants were then further characterised using scanning electron and fluorescence microscopy to visualise the structure of the respective biofilms.

5.2 Materials and Methods

5.2.1 Bacterial strains and growth conditions

The wild-type strain used in this study was *L. monocytogenes* 15G01, a representative of the persistent pulsotype 5132 obtained in a New Zealand seafood-processing facility during a 2.5 year sampling programme (Cruz and Fletcher 2011). *L. monocytogenes* 15G01 was kept as glycerol stocks in a -80°C freezer and recovered in a three-step process by growing in Tryptic Soy Broth (TSB) enriched with 0.6% yeast extract (TSBYE) (Difco, BD, USA) overnight at 37°C, then plating on Tryptic Soy Agar enriched with 0.6% yeast extract (TSAYE) (Difco, BD, USA) and lastly, subculturing and storing on Columbian sheep blood agar (Fort Richard, New Zealand) at 4°C. A library of 6500 mutants of *L. monocytogenes* 15G01, created by the New Zealand Plant & Food Research Ltd using the Himar1 mariner-based transposition system in 2012

(unpublished) according to a method described previously (Cao et al. 2007), were kept on 96-well master plates in glycerol at -80°C, subcultured twice before use and stored on TSAYE plates supplemented with 5 ppm erythromycin ((Duchefa, Biochemie, The Netherlands) final concentration)). Media and agar plates were supplemented with erythromycin at a final concentration of 5 ppm for the studies with the mutants and with additional kanamycin (MP Biomedicals, Illkirch, France) at a concentration of 50 ppm for the complemented strains. The optical density measurements for the growth studies were taken at a wavelength of 595 nm using a microplate reader (Multiskan EX, ThermoFisher) or the automated microplate reader (SPECTROstar Omega, BMG Labtech).

Table 5.1. Bacterial strains and plasmids used in this study

Bacterial strains and plasmids		***Mutation***	***Source***
<u>*Bacterial strains*</u>			
Listeria monocytogenes 15G01	Wild-type serotype 1/2a; Em^S, Kan^S		Cruz and Fletcher (2011)
34F11	15G01 with transposon inserted in the *clsA* gene (*LMON_2515)*; Em^R, Kan^S	15G01 *clsA*::himar1	This study
33E11	15G01 with transposon inserted in the *uvrB* gene (*LMON_250*1); Em^R, Kan^S	15G01 *uvrB*::himar1	This study
33E11-C	33E11 containing the pIMK-uvrB plasmid; Em^R, Kan^R	15G01 *uvrB*::himar1/pIMK*uvrB*	This study
33E11-EV	33E11 containing the pIMK plasmid; Em^R, Kan^R	15G01 *uvrB*::himar1/pIMK	This study
39G5	15G01 with transposon inserted in the *mltD* gene (*LMON_2714*); Em^R, Kan^S	15G01 *mltD*::himar1	This study
39G5-C	39G5 containing the pIMK-mltD plasmid; Em^R, Kan^R	15G01 *mltD*::himar1/pIMK*mltD*	This study
39G5-EV	39G5 containing the pIMK plasmid; Em^R, Kan^R	15G01 *mltD*::himar1/pIMK	This study
44D3	15G01 with transposon inserted in the mgtB gene (*LMON_2712*); Em^R, Kan^S	15G01 *mgtB*::himar1	This study
41H7	15G01 with transposon inserted in the *flaA* gene (*LMON_0695*); Em^R, Kan^S	15G01 *flaA*::himar1	This study
<u>*Plasmids*</u>			
pIMK	Site-specific listerial integrative vector, 5.1 kb, Kan^R		Monk et al. (2008)
pIMK-uvrB	Site-specific plasmid carrying the *LMON_2501* gene; Kan^R		This study
pIMK-mltD	Site-specific plasmid carrying the *LMON_2714* gene, Kan^R		This study

5.2.2 Biofilm formation assay

A biofilm formation assay was performed according to the method described by Djordjevic et al (Djordjevic et al. 2002) with some modifications. Briefly, overnight cultures were grown at 37°C in TSBYE in a sterile 96-well plate (polystyrene, U-bottom, Interlab, New Zealand) and transferred to new 96-well plates with a 96-well replicator, each well containing 200 µl modified Welshimer's broth (MWB) (Himedia, India). The cultures were incubated for 48 h at 30°C and then washed three times with 200 µl double-distilled H_2O (ddH_2O) using a microplate strip washer (ELx50, Biotek). After air-drying at ambient temperature for 30 min, 150 µl of a 1% aqueous crystal violet solution was added to the plates. After 45 min of incubation at 30°C, the crystal violet solution was removed and the cultures were washed six times with 150 µl ddH_2O. After drying for 30 min at 30°C, 150 µl of 96% ethanol was added to each well to de-stain the crystal violet stained cells. The optical density was measured after 1 h at 595nm with a microplate reader (Multiskan EX, Thermo Fisher). The OD_{595nm} values obtained were corrected by subtracting the OD_{595nm} value of uninoculated media. To screen the library of transposon mutants, mutants were stored in 96-well master-plates and subcultured twice before tests for biofilm formation. Biofilm formation for each mutant was measured three times and compared to the wild-type strain. Statistical analysis (two sample t-test; $p \leq 0.05$) was performed to select mutants of interest. The OD_{595nm} values of selected mutants were at least 2 standard deviations (SD) above or below that of the wild-type strain. To eliminate variability caused by growth deficiency biofilm formation and turbidity measurements were repeated for 120 selected mutants in two independent experiments with eight replicates each. Turbidity was measured at 595 nm with a microplate reader before the washing and staining process. The growth of the mutants was compared to the growth of the wild-type in MWB to confirm that the altered biofilm formation observed in the initial screen was not due to differences in the ability of the mutants to grow in this media (Supplementary Table S5.1 and Supplementary Figure S5.1).

5.2.3 Identification of transposon insertion sites in selected mutants

To locate the transposon insertion sites in the genomes of the mutants of interest, a nested arbitrary PCR was performed using one transposon specific primer and one arbitrary primer (Table 5.2) to amplify the regions flanking the transposon from the right and the left end. The PCR was performed in two steps with the Mastercycler gradient (Eppendorf, Germany). BioMix Red (Bioline) was used as the mastermix in the first round and the second round was run using AccuPrime Hifi Taq polymerase (Invitrogen). The first PCR round was performed with primer pairs Marq207/255 and Marq207/269 (Cao et al. 2007) to amplify the left end and right end of

the transposon, respectively. The following cycle conditions were used: 1 cycle at 94°C for 2 min; then 30 cycles at 94°C for 30s, 50°C for 30s and 72°C for 1 min; last cycle at 72°C for 7 min. One microlitre of the PCR product from the first round was used as template in the second step and amplified using primer pairs Marq208/256 and Marq208/270 to flank the transposon insertion region from the left and the right end, respectively with the following conditions: PCR conditions: 1 cycle at 94°C for 1 min; 35 cycles at 94°C for 30s, 55°C for 30s and 72°C for 1 min; last cycle at 72°C for 7 min. The annealing temperature was adjusted for each mutant if necessary to minimise non-specific annealing or to increase annealing. A final concentration of 3mM Mg^{2+} was used for all PCR reactions. Five microlitres of each amplified product was visualised on a 1.5% Agarose Gel (settings 100V for 30 min) using Redsafe (Intron Biotechnology) under UV light. The PCR products obtained were subsequently purified and sequenced by Macrogen Ltd. (South Korea) and analysed using the NCBI BLAST program version 2.2.30 (available from https://www.ncbi.nlm.nih.gov) and the Geneious® 7 program (available from http://www.geneious.com) (Kearse et al. 2012). The reference strain *L. monocytogenes* EGD (accession HG421741) (Becavin et al. 2014) was used to identify the coordinates at the point of the transposon insertion site and the orientation of the transposon in the chromosome. This reference strain was chosen as it is the same serovar as the *L. monocytogenes* 15G01 strain (serovar 1/2a).

5.2.4 Complementation of selected mutants

The site-specific integrative vector pIMK (Monk et al. 2008) was used for the genetic complementation of selected mutants. Vector pIMK is a derivative of pPL2 (1Kb smaller), originally constructed by Lauer et al. (2002) and facilitates the insertion at the $tRNA_{Arg}$ locus. Genetic complementation constructs using this plasmid were constructed by amplifying the target genes from wild-type 15G01 using gene-specific primers in PCR (Table 5.2). PCR reactions were conducted in the following cycle conditions: 1 cycle at 94°C for 2 min; then 30 cycles at 94°C for 30s, 55°C for 30s and 72°C for 1 min; last cycle at 72°C for 7 min. The gene-specific PCR products and the pIMK vector were then digested with *Pst*I and *Bam*HI and ligated to one another using the LigaFast™ Rapid DNA Ligation System (Promega), following the manufacturer's instructions, in a molar ratio 3:1 (vector to insert). One microlitre of the recombinant plasmid was then introduced into chemically competent *Escherichia coli* S17 cells by heat shock. Transformants were selected on Luria Bertani (LB) agar plates supplemented with kanamycin. A colony was picked from the agar plate with a sterile toothpick and dipped in the PCR-mix. Colony PCR was performed with one gene-specific primer and T7 (T7 binding site

present in the pIMK plasmid) to confirm successful transformation. Recombinant plasmids were extracted from colony PCR-positive cultures and the gene inserts were sequenced to confirm the authenticity of the constructs. Authentic transformants were then used for the genetic complementation of *L. monocytogenes* 15G01 mutants. Conjugation was performed according to Azizoglu et al. (2014) with some modifications. Single colonies of the donor (*E. coli* transformants containing the construct pIMK:gene or a control containing only the pIMK vector) were resuspended overnight in LB broth containing kanamycin and incubated at 30°C, at 100 rpm to an OD_{595nm} of approximately 0.55. At the same time, a colony of the recipient (*L. monocytogenes* 15G01 transposon mutant) was resuspended in Brain-Heart-Infusion (BHI) (Difco, BD, USA) medium and incubated overnight at 37°C with shaking. The donor culture (3 mL) and a pre-warmed (45°C; 10 min) recipient culture (1.5 mL) were mixed together and centrifuged at 2050 g for 8 min; the bacterial pellet was then washed with 10 mL of BHI broth and centrifuged again using the same conditions. After washing, the pellet was resuspended in 500 µL of fresh BHI broth, deposited in the centre of a BHI agar plate and left overnight at 37°C. The drop was then resuspended in 2 mL BHI broth and spread-plated on BHI agar plates containing kanamycin and nalidixic acid (Fort Richard, New Zealand) (20 µg/mL). *L. monocytogenes* strains are naturally resistant against nalidixic acid and therefore it was used for counterselection. The authenticity of transconjugants was confirmed by colony PCR using the corresponding gene-specific primers. Their identity as *L. monocytogenes* was also confirmed using 16S-rRNA specific primers for *L. monocytogenes* (Table 5.2). To confirm that the empty vector had no effect on the phenotype, the parent plasmid was transformed in each mutant as well as the wild-type strain 15G01.

Table 5.2. Primers used in this study

Primer name	Nucleotide sequence of primer (5'-3')	Product size	Reference	Usage
clsA_Fwd	CAA*CTGCAG*AGAAATTTTTCA ATTCTAACCAAGGTG (*Pst*I)	1842	This study	Complementation of 34F11
clsA_Rev	AAC*GGATCC*CAATCGACAGG ACAACATCTACA (*Bam*HI)		This study	
uvrB_Fwd	CAA*CTGCAG*CCTTCAATTAAA TCCACATCTGGT (*Pst*I)	2528	This study	Complementation of 33E11
uvrB_Rev	AAC*GGATCC*TGTGCTTGCAAC GTATATGCT (*Bam*HI)		This study	
mltD_Fwd	CAA*CTGCAG*TTGACGTAGAAA CACCTTAGCAC (*Pst*I)	2683	This study	Complementation of 39G5
mltD_ Rev	AAC*GGATCC*AAAGGCAATTTC GGTGCGAC (*Bam*HI)		This study	
mgtB_Fwd	CAA*CTGCAG*TGCGATTACTAG TATCAAAAGCAAT (*Pst*I)	3222	This study	Complementation of 44D3
mgtB_Rev	AAC*GGATCC*TAGTATGCGTAT AAACTCAGAAGTCCT (*Bam*HI)		This study	
16S_Fwd	CAGCAGCCGCGGTAATAC	938	Park et al. (2012)	Identification of *L. monocytogenes*
16S_Rev	CTCCATAAAGGTGACCCT		Park et al. (2012)	
Marq254	CGTGGAATACGGGTTTGCTAA AAG		Cao et al. (2007)	Arbitrary PCR
Marq255	CAGTACAATCTGCTCTGATGC CGCATAGTT		Cao et al. (2007)	
Marq206	TGTCAGACATATGGGCACACG AAAAACAAGT		Cao et al. (2007)	
Marq207	GGCCACGCGTCGACTAGTAC NNNNNNNNNNGTAAT		Cao et al. (2007)	
Marq208	GGCCACGCGTCGACTAGTAC		Cao et al. (2007)	
Marq257	CTTACAGACAAGCTGTGACCG TCT		Cao et al. (2007)	
Marq270	TGTGAAATACCGCACAGATGC GAAGGGCGA		Cao et al. (2007)	
Marq271	GGGAATCATTTGAAGGTTGGT ACT		Cao et al. (2007)	
T7promoter	TAATACGACTCACTATAGGG		Macrogen, Inc (South Korea)	Presence of vector pIMK

Italics represent restriction enzyme sites; used restriction enzymes are shown in brackets; N= A or C or G or T

5.2.5 Microscopy

Fluorescence microscopy and scanning electron microscopy (SEM) were used to visualise biofilm formation of bacterial strains on polystyrene and stainless steel, respectively. For visualisation under fluorescence, the fluorescent LIVE/DEAD *Bac*Light bacterial viability kit (Life Technologies, Thermo Fisher, NZ) was used to label living cells green (SYTO9, membrane-permeable stain) and dead cells red (propidium iodide, a non-membrane permeable stain). Six-well plates (tissue treated, Greiner, Germany) were filled with 2.97 mL MWB and inoculated with 30 µL of an overnight bacterial culture grown in TSBYE at 37°C and were incubated at 30°C for 48 h. The biofilms were then washed twice with 0.8% NaCl to remove loosely attached cells and stained with 1 mL fluorescent stain prepared according to the manufacturer's manual. A fluorescence microscope (Olympus, BX51 fitted with the XC30 digital camera) was used at 10 x 100 magnification to take images.

SEM was performed on stainless steel coupons (5x5mm, food grade) as described previously (Borucki et al. 2003). The coupons were coated with cooked mussel juice (CMJ) produced as described previously (Nowak et al. 2015), but with some modifications. Briefly, Greenshell™ mussels obtained from the local supermarket were stored for 24 h at 10°C in a fridge and then boiled in a wok closed with a lid without addition of water. When all mussels opened and released the intervalvular juice, the liquid was collected and autoclaved at 121°C for 15 min. The coupons were pre-treated with alkali detergent for 2 h at 45°C and then rinsed with ddH_20 and autoclaved at 121°C for 15 min in deionised water. The coupons were coated by immersion in CMJ in a 6-well plate (tissue treated, Greiner, Germany) for 4 h at 60°C (or until dried). The coupons were then placed into a fresh 6-well plate containing MWB (2.97 mL) and were inoculated with 30 µL of an overnight culture of the bacterium ($10^{7.5}$–10^8 CFU). After incubation for 7 d at 30°C, phosphate buffered saline (PBS) (pH 7.2) was used to remove loosely attached cells on the coupons. After rinsing with 100 mM cacodylate buffer (pH 7.2) (Acros Organics, NJ, USA) the coupons were fixed overnight at 4°C in 2% glutaraldehyde (Acros Organics, NJ, USA) and 0.1% ruthenium red solution (Acros Organics, NJ, USA) in 100 mM cacodylate buffer. The next morning coupons were rinsed to remove unbound dye and then dehydrated in serial dilutions of ethanol for 10 min each (30, 50, 60, 70, 90% v/v) with three final 10 min rinses in absolute ethanol. The coupons were then critical point dried (BalTec CPD030 (BalTec AG, Balzers, Liechtenstein)) and sputter coated with gold (Leica EM ACE200, (Leica Microscopy Systems Ltd, Heerbrugg, Switzerland)) for visualisation using a scanning electron microscope (FEI Quanta 250 SEM (Fei Company, Hillsboro, OR)).

5.2.6 Motility assay

A motility assay was performed according to the method of Knudsen et al. (2004). Briefly, semi-solid agar plates (TSB+0.25% Agar (Difco, BD, USA)) were inoculated with *L. monocytogenes* 15G01 or the mutants using a sterile pick and incubated at 30°C or 37°C for 48 h. The diameter of the halo formed around the colony was then measured and compared to halo surrounding the wild-type strain (15G01). Three independent experiments were performed with each treatment repeated in triplicate.

5.2.7 Autolysis assay

The assay was performed according to Huang et al. (2012) with minor modifications. Briefly, single colonies of the wild-type and five selected mutants were picked from the TSAYE agar plate and grown in BHI at 37°C overnight. OD_{595nm} was measured in a microplate reader (SPECTROstar Omega, BMG Labtech) and adjusted to 0.6 ± 0.05 for each culture. Each culture (1.5 mL) was transferred to 2 mL microtubes and centrifuged at 4°C at 4500g for 10 min. The supernatant was discarded, and the cell pellet washed twice with ice cold ddH_2O, then resuspended in the same volume of Tris-HCl (pH 7.2) containing 0.05% Triton-X-100. Solutions with cells were incubated at 30°C in a 96-well plate and the OD_{595nm} was measured in 5 min intervals using an automated microplate reader (SPECTROstar Omega, BMG Labtech).

5.3 Results

5.3.1 An *in vitro* biofilm assay identified a multitude of mutants that have either greater or lower biofilm formation than the wild-type

A part (4,500) of an existing library of approximately 6,500 mutants of *L. monocytogenes* 15G01, created with the mariner transposition system, was screened at 30°C in MWB for mutants with altered biofilm formation using the crystal violet assay. MWB and a temperature of 30°C were used as they were previously shown to induce biofilm formation in *L. monocytogenes* 15G01 (Nowak et al. 2015). In total, 36 mutants were found with greater (10 mutants) or lower (26 mutants) biofilm formation ability under these conditions compared to the wild-type (Table 5.3), with the average OD_{595nm} of the mutants being at least 2 SD below or above the average OD_{595nm} value (1.197) of the wild-type *L. monocytogenes* 15G01. The growth of the mutants was compared to the growth of the wild-type strain in MWB during the screen to confirm that the altered biofilm formation observed was not due to differences in the ability of the mutants to grow in this media and only mutants showing equal or higher OD_{595nm} than the wild-type after 48 h were included.

5.3.2 Characterisation of the transposon insertion sites in mutants with altered biofilm formation identified 27 loci potentially involved in this process

Nested semi-arbitrary PCR enabled the amplification of the genome sequences flanking the transposons in the 36 mutants. DNA sequencing of the flanking regions and subsequent BlastN and megablast comparisons of the sequences with the genome of reference strain *L. monocytogenes* EGD permitted the locations and directions of the insertions in 15G01 to be estimated for all mutants with identification of the exact insertion sites for 28 mutants. Overall, the transposon insertion sites in the mutants were distributed across the genome of the reference strain *L. monocytogenes* EGD. However, transposon insertions seemed to predominantly occur in the second half of the genome questioning random transposon insertions (Figure 5.1). In the 36 mutants examined, 27 genes were disrupted by a transposon insertion, with six loci disrupted in two independent mutants and one disrupted in three independent mutants (Table 5.3). A further two mutants had an insertion of the transposon in an intergenic region (Table 5.3). Two transposons were inserted in genes that were not present in the reference genome of *L. monocytogenes* EGD, but were present in the parental strain *L. monocytogenes* 15G01.

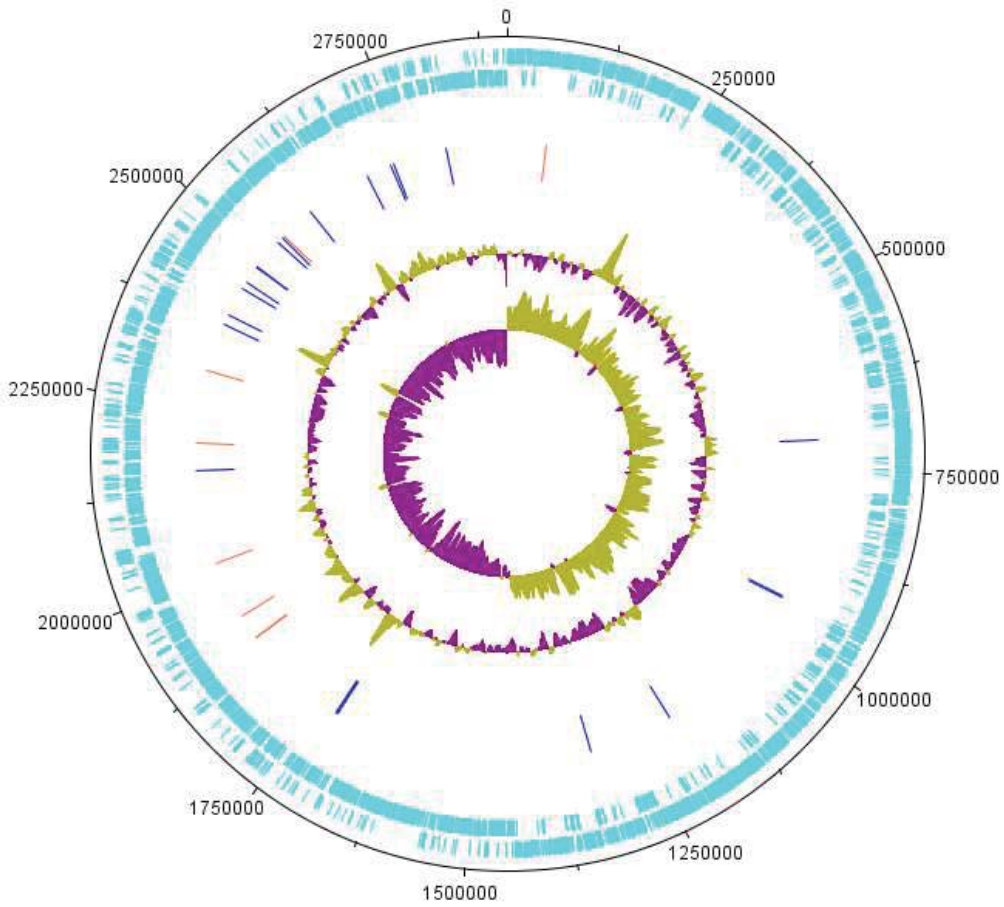

Figure 5.1. A representation of the genome of *Listeria monocytogenes* EGD showing the locations of transposon insertion sites associated with biofilm formation in *L. monocytogenes* 15G01. The outer ring represents the scale in bp, protein coding sequences are shown in turquoise, the middle ring highlights genes with a transposon insertion leading to greater biofilm formation (orange) and low biofilm formation (blue) in 15G01 and the inner ring (purple/green) shows the G+C% content plot with the GC skew. The image was generated using DNAPlotter available from www.sanger.ac.uk.

5.3.3 Functional analysis of the disrupted genes identified multiple functional groups are involved in biofilm formation in *L. monocytogenes* 15G01

Comparison of the disrupted genes in each of the mutants affected in biofilm formation with homologous genes in *L. monocytogenes* EGD and other *L. monocytogenes* strains in the GenBank database divided them into seven diverse functional groups (Table 5.3). Of particular note, five genes annotated as being involved in cell wall/membrane synthesis or integrity were identified, including genes encoding a putative peptidoglycan bound protein disrupted in three mutants that had low biofilm formation, cardiolipin synthetase (designated as *clsA*), disrupted

in two mutants with greater biofilm formation and the glycosyltransferase LafA in two mutants with low biofilm formation.

Genes involved in transport systems, stress response, autolysis and motility also influenced biofilm formation, including a gene predicted to encode a P-type $Mg^{(2+)}$ transport ATPase (designated as *mgtB*) and a gene annotated as encoding the ABC transporter permease protein EscB, which were both disrupted in two mutants. Disruption of the P-type $Mg^{(2+)}$ transport ATPase resulted in decreased biofilm formation, whilst the insertion of the transposon into *escB* resulted in increased biofilm formation.

Table 5.3. Biofilm-related genes identified in *Listeria monocytogenes* 15G01 through transposon insertions based on DNA homologies with *L. monocytogenes* EGD genome database, accession HG421741. Homologies were identified by megablast and Geneious® 7 software using default settings. The biofilm mass produced by the mutants was calculated relative to the biofilm formation of the wild-type in two independent experiments with eight replicates. Putative gene functions are based on the information from http://www.genome.jp/.

Function group and Putative gene function	Transposon insertion site	Biofilm mass relative to wt strain [%] mean ± SD	Coordinates for insertion in *L. monocytogenes* EGD	Number of hits	Orientation of transposon insertion	Mutant strain [a]
Biosynthesis						
Adenylosuccinate synthase	*LMON_0057 (purA)*	135.7 ± 5.12	59174	1	3'-5'	24A10
Dihydroxyacetone kinase family protein	*LMON_1882*	173.03 ± 7.82	1888930	1	3'-5'	35H9
Acetyltransferase, GNAT family	*LMON_2362*	27.32 ± 6.92	2377834	1	5'-3'	31E7
Glycosyl hydrolase, family 31	*LMON_2457*	19.90 ± 4.93	[c]	1	3'-5'	32E5
Cell wall / membrane						
Sortase A, LPXTG specific SrtA	*LMON_0935*	7.49 ± 0.59	948604	1	3'-5'	31C7
Glycosyltransferase	*LMON_0939*	18.74 ± 3.36	[c]	1	5'-3'	35C1
Putative peptidoglycan bound protein (LPXTG motif) Lmo1666 homologue	*LMON_1733*	29.70 ± 5.66	1723653, 1721718, 1721318	3	5'-3', 3'-5', 3'-5'	24H3, 25D2, 64B3

Cardiolipin synthetase ClsA	*LMON_2515*	163.02 ± 15.27	2538121, 2538525	2	3'-5', 5'-3'	30A9, 34F11
Glycosyltransferase LafA	*LMON_2570*	16.10 ± 2.43	2591134, 2590370	2	3'-5', 3'-5'	28G11, 32A9
Translation and Transcription						
GTP-binding protein HflX	*LMON_1358*	16.06 ± 6.92	[c]	1	3'-5'	43C9
SSU ribosomal protein S1p	*LMON_2007 (rpsA)*	140.68 ± 10.74	2012734	1	3'-5'	28A2
Transporter systems						
Manganese ABC transporter, ATP-binding protein SitB	*LMON_1917*	133.83 ± 7.38	1925244	1	3'-5'	47H11
Bacitracin export ATP-binding protein BceA	*LMON_2188*	138.01 ± 1.19	2194403	1	3'-5'	31G1
ABC transporter, permease protein EscB	*LMON_2290*	142.51 ± 7.66	2305608, 2305511	2	3'-5', 3'-5'	32E2, 47H10
Phosphate ABC transporter, periplasmic phosphate-binding protein PstS	*LMON_2511*	30.81 ± 0.50	2532962	1	3'-5'	36A2
PTS system, IIA component	*LMON_2675*	32.69 ± 3.94	2692293	1	3'-5'	26C10
$Mg^{(2+)}$ transport ATPase, P-type	*LMON_2712*	7.16 ± 1.47	2731046, 2731895	2	3'-5', 3'-5'	30H2, 44D3
ABC transporter, ATP-binding protein	*LMON_2792*	6.93 ± 0.27	2816670	1	3'-5'	28D10

Motility						
Flagellin protein FlaA	*LMON_0695 (flaA)*	23.74 ± 5.93	[c]	1	3'-5'	41H7
Autolysis						
Membrane-bound lytic murein transglycosylase D precursor MltD	*LMON_2714*	2.11 ± 0.35	2734305	1	5'-3'	39G5
DNA repair and stress response						
Glutamate decarboxylase	*LMON_2376*	6.81 ± 0.93	2393310	1	3'-5'	35F3
Excinuclease ABC subunit B UvrB	*LMON_2501 (uvrB)*	18.33 ± 2.58	2525427, 2525564	2	3'-5', 3'-5'	33E11, 42G3
Unknown						
FIG00774663: hypothetical protein	*LMON_1212*	21.32 ± 0.69	[c]	1	5'-3'	36B9
FIG00774466: hypothetical protein	*LMON_2144*	28.89 ± 3.37	2153693	1	5'-3'	26H1
COG1801: Uncharacterized conserved protein	*LMON_2417*	15.37 ± 0.51	[c]	1	3'-5'	40C12
Hypothetical protein (mutant strain 44F5)	[b]	10.70 ± 5.02	[c]	1	3'-5'	44F5
Hypothetical protein (mutant strain 41A8)	[b]	21.88 ± 7.19	[c]	1	3'-5'	41A8

Intergenic						
Methionine ABC transporter ATP-binding protein and hypothetical protein	*LMON_2430 & LMON_2431*	18.27 ± 1.96	2450096	1	3'-5'	67C5
Dihydroxyacetone kinase family protein and putative alkaline-shock protein	*LMON_1882 & LMON_1883*	173.44 ± 15.52	1889436	1	3'-5'	33F8

[a] Internal numbering of the transposon library

[b] No counterpart in *L. monocytogenes* EGD, but present in parental strain

[c] Exact insertion site could not be determined

5.3.4 Complementation of selected genes confirms a role for *mltD* in biofilm formation of *L. monocytogenes* 15G01

Four mutants (33E11, 34F11, 39G5 and 44D3) were examined further to confirm the role of the disrupted genes in biofilm formation of *L. monocytogenes* 15G01. The first, the *clsA* mutant (34F11) was selected because this gene had been disrupted in multiple mutants with increased biofilm formation and the *clsA* gene was known to be involved in biofilm formation of other bacterial species (Lin et al. 2015, Munoz-Elias et al. 2008, Puttamreddy et al. 2010). The *uvrB* mutant (33E11) was selected as multiple mutants in this excinuclease ABC subunit B were identified in the screen and *uvrB* is part of an operon consisting of *uvrA*, which, when disrupted in *L. monocytogenes* in a previous study, showed increased biofilm formation at 15°C (Piercey et al. 2016). The *mltD* mutant (39G5) was studied because it was predicted to affect autolysis, which has been implicated in changes in biofilm formation *in L. monocytogenes* (Machata et al. 2005, Machata 2008) and other bacteria (Bao et al. 2015), whilst the *mgtB* mutant (44D3) was included because it has also been disrupted in multiple mutants and has not been associated with biofilm formation in *L. monocytogenes* before.

Growth curves of the selected 36 mutants in MWB at 30°C were then produced by manual measurements to further examine growth behaviour (Supplementary Table S5.1 and Supplementary Figure S5.1) in the presence of selective antibiotics. Additionally, growth studies with an automated plate reader were carried out for the four selected mutants without selective antibiotics to further rule out the possibility that the changes in biofilm formation were due to impaired growth (Supplementary Figure S5.2). The two mutants 39G5 and 44D3 formed cell aggregates during growth which is reflected by the high OD_{600nm} values, however, growth pattern was not affected. 39G5 had an extended exponential phase compared to the other strains, however all five examined strains were in stationary phase at 48 h, the time point at which biofilm formation was measured.

Complementation studies were subsequently carried out on the four mutants. In these studies, introduction of the pIMK vector containing the wild-type *mltD* gene into the *mltD* mutant resulted in the restoration of biofilm formation to levels produced by the wild-type (Figure 5.2). Conjugation of the empty vector into this mutant had no obvious effect on biofilm formation (Figure 5.2). These data confirmed that the change in biofilm formation in the *mltD* mutant was a result of inactivation of the gene. Thus, *mltD* is required for biofilm formation in *L. monocytogenes* 15G01.

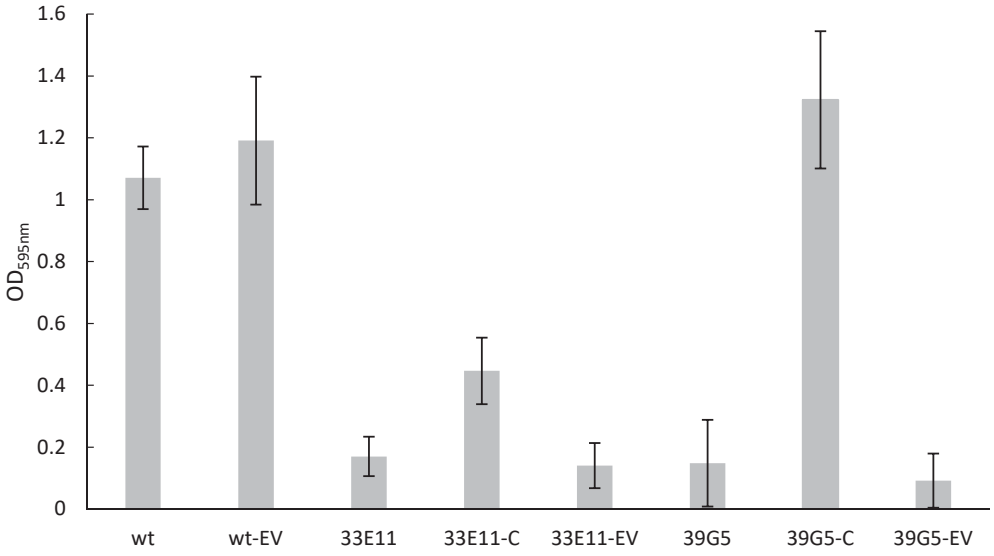

Figure 5.2. A comparison of biofilm formation in *Listeria monocytogenes* 15G01 (wt), the *uvrB* and *mltD* mutants (33E11 and 39G5), and mutants containing a wild type copy of the corresponding gene (complemented strains (-C)) or the empty vector pIMK (-EV). Error bars represent the standard deviation for three independent experiments (n=6). Biofilm formation was determined by measuring the OD_{595nm} as part of the crystal violet assay.

In contrast to the successful complementation of the *mltD* mutant, introduction of the pIMK vector containing the wild-type *uvrB* gene into the *uvrB* mutant only partially restored (41.7%) the wild-type phenotype (Figure 5.2), whilst attempts to complement the *mgtB* and *clsA* mutants using a similar process failed to restore the wild-type phenotype altogether (data not shown). Partial complementation of *uvrB* may have occurred because this gene is part of an operon with *uvrA*, which may have been affected by the transposon insertion and requires its entire presence for full expression. The inability to restore the wild-type phenotypes in the *mgtB* and *clsA* mutants may have resulted from differential expression of the genes upon site-specific integration of the pIMK vector or even due to polar effects upon insertion of the transposon. Nevertheless, transposon insertions in both *mgtB* and *clsA* were identified twice in the screen of the transposon library (Table 5.3), with transposon insertions in different positions (identified insertions 404 base pairs apart for *clsA* and 849 base pairs apart for *mgtB* (Table 5.3)) and in the opposite direction for *clsA* mutants. Together with literature linking these genes to biofilm formation in other studies, their repeated identification provided strong evidence that they are involved in biofilm formation by *L. monocytogenes* 15G01 despite our being unable to complement the associated phenotype.

5.3.5 Microscopy confirmed that the *mltD* mutant (39G5) has a dramatic loss in both viable and non-viable cells

Despite the failure to complement all the selected mutants, the weight of evidence associating each of the disrupted genes with biofilm formation led to their inclusion in microscopic analysis. Fluorescence microscopy showed that after 48 h incubation of the mutants on polystyrene surfaces in MWB at 30°C, all appeared to show some differences in the structure of their biofilms when compared to the wild-type (Figure 5.3). The biofilm of the *mltD* mutant consisted of few mainly individual cells, which seemed to be elongated and in chains. The lack of visible cells (whether alive or dead) was consistent with the low biofilm formation observed in the initial screen. The biofilm produced by the *mgtB* mutant also contained few live cells, although it did appear to have a cloudy structure under fluorescence, possibly because extracellular DNA (eDNA) was present in the biofilm. Consistent with this, there appeared to be greater numbers of dead cells associated with the biofilm produced by the *mgtB* mutant. Harmsen et al. (2010) identified extracellular DNA (of chromosomal origin) to be a major component of biofilms formed by *L. monocytogenes* (Harmsen et al. 2010). The *uvrB* mutant was a low biofilm former and exhibited only sparse biofilm formation after 48 h, which was consistent with the approximately 80% reduction in biofilm formation observed in *in vitro* assays. The *clsA* mutant showed a biofilm with a high number of living cells, but also showed a higher amount of dead, red-stained cells.

Scanning electron microscopy was used to analyse further the structure of the biofilms produced by the mutants after growth of the bacteria on stainless steel (SS) coupons coated with mussel juice for 7 d at 30°C. A SS coupon coated with mussel juice was used as a control and organic debris was clearly visible (Figure 5.4). Bacterial cells attached and formed biofilms on the coupons preferably where organic debris of the mussel juice was present (Figure 5.4).

SEM images of 39G5 confirmed the long chain phenotype with some elongated cells (Figure 5.4). The 33E11 was a low biofilm former and exhibited only sparse biofilm formation on SS coupons after 7 d although extracellular matrix was present. 33E11 appeared to have a different cell morphology compared to the wild-type with cocci-shaped rather than rod-shaped cells (Figure 5.4). 34F11 identified as a high biofilm former in both the microtitre plate screening test and under the fluorescence microscope, also exhibited greater biofilm formation on the SS coupons compared to the wild-type (Figure 5.4). In addition, 34F11 exhibited an extensive thread structured biofilm attached to organic mussel debris (Figure 5.5).

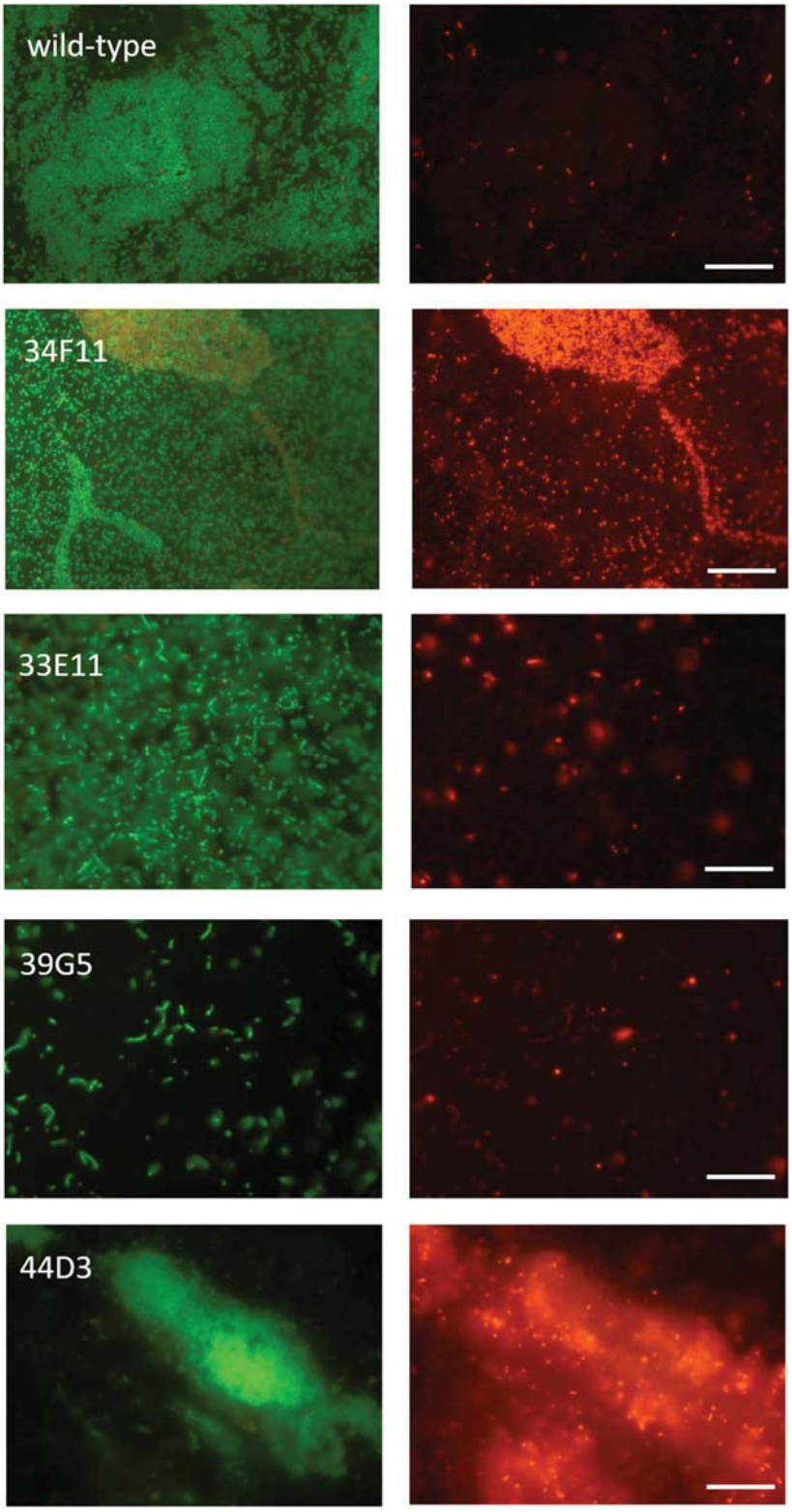

Figure 5.3. Images of the biofilms produced by *Listeria monocytogenes* 15G01 (wild-type) and selected transposon mutants with altered biofilm formation (34F11, 33E11, 39G5 and 44D3) grown on polystyrene surfaces in MWB for 48 h at 30°C. The biofilms were stained with the LIVE/DEAD *Bac*Light bacterial viability kit according to the manufacturer's instruction (Life Technologies, Thermo Fisher, New Zealand). Living cells were labelled with SYTO9 (green) and dead cells with propidium iodide (red). Scale bars represent 20 µm.

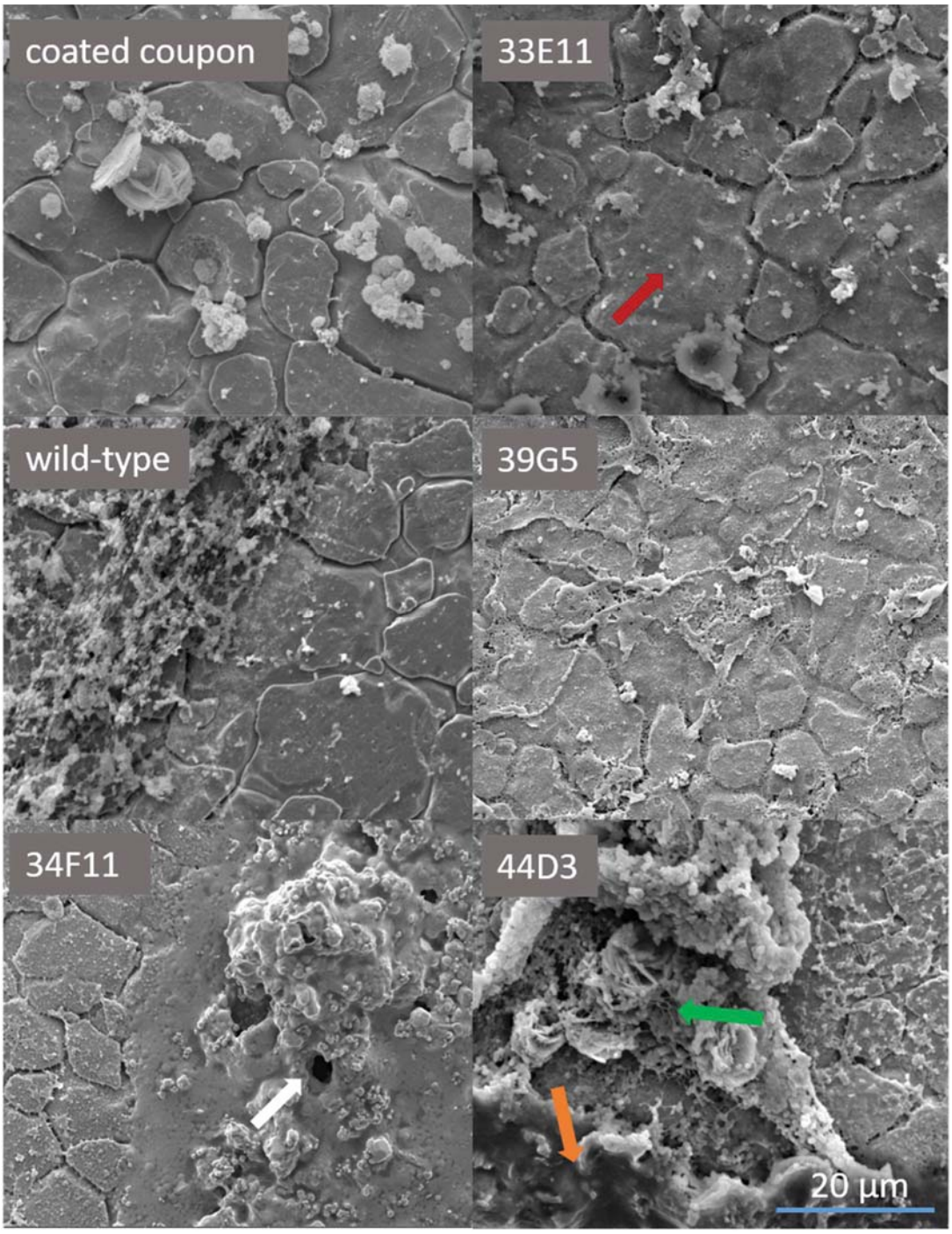

Figure 5.4. Images of the biofilms produced by *Listeria monocytogenes* 15G01 (wild-type) and selected transposon mutants with altered biofilm formation (34F11, 33E11, 39G5 and 44D3) grown on stainless steel coupons coated with mussel juice for 7 d at 30°C. The images were obtained with a scanning electron microscope with 5000x magnification. The cracks are features of the stainless steel surface.

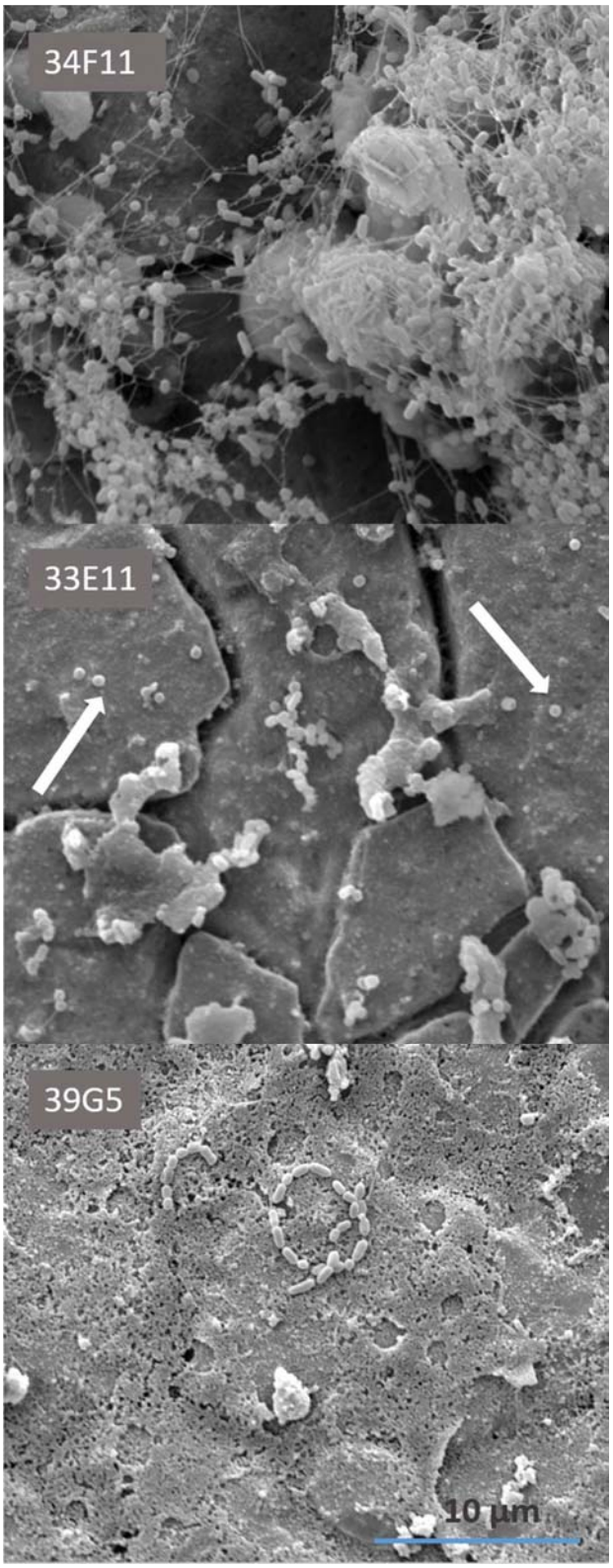

Figure 5.5. High magnification images of the biofilms produced by 34F11, 33E11 and 39G5 grown on stainless steel coupons coated with mussel juice for 7 d at 30°C. The images were obtained with a scanning electron microscope with 10000x magnification. The white arrows point at coccoid-shaped bacteria.

5.3.6 The *mltD* mutant (39G5) was defective in autolysis, motility as well as biofilm formation

Inactivation of *mltD* (*murA*-homologue) has previously been shown to result in loss of motility in *L. monocytogenes* (Machata 2008). Consistent with these findings, the *mltD* mutant created in this study not only produced a low biofilm mass, but also exhibited no motility (Figure 5.6 b), which was restored upon gene complementation (Figure 5.6 b) The Triton X-100 induced autolysis rate was reduced for the *mltD* mutant compared to the wild-type (Figure 5.6 a), but not for the remaining mutants (data not shown), suggesting that the observed changes in biofilm formation in the remaining mutants are not due to autolytic processes.

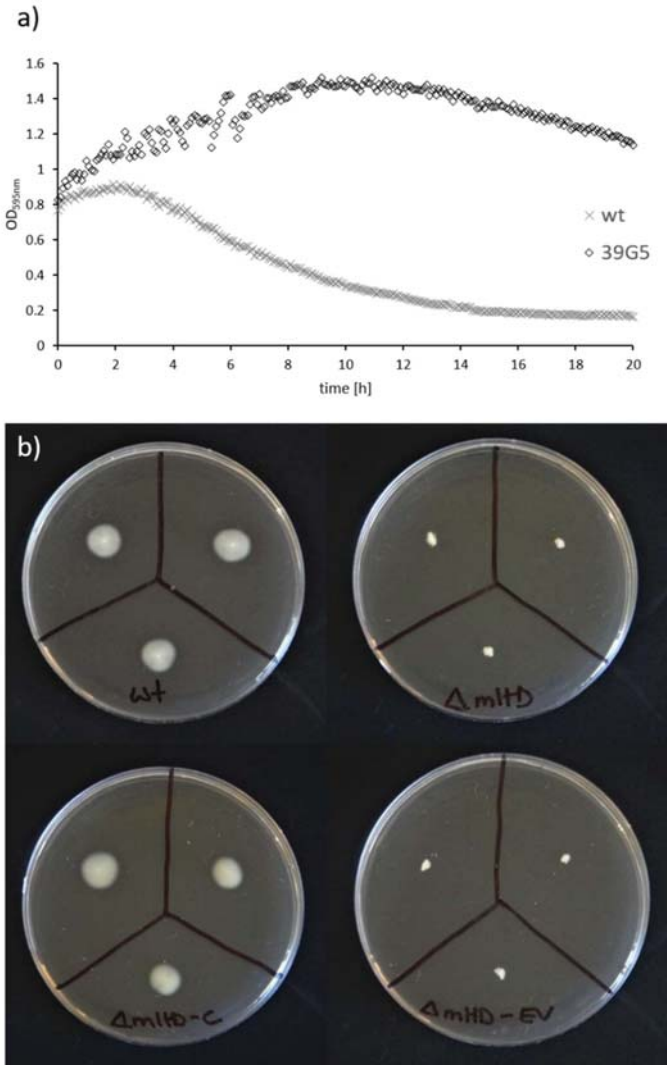

Figure 5.6. Triton X-100 induced autolysis of *Listeria monocytogenes* 15G01 (wt) and the *mltD* mutant (39G5) determined by optical density measurement at 595nm (a) and motility of *L. monocytogenes*, 39G5 (Δ*mltD*) and the *mltD* mutant containing a wild-type copy of the corresponding gene (complemented strains (-C)) or the empty vector pIMK (-EV) after 24 h at 30°C (b).

5.4 Discussion

L. monocytogenes can persist in seafood-processing plants in New Zealand, increasing the risk of contamination with this food borne pathogen (Cruz and Fletcher 2011). Biofilm formation is believed to be a major factor in survival and persistence of *L. monocytogenes* (Van Houdt and Michiels 2010). In this study, the screening of a transposon library created from *L. monocytogene*s 15G01, a persistent isolate from a seafood-processing plant in New Zealand, identified 27 genes as involved in biofilm formation. Four other research groups have also used transposon mutagenesis to identify genes involved in biofilm formation in *L. monocytogenes* (Alonso et al. 2014, Chang et al. 2012, Ouyang et al. 2012, Piercey et al. 2016). In each case, a large number of loci were reportedly associated with biofilm formation; Alonso et al. (2014) identifying 38 genes, Ouyang et al. (2012) 15 genes, Chang et al. (2012) 24 genes and Piercey et al. (2016) 19 genes. The large number of loci identified in each of the studies to date, and the variety of cellular processes the loci were predicted to be involved in (e.g. primary metabolism, translation and transcription and DNA repair) suggest that biofilm formation is very important for this pathogen, regardless of the environmental niche inhabited by the isolate. Many of the loci were only identified in a single study, however, probably because different isolates and different assay conditions were used. For example, Piercey et al. (2016) performed the assays at 15°C while other groups used higher temperatures (32°, 35° or 37°C). Ouyang et al. (2012) and Alonso et al. (2014) both generated a mutant library using *L. monocytogenes* 10403S whereas Chang et al. (2012) created a library of *L. monocytogenes* Scott A mutants and Piercey et al. (2016) used a serotype 1/2a strain isolated from a processing plant. This suggests that various environmental cues may regulate biofilm formation and that the regulatory cascade controlling biofilm formation may be specific to certain environmental triggers.

A number of genes identified as involved in the process of biofilm formation in these previous studies were also identified in the current study, despite the differences in assay conditions. For example, all studies, including this one, showed that a transposon insertion in lafA (gene ID lmo2555 in *L. monocytogenes* EGDe) caused a reduction in biofilm formation. Alonso et al. (2014) and Ouyang et al. (2012) also showed that an insertion in the gene (lmo2554) downstream of lafA led to decreased biofilm formation. Lmo2555 and lmo2554 are part of the same operon and encode glycosyltransferases that are required for the formation of the glycolipid that links the lipoteichoic acid to the bacterial membrane (Webb et al. 2009). Four transposants with insertions in the genes encoding for the glycosyltransferase LafA, sortase A, a peptidoglycan linked protein (LPXTG) and the flagella protein FlaA had previously been

identified by Chang et al. (2012). The detection of common genes associated with changes in biofilm formation under such variable conditions suggests that they are critical to biofilm formation regardless of the environmental conditions the bacterium experiences.

Transporter systems are essential in living organisms. The transition from a planktonic to a sessile lifestyle requires changes in metabolism and energy generation as resources in biofilms become scarce (Parsek and Fuqua 2004). Thus, during the screening of the *L. monocytogene*s 15G01 mutants, it was not surprising to identify seven genes involved in these broad processes that influence biofilm formation. These seven genes encode for ABC tranporters, ATPases and a phosphotransferase system, with inactivation of three leading to enhanced biofilm formation (a manganese ABC transporter SitB, the Bacitracin export ATP-binding protein BceA, and the ABC transporter EscB) and four causing a decrease in biofilm formation (a phosphate ABC transporter, a periplasmic phosphate-binding protein PstS, a PTS system, IIA component, and a $Mg^{(2+)}$ transport ATPase known as MgtB).

ABC transporters are one of the largest protein families known. These transporter systems facilitate the transport of compounds through a membrane under ATP-hydrolysis to maintain homeostasis. ABC-transporters have also been found to be involved in biofilm formation of other species (Hinsa et al. 2003, Vanderlinde et al. 2010, Varahan et al. 2014). The involvement of the Manganese ABC transporter protein SitB in biofilm formation is supported by the findings of Ceruso et al. (2014), who showed increased biofilm formation for an in-frame deletion mutant of LMOf2365_1877 (LMON_1917 homologue) while studying the ABC transport system. Zhu et al. (2011) investigated the involvement of a putative ABC transporter permease gene (lm.G_1771) in biofilm formation by generating a deletion mutant. This gene is part of an operon which consists of two genes (Zhu et al. 2011) and is the homologue for LMON_1812 in *L. monocytogenes* EGD, which encodes for a bacitracin export permease. The *L. monocytogenes* EGD strain harbours two of those operons encoding for bacitracin export permease protein BceB and BceA, situated on two distinct locations in the genome (LMON_1812 and LMON_1814; LMON_2188 and LMON_2189). Both, the greater biofilm formation observed for the *bceA* mutant (LMON_2188) in the present screen and the increase in biofilm formation for the lm.G_1771 mutant observed by Zhu et al. (2011), support its involvement in the biofilm formation process. By deletion of the lmo2115 gene (LMON_2189) it was shown that this multi-drug resistance transporter system not only affects biofilm formation but also nisin sensitivity (Collins et al. 2010a).

Another transporter system of particular interest is the P-type ATPase which takes up Mg^{2+} upon ATP-hydrolysis (MgtB). The Mg^{2+} P-type ATPase MgtB has not been associated with biofilm formation before. Given that Mg^{2+} is one of the most abundant cations present in cells and is involved in several vital processes in bacteria ranging from acting as a cofactor in ATP-dependent phosphorylations and other enzymatic reactions to stabilising ribosomes and influencing nucleic acid protein interactions and RNA folding (Groisman et al. 2013), the *mgtB* mutant was chosen for further investigation. Previous studies showed that magnesium deprivation triggers biofilm formation (Mulcahy and Lewenza 2011) and a temperature-dependent expression (Kehres and Maguire 2003) of *mgtB* regulated by the PhoP/PhoQ two component system in Gram-negative bacteria (Chamnongpol and Groisman 2002, Groisman et al. 2006). However, knowledge about the function in Gram-positive bacteria, in particular *L. monocytogenes*, is scarce. No data about the involvement of *mgtB* in biofilm formation are currently available. According to Nielsen et al. (2012) *mgtB* (lmo2689) is regulated by the two component system CesRK as CesR binding boxes were found upstream of that gene which, according to Nielsen et al. (2012), suggests direct control of *mgtB*. *mgtB* is part of an operon including genes encoding for cell division proteins which are also regulated by CesR (Nielsen et al. 2012). CesRK is associated with virulence, ethanol sensitivity and cephalosporine resistance (Kallipolitis et al. 2003). The transposon insertion in the *mgtB* gene (44D3) resulted in the production of a low biofilm phenotype in this study, suggesting an involvement of *mgtB* in biofilm formation of *L. monocytogenes*. ABC transporter systems do not only seem to be involved in biofilm formation and drug resistance, but also in stress tolerance of *L. monocytogenes* (Suo et al. 2012) and other species (Seaton et al. 2011). The high number of identified mutants associated with ABC transporter systems in this screen and the widespread functions of these systems highlight their importance in the survival of *L. monocytogenes*. Identification of the molecular mechanisms involved will help to understand and reduce survival of *L. monocytogenes* strains in food-processing plants.

Magnesium is not only important for bacterial homeostasis but has also been found to alter autolysis by inhibiting induced autolysis in *E.coli* upon its addition (Leduc et al. 1982). Autolysis is a very strictly regulated process ensuring that no self-destruction of the cell takes place. One mutant with a disruption of *mltD* produced low biofilm in this screen. The membrane-bound lytic murein transglycosylase D precursor (*mltD*) encodes for a murein degrading enzyme (autolysin) and belongs to the class of lytic transglycosylases which are important for cell division, insertion of proteins in the cell envelope and also for maintenance of bacterial morphology (Scheurwater et al. 2008). Observations of planktonic cells as well as biofilm cells

in the present study using fluorescence microscopy (Figure 5.3) and SEM (Figure 5.4) showed a high number of elongated cells in long chains for the *mltD* mutant, which is in line with a previous finding (Carroll et al. 2003). Carroll et al. (2003) observed long chains in exponential phase but not in stationary phase and no change in growth behaviour of the mutant. However, in the present study long chains in both exponential and stationary phases and slight differences in growth pattern were observed (Supplementary Figure S5.1 and S5.2 and Supplementary Table S5.1). In agreement with a previous study (Renier et al. 2014), the *mltD* mutant was found to produce increased cell aggregation, which interfered with optical density measurements for growth behaviour. However, cell counts after 24 h growth were similar to the wild-type levels (>9.0 $\log_{10}$ CFU/mL) (Supplementary Table S5.1). The *mltD* mutant (39G5) was one of the few in the screen that exhibited no motility, which is in agreement with other studies (Machata et al. 2005, Machata 2008). Two other low biofilm formers with insertions in the *flaA* gene and a gene encoding for an unknown protein (44F5) were also motility deficient (data not shown), which might account for their low biofilm production. The complemented *mltD* mutant restored biofilm formation and motility to the level of the wild-type (Figure 5.2 and 5.5b), confirming the gene's direct involvement in biofilm formation and motility. The reduction in motility and production of long chains with elongated cells might impair the ability of the mutant to move freely and to attach to surfaces, thus resulting in biofilm reduction. The observed lower autolysis rate after exposure of 39G5 to Triton X-100 as also seen in another study (Carroll et al. 2003) further confirmed the autolytic function of the gene. Autolysin was found to be involved in biofilm formation of other species through autolysin mediated release of extracellular DNA (Qin et al. 2007). It is known that eDNA plays a role in biofilm formation in *L. monocytogenes* by enabling attachment to surfaces as shown by Harmsen et al. (2010). It is possible that disruption of the *mltD* gene might lead to decreased release of eDNA and therefore less biofilm production. However, further tests need to examine the role of eDNA in biofilm formation of the *mltD* mutant.

The cell wall and membrane also play an important role in damage control of bacteria as they function as barriers against environmental damage and regulate homeostasis. Thus, when bacteria adapt to changing environmental conditions, the composition of the cell membrane changes. Therefore, it was not a surprise that this screen identified a number of mutants with defects in cell wall and membrane function that showed changes in biofilm formation. Of particular note, a transposon insertion in the *clsA* gene, encoding for the cardiolipin synthetase, led to enhanced biofilm formation in the present study. The cardiolipin synthetase catalyses the formation of cardiolipin from phosphatidylglycerol and is predominantly active in stationary

phase (Koprivnjak et al. 2011). Previous research showed that gene disruption of *clsA* resulted in decreased biofilm formation in other Gram-negative and Gram-positive species (Lin et al. 2015, Munoz-Elias et al. 2008, Puttamreddy et al. 2010), which indicates that *clsA* might be differentially regulated in different species, possibly due to differences in membrane composition. The hypothesis of differential regulation in multiple species is supported by studies which showed that changes in the environmental conditions, such as osmotic stress or desiccation, led to activation of *clsA* in *E. coli* and *Staphylococcus aureus* (Maudsdotter et al. 2015, Romantsov et al. 2009), but butanol stress induced downregulation of *clsA* in *Bacillus subtilis* (Vinayavekhin et al. 2015). By screening a mutant library of *L. monocytogenes* for desiccation survival, Hingston et al. (2015) found that a transposon insertion in the gene encoding for *clsA* resulted in decreased desiccation survival compared to the wild-type in *L. monocytogenes*.

Changes in environmental conditions not only trigger activation of genes involved in membrane composition to protect cells from damage but also trigger other stress response mechanisms. Many studies showed that stress response is somehow linked to biofilm formation (Huang et al. 2013, Suo et al. 2012, Taylor et al. 2002, van der Veen and Abee 2010a, van der Veen and Abee 2010b, van der Veen and Abee 2010c, van der Veen and Abee 2011b). One well-described system is the SOS response which is induced upon replication fork stalling caused by DNA damage through reactive oxygen species (ROS) (van der Veen and Abee 2011a). The *uvrB* is part of an enzyme complex that mediates excision and incision steps of DNA repair and is induced as part of the SOS response (van der Veen et al. 2010). In this study, a gene disruption of *uvrB* resulted in a low biofilm phenotype. The SOS-response has been linked to biofilm formation in several bacterial species including *L. monocytogenes*, *Pseudomonas aeruginosa* and *Streptococcus mutans* (Inagaki et al. 2009, van der Veen and Abee 2011a, van der Veen et al. 2010). Gene expression of *uvrB* and stress response associated genes were found to be upregulated in planktonic cells after heat exposure (van der Veen et al. 2007 Microscopic analysis of 33E11 showed changes in phenotype and produced coccoid-shaped bacteria (Figure 5.4 and 5.5). The formation of coccoid-shaped bacteria has been reported previously for *Listeria* cells after exposure to stresses such as starvation due to change from log-growth to long-term survival (Doijad et al. 2015, Wen et al. 2009). Tremoulet et al. (2002) found that the bacterial cells of seven-day-old biofilms of *L. monocytogenes* were more coccoid-shaped than rod-shaped, which is in agreement with our findings. The changes in phenotype might be due to maturation of the biofilm as suggested by Tremoulet et al. (2002) as they did not observe this phenotype for biofilm grown for 24 h.

In 44D3 and 34F11, some biofilms cells were found anchored in the biofilm matrix by thread-like structures and some embedded in an extrapolymeric substance with water channels (Figure 5.4 and 5.5), which showed that *L. monocytogenes* strains are capable of forming two different types of biofilm and not only thread-like structures as suggested by Marsh et al. (2003). The thread-like structures observed in the SEM images of the wild-type and the mutants could be flagella as suggested by Guilbaud et al. (2015), who found that the non-motile mutant (Δ*flaA*) did not exhibit such a structure when analysed with SEM in their study. However, SEM images of the flagella-deficient mutant (41H7) generated in the present study (Supplementary Figure S5.3), also showed filaments, suggesting that these are not flagella but rather an extracellular polymeric substance as also observed by Alonso et al. (2014). Despite these findings, discussion about whether *L. monocytogenes* forms a real biofilm or rather simply attaches is ongoing (Ferreira et al. 2014), however, the presented results strongly suggest that *L. monocytogenes* is able to produce a complex biofilm.

In the present study bacteria cells attached preferably on mussel juice debris rather than on plain stainless steel, which not only confirms the ability of the bacteria to attach to biotic surfaces, but also emphasises the necessity of proper cleaning and sanitation in food-processing plants as organic residues might encourage attachment and survival of bacteria. Despite the disadvantage in being an indirect method of measurement, the crystal violet assay is a fast and easy to use tool to screen large numbers of strains for their ability to form biofilms. The microscope analysis confirmed the findings of the crystal violet assay which corroborates its use as a screening method.

Other transposants identified in this study were the Lmo1666 homologue and *srtA*. It is known that Lmo 1666 contains a LPXTG motif and is covalently linked to the cell surface by Sortase A (Cascioferro et al. 2014) linking two of these identified genes with each other. Biofilm formation for the *srtA* mutant was lower (OD_{595nm} = 0.09) than the *lmo1666* homologue mutant (OD_{595nm} =0.355) implying that additional genes apart from the *lmo1666* homologue that are linked to the cell surface by Sortase A affect biofilm formation.

A disruption in the gene encoding for a dihydroxyacetone kinase resulted in enhanced biofilm formation in this study and the intergenic transposon insertion downstream of this gene and upstream of a gene encoding for a putative alkaline shock protein showed a similar increase in biofilm production, which leads to the speculation that the intergenic insertion interfered with the promoter binding site rather than causing a polar effect. Both mutants (intergenic and

dihydroxyacetone kinase) exhibited nearly identical biofilm formation (OD_{595nm} = 2.071 vs 2.069) strengthening the evidence for involvement of this gene in biofilm formation.

To this date numerous genes have been found to be involved in biofilm formation of *L. monocytogenes*. However, this screen did not reveal all of them which might be due to the selection process. Only mutants that showed significant increase or decrease were chosen to be further analysed. Moreover, it is known that environmental conditions influence a phenotype, so different screening methods and media might influence biofilm formation and consequently, limit the number of transposants found to alter biofilm formation. This screen identified transposon insertions in several genes associated with multiple cellular processes, including autolysis, stress response, transporter systems, motility, biosynthesis, translation and transcription and cell wall/membrane.

Although complementation remained unsuccessful for two of the mutants in this study (*clsA* and *mgtB*), evidence for their involvement in biofilm formation was strengthened due to their repeated identification in the screen harbouring transposon insertions in different spots (*mgtB* and *clsA*) and orientations within the gene (*clsA*). In addition both *mgtB* mutants (30H2 and 44D3, Table 5.3) identified in this screen behaved similarly in the biofilm formation assay, in growth studies (Supplementary Figure S5.1 and Supplementary Table S5.1) and in motility tests (data not shown). The two *clsA* mutants (30A9 and 34F11) produced the same amount of biofilm and were also similar in growth behaviour, but they had differences in their motility. The *clsA* gene consists of 1449 bp and the mutant 30A9 which exhibited higher motility than the mutant 34F11 had the transposon inserted after the 1133rd bp, whereas 34F11 had the insertion after the 729th bp. Therefore, the *clsA* gene might be partially functional in 30A9 resulting in higher motility than 34F11. The membrane protein cardiolipin is predominantly found at the cell poles of rod-shaped bacteria (Dworkin 2009) and lack of cardiolipin might affect incorporation or attachment of specific proteins, such as flagella, into the cell poles resulting in decreased motility. A previous study found that the swimming motility of *Rhodobacter sphaeroides* was not affected by cardiolipin deficiency (Lin et al. 2015). However, in contrary to *L. monocytogenes*, which has 4-6 peritrichous flagella, *R. sphaeroides* has one single flagellum usually not situated at the cell pole but medially on the cell body (Armitage and Macnab 1987). This strengthens the evidence for the involvement of *clsA* in motility.

It is worth noting that a variety of authors published phenotypic data on genes associated with biofim formation in lieu of complementation (Ouyang et al. 2012, Piercey et al. 2016).

5.5 Conclusion

In this study insertions in 27 genes were found to alter biofilm formation in *L. monocytogenes* 15G01. Genes previously known to be involved in biofilm formation by *L. monocytogenes* and/or other bacterial species were found, corroborating the method of transposon mutagenesis for functional analysis of biofilm formation. Two genes, *clsA* and *mgtB*, were identified to be involved in biofilm formation. Both have, to the best of our knowledge, not previously been associated with biofilm formation in *L. monocytogenes* before. *uvrB*, a stress response gene is clearly involved in biofilm formation, strengthening the link between biofilm formation and stress response. Further studies to analyse gene regulation are required to assess the exact involvement of the biofilm-associated genes.

5.6 Acknowledgements

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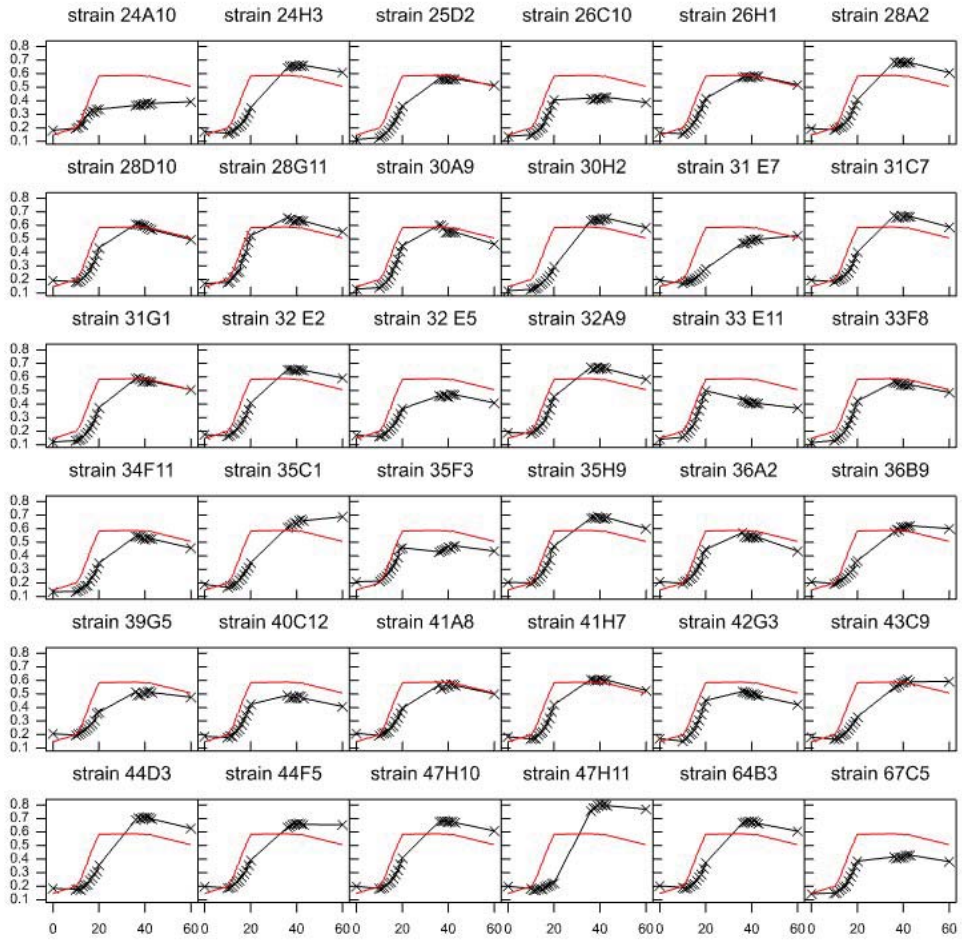
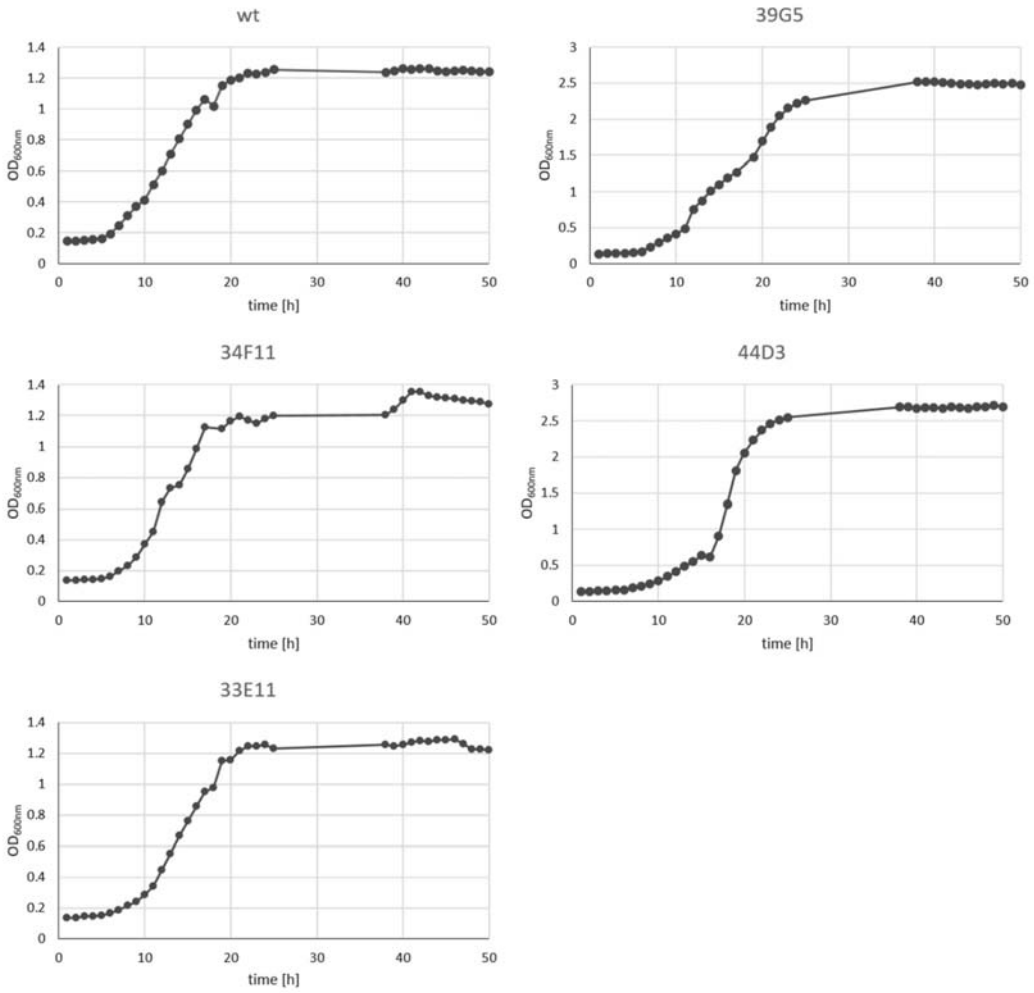
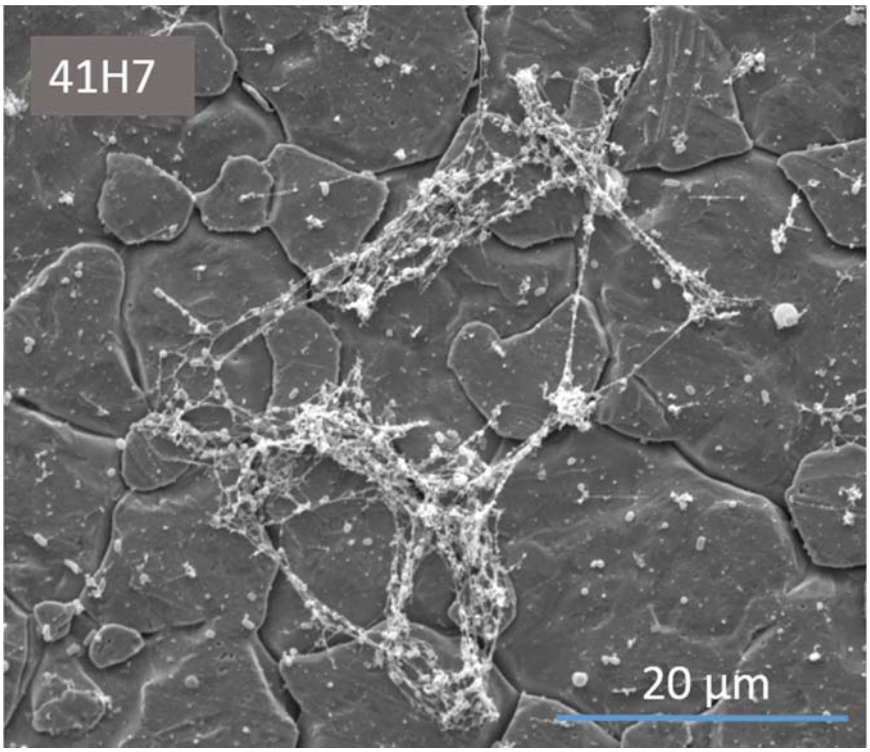
5.7 Supplementary information



Supplementary Figure S5.1. Growth of 36 transposon mutants of the *Listeria monocytogenes* 15G01 strain in MWB at 30°C determined by optical density measurements at 595 nm. A 96-well plate with each well containing 200 µl of MWB was inoculated with an overnight culture of *L. monocytogenes* 15G01 and mutants grown in TSBYE at 37°C using a 96-well replicator. The turbidity of the wells was measured with a microplate reader at a wavelength of 595nm at given time points. The readings were averaged and plotted against measured time points to produce a growth curve. OD_{595nm} values of the samples were corrected by subtracting the OD_{595nm} values for uninoculated media. The wild-type growth curve is shown in red.



Supplementary Figure S5.2. Growth of the *L. monocytogenes* strain 15G01 (wt) and its four mutants in MWB at 30°C measured with an automated microplate reader at defined time points at 600nm. The mutants have been grown without selective antibiotics to eliminate its effects on growth. The growth curves pictured are the means of two measurements.



Supplementary Figure S5.3. Scanning electron microscopy image of a biofilms formed by the *flaA* mutant (41H7) after 7 d incubation at 30°C on stainless steel coupons coated with mussel juice at a 5000x magnification.

Supplementary Table S5.1. Growth of 36 transposon mutants of the *L. monocytogenes* 15G01 strain in MWB at 30°C determined by optical density measurements at 595 nm.

To compare the mutant strains to the wild-type, logistic growth curves were fitted to each repetition of each strain (using the FITCURVE procedure in Genstat version 17, 2014). Because of the declines noted for some strains at 60 h, the 60 h data were excluded. The parameters of the curves were saved, and then compared to those for the wild-type replicates using t-tests (since the parameters are correlated with each other, multivariate ANOVA was also used to compare each strain with the wild-type; the results were similar)

The logistic curve is an S shaped curve often used as a basic growth model. It has four parameters: a, the starting value or left hand asymptote; c, the difference between the right and left hand asymptotes; and m, the horizontal value (here, the time) which is the mid-point of the rise. All the strains were significantly different from the wild-type on at least one parameter. The most consistent difference was that all but two strains (24A10 and 33E11) had significantly higher m: they took longer to start growing. The other notable feature was that most strains grew more slowly (had lower b) than the wild-type, which is quite likely due to antibiotic presence.

The mean parameters (from the eight repetitions) are tabulated below, along with standard errors (SEs – based on the difference between the repetitions) and p values for the difference from the wild-type. P values are colour coded yellow (significant at p = 0.05 and value is higher than the wild-type) and turquoise (significant at p = 0.05 and value is lower than the wild-type).

	a (starting level)			Slope at time = m			c (how much line rises)			m (midpoint of increasing phase)		
Strain	Mean	s.e.	p	Mean	s.e.	p	Mean	s.e.	p	Mean	s.e.	p
Wild-type	0.15	0.004		0.053	0.001		0.44	0.012		14.6	0.55	
24A10	0.17	0.001	<.001	0.021	0.001	<.001	0.20	0.001	<.001	14.5	0.34	0.949
24H3	0.16	0.003	0.066	0.043	0.001	<.001	0.50	0.009	0.001	21.4	0.21	<.001
25D2	0.11	0.002	<.001	0.037	0.001	<.001	0.45	0.011	0.459	19.3	0.20	<.001
26C10	0.15	0.005	0.989	0.043	0.002	0.004	0.27	0.002	<.001	17.0	0.39	0.003
26H1	0.15	0.001	0.589	0.040	0.001	<.001	0.43	0.008	0.404	18.6	0.15	<.001
28A2	0.19	0.001	<.001	0.049	0.001	0.108	0.50	0.011	0.002	20.5	0.31	<.001
28D12	0.18	0.002	<.001	0.048	0.001	0.013	0.41	0.006	0.056	19.1	0.33	<.001
28G11	0.17	0.004	0.001	0.054	0.001	0.374	0.47	0.011	0.086	17.8	0.12	0.001
30A9	0.13	0.003	0.002	0.048	0.001	0.015	0.44	0.006	0.889	17.8	0.24	<.001
30H2	0.11	0.004	<.001	0.036	0.001	<.001	0.54	0.004	<.001	22.9	0.50	<.001
31 E7	0.17	0.003	<.001	0.022	0.002	<.001	0.32	0.011	<.001	23.1	0.60	<.001
31C7	0.19	0.002	<.001	0.050	0.001	0.123	0.48	0.006	0.009	20.5	0.32	<.001
31G1	0.12	0.002	<.001	0.046	0.001	0.001	0.45	0.004	0.211	19.5	0.53	<.001
32 E2	0.16	0.004	0.032	0.047	0.001	0.006	0.49	0.011	0.006	20.0	0.21	<.001
32 E5	0.16	0.003	0.019	0.034	0.001	<.001	0.30	0.005	<.001	18.5	0.28	<.001
32A9	0.18	0.001	<.001	0.049	0.001	0.071	0.48	0.007	0.005	19.3	0.18	<.001
33 E11	0.16	0.002	0.020	0.055	0.001	0.220	0.27	0.011	<.001	15.5	0.08	0.151
33F8	0.12	0.003	<.001	0.045	0.002	0.003	0.43	0.014	0.589	18.1	0.14	<.001
34F11	0.13	0.003	0.004	0.038	0.001	<.001	0.40	0.010	0.034	19.7	0.25	<.001
35C1	0.16	0.004	0.050	0.035	0.002	<.001	0.49	0.006	0.002	22.0	0.41	<.001
35F3	0.22	0.002	<.001	0.040	0.000	<.001	0.24	0.003	<.001	16.2	0.14	0.025
35H9	0.20	0.003	<.001	0.052	0.001	0.818	0.49	0.009	0.007	19.2	0.41	<.001
36A2	0.20	0.002	<.001	0.044	0.001	<.001	0.34	0.004	<.001	18.2	0.32	<.001
36B9	0.19	0.002	<.001	0.034	0.001	<.001	0.41	0.010	0.135	21.0	0.13	<.001

39G5	0.20	0.002	<.001	0.031	0.001	<.001	0.31	0.010	<.001	19.4	0.18	<.001
40C12	0.18	0.003	<.001	0.039	0.001	<.001	0.30	0.009	<.001	17.5	0.40	0.001
41A8	0.20	0.003	<.001	0.038	0.001	<.001	0.36	0.003	<.001	19.5	0.20	<.001
41H7	0.17	0.001	0.001	0.050	0.001	0.084	0.43	0.007	0.756	19.3	0.29	<.001
42G3	0.16	0.007	0.296	0.045	0.003	0.044	0.35	0.013	<.001	17.4	0.23	0.001
43C9	0.16	0.003	0.115	0.030	0.002	<.001	0.43	0.009	0.496	21.8	0.50	<.001
44D3	0.17	0.002	<.001	0.044	0.001	<.001	0.53	0.006	<.001	22.0	0.42	<.001
44F5	0.18	0.004	<.001	0.040	0.001	<.001	0.47	0.017	0.112	20.7	0.18	<.001
47H10	0.18	0.002	<.001	0.050	0.001	0.194	0.49	0.008	0.002	20.4	0.26	<.001
47H11	0.18	0.003	<.001	0.050	0.000	0.104	0.63	0.009	<.001	28.1	0.33	<.001
64B3	0.19	0.003	<.001	0.051	0.001	0.449	0.49	0.006	0.003	21.1	0.23	<.001
67C5	0.15	0.004	0.858	0.038	0.002	<.001	0.27	0.003	<.001	17.3	0.32	0.001

5.8 References

Alonso, A. N., K. J. Perry, J. M. Regeimbal, P. M. Regan and D. E. Higgins (2014). Identification of *Listeria monocytogenes* determinants required for biofilm formation. PLoS One **9**(12): e113696.

Armitage, J. P. and R. M. Macnab (1987). Unidirectional, intermittent rotation of the flagellum of *Rhodobacter sphaeroides*. J Bacteriol **169**(2): 514-518.

Azizoglu, R. O., D. Elhanafi and S. Kathariou (2014). Mutant construction and integration vector-mediated gene complementation in *Listeria monocytogenes*. Methods Mol Biol **1157**: 201-211.

Bao, Y., X. Zhang, Q. Jiang, T. Xue and B. Sun (2015). Pfs promotes autolysis-dependent release of eDNA and biofilm formation in *Staphylococcus aureus*. Med Microbiol Immunol **204**(2): 215-226.

Becavin, C., C. Bouchier, P. Lechat, C. Archaumbaud, S. Creno, E. Gouin, Z. Wu, A. Kuhbacher, S. Brisse, M. G. Pucciarelli, F. Garcia del Portillo, T. Hain, D. A. Portnoy, T. Chakraborty, M. Lecuit, J. Pizarro Cerda, I. Moszer, H. Bierne and P. Cossart (2014). Comparison of widely used *Listeria monocytogenes* strains EGD, 10403S, and EGD-e highlights genomic variations underlying differences in pathogenicity. MBio **5**(2): e00969-00914.

Borucki, M. K., J. D. Peppin, D. White, F. Loge and D. R. Call (2003). Variation in biofilm formation among strains of *Listeria monocytogenes*. Appl Environ Microbiol **69**(12): 7336-7342.

Cao, M., A. P. Bitar and H. Marquis (2007). A mariner-based transposition system for *Listeria monocytogenes*. Appl Environ Microbiol **73**(8): 2758-2761.

Carpentier, B. and O. Cerf (2011). Review--Persistence of *Listeria monocytogenes* in food industry equipment and premises. Int J Food Microbiol **145**(1): 1-8.

Carroll, S. A., T. Hain, U. Technow, A. Darji, P. Pashalidis, S. W. Joseph and T. Chakraborty (2003). Identification and characterization of a peptidoglycan hydrolase, MurA, of *Listeria monocytogenes*, a muramidase needed for cell separation. J Bacteriol **185**(23): 6801-6808.

Cascioferro, S., M. Totsika and D. Schillaci (2014). Sortase A: An ideal target for anti-virulence drug development. Microb Pathog **77c**: 105-112.

Ceruso, M., P. Fratamico, C. Chirollo, R. Taglialatela, M. L. Cortesi and T. Pepe (2014). The capacity of *Listeria monocytogenes* mutants with in-frame deletions in putative ATP-binding cassette transporters to form biofilms and comparison with the wild type. Ital J Food Saf **3**(1): 1657.

Chamnongpol, S. and E. A. Groisman (2002). Mg^{2+} homeostasis and avoidance of metal toxicity. Mol Microbiol **44**(2): 561-571.

Chang, Y., W. Gu, N. Fischer and L. McLandsborough (2012). Identification of genes involved in *Listeria monocytogenes* biofilm formation by mariner-based transposon mutagenesis. Appl Microbiol Biotechnol **93**(5): 2051-2062.

Collins, B., N. Curtis, P. D. Cotter, C. Hill and R. P. Ross (2010a). The ABC transporter AnrAB contributes to the innate resistance of *Listeria monocytogenes* to nisin, bacitracin, and various β-lactam antibiotics. Antimicrob Agents Chemother **54**(10): 4416-4423.

Collins, B., S. Joyce, C. Hill, P. D. Cotter and R. P. Ross (2010b). TelA contributes to the innate resistance of *Listeria monocytogenes* to nisin and other cell wall-acting antibiotics. Antimicrob Agents Chemother **54**(11): 4658-4663.

Cruz, C. D. and G. C. Fletcher (2011). Prevalence and biofilm-forming ability of *Listeria monocytogenes* in New Zealand mussel (*Perna canaliculus*) processing plants. Food Microbiol **28**(7): 1387-1393.

Djordjevic, D., M. Wiedmann and L. A. McLandsborough (2002). Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl Environ Microbiol **68**(6): 2950-2958.

Doijad, S. P., S. B. Barbuddhe, S. Garg, K. V. Poharkar, D. R. Kalorey, N. V. Kurkure, D. B. Rawool and T. Chakraborty (2015). Biofilm-forming abilities of *Listeria monocytogenes* serotypes isolated from different sources. PLoS One **10**(9): e0137046.

Dworkin, J. (2009). Cellular Polarity in Prokaryotic Organisms. Cold Spring Harb Perspect Biol **1**(6): a003368.

Ferreira, V., M. Wiedmann, P. Teixeira and M. J. Stasiewicz (2014). *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. J Food Prot **77**(1): 150-170.

Fletcher, G., M. Rogers and R. Wong (1994). Survey of *Listeria monocytogenes* in New Zealand seafood. J Aquat Food Prod T **3**(2): 13-24.

Groisman, E. A., M. J. Cromie, Y. Shi and T. Latifi (2006). A Mg^{2+}-responding RNA that controls the expression of a Mg^{2+} transporter. Cold Spring Harb Symp Quant Biol **71**: 251-258.

Groisman, E. A., K. Hollands, M. A. Kriner, E. J. Lee, S. Y. Park and M. H. Pontes (2013). Bacterial Mg^{2}+ homeostasis, transport, and virulence. Annu Rev Genet **47**: 625-646.

Guilbaud, M., P. Piveteau, M. Desvaux, S. Brisse and R. Briandet (2015). Exploring the diversity of *Listeria monocytogenes* biofilm architecture by high-throughput confocal laser scanning microscopy and the predominance of the honeycomb-like morphotype. Appl Environ Microbiol **81**(5): 1813-1819.

Harmsen, M., M. Lappann, S. Knochel and S. Molin (2010). Role of extracellular DNA during biofilm formation by *Listeria monocytogenes*. Appl Environ Microbiol **76**(7): 2271-2279.

Hingston, P. A., M. J. Piercey and L. Truelstrup Hansen (2015). Genes associated with desiccation and osmotic stress in *Listeria monocytogenes* as revealed by insertional mutagenesis. Appl Environ Microbiol **81**(16): 5350-5362.

Hinsa, S. M., M. Espinosa-Urgel, J. L. Ramos and G. A. O'Toole (2003). Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. Mol Microbiol **49**(4): 905-918.

Huang, Y., C. Shi, S. Yu, K. Li and X. Shi (2012). A putative MerR family regulator involved in biofilm formation in *Listeria monocytogenes* 4b G. Foodborne Pathog Dis **9**(8): 767-772.

Huang, Y., Y. Suo, C. Shi, J. Szlavik, X. M. Shi and S. Knochel (2013). Mutations in *gltB* and *gltC* reduce oxidative stress tolerance and biofilm formation in *Listeria monocytogenes* 4b G. Int J Food Microbiol **163**(2-3): 223-230.

Inagaki, S., M. Matsumoto-Nakano, K. Fujita, K. Nagayama, J. Funao and T. Ooshima (2009). Effects of recombinase A deficiency on biofilm formation by *Streptococcus mutans*. Oral Microbiol Immunol **24**(2): 104-108.

Kallipolitis, B. H., H. Ingmer, C. G. Gahan, C. Hill and L. Søgaard-Andersen (2003). CesRK, a two-component signal transduction system in *Listeria monocytogenes*, responds to the presence of cell wall-acting antibiotics and affects β-lactam resistance. Antimicrob Agents Chemother **47**(11): 3421-3429.

Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A. Cooper, S. Markowitz, C. Duran, T. Thierer, B. Ashton, P. Meintjes and A. Drummond (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics **28**(12): 1647-1649.

Kehres, D. G. and M. E. Maguire (2003). The unusual nature of magnesium transporters. Microbial Transport Systems, Wiley-VCH Verlag GmbH & Co. KGaA**:** 347-360.

Knudsen, G. M., J. E. Olsen and L. Dons (2004). Characterization of DegU, a response regulator in *Listeria monocytogenes*, involved in regulation of motility and contributes to virulence. FEMS Microbiol Lett **240**(2): 171-179.

Koprivnjak, T., D. Zhang, C. M. Ernst, A. Peschel, W. M. Nauseef and J. P. Weiss (2011). Characterization of *Staphylococcus aureus* cardiolipin synthases 1 and 2 and their contribution to accumulation of cardiolipin in stationary phase and within phagocytes. J Bacteriol **193**(16): 4134-4142.

Lauer, P., M. Y. Chow, M. J. Loessner, D. A. Portnoy and R. Calendar (2002). Construction, characterization, and use of two *Listeria monocytogenes* site-specific phage integration vectors. J Bacteriol **184**(15): 4177-4186.

Lecuit, M. (2005). Understanding how *Listeria monocytogenes* targets and crosses host barriers. Clin Microbiol Infect **11**(6): 430-436.

Leduc, M., R. Kasra and J. van Heijenoort (1982). Induction and control of the autolytic s ystem of *Escherichia coli*. J Bacteriol **152**(1): 26-34.

Lin, T. Y., T. M. Santos, W. S. Kontur, T. J. Donohue and D. B. Weibel (2015). A cardiolipin-deficient mutant of Rhodobacter sphaeroides has an altered cell shape and is impaired in biofilm formation. J Bacteriol **197**(21): 3446-3455.

Machata, S. (2008). Molecular investigations of peptidoglycanbinding proteins in *Listeria monocytogenes* PhD, Justus-Liebig-Universitaet Giessen.

Machata, S., T. Hain, M. Rohde and T. Chakraborty (2005). Simultaneous deficiency of both MurA and p60 proteins generates a rough phenotype in *Listeria monocytogenes.* J Bacteriol **187**(24): 8385-8394.

Marsh, E. J., H. Luo and H. Wang (2003). A three-tiered approach to differentiate *Listeria monocytogenes* biofilm-forming abilities. FEMS Microbiol Lett **228**(2): 203-210.

Maudsdotter, L., S. Imai, R. L. Ohniwa, S. Saito and K. Morikawa (2015). *Staphylococcus aureus* dry stress survivors have a heritable fitness advantage in subsequent dry exposure. Microbes Infect **17**(6): 456-461.

Monk, I. R., C. G. Gahan and C. Hill (2008). Tools for functional postgenomic analysis of *Listeria monocytogenes*. Appl Environ Microbiol **74**(13): 3921-3934.

Mulcahy, H. and S. Lewenza (2011). Magnesium limitation is an environmental trigger of the *Pseudomonas aeruginosa* biofilm lifestyle. PLoS One **6**(8): e23307.

Munoz-Elias, E. J., J. Marcano and A. Camilli (2008). Isolation of *Streptococcus pneumoniae* biofilm mutants and their characterization during nasopharyngeal colonization. Infect Immun **76**(11): 5049-5061.

Nielsen, P. K., A. Z. Andersen, M. Mols, S. van der Veen, T. Abee and B. H. Kallipolitis (2012). Genome-wide transcriptional profiling of the cell envelope stress response and the role of LisRK and CesRK in *Listeria monocytogenes*. Microbiology **158**(Pt 4): 963-974.

Nowak, J., C. D. Cruz, J. Palmer, G. C. Fletcher and S. Flint (2015). Biofilm formation of the *L. monocytogenes* strain 15G01 is influenced by changes in environmental conditions. J Microbiol Methods **119**: 189-195.

Ouyang, Y., J. Li, Y. Dong, L. V. Blakely and M. Cao (2012). Genome-wide screening of genes required for *Listeria monocytogenes* biofilm formation. J Biotech Res **4**: 13-25.

Park, S., J. Jung, S. Choi, Y. Oh, J. Lee, H. Chae, S. Ryu, H. Jung, G. Park and S. Choi (2012). Molecular characterization of *Listeria monocytogenes* based on the PFGE and RAPD in Korea.

Parsek, M. R. and C. Fuqua (2004). Biofilms 2003: emerging themes and challenges in studies of surface-associated microbial life. J Bacteriol **186**(14): 4427-4440.

Piercey, M. J., P. A. Hingston and L. Truelstrup Hansen (2016). Genes involved in *Listeria monocytogenes* biofilm formation at a simulated food processing plant temperature of 15°C. Int J Food Microbiol **223**: 63-74.

Puttamreddy, S., N. A. Cornick and F. C. Minion (2010). Genome-wide transposon mutagenesis reveals a role for pO157 genes in biofilm development in *Escherichia coli* O157:H7 EDL933. Infect Immun **78**(6): 2377-2384.

Qin, Z., Y. Ou, L. Yang, Y. Zhu, T. Tolker-Nielsen, S. Molin and D. Qu (2007). Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. Microbiology **153**(Pt 7): 2083-2092.

Renier, S., C. Chagnot, J. Deschamps, N. Caccia, J. Szlavik, S. A. Joyce, M. Popowska, C. Hill, S. Knochel, R. Briandet, M. Hebraud and M. Desvaux (2014). Inactivation of the SecA2 protein export pathway in *Listeria monocytogenes* promotes cell aggregation, impacts biofilm architecture and induces biofilm formation in environmental condition. Environ Microbiol **16**(4): 1176-1192.

Rocourt, J., C. Jacquet and A. Reilly (2000). Epidemiology of human listeriosis and seafoods. Int J Food Microbiol **62**(3): 197-209.

Romantsov, T., Z. Guan and J. M. Wood (2009). Cardiolipin and the osmotic stress responses of bacteria. Biochim Biophys Acta **1788**(10): 2092-2100.

Scheurwater, E., C. W. Reid and A. J. Clarke (2008). Lytic transglycosylases: bacterial space-making autolysins. Int J Biochem Cell Biol **40**(4): 586-591.

Seaton, K., S.-J. Ahn, A. M. Sagstetter and R. A. Burne (2011). A transcriptional regulator and ABC transporters link stress tolerance, (p)ppGpp, and genetic competence in *Streptococcus mutans*. J Bacteriol **193**(4): 862-874.

Srey, S., I. K. Jahid and S. D. Ha (2013). Biofilm formation in food industries: A food safety concern. Food Control **31**(2): 572-585.

Suo, Y., Y. Huang, Y. Liu, C. Shi and X. Shi (2012). The expression of superoxide dismutase (SOD) and a putative ABC transporter permease is inversely correlated during biofilm formation in *Listeria monocytogenes* 4b G. PLoS One **7**(10): e48467.

Taylor, C. M., M. Beresford, H. A. Epton, D. C. Sigee, G. Shama, P. W. Andrew and I. S. Roberts (2002). *Listeria monocytogenes relA* and *hpt* mutants are impaired in surface-attached growth and virulence. J Bacteriol **184**(3): 621-628.

Tremoulet, F., O. Duche, A. Namane, B. Martinie, J. C. Labadie and the European Listeria Genome Consortium (2002). Comparison of protein patterns of *Listeria monocytogenes* grown in biofilm or in planktonic mode by proteomic analysis. FEMS Microbiol Lett **210**(1): 25-31.

van der Veen, S. and T. Abee (2010a). Dependence of continuous-flow biofilm formation by *Listeria monocytogenes* EGD-e on SOS response factor YneA. Appl Environ Microbiol **76**(6): 1992-1995.

van der Veen, S. and T. Abee (2010b). HrcA and DnaK are important for static and continuous-flow biofilm formation and disinfectant resistance in *Listeria monocytogenes*. Microbiology **156**(Pt 12): 3782-3790.

van der Veen, S. and T. Abee (2010c). Importance of SigB for *Listeria monocytogenes* static and continuous-flow biofilm formation and disinfectant resistance. Appl Environ Microbiol **76**(23): 7854-7860.

van der Veen, S. and T. Abee (2011a). Bacterial SOS response: a food safety perspective. Curr Opin Biotechnol **22**(2): 136-142.

van der Veen, S. and T. Abee (2011b). Generation of variants in *Listeria monocytogenes* continuous-flow biofilms is dependent on radical-induced DNA damage and RecA-mediated repair. PLoS One **6**(12): e28590.

van der Veen, S., T. Hain, J. A. Wouters, H. Hossain, W. M. de Vos, T. Abee, T. Chakraborty and M. H. Wells-Bennik (2007). The heat-shock response of *Listeria monocytogenes* comprises genes involved in heat shock, cell division, cell wall synthesis, and the SOS response. Microbiology **153**(Pt 10): 3593-3607.

van der Veen, S., S. van Schalkwijk, D. Molenaar, W. M. de Vos, T. Abee and M. H. Wells-Bennik (2010). The SOS response of *Listeria monocytogenes* is involved in stress resistance and mutagenesis. Microbiology **156**(Pt 2): 374-384.

Van Houdt, R. and C. W. Michiels (2010). Biofilm formation and the food industry, a focus on the bacterial outer surface. J Appl Microbiol **109**(4): 1117-1131.

Vanderlinde, E. M., J. J. Harrison, A. Muszyński, R. W. Carlson, R. J. Turner and C. K. Yost (2010). Identification of a novel ABC transporter required for desiccation tolerance, and biofilm formation in *Rhizobium leguminosarum* bv. *viciae* 3841. FEMS Microbiol Ecol **71**(3): 327-340.

Varahan, S., N. Harms, M. S. Gilmore, J. M. Tomich and L. E. Hancock (2014). An ABC transporter is required for secretion of peptide sex pheromones in *Enterococcus faecalis*. MBio **5**(5): e01726-01714.

Vinayavekhin, N., G. Mahipant, A. S. Vangnai and P. Sangvanich (2015). Untargeted metabolomics analysis revealed changes in the composition of glycerolipids and phospholipids in *Bacillus subtilis* under 1-butanol stress. Appl Microbiol Biotechnol **99**(14): 5971-83

Webb, A. J., M. Karatsa-Dodgson and A. Grundling (2009). Two-enzyme systems for glycolipid and polyglycerolphosphate lipoteichoic acid synthesis in *Listeria monocytogenes*. Mol Microbiol **74**(2): 299-314.

Wen, J., R. C. Anantheswaran and S. J. Knabel (2009). Changes in barotolerance, thermotolerance, and cellular morphology throughout the life cycle of *Listeria monocytogenes.* Appl Environ Microbiol **75**(6): 1581-1588.

Yousef, A. E. (1999). Characteristics of *Listeria monocytogenes* important to food processors. Listeria: Listeriosis, and Food Safety: 131.

Zhu, X., W. Liu, R. Lametsch, F. Aarestrup, C. Shi, Q. She, X. Shi and S. Knochel (2011). Phenotypic, proteomic, and genomic characterization of a putative ABC-transporter permease involved in *Listeria monocytogenes* biofilm formation. Foodborne Pathog Dis **8**(4): 495-501.

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Jessika Nowak

Name/Title of Principal Supervisor: Steve Flint

Name of Published Research Output and full reference:

Nowak, J., Cruz, C., Visnovsky, S., Palmer, J., Fletcher, G., Pitman, A. & Flint, S. (2017). Biofilm formation of Listeria monocytogenes 15G01, a persistent isolate from a seafood processing plant in New Zealand, is influenced by inactivation of multiple genes belonging to different functional groups. (to be submitted).

In which Chapter is the Published Work: Chapter 5

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate carried out the laboratory work and the analysis (the statistical analysis for the growth curves was carried out by D. Hedderley) and prepared the manuscript with input in guidance of direction and editorial help from the co-authors and supervisors.

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Chapter 6

The *mgtB* mutant of the persistent *Listeria monocytogenes* 15G01 strain produces a unique biofilm sandwich structure which changes its phenotype upon addition of magnesium

Prepared for submission to the Journal of Bacteriology

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Abstract

Listeria monocytogenes is a food borne pathogen which can lead to severe bacterial infections in immunocompromised people, the elderly and in pregnant women. Biofilm formation is thought to be a major factor for contamination of food in the food-processing environment. Five genes identified in a previous screen to be associated with biofilm formation in a persistent strain *L. monocytogenes* 15G01 were investigated for their effect on surface characteristics and biofilm composition. Three different assays (crystal violet assay, cell enumeration and a tetrazolium salt based assay) were used to determine the amount of biofilm produced, and all of them significantly correlated with each other. However, surface properties determined by the MATH test, hydrophobic interaction chromatography and cytochrome C assay did not correlate with biofilm formation or attachment. The influence of cations on biofilm formation and the involvement of stress response were also investigated. Increased magnesium presence led to variable effects on biofilm formation at mammalian and environmental temperatures resulting in a decrease in biofilm formation for a *mltD* mutant and in an increase for a *mgtB* mutant. Confocal microscopy revealed a unique biofilm structure for the *mgtB* mutant exhibiting a sandwich structure which was reversed to the wild-type phenotype with increased magnesium presence. This study identifies, for the first time, the involvement of magnesium in biofilm formation of *L. monocytogenes* and contributes to understanding the related mechanism.

6.1 Introduction

Listeria monocytogenes is a rod-shaped bacterium which is ubiquitous in the environment. It is the causative agent for the bacterial infection listeriosis. *L. monocytogenes* can lead to severe and life-threatening infections in immunocompromised people, the elderly, pregnant women and their unborn as well as newborn babies (Rocourt et al. 2000). Infection with *L. monocytogenes* usually occurs through consumption of contaminated food, including ready-to-eat products, dairy products, seafood and vegetables and fruit. Contamination primarily occurs in the food-processing plant and it is believed that biofilms are a major factor in (re)-contamination of surfaces in food processing plants and products (Srey et al. 2013).

Biofilms are defined as a community of microorganisms which are attached to a surface and embedded in a self-produced matrix that helps protect them from the environment. This matrix can consist of extracellular DNA (eDNA), lipids, polysaccharides and other components. The extracellular matrix can make up about 90% of the whole biofilm mass and differs enormously between species and between strains. In nature, biofilms are usually composed of fungal or bacterial cells, or a mixture of thereof. It is well known that the majority of microorganisms in the environment exist in form of established biofilms rather than in the planktonic form (Flemming and Wingender 2010).

Biofilm formation requires bacterial cells to attach to surfaces. Attachment is dependent on several environmental factors, which include but are not limited to the surface charge and hydrophobicity of the cell and the attachment site and to the cation concentration (Chmielewski and Frank 2003). The first step of the initial attachment of planktonic cells to surfaces is driven by van der Waals forces and electrostatic forces (Garrett et al. 2008). To become a biofilm the cells attach irreversibly through dipole-dipole interaction, ionic and covalent bonding (Garrett et al. 2008). The charge of the individual cell and the attachment site will determine whether a cell can adhere to the surface and establish as a biofilm or whether repulsion is taking place. After attaching irreversibly to the surface, cells start to produce extracellular polymeric substances which are responsible for the structure of the biofilm.

Crystal violet (CV) based assays are commonly used as a fast and efficient method to determine the ability of bacterial cells to form biofilm (Alonso et al. 2014, Chang et al. 2012, Djordjevic et al. 2002, Ochiai et al. 2014). Crystal violet can stain any organic matter by binding to negatively charged surface molecules, including live and dead cells as well as the extracellular matrix of a biofilm. Although it is widely used as a means of determining biofilm, it does not give any

indication about the numbers of viable or metabolically active cells in the biofilm and does not differentiate between living and dead cells. Viable cells are of most risk to food safety and biofilm can be a source of food contamination through the release of cells from the biofilm. Plate counting can estimate the presence of culturable cells, but will not detect viable but non-culturable (VBNC) strains. Therefore, tetrazolium salt based assays can be used to measure the metabolic activity of cells present in the biofilm including VBNC strains (Celis 2014, Koban et al. 2012). XTT (sodium 3'-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate), a tetrazolium salt, is reduced by enzymes of the respiratory chain in the cytoplasmatic membrane. Using CV-staining as well as plate counting and the XTT assay can give a good indication of the composition of the biofilm. Extracellular DNA (eDNA) has been found to be a vital part of biofilms of *L. monocytogenes* (Harmsen et al. 2010), however, the mechanism of eDNA release remains elusive. It is postulated that it is either active secretion (Liao et al. 2014) or autolysin mediated release (Qin et al. 2007). Determination of the amount of eDNA present in a biofilm can help to elucidate the biofilm's structure. eDNA is negatively charged, able to interact with positively charged molecules and can increase the hydrophobicity of bacterial cells and therefore influence attachment (Das et al. 2011).

Attachment and biofilm formation can also be influenced by cations, which can alter the biofilm structure by binding to negatively charged surface molecules. It is known that cations such as Ca^{2+}, Na^{+} and Mg^{2+} play a vital role in bacterial homeostasis. Therefore, those cations have been studied with regard to biofilm formation of several bacterial species (Mulcahy and Lewenza 2011, Oknin et al. 2015, Somerton et al. 2015, Song and Leff 2006), however, little is known about their role in biofilm formation of *Listeria*.

L. monocytogenes 15G01 is a high biofilm forming isolate of a persistent strain that was originally collected from a mussel processing facility. Five transposon mutants of 15G01 were selected for subsequent studies to identify the genes' involvement in biofilm formation by testing for changes in biofilm composition and surface properties. As it has been reported that biofilm formation is closely connected to stress response (Gotoh et al. 2010, Landini 2009), planktonic cultures were subjected to heat treatment, and exposure to acid and an oxidative reagent (H_2O_2).

6.2 Materials and Methods

6.2.1 Strains and growth conditions

The strains used in this study are listed in Table 6.1.

Table 6.1. *Listeria monocytogenes* strains used in this study

Strain	Details	Source
Wild-type	*L. monocytogenes* 15G01, serotype 1/2a, persistent isolate	Cruz and Fletcher (2011)
6B4	15G01 with transposon inserted in the *LMON_1762* gene; Em^R	(unpublished)
34F11	15G01 with transposon inserted in the *clsA* gene (*LMON_2515*); Em^R	Chapter 5
33E11	15G01 with transposon inserted in the *uvrB* gene (*LMON_2501*); Em^R	Chapter 5
39G5	15G01 with transposon inserted in the *mltD* gene (*LMON_2714*); Em^R	Chapter 5
44D3	15G01 with transposon inserted in the *mgtB* gene (*LMON_2712*); Em^R	Chapter 5
41H7	15G01 with transposon inserted in the *flaA* gene; Em^R	Chapter 5

To determine growth, *L. monocytogenes* strains were grown overnight in tryptic soy broth enriched with 0.6% yeast extract (TSBYE) at 37°C. A 96 pin replicator was used to inoculate a 96-well plate filled with 200 µL of modified Welshimer's broth (MWB) (Himedia, India) in each well. The OD_{595nm} was measured by an automated plate reader (SPECTROstar Omega, BMG Labtech) every 5 min for 48 h.

6.2.2 Growth, biofilm formation and attachment

Biofilm formation was measured as described previously using the crystal violet assay (Djordjevic et al. 2002). Briefly, overnight cultures were grown at 37°C in TSBYE in a sterile 96-well plate (polystyrene, U-bottom, Interlab, New Zealand) and used to inoculate new 96-well plates with each well containing 200 µL medium using a 96-pin replicator. The cultures were incubated for 48 h at 30°C in MWB and then washed thrice with 200 µL sterile water using a microplate strip washer (Elx50, Biotek). After air drying at ambient temperature for 30 min,

150 µL of a 1% aqueous crystal violet solution was added to the plates. After 45 min of incubation at 30°C the crystal violet solution was removed and the cultures were washed six times with 150 µL sterile water. After drying for 30 min at 30°C, 96% ethanol was added to destain the cells. The optical density was measured after 1 h at 595nm with a microplate reader (SPECTROstar Omega, BMG Labtech). The obtained OD_{595nm} values were corrected by subtracting the OD_{595nm} value of uninoculated media. As one of the biofilm impaired mutants showed a transposon insertion in a gene encoding for a Mg^{2+} P-type ATPase, biofilm formation assays were performed for the wild-type and selected mutants with added magnesium (Mg^{2+}) to determine its influence. MWB contains 1.67mM Mg^{2+} and to test for Mg^{2+} involvement in biofilm formation the concentration of Mg^{2+} was adjusted with $MgSO_4*7H_20$ (Merck KgaA, Darmstadt, Germany) to final concentrations of 2.5 mM, 5 mM and 10 mM Mg^{2+} and incubated for 48 h at 30°C.

For the viable cell enumeration of the biofilm, the 6 x 6 drop plate method was used (Chen et al. 2003). Biofilm cells were grown in 96-well plates for 48 h and gently washed three times with double-distilled H_20 (ddH_20) and then resuspended in 200 µL Dulbecco's phosphate buffered saline (DPBS (-Mg^{2+} and Ca^{2+})) (Thermo Fisher, New Zealand) by rigorous pipetting. The suspension was transferred to a new 96-well plate. Tenfold serial dilutions were prepared with a multichannel pipette by mixing 20 µL cell solution with 180 µL DPBS. Ten µL drops of each dilution were plated on a brain heart infusion (BHI) agar plate and incubated at 37°C for 24 h. Colonies were enumerated, counts averaged and plotted against the OD_{595nm} values from CV staining. The experiment was performed in duplicate with five replicates for each strain.

For the attachment studies the wild-type strain 15G01 and the mutants were grown overnight in TSBYE at 37°C without shaking. The next day cultures were centrifuged (4000g, 20°C, 10 min) and resuspended in MWB to an OD_{595nm} of 1.0. Then cultures were transferred to 96-well plates (Costar, U-bottom) and incubated for 30 min at 30°C. The medium was removed after incubation and the wells were washed three times with ddH_20 to remove loosely attached cells. An aliquot of 150 µL of an aqueous 1% solution of CV was added and the plates were incubated for 45 min at 30°C. Before destaining the CV with 96% EtOH, the wells were washed six times with ddH_20 to remove unbound dye. Absorption was determined at OD_{595nm}, averaged and blank corrected. Attachment is here defined as the levels of cells and extracellular material adhering to the wells after 30 min incubation.

6.2.3 Determination of metabolic activity of biofilm cells

Biofilms were grown as described previously for 48 h in MWB at 30°C. The metabolic activity of the cells in the biofilm was examined by addition of the tetrazolium salt XTT (sodium 3'-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate) (Sigma-Aldrich, USA) and phenazine methosulfate (PMS) (Sigma-Aldrich, USA) as described previously with minor modifications (Celis 2014, Koban et al. 2012). XTT solution was prepared at a concentration of 1mg/mL in phosphate buffered saline (PBS, pH 7.2) and a stock solution of PMS at a concentration of 5 mM. The PMS solution (25 µL) was added to 5 mL XTT solution and 200 µL were added into each well and incubated in the dark at 37°C for 3h. The optical density was measured at 450 nm and the obtained values were blank corrected.

6.2.4 Determination of eDNA and polysaccharides (carbohydrate assay) of the extracellular polymeric substance

Biofilms were grown in 96-well plates as described previously (Djordjevic et al. 2002). Briefly, an overnight culture grown in TSBYE at 37°C was used to inoculate a 96-well plate filled with 200 µL MWB using a 96-well replicator. After incubation at 30°C for 48 h the 96-well plates were washed three times with ddH_2O. To disrupt the biofilm and measure the eDNA content a method described by Wu and Xi (2009) was used with slight modifications. N-Glycosidase solution (200 µL of a 1U/mL 0.9% NaCl- solution) was added to each well and then incubated for 30 min at 37°C. Proteinase K was then added in a final concentration of 5 µg/mL and incubated for another 30 min at 37°C. Disruption of the biofilm was supported by rigorous pipetting. Eighteen wells per strain were pooled together and filtered through a 0.2 µm membrane (cellulose acetate). Five hundred µL of the obtained solution was used to determine extracellular carbohydrates present in a biofilm using the phenol-sulphuric assay (DuBois et al. 1956). An aqueous 5% phenol solution (500 µL) was added to a 500 µL sample. Concentrated H_2SO_4 (2.5 mL) was quickly added and the solution incubated at room temperature for 30 min. Glucose content was determined with a spectrometer at 490 nm. A standard curve was produced with glucose in concentrations ranging from 40 µg to 200 µg/mL. To determine the eDNA amount of the biofilm the extracellular DNA was precipitated using the CTAB (cetyltrimethylammonium bromide) – method as described by Corinaldesi et al. (2005) and used by Wu and Xi (2009).

The supernatants of the biofilms of 12 wells were pooled together and centrifuged at 5225 g for 10 min at room temperature. The eDNA of the supernatant (200 µL) was measured with the

NanoDrop 2000 (Thermo Fisher, New Zealand) after using a commercially available kit (DNeasy blood and tissue kit, Qiagen) for extraction.

6.2.5 Determination of surface charge

6.2.5.1 MATH test

The MATH (microbial adhesion to hydrocarbon) test evaluates the bacterial cell surface hydrophobicity through separation of bacterial cells to an aqueous phase and a hydrocarbon phase. A fresh colony of the mutants and the wild-type strains was picked from the TSAYE plates and used to inoculate 10 mL of TSBYE. The cultures were incubated overnight (18h) at 35°C under shaking (200 rpm, Orbit 19 Labshaker, Labnet, NJ). Five mL of each overnight culture was washed three times with PBS (2050 g, 10 min, 20°C) and then resuspended in the same volume of PBS. The optical density (600nm) was adjusted to 0.8. The culture (2.5 mL) was then mixed with 2.5 mL hexadecane (Fisher Scientific, UK), a non-polar solvent, and vortexed for one minute. The optical density at 600 nm of the aqueous phase was measured after 15 min incubation at room temperature with a microplate reader. The hydrophobicity was calculated using following equation:

$$hydrophobicity[\%] = \frac{(A_0 - A_1)}{A_0 * 100}$$

with A_0 being the OD of the bacterial suspension before addition of hexadecane and A_1 the OD of the aqueous phase after mixing with hexadecane.

6.2.5.2 Hydrophobic interactive chromatography

As another method to evaluate hydrophobicity, cultures picked from a TSAYE agar plate were used to grow a liquid culture in TSBYE overnight at 37°C. The overnight cultures (100 µL) were used to inoculate 9.9 mL MWB and grown at 35°C under shaking (200 rpm, Orbit 19 Labshaker, Labnet, NJ) until mid-log phase. Cultures were centrifuged at 1000 g for 20 min and resuspended in 0.05 M phosphate buffer (pH 7.0) to an OD_{600nm} of 0.5. Chromatography columns were created from pasteur pipettes. These were plugged with glass wool, filled with one 1 mL of the resin (Phenyl-sepharose fast flow (Sigma-Aldrich, USA)) and washed ten times with 0.05 M phosphate buffer (pH 7.0). The resuspended culture (300 µL) was loaded into each column and washed with 900 µL 0.05 M phosphate buffer. The absorbance of the eluent and of a ¼ dilution of the original culture were determined at 600 nm and the percentage of bacteria retained in the hydrophobic column was calculated as below.

$$\%\ retained = \frac{(A_0 - A_1)}{(A_0 * 100)}$$

A_0= OD_{600nm} of ¼ dilution of bacterial culture resuspended in phosphate buffer

A_1= OD_{600nm} of the eluted bacterial suspension

6.2.5.3 Cytochrome C binding assay

Cytochrome C is a cationic polymer that will bind to anionic structures on the bacterial cell. Unbound cytochrome C can be assayed through optical density measurements. The cytochrome C assay was performed as described previously (Kang et al. 2015) with minor modifications. An aliquot of 100 µL of an overnight culture grown in TSBYE at 37°C was used to inoculate 10 mL of MWB. The culture was grown overnight at 30°C and cells were harvested by centrifugation at 2050 g for 10min. The obtained cells were washed twice with 20 mM MOPS (Sigma-Aldrich, USA) buffer, pH 7.1 and resuspended to an OD_{600nm} of 0.7. The culture (1.5 mL) was transferred to a 2 mL Eppendorf tube and centrifuged (1485g, 10 min). The cells were then resuspended in half the volume of 0.5 mg/mL cytochrome C in MOPS buffer (20mM, pH 7.1) and incubated for 10 min at room temperature. The cells were again centrifuged for 5 min (6000 g), the supernatant was transferred to a 96-well plate and the optical density measured at 410 nm. The bound cytochrome C amount was calculated using the following equation:

$$bound\ Cytochrome\ C\ [\%] = 100 - \frac{A_1}{A_0} * 100$$

with A_0 being the optical density measurement of the supernatant of the blank at 410 nm and A_1 the optical density measurement of the supernatant of the sample at 410 nm.

6.2.6 Microscopic analysis – Confocal laser scanning microscopy (CLSM)

The biofilms were grown on glass-bottom dishes (35mm petri dish, 10mm Microwell No. 0 coverglass, MatTek Corporation, USA). First, single colonies were used to inoculate TSBYE and then incubated overnight at 37°C. The glass-bottom dishes were filled with 2.97 mL MWB (Mg^{2+} concentration 1.67 mM or 5 mM) and inoculated with 30 µL of the overnight culture. After 7 d incubation at 30°C (or 37°C for 39G5), the medium was carefully removed and the biofilm on the plates washed twice with 0.8% NaCl solution. The fluorescent LIVE/DEAD *Bac*Light bacterial viability kit (Life Technologies, Thermo Fisher, New Zealand) was used to stain the biofilms according to the manufacturer's instructions. Three images (246.03x246.03 µm) per sample were taken with a Leica DM6000B scanning confocal microscope running LAS AF software

version 2.7.3.9723. Excitation and emission were as follows: Stains: SYTO9, excitation @ 488 nm (argon laser), emission collection @ 498-550 nm; propidium iodide, excitation @ 561 nm (DPSS 561 laser), emission collection @ 571-700 nm.

Images were analysed using ImageJ software and/or Imaris (Bitplane, Zurich). COMSTAT (available from www.comstat.dk) was used to calculate biomass, roughness, maximum and average thickness of the biofilms (Heydorn et al. 2000, Vorregaard 2008).

6.2.7 Stress resistance

Stress resistance was examined according to a method described previously (van der Veen et al. 2010). The wild-type and the mutants were grown overnight in TSBYE at 37°C. The overnight cultures (100 µL) were used to inoculate 9.9 mL BHI. The mid-exponential cultures were harvested after about 5-6 h growth (OD_{595nm} ca. 0.4) and exposed to different stresses. H_2O_2 was added at a final concentration of 60mM to determine oxidative stress response and incubated for 1 h at room temperature. To determine acid resistance, the cultures were centrifuged (10 min, 2050 g, room temperature) and resuspended in BHI, pH 3.4 (adjusted with concentrated HCl) and then incubated at room temperature for 1 h. Assessment of heat response was performed by exposing the cultures to 55°C in a water bath for 1 h. The untreated control was incubated at room temperature. All cultures were incubated under shaking (200 rpm, Orbit 19 Labshaker, Labnet, NJ). After 1 h decimal dilutions were prepared in PBS and plated on BHI-Agar. Surviving cells were enumerated after 24 h incubation at 30°C.

6.2.8 Statistical analysis

Statistical differences between the wild-type and the mutants was calculated using ANOVA. A significant difference was concluded for $p<0.05$. Correlation analysis was performed using regression analysis (Pearson) and a significant difference was concluded for $p<0.05$, two-tailed. Analyses were performed with Genstat version 17 (2016) and Microsoft Excel software.

6.3 Results

6.3.1 Biofilm formation does not correlate to surface properties

The two high biofilm forming transposants with insertions in the genes encoding for a lysyl-phosphatidyglycerol synthetase (*mprF*) and a cardiolipin synthetase (*clsA*) are both associated with membrane synthesis. Gene disruptions of the three low biofilm producer were located in *uvrB* (excinuclease B), *mltD*, which is associated with autolysis, and a gene encoding for Mg^{2+} P-type ATPase (*mgt*B).

To elucidate biofilm formation and composition, three different biofilm formation assays were used (CV assay, cell enumeration by plate counting and metabolic activity (XTT assay)) (Table 6.2). Koban et al. (2012) found that the cell counts need to be at least 4.5 $\log_{10}$ CFU/mL to detect metabolic activity with the XTT assay, which made this assay suitable for the present study as the cell counts measured were well above that limit. The XTT assay is based on reduction of the XTT to a water soluble formazan salt by dehydrogenases, which are present in metabolically active cells but not in dead cells.

By plotting the obtained values for cell enumeration against the values for the CV staining, a significant correlation was found (0.98), indicating that biofilm mass highly correlates with viable cell numbers and both methods may be used interchangeably in our case. Furthermore, the XTT assay showed a significant correlation with the CV assay and the cell enumeration (0.89 and 0.82, respectively).

34F11 showed highest metabolic activity and also produced greater biofilm determined by CV and cell enumeration. The increased metabolic activity compared to the wild-type suggests that cells that were stained by propidium iodide (PI) in a previous study (Chapter 5) using the LIVE/DEAD stain were actually not dead but rather had a defect in their membrane allowing PI to penetrate the membrane. 6B4 showed greater biofilm formation in all three assays, suggesting that increased biofilm mass is likely due to increased cell numbers and hence higher metabolic activity (Table 6.2). Interestingly, 39G5 showed the same amount of metabolic activity compared with the wild-type but showed only low amounts of biofilm using the CV assay. Plate counts (6.087 $\log_{10}$ CFU/well) showed the presence of viable cells for 39G5 indicating that CV has a lower detection limit. Kadam et al. (2013) evaluated the correlation between the CV assay and cell enumeration and found nearly no biofilm through the CV assay when viable cells of 6.5 $\log_{10}$ CFU/mL or lower were detected. which is in line with our observations. 33E11 showed lower metabolic activity, CV staining and plate counts compared with the wild-type. Reduction in biofilm formation compared to the wild-type is due to lower number of attached cells. 44D3 demonstrated higher metabolic activity, which is interesting considering it produced less biofilm when analysed with the CV assay and through plating compared to the wild-type.

The amount of eDNA present in the biofilm and supernatant and the carbohydrate amount were standardized against the OD_{595} values obtained after crystal violet staining for all strains tested. Determination of eDNA in the biofilm and the supernatant of the wild-type and the mutants showed significant differences for the low biofilm producer 33E11 and 39G5: eDNA content of

the disrupted biofilm of 33E11 and 39G5 was higher compared to the wild-type and the high biofilm producer (Table 6.2). 33E11 and 39G5 are both low biofilm formers and involved in stress response or autolysis, respectively. The gene disruptions might have caused a dysfunctional stress response and autolysis regulation resulting in damage to the cell which could have induced an increase in eDNA. Furthermore, the gene disruption of *uvrB* might have also led to an increase in ssDNA which could have been detected as eDNA. The other low biofilm former 44D3 produced not significantly more eDNA compared with the wild-type (Figure 6.2), which rebuts the suggestion that the cloudy structure observed for this mutant with the fluorescence microscope using LIVE/DEAD stain was due to presence of eDNA. No difference in eDNA production was observed for the two high biofilm formers 6B4 and 34F11 compared to the wild-type (Figure 6.2).

Determination of carbohydrates in the biofilm resulted in a significant difference for 39G5 (Table 6.2). Defects in autolysis obviously affect carbohydrate production and eDNA presence significantly. Surprisingly, biofilm formation measured by the crystal violet assay did not correlate to the carbohydrates present in the biofilm. The crystal violet assay measures biofilm formation by staining any organic matter including carbohydrates. Greater biofilm formation should therefore result in higher carbohydrate amounts, however, this was not the case suggesting the involvement of the disrupted genes in carbohydrate production/metabolism.

Table 6.2. Composition of the biofilm determined through crystal violet assays (whole biofilm mass), cell count (viable cells) and metabolic activity (viable cells and viable but non culturable cells); eDNA amount was measured through ethanol precipitation and by NanoDrop after extraction with the DNeasy blood and tissue kit (Qiagen) and carbohydrate amount with the phenol sulphuric assay; means of three experiments with n=1 are shown for the eDNA and carbohydrate measurements and means of two experiments with n=5-6 are shown for the crystal violet assay, the XTT assay and the cell enumeration. The standard deviations are shown in brackets underneath the respective mean.

	wild-type	6B4	34F11	33E11	39G5	44D3
CV [OD_{595nm}]	1.205	1.921	1.762	0.263	0.153	0.756
	(0.079)	(0.266)	(0.020)	(0.022)	(0.116)	(0.017)
Cell enumeration [log_{10} CFU/well]	6.994	7.391	7.226	6.418	6.087	6.571
	(0.160)	(0.067)	(0.072)	(0.224)	(0.155)	(0.207)
Metabolic activity [OD_{450nm}]	0.079	0.217	0.201	0.055	0.070	0.178
	(0.018)	(0.084)	(0.002)	(0.036)	(0.014)	(0.012)
eDNA biofilm [ng/OD_{595nm}]	6.55^{c}	4.87^{c}	4.34^{c}	$38.34^{a,b}$	51.92^{a}	$14.62^{b,c}$
	(0.29)	(1.29)	(1.15)	(11.42)	(17.74)	(2.21)
eDNA supernatant [µg/OD_{595nm}]	1.24^{b}	0.53^{b}	0.58^{b}	8.50^{a}	12.23^{a}	1.30^{b}
	(0.08)	(0.06)	(0.10)	(2.76)	(2.94)	(0.28)
carbohydrate content biofilm [µg/OD_{595nm}]	7.83^{c}	5.19^{c}	5.46^{c}	35.04^{b}	57.90^{a}	10.38^{c}
	(0.96)	(0.82)	(0.72)	(5.48)	(9.88)	(0.86)

To identify surface characteristics and possible correlations to biofilm formation, the wild-type and the mutants were subject to the MATH test and the hydrophobic interchange chromatography, which both determine hydrophobicity, and the cytochrome C assay to measure surface charge of the bacterial cells.

Hydrophobicity measured with the MATH test was highest for the wild-type and lowest for 33E11 (Table 6.3). One way ANOVA revealed a significant difference for the strains (p=0.019) (Table 6.3). Di Bonaventura et al. (2008) classified *L. monocytogenes* strains with a value above 50% as highly hydrophobic, between 20–50% as moderately hydrophobic and strains with a value of less than 20% are considered hydrophilic. Therefore, all strains tested in this study can be considered hydrophilic.

Hydrophobic interactive chromatography (HIC) revealed a hydrophobicity ranging between 43.79% and 81.98% for the six strains. 39G5 was significantly different ($p<0.01$) compared to the other strains. However, this result needs to be interpreted with caution- 39G5 is a low biofilm former with a long chain phenotype (Nowak et al. n.d.). Long chains might get caught in the resin and held back from elution causing a false high hydrophobic result. Excluding the result for 39G5, there was a significant negative correlation between the MATH test and HIC (-0.93).

34F11, 39G5 and 44D3 had higher negative surface charge compared to the wild-type ($p<0.01$) when measured with the cytochrome C assay (Table 6.3). *clsA* catalyses the formation of cardiolipin from phosphatidylglycerol (PG). The lack of *clsA* might result in higher amounts of PG and therefore a higher negative charge (PG is negatively charged). However, the increased negative surface charge cannot explain greater biofilm formation for the *clsA* mutant (34F11) as 39G5 and 44D3, both low biofilm formers, showed the highest negative surface charge. No correlation with biofilm formation could be observed for all surface property tests.

Surface charge is especially important for the first steps of biofilm development as electrostatic attraction and repulsion will determine attachment. As surface characteristics are more likely to influence attachment than biofilm development, the attachment ability of the wild-type and the mutants was evaluated after 30 min in MWB (Figure 6.1a). No correlation between attachment and surface properties was observed, suggesting that both, attachment and biofilm formation, are independent of surface properties for the six examined strains. However, there was a significant correlation between cell enumeration and attachment (0.88), but not between attachment and the XTT or crystal violet assays.

Table 6.3. Surface properties of planktonic cultures of the wild-type and the mutants. The surface properties were determined with the MATH test, hydrophobic interaction chromatography and the cytochrome C assay. Tukey's 95% confidence interval is indicated for each treatment in superscript. The cytochrome C assay was performed twice with n=3 and MATH and HIC was performed three times with n=1. Standard deviation is shown in brackets underneath its respective mean.

Surface properties	wild-type	6B4	34F11	33E11	39G5	44D3
MATH test	18.96^{b}	$12.37^{a,b}$	$9.14^{a,b}$	4.20^{a}	$9.22^{a,b}$	7.29^{a}
Hydrophobicity [%]	(6.49)	(5.02)	(1.24)	(4.86)	(3.48)	(1.84)
Hydrophobic interaction chromatography	43.79^{a}	47.96^{a}	48.49^{a}	56.20^{a}	81.98^{b}	55.01^{a}
Hydrophobicity [%]	(5.08)	(3.31)	(5.78)	(4.54)	(1.30)	(8.30)
Cytochrome C assay	29.39^{a}	$33.26^{a,b}$	$39.01^{b,c}$	$38.20^{a,b,c}$	45.93^{c}	47.14^{c}
bound cytochrome C [%]	(3.21)	(1.62)	(1.02)	(11.98)	(0.28)	(1.19)

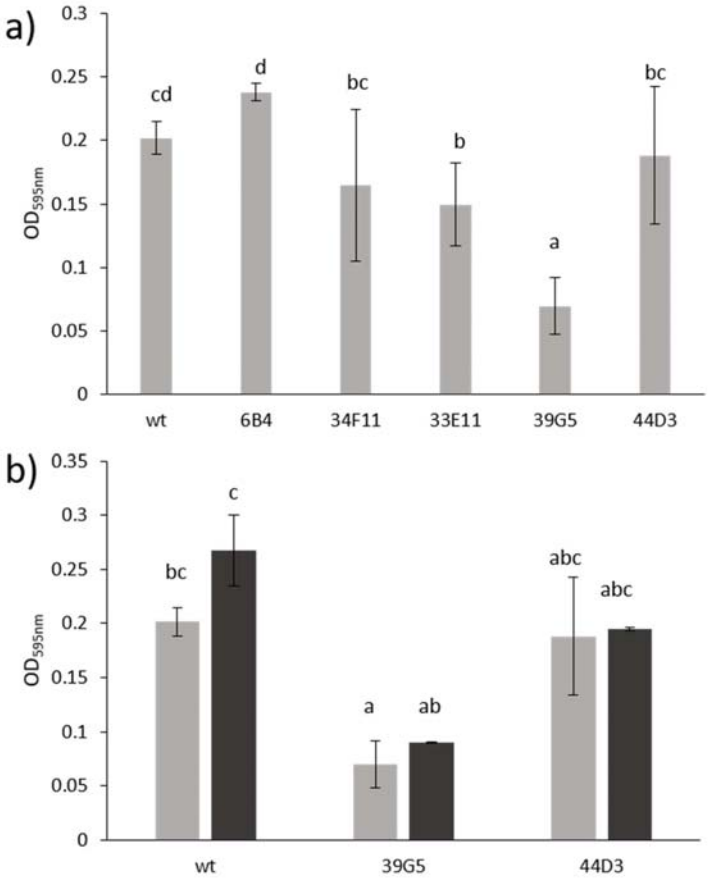

Figure 6.1. Attachment of the *Listeria monocytogenes* 15G01 (wt) strain and the five mutant strains on polystyrene after 30 min at 30°C in MWB (a) and attachment of *L. monocytogenes* 15G01 (wt) and the mutants in MWB (light grey bars) and in MWB-5mM Mg^{2+} (dark grey bars) (b). Error bars represent the standard deviation of two experiments with n=12. Letters in common indicate no significant difference.

6.3.2 Magnesium increases biofilm production for the wild-type and the *mgtB* mutant (44D3), but decreases the biofilm mass for the *mltD* mutant (39G5)

A transposon insertion in a Mg^{2+}-ATPase gene led to reduced biofilm formation (44D3*)*. To identify whether Mg^{2+} influences biofilm formation, the biofilm formation assay was performed with the wild-type in MWB at 30°C with final Mg^{2+} concentrations of 2.5, 5 and 10 mM. ANOVA revealed that biofilm formation of the wild-type in MWB with a concentration of 5mM Mg^{2+} was significantly increased compared to other Mg^{2+} concentrations (Figure 6.2). Therefore, the mutants were tested in MWB with a final concentration of 5mM Mg^{2+}. Biofilm formation of the mutant 44D3 was greater in the presence of 5 mM Mg^{2+} (Figure 6.3 a) compared to the wild-type level.

39G5 showed low biofilm formation at 30°C with a reduction in biofilm mass after magnesium addition (Figure 6.3a). At 37°C, 39G5 showed greater biofilm formation compared with the wild-type, but the addition of magnesium reversed that effect, resulting in low biofilm mass (Figure 6.3b). *mltD* is obviously temperature dependent and gene disruption exhibits a different phenotype (greater biofilm formation) when incubated at 37°C. The growth of the selected strains was tested in MWB with added magnesium and it was observed that growth in MWB with magnesium addition did not differ from growth without additional magnesium (data not shown). Therefore, the effect of magnesium on biofilm formation can be considered growth independent.

Mg^{2+} addition did not increase attachment significantly for any of the tested strains (Figure 6.1b). 39G5 showed no motility in previous experiments (Nowak et al. n.d.) and as flagella have been found to be influenced by the presence of Mg^{2+} (Robinson et al. 1992), the *flaA* mutant was tested under the same conditions. Flagella are generally produced at temperatures of 30°C and below but not at 37°C. Biofilm formation increased slightly at 37°C, supporting no involvement for *flaA* in biofilm reduction of 39G5 in the presence of 5mM Mg^{2+} (Supplementary Figure S6.1).

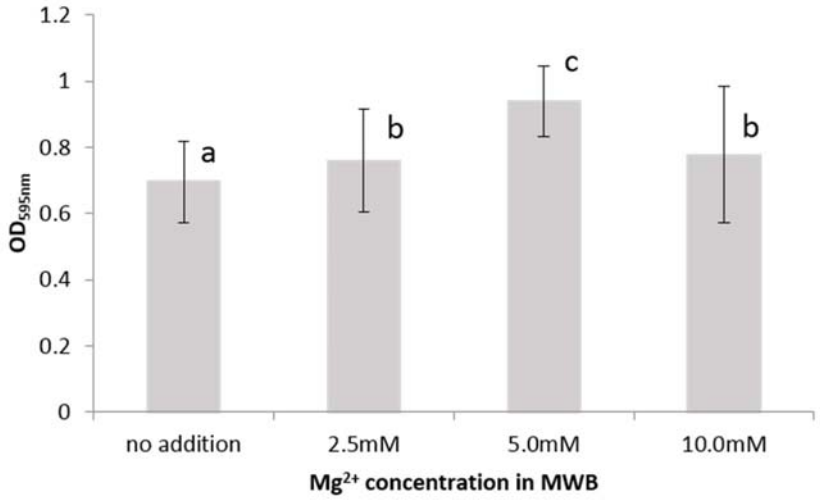

Figure 6.2. Biofilm formation of *Listeria monocytogenes* 15G01 in MWB plus Mg^{2+} addition at 30°C after 48 h of incubation. Error bars represent standard deviation of 2 independent experiments with n=16. Letters in common indicate no significant difference.

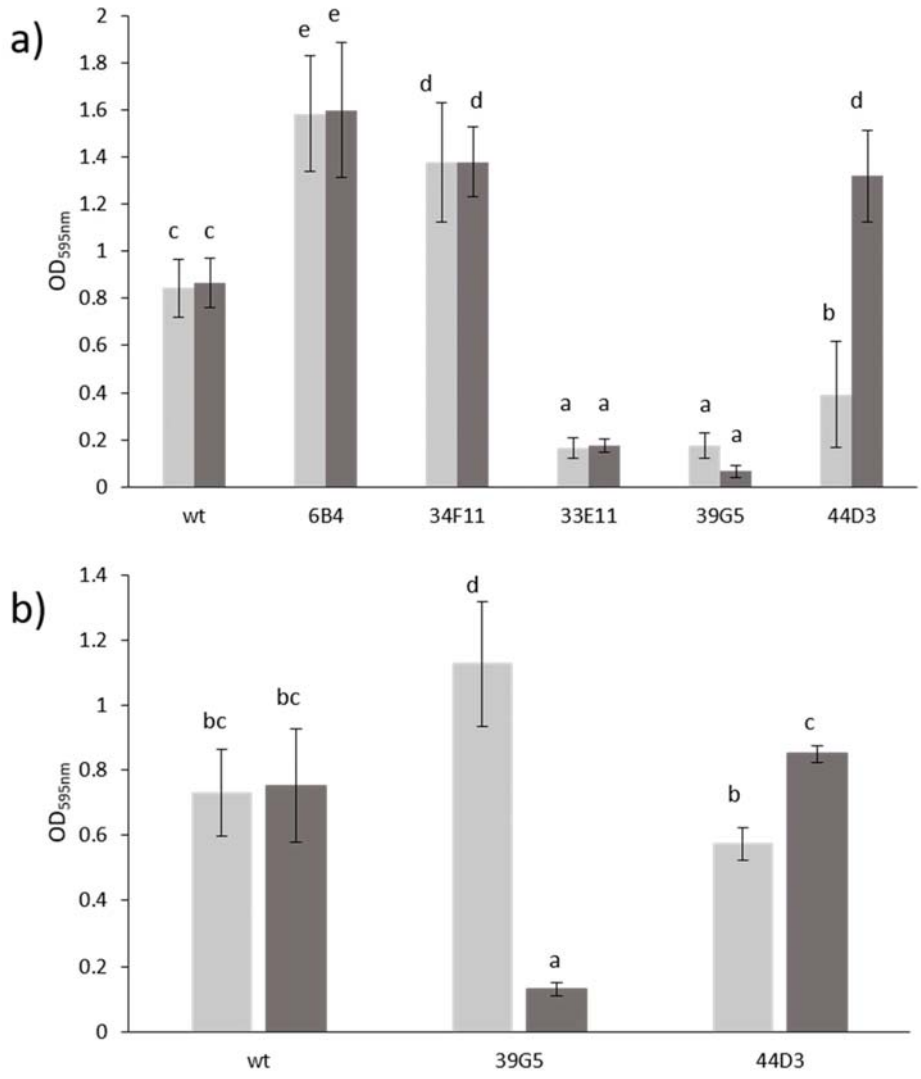

Figure 6.3. Biofilm formation of the wild-type and selected mutants in MWB (light grey bars) and MWB with a Mg^{2+} concentration of 5 mM (dark grey bars) at 30°C (a) and 37°C (b) after 48 h of incubation. Error bars represent standard deviation of three independent experiments (n=6). Letters in common indicate no significant difference.

6.3.3 Effect of calcium on biofilm formation at 30°C and 37°C

To test the influence of calcium on biofilm formation, $CaCl_2$ was also added to MWB in final concentrations of 2.5 mM, 5 mM and 10 mM. Biofilms were incubated for 48 h at 30°C and 37°C.

All tested strains showed decreased biofilm formation in MWB after calcium addition. During preparation of MWB with calcium, a precipitate formed in the medium. Literature states that calcium phosphorus precipitation is a common method of phosphorus removal in wastewater systems (de-Bashan and Bashan 2004). According to de-Bashan and Bashan (2004) the calcium concentration must be at least 100 mg/L and the phosphate concentration at least 50 mg/L to

start precipitation at a neutral pH. As MWB is high in phosphate content (monopotassium phosphate 6.560 g/L; disodium phosphate *$7H_20$ 30.960 g/L), it is quite likely that the observed precipitation was due to formation of calcium phosphate. Calcium addition in MWB resulted in decreased biofilm formation which might either be due to the addition of calcium or phosphate depletion due to calcium phosphate formation and precipitation as phosphate has previously been found to be involved in biofilm formation (Jang et al. 2012). To minimise the influence of phosphate, the experiment was repeated using TSB instead of MWB.

Biofilm formation in TSB increased significantly at a calcium concentration of 10 mM at 30°C for the wild-type and 39G5 (Figure 6.4 a) ($p<0.001$) compared to no calcium presence. 6B4 and 33E11 followed a similar pattern, but the difference were not significant; while 34F11 and 44D3 showed no significant changes. At 37°C, biofilm formation was lower in the presence of 10 mM Ca^{2+} for all strains tested compared to TSB without calcium addition ($p<0.001$) except for 44D3. Calcium addition led to a reduction in biofilm mass at 37°C for all strains.

In conclusion, calcium had a similar effect on biofilm formation for the wild-type strain and the tested mutants. It therefore does not appear to have a specific role in the function of the disrupted genes of the mutants.

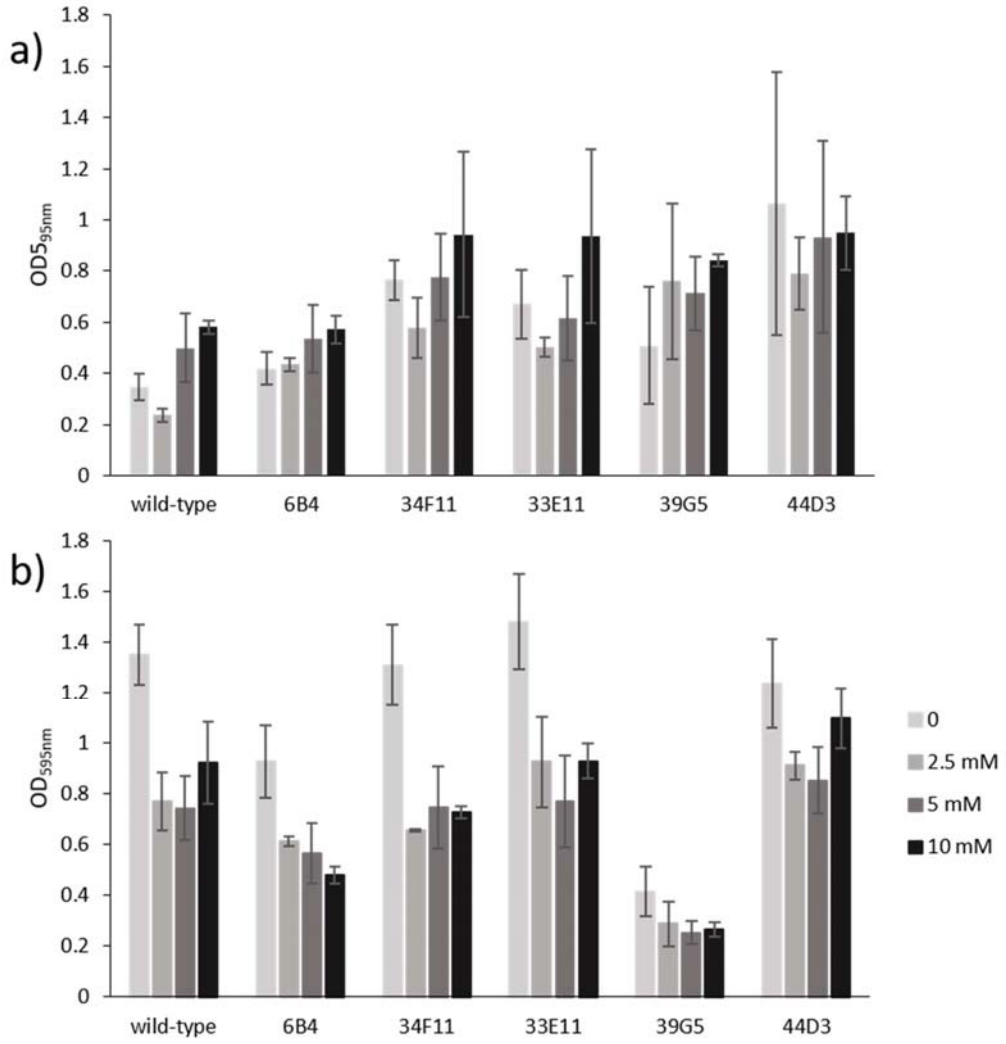

Figure 6.4. Biofilm formation of the wild-type and the selected mutant strains in TSB after 48 h incubation at 30°C (a) and 37°C (b) with increasing calcium concentration. Error bars represent standard deviation of n=3

6.3.4 Confocal analysis reveals a unique sandwich structure for the biofilm of the *mgtB* mutant

The influence of magnesium on the biofilm structure was investigated using confocal analysis. COMSTAT was used to calculate biomass, roughness and maximum and average thickness of the biofilms (Table 6.4). COMSTAT defines biomass as the volume of all voxels (the smallest 3D picture element with the base of a pixel (x and y) and a height of the slight distance) that contain biomass divided by the area. The number of voxels is multiplied by the height (z) and width (x) and length (y) in µm of a voxel (= Biovolume). The roughness coefficient ($Ra*$) is a measure for

the variability in the height of the biofilm and has no dimension. The maximum thickness is defined as the thickest piece of the biofilm taking holes and cavities in the structure into account.

Isosurface images of the biofilms stained with SYTO 9 were generated with Imaris (Bitplane, Zurich, Switzerland). The wild-type formed microcolonies on glass after 7 d incubation in MWB at 30°C (Figure 6.5). In contrast to the CV assay, biomass was reduced in the presence of 5 mM Mg^{2+} for the wild-type, however, maximum thickness and average thickness of the biomass remained the same. Magnesium led either to increased production or stabilisation of extracellular polymeric substance (which is not detected by CLSM) and/or decreased attachment of bacterial cells. Furthermore, differences in methodology could have led to the observed differences: the biofilm was washed with a microplate washer for the crystal violet assay, whereas the biofilms grown for CLSM analysis were manually washed. This might have resulted in less disturbance of the biofilm produced. In addition, biofilms were formed on two different surfaces (polystyrene and glass), which could have also contributed to the observed differences.

As the differences for the biofilm mass in the presence of 5 mM Mg^{2+} was greatest when 39G5 was incubated at 37°C (Figure 6.3b), this temperature was used for the CLSM analysis. Magnesium presence led to a decrease in biofilm and attached cells for 39G5 after 7 d incubation at 37°C (Figure 6.5c and d). This was also observed with the CV assays after 48 h (Figure 6.3b). Maximum thickness and average thickness were more than halved in the presence of 5 mM Mg^{2+} (46.71% and 40.84% of the thicknesses without additional magnesium, respectively) (Table 6.4).

44D3 produced more biofilm mass in the presence of 5 mM Mg^{2+} compared with 1.67 mM Mg^{2+} and less biomass than the wild-type in MWB (1.67 mM Mg^{2+}) (Table 6.4), which is in line with the observations made with the CV assay (Figure 6.3a). Calculations with COMSTAT revealed double maximum thickness for the biofilm of 44D3 compared with biofilms formed by the wild-type and 44D3 in the presence of 5mM Mg^{2+} (Table 6.4). This suggests that Mg^{2+} restored the wild-type phenotype for the *mgtB* mutant (44D3) (Figure 6.5f, Table 6.4). 44D3 produced a unique sandwich structure for the biofilm (Figure 6.6b) with monolayers of bacterial cells at the top and bottom and EPS or fluid in between. This structure has, to the best of our knowledge, not been reported for a biofilm before. In the presence of 5 mM Mg^{2+}, 44D3 produced a biofilm similar in structure to the wild-type (Figure 6.6a and c) further strengthening the hypothesis of restoration of the wild-type's phenotype.

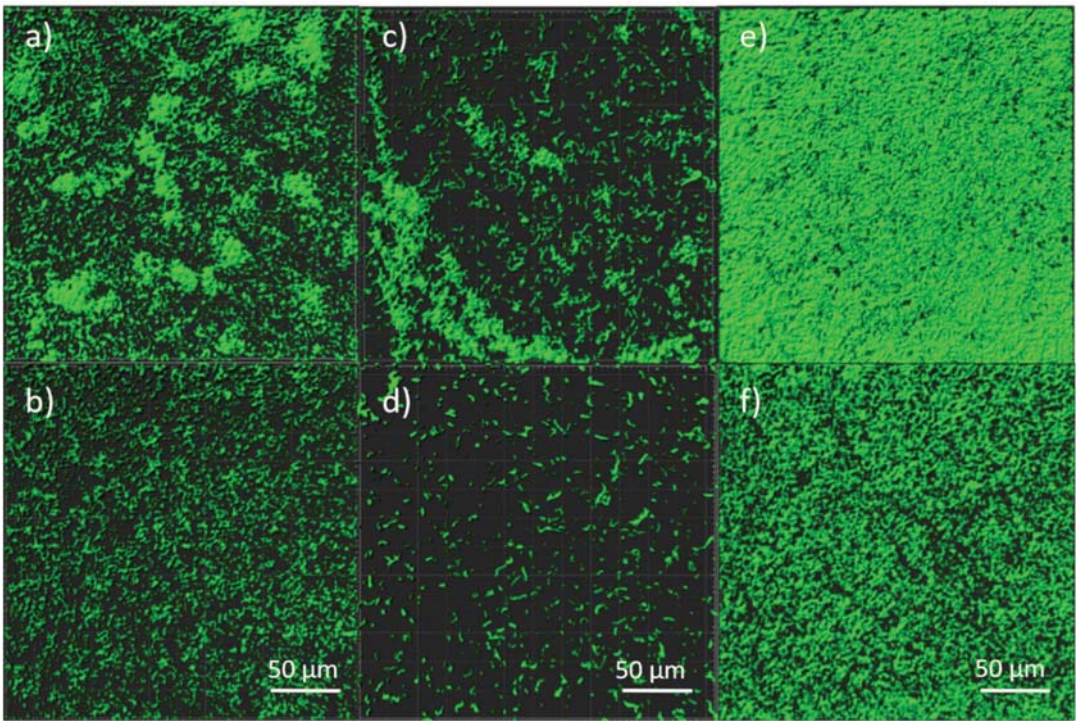

Figure 6.5. Isosurface images of biofilms of the wild-type (a), 39G5 (c) and 44D3 (e) formed on glass after 7 d incubation in MWB at 30°C and in MWB with a final Mg concentration of 5 mM (wild-type (b), 39G5 (d) and 44D3 (f)). The biofilms were stained with SYTO9.

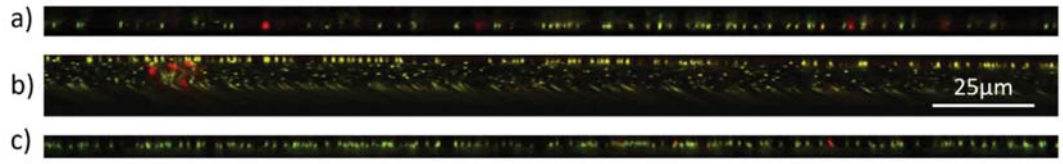

Figure 6.6. Orthogonal view of biofilms formed on a glass surface after 7 days at 30°C by the wild-type in MWB (a), by the *mgt*B mutant (44D3) in MWB (b) and by the *mgtB* mutant (44D3) in presence of 5 mM Mg^{2+} (c). Images were taken after removal of media and staining with the LIVE/DEAD *Bac*Light kit with a confocal laser scanning microscope

Table 6.4. Biomass, roughness coefficient, maximum thickness and average thickness of biofilms formed by the wild-type (wt) and the mutants 39G5 and 44D3 in MWB and MWB with a final concentration of 5mM Mg^{2+} after incubation at 30°C for 7 d calculated using COMSTAT. The standard deviation was calculated from three analysed images taken of each sample and are shown in brackets.

	wt	wt-Mg^{2+}	39G5	39G5-Mg^{2+}	44D3	44D3-Mg^{2+}
Biomass ($\mu m^3/\mu m^2$)	1.145 (0.054)	0.497 (0.409)	1.813 (1.620)	0.191 (0.043)	0.841 (1.130)	1.626 (0.950)
Roughness coefficient (Ra*)	0.639 (0.074)	1.515 (0.256)	0.891 (0.750)	1.787 (0.009)	1.284 (0.874)	0.654 (0.455)
Maximum thickness (µm)	4.784 (1.452)	4.951 (0.317)	9.162 (3.767)	4.280 (0.00)	10.966 (6.764)	5.077 (0.262)
Average thickness (Entire area) (µm)	2.295 (0.776)	0.840 (0.561)	4.063 (2.399)	0.320 (0.089)	3.107 (4.807)	2.398 (1.340)
Average thickness (Biomass) (µm)	3.357 (1.377)	3.281 (0.533)	7.408 (2.243)	3.026 (0.913)	4.904 (4.283)	3.238 (1.082)

6.3.5 Oxidative stress significantly reduces survival of 34F11 compared to the wild-type

Planktonic cultures were exposed to a variety of stresses to determine the involvement of biofilm-related genes in stress response. All cultures showed a reduction in cell numbers after stress exposure compared to the untreated counterparts (Table 6.5). 39G5 was the most stable strain, exhibiting only slight reduction in cell numbers (maximum reduction of 1 $\log_{10}$ CFU/mL after heat treatment). 34F11 and 44D3 were most affected by the oxidative reagent (reduction of 4.25 $\log_{10}$ CFU/mL and 1.81 $\log_{10}$ CFU/mL, respectively), but only 34F11 was significantly different as determined by the post-hoc Tukey's test after 2-way ANOVA. Hydrogen peroxide is cytotoxic due to the formation of hydroxyl radicals, therefore the membrane structure of 34F11 must be more susceptible to oxidative reagents. A possible reason for the higher susceptibility might be enhanced membrane permeability. However, staining the cultures of the wild-type and 34F11 after oxidative stress exposure with propidium iodide did not reveal any difference (data not shown).

Interestingly, the wild-type exhibited the highest reduction in cell numbers after heat-treatment, which was significantly different from the untreated culture of the wild-type

(p<0.001). None of the other strains showed a significant difference, suggesting that the genes might be involved in heat resistance. All treatments resulted in a significant decrease in cell numbers for all strains tested (p<0.001).

Table 6.5. Cell enumeration of surviving cells after treatment with an oxidiser, acid and heat for 1 h at room temperature. Means and standard deviations (in brackets) of three independent experiments are shown in $\log_{10}$ CFU/mL. Superscript letters in common indicate no significant difference.

	wt	**6B4**	**34F11**	**33E11**	**39G5**	**44D3**
Untreated	9.41^{c} (0.23)	$9.26^{b,c}$ (0.21)	$9.13^{b,c}$ (0.13)	$9.24^{b,c}$ (0.11)	$9.02^{b,c}$ (0.41)	$9.24^{b,c}$ (0.17)
60mM H_2O_2	$8.81^{b,c}$ (0.25)	$8.41^{b,c}$ (0.69)	4.88^{a} (0.85)	$8.53^{b,c}$ (0.55)	$8.58^{b,c}$ (0.26)	$7.43^{b,c}$ (1.75)
PH 2.5	$8.11^{b,c}$ (1.02)	$8.05^{b,c}$ (1.16)	$8.63^{b,c}$ (0.79)	$8.79^{b,c}$ (0.62)	$8.45^{b,c}$ (0.27)	$8.64^{b,c}$ (0.31)
55°C	7.12^{b} (0.17)	$8.34^{b,c}$ (0.41)	$7.32^{b,c}$ (0.22)	$8.06^{b,c}$ (0.35)	$8.03^{b,c}$ (0.59)	$8.07^{b,c}$ (0.64)

6.4 Discussion

Mg^{2+} presence (5 mM) enhanced biofilm formation for the mutant 44D3 above the wild-type level when measured by the CV assay as well as by CLSM which could either be due to restoration of the wild-type phenotype or to stabilisation of the biofilm matrix due to binding to negatively charged surface molecules, such as eDNA. Previous experiments with the LIVE/DEAD stain showed a cloudy structure stained in red by PI for the biofilm of the *mgtB* mutant, which was thought to be eDNA (Chapter 5). However, the eDNA measurements in this study could not confirm this. The restoration of the *mgtB* mutant (44D3) to the wild-type phenotype with the addition of Mg^{2+}demonstrates the putative function of this gene.

Confocal analysis was used to observe the influence of magnesium on biofilm production. Of particular interest was the observed sandwich structure of the biofilm formed by 44D3. The CLSM exposed a biofilm with static monolayers at the top and bottom and movement in the fluid in between (Figure 6.6). Guilbaud et al. (2015) also observed movement in the fluid in hollow structures of the honeycomb-like structured biofilm of *L. monocytogenes* when analysing it with CLSM. Furthermore, it is possible that the observed structure in the present

study is rather a mushroom-like structure with the top layer connected to the bottom layer by a strand of biofilm. This is the first time that such a biofilm structure has been reported.

Expression of *mgtB*, a gene encoding for a Mg^{2+} transport ATPase, is induced upon Mg^{2+} deprivation sensed by the PhoP/PhoQ two component systems (Chamnongpol and Groisman 2002, Groisman et al. 2006) in Gram-negative bacteria. The magnesium transporter system is well studied in Gram-negative organisms (Groisman et al. 2013), however little is known about its function and involvement in biofilm formation in Gram-positive bacteria, especially in *L. monocytogenes*. Previous research has found that *mgtB* is regulated by CesR (Nielsen et al. 2012) in *L. monocytogenes*. The two component system CesRK also regulates genes involved in virulence (Kallipolitis et al. 2003) and is involved in ethanol and antibiotic sensitivity (Gottschalk et al. 2008).

In the present study, the *mltD* mutant, 39G5, produced less biofilm than the wild-type at 30°C but more than the wild-type at 37°C. At both temperatures the presence of magnesium (5 mM) reduced biofilm production to a minimum when measured with the CV assay and by CLSM. Attachment studies revealed that the presence of magnesium did not alter the attachment ability of 39G5, suggesting that magnesium influences the biofilm maturation process rather than surface characteristics or initial attachment. Another study has found that autolysin is involved in biofilm formation of *S. epidermidis* through autolysin-mediated release of extracellular DNA (Qin et al. 2007). It is known that eDNA plays a role in biofilm formation in several bacterial species including *L. monocytogenes* (Harmsen et al. 2010, Okshevsky and Meyer 2015). Harmsen et al. (2010) showed that high molecular weight DNA it is essential for attachment to surfaces. In the present study it was found that the *mltD* mutant produced more eDNA possibly due to disruption of the autolytic process. However, this cannot explain the low biofilm formation of this mutant. Further research needs to be conducted to elucidate the exact role of *mltD* in biofilm formation.

The eDNA concentration and the biofilm formation measured by the crystal violet assay did not correlate. This can either be due to the genes being involved in carbohydrate metabolism or to the method itself. In this study the concentration of the extracellular DNA was measured with the NanoDrop, which calculates nucleic acid conenctrations in a range of 2 – 15000 ng/µL and determines its purity by measuring the absorbance ratios at 260/230nm and 260/280nm. The eDNA concentrations were in the lower detection range, so the results may not be accurate. To further clarify the eDNA involvement, other measurements, such as Qubit or fluorescent marking are advised.

Magnesium has been shown to influence biofilm formation in other bacterial species (Mulcahy and Lewenza 2011, Oknin et al. 2015), where high magnesium concentrations (50 mM and higher) led to reduced biofilm formation in *Bacillus subtilis* and *Bacillus cereus* (Oknin et al. 2015) without affecting the growth. However, lower concentration of 5 mM and 10 mM led to an increase in biofilm formation of *B. subtilis* in the same study, whereas Ca^{2+} presence had no significant effect on biofilm formation and growth. These results are in line with our findings. Other studies showed that the presence of Mg^{2+} led to increased attachment of *P. fluorescence* cells to glass (Song and Leff 2006). In contrast, limiting Mg^{2+} resulted in increased biofilm formation in *Pseudomonas aeruginosa* through repression of *retS*, a gene responsible for EPS biosynthesis (Mulcahy and Lewenza 2011). Although the findings are contradictory, it is clear that magnesium plays a vital role in biofilm formation and should be focus of further investigations.

Interestingly, the two genes influenced by magnesium in this study (*mgtB* and *mltD*) are both situated in very close proximity on the genome only 1282 base pairs apart. The coding region for *mltD* is situated on the positive strand and for *mgtB* on the negative strand. A gene encoding for a transcriptional regulator of the TetR family is situated between these two genes. *mltD* is suggested to be regulated by this gene (Chatterjee et al. 2006), which is known to be influenced by magnesium (Leypold et al. 2004, Scholz et al. 2000, Werten et al. 2014). The *mgtB* mutant (44D3) showed a low biofilm phenotype which was reversed to the wild-type level upon magnesium addition, whereas the *mltD* mutant (39G5) showed a further reduction in biofilm mass upon magnesium addition. This and the close proximity of the genes suggest a common regulative mechanism, possibly through TetR, although different regulative systems for the two genes have been suggested (Chatterjee et al. 2006, Nielsen et al. 2012). The hypothesis of a common regulative mechanism is strengthened by the association with cell division for both genes: *mltD* in a previous study (Machata et al. 2005) and *mgtB* being part of an operon which also encodes for cell division proteins. In addition, both mutants showed enhanced cell aggregation when grown in liquid culture (data not shown), further reinforcing the link between both genes.

Poimenidou et al. (2016) investigated the correlation of the CV assay and cell enumeration of 12 *L. monocytogenes* strains and they showed a significant correlation upon exclusion of two outliers, which is in line with our findings. Furthermore, they found no correlation between cell surface properties and biofilm formation in *L. monocytogenes* (Poimenidou et al. 2016), which was also observed in this study. Surface properties depend on environmental conditions, as

shown by Di Bonaventura et al. (2008) who found that hydrophobicity increased with increasing temperature for 44 *L. monocytogenes* strains. Out of those 44 strains (including animal, human, food and environmental origin), four were highly hydrophobic, 32 moderately hydrophobic and eight hydrophilic. Hydrophobicity was significantly increased at 37°C compared to 4°C, 12°C and 22°C in their study. They showed a positive correlation between biofilm formation on glass and hydrophobicity level at 12°C, but not for the other temperatures tested. In the present study hydrophobicity was tested at 30°C to identify a possible correlation between hydrophobicity and biofilm formation or attachment. However, no correlation could be observed between surface properties and attachment and biofilm formation, which is consistent with the findings of Chae et al. (2006) who also found no correlation between hydrophobicity and bacterial attachment (3h) or biofilm formation (24h).

The influence of Ca^{2+} was examined in the present study, however, MWB was not suitable as the medium to test Ca^{2+} influence as a precipitate formed upon addition of Ca^{2+}. Therefore, TSB was used and this showed an influence of Ca^{2+} on biofilm formation for all six strains tested. However, biofilm formation is highly dependent on nutrient availability and using the nutrient-rich medium TSB instead of MWB might mask effects that would have been observed in MWB.

clsA seems to not only play a role in biofilm formation but also in stress response, as observed upon exposure to an oxidative reagent. Oxidative reagents can lead to DNA damage and heat stress to replication fork stalling (van der Veen et al. 2010). A previous study showed that *clsA* is susceptible to damage caused by ROS (reactive oxygen species) in human mitochondria (Paradies et al. 2014), which supports the observations made in this study. The reason for enhanced susceptibility of the *clsA* mutant remains unclear as staining with the LIVE/DEAD stain did not reveal an enhanced membrane permeability.

A recent publication found an interesting link between cardiolipin and MgtA (which belongs to the same transporter class as MgtB) in *E. coli*: both MgtA and cardiolipin were found together in the bacterial membrane (Subramani et al. 2016). Subramani et al. (2016) suggested that the head group of cardiolipin contributes to MgtA activation by possibly acting as a chaperone for MgtA. Whether a similar link is present in Gram-positive bacteria will need further investigation.

In conclusion, the results of this study show that Mg^{2+} is an influencing factor for biofilm formation in *L. monocytogenes*, and this appears to be linked to two closely located genes (*mltD* and *mgtB*) on the chromosome involved in Mg^{2+} transport and cell autolysis, respectively.

6.5 Acknowledgements

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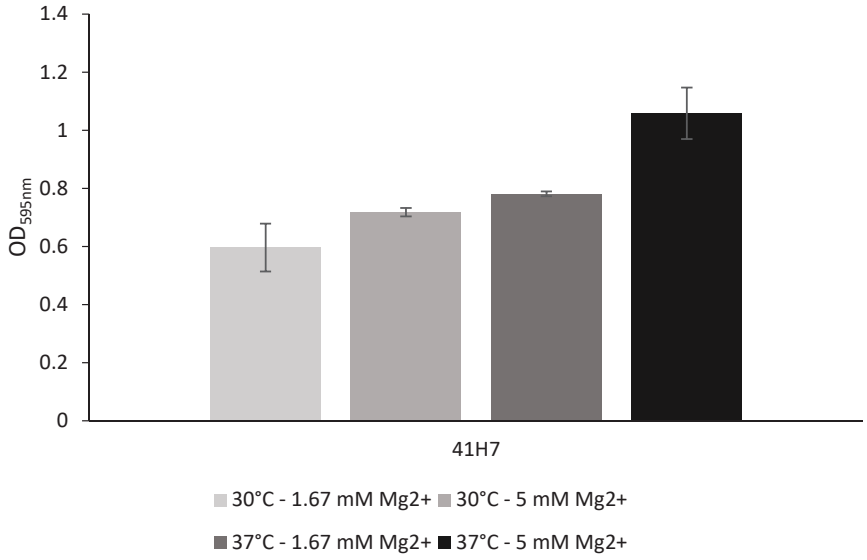
6.6 Supplementary information



Supplementary Figure S6.1. Biofilm formation of the *flaA* mutant (41H7) after 48 h incubation at 30°C and 37°C with and without magnesium addition. Error bars represent the standard deviation of 1 experiment n=3

6.7 References

Alonso, A. N., K. J. Perry, J. M. Regeimbal, P. M. Regan and D. E. Higgins (2014). Identification of *Listeria monocytogenes* determinants required for biofilm formation. PLoS One **9**(12): e113696.

Celis, J. E. (2014). Cell Biology: A Laboratory Handbook, Elsevier Science.

Chae, M. S., H. Schraft, L. Truelstrup Hansen and R. Mackereth (2006). Effects of physicochemical surface characteristics of *Listeria monocytogenes* strains on attachment to glass. Food Microbiol **23**(3): 250-259.

Chamnongpol, S. and E. A. Groisman (2002). Mg^{2+} homeostasis and avoidance of metal toxicity. Mol Microbiol **44**(2): 561-571.

Chang, Y., W. Gu, N. Fischer and L. McLandsborough (2012). Identification of genes involved in *Listeria monocytogenes* biofilm formation by mariner-based transposon mutagenesis. Appl Microbiol Biotechnol **93**(5): 2051-2062.

Chatterjee, S. S., H. Hossain, S. Otten, C. Kuenne, K. Kuchmina, S. Machata, E. Domann, T. Chakraborty and T. Hain (2006). Intracellular gene expression profile of *Listeria monocytogenes*. Infect Immun **74**(2): 1323-1338.

Chen, C. Y., G. W. Nace and P. L. Irwin (2003). A 6 x 6 drop plate method for simultaneous colony counting and MPN enumeration of *Campylobacter jejuni*, *Listeria monocytogenes*, and *Escherichia coli*. J Microbiol Methods **55**(2): 475-479.

Chmielewski, R. A. N. and J. F. Frank (2003). Biofilm formation and control in food processing facilities. Compr Rev Food Sci Food Saf **2**(1): 22-32.

Corinaldesi, C., R. Danovaro and A. Dell'Anno (2005). Simultaneous recovery of extracellular and intracellular DNA suitable for molecular studies from marine sediments. Appl Environ Microbiol **71**(1): 46-50.

Cruz, C. D. and G. C. Fletcher (2011). Prevalence and biofilm-forming ability of *Listeria monocytogenes* in New Zealand mussel (*Perna canaliculus*) processing plants. Food Microbiol **28**(7): 1387-1393.

Das, T., B. P. Krom, H. C. van der Mei, H. J. Busscher and P. K. Sharma (2011). DNA-mediated bacterial aggregation is dictated by acid–base interactions. Soft Matter **7**(6): 2927-2935.

de-Bashan, L. E. and Y. Bashan (2004). Recent advances in removing phosphorus from wastewater and its future use as fertilizer (1997-2003). Water Res **38**(19): 4222-4246.

Di Bonaventura, G., R. Piccolomini, D. Paludi, V. D'Orio, A. Vergara, M. Conter and A. Ianieri (2008). Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: relationship with motility and cell surface hydrophobicity. J Appl Microbiol **104**(6): 1552-1561.

Djordjevic, D., M. Wiedmann and L. A. McLandsborough (2002). Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl Environ Microbiol **68**(6): 2950-2958.

DuBois, M., K. A. Gilles, J. K. Hamilton, P. Rebers and F. Smith (1956). Colorimetric method for determination of sugars and related substances. Anal Chem **28**: 350-356.

Flemming, H. C. and J. Wingender (2010). The biofilm matrix. Nat Rev Microbiol **8**(9): 623-633.

Garrett, T. R., M. Bhakoo and Z. Zhang (2008). Bacterial adhesion and biofilms on surfaces. Prog Nat Sci **18**(9): 1049-1056.

Gotoh, H., N. Kasaraneni, N. Devineni, S. F. Dallo and T. Weitao (2010). SOS involvement in stress-inducible biofilm formation. Biofouling **26**(5): 603-611.

Gottschalk, S., I. Bygebjerg-Hove, M. Bonde, P. K. Nielsen, T. H. Nguyen, A. Gravesen and B. H. Kallipolitis (2008). The two-component system CesRK controls the transcriptional induction of cell envelope-related genes in *Listeria monocytogenes* in response to cell wall-acting antibiotics. J Bacteriol **190**(13): 4772-4776.

Groisman, E. A, M. J. Cromie, Y. Shi and T. Latifi (2006). A Mg^{2+}-responding RNA that controls the expression of a Mg^{2+} transporter. Cold Spring Harb Symp Quant Biol **71**: 251-258.

Groisman, E. A., K. Hollands, M. A. Kriner, E. J. Lee, S. Y. Park and M. H. Pontes (2013). Bacterial Mg^{2+} homeostasis, transport, and virulence. Annu Rev Genet **47**: 625-646.

Guilbaud, M., P. Piveteau, M. Desvaux, S. Brisse and R. Briandet (2015). Exploring the diversity of Listeria monocytogenes biofilm architecture by high-throughput confocal laser scanning microscopy and the predominance of the honeycomb-like morphotype. Appl Environ Microbiol **81**(5): 1813-1819.

Harmsen, M., M. Lappann, S. Knochel and S. Molin (2010). Role of extracellular DNA during biofilm formation by *Listeria monocytogenes*. Appl Environ Microbiol **76**(7): 2271-2279.

Heydorn, A., A. T. Nielsen, M. Hentzer, C. Sternberg, M. Givskov, B. K. Ersboll and S. Molin (2000). Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology **146 (Pt 10)**: 2395-2407.

Jang, H. J., Y. J. Choi, H. M. Ro and J. O. Ka (2012). Effects of phosphate addition on biofilm bacterial communities and water quality in annular reactors equipped with stainless steel and ductile cast iron pipes. J Microbiol **50**(1): 17-28.

Kadam, S. R., H. M. den Besten, S. van der Veen, M. H. Zwietering, R. Moezelaar and T. Abee (2013). Diversity assessment of *Listeria monocytogenes* biofilm formation: impact of growth condition, serotype and strain origin. Int J Food Microbiol **165**(3): 259-264.

Kallipolitis, B. H., H. Ingmer, C. G. Gahan, C. Hill and L. Søgaard-Andersen (2003). CesRK, a two-component signal transduction system in *Listeria monocytogenes*, responds to the presence of cell wall-acting antibiotics and affects β-lactam resistance. Antimicrob Agents Chemother **47**(11): 3421-3429.

Kang, J., M. Wiedmann, K. J. Boor and T. M. Bergholz (2015). VirR-mediated resistance of *Listeria monocytogenes* against food antimicrobials and cross-protection induced by exposure to organic acid salts. Appl Environ Microbiol **81**(13): 4553-4562.

Koban, I., R. Matthes, N. O. Hubner, A. Welk, R. Sietmann, J. Lademann, A. Kramer and T. Kocher (2012). XTT assay of ex vivo saliva biofilms to test antimicrobial influences. GMS Krankenhhyg Interdiszip **7**(1): Doc06.

Landini, P. (2009). Cross-talk mechanisms in biofilm formation and responses to environmental and physiological stress in *Escherichia coli*. Res Microbiol **160**(4): 259-266.

Leduc, M., R. Kasra and J. van Heijenoort (1982). Induction and control of the autolytic system of *Escherichia coli*. J Bacteriol **152**(1): 26-34.

Leypold, C. F., D. T. Marian, C. Roman, S. Schneider, P. Schubert, O. Scholz, W. Hillen, T. Clark and H. Lanig (2004). How does $Mg^{(2+)}$ affect the binding of anhydrotetracycline in the TetR protein? Photochem Photobiol Sci **3**(1): 109-119.

Liao, S., M. I. Klein, K. P. Heim, Y. Fan, J. P. Bitoun, S. J. Ahn, R. A. Burne, H. Koo, L. J. Brady and Z. T. Wen (2014). *Streptococcus mutans* extracellular DNA is upregulated during growth in biofilms, actively released via membrane vesicles, and influenced by components of the protein secretion machinery. J Bacteriol **196**(13): 2355-2366.

Machata, S., T. Hain, M. Rohde and T. Chakraborty (2005). Simultaneous deficiency of both MurA and p60 proteins generates a rough phenotype in *Listeria monocytogenes*. J Bacteriol **187**(24): 8385-8394.

Mulcahy, H. and S. Lewenza (2011). Magnesium limitation is an environmental trigger of the *Pseudomonas aeruginosa* biofilm lifestyle. PLoS One **6**(8): e23307.

Nowak J., Cruz C., Visnovsky S., Palmer J., Fletcher G., Pitman A., Flint S. (n.d.). Biofilm formation of *Listeria monocytogenes* 15G01, a persistent isolate from a seafood-processing plant in New Zealand, is influenced by inactivation of multiple genes belonging to different functional groups. (in advanced preparation for submission)

Nielsen, P. K., A. Z. Andersen, M. Mols, S. van der Veen, T. Abee and B. H. Kallipolitis (2012). Genome-wide transcriptional profiling of the cell envelope stress response and the role of LisRK and CesRK in *Listeria monocytogenes*. Microbiology **158**(Pt 4): 963-974.

Ochiai, Y., F. Yamada, M. Mochizuki, T. Takano, R. Hondo and F. Ueda (2014). Biofilm formation under different temperature conditions by a single genotype of persistent *Listeria monocytogenes* strains. J Food Prot **77**(1): 133-140.

Oknin, H., D. Steinberg and M. Shemesh (2015). Magnesium ions mitigate biofilm formation of *Bacillus* species via downregulation of matrix genes expression. Front Microbiol **6**: 907.

Okshevsky, M. and R. L. Meyer (2015). The role of extracellular DNA in the establishment, maintenance and perpetuation of bacterial biofilms. Crit Rev Microbiol **41**(3): 341-352.

Paradies, G., V. Paradies, F. M. Ruggiero and G. Petrosillo (2014). Oxidative stress, cardiolipin and mitochondrial dysfunction in nonalcoholic fatty liver disease. World J Gastroenterol **20**(39): 14205-14218.

Poimenidou, S. V., M. Chrysadakou, A. Tzakoniati, V. C. Bikouli, G. J. Nychas and P. N. Skandamis (2016). Variability of *Listeria monocytogenes* strains in biofilm formation on stainless steel and polystyrene materials and resistance to peracetic acid and quaternary ammonium compounds. Int J Food Microbiol **237**: 164-171.

Qin, Z., Y. Ou, L. Yang, Y. Zhu, T. Tolker-Nielsen, S. Molin and D. Qu (2007). Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. Microbiology **153**(Pt 7): 2083-2092.

Robinson, J. B., O. H. Tuovinen and W. D. Bauer (1992). Role of divalent cations in the subunit associations of complex flagella from *Rhizobium meliloti*. J Bacteriol **174**(12): 3896-3902.

Rocourt, J., C. Jacquet and A. Reilly (2000). Epidemiology of human listeriosis and seafoods. Int J Food Microbiol **62**(3): 197-209.

Scholz, O., P. Schubert, M. Kintrup and W. Hillen (2000). Tet repressor induction without Mg^{2+}. Biochemistry **39**(35): 10914-10920.

Somerton, B., D. Lindsay, J. Palmer, J. Brooks and S. Flint (2015). Changes in sodium, calcium, and magnesium ion concentrations that inhibit *Geobacillus* biofilms have no effect on *Anoxybacillus flavithermus* biofilms. Appl Environ Microbiol **81**(15): 5115-5122.

Song, B. and L. G. Leff (2006). Influence of magnesium ions on biofilm formation by *Pseudomonas fluorescens*. Microbiol Res **161**(4): 355-361.

Srey, S., I. K. Jahid and S.-D. Ha (2013). Biofilm formation in food industries: A food safety concern. Food Control **31**(2): 572-585.

Subramani, S., H. Perdreau-Dahl and J. P. Morth (2016). The magnesium transporter A is activated by cardiolipin and is highly sensitive to free magnesium in vitro. Elife **5**.

van der Veen, S., S. van Schalkwijk, D. Molenaar, W. M. de Vos, T. Abee and M. H. Wells-Bennik (2010). The SOS response of *Listeria monocytogenes* is involved in stress resistance and mutagenesis. Microbiology **156**(Pt 2): 374-384.

Vorregaard, M. (2008). Comstat2-a modern 3D image analysis environment for biofilms, Technical University of Denmark, DTU, DK-2800 Kgs. Lyngby, Denmark.

Werten, S., D. Dalm, G. J. Palm, C. C. Grimm and W. Hinrichs (2014). Tetracycline repressor allostery does not depend on divalent metal recognition. Biochemistry **53**(50): 7990-7998.

Wu, J. and C. Xi (2009). Evaluation of different methods for extracting extracellular DNA from the biofilm matrix. Appl Environ Microbiol **75**(16): 5390-5395.

DRC 16

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Jessika Nowak

Name/Title of Principal Supervisor: Steve Flint

Name of Published Research Output and full reference:

Nowak, J., Visnovsky, S., Cruz, C., Palmer, J., Fletcher, G., Pitman, A. & Flint, S. (2017). The ΔmgtB mutant of the persistent Listeria monocytogenes 15G01 strain produces a unique biofilm sandwich structure which changes its phenotype upon magnesium addition. (to be submitted).

In which Chapter is the Published Work: Chapter 6

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate carried out the laboratory work and the analysis and prepared the manuscript with input in guidance of direction and editorial help from the co-authors and supervisors.

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Chapter 7

Overall Summary and Outlook

7.1 Introduction

Listeria monocytogenes is a food borne pathogen that may cause listeriosis in immunocompromised people (Rocourt et al. 2000). Listeriosis is a severe bacterial infection which can lead to death in up to 30% of the cases (Rocourt et al. 2000). In 2015, 26 cases of listeriosis were reported in New Zealand (The Institute of Environmental Science and Research Ltd. 2016). Although it is a small number in comparison to other food-related illnesses, *L. monocytogenes* is a major concern for the New Zealand food export industry as some countries have a zero-tolerance policy (US Food and Drug Administration 2015) for *L. monocytogenes* in food. The seafood industry and especially aquaculture, is a growing industry with increasing value for the New Zealand economy (Seafood New Zealand n.d.) and most of the produce is exported to countries such as China, Japan, Australia and the USA. Biofilms and persistence are thought to be the major reasons for food contamination with *L. monocytogenes*.

The literature review highlights the risks of *L. monocytogenes* and biofilm formation for the seafood industry and summarises genes that are associated with biofilm formation of *L. monocytogenes*. So far, several genes with different functions have been found to influence biofilm formation, however, there is still lack of knowledge about its mechanism. To investigate the major factors contributing to persistence and biofilm formation, this study focused on a selection of sporadic and persistent strains obtained from mussel-processing facilities. *L. monocytogenes* 15G01, a persistent strain, which showed great biofilm formation in a nutrient-rich medium using a simple monoculture assay, was selected for detailed studies. A transposon library was screened and five selected mutants, including two high and three low biofilm formers, were subjected to phenotypic assays to identify the genes' involvement in biofilm formation.

7.2 Highlights

- Biofilm formation was greater for persistent strains compared to sporadic strains
- Biofilm formation increased with extended incubation time in nutrient-rich media (up to 48 h), but reached its maximum after 24 h in defined minimal media
- Three different assays for biofilm determination (crystal-violet assay, cell enumeration and XTT assay) correlated highly with each other supporting the reliability of the crystal violet assay

- Transposon insertions in 27 genes and two intergenic regions resulted in altered biofilm formation
- Two novel genes involved in biofilm formation of *L. monocytogenes* were identified: *clsA* and *mgtB*
- SEM analysis showed two different types of biofilm for 34F11 and 44D3: single cells attached by thread-like structures and cells embedded in extracellular polymeric substance
- Gene disruption of *mltD* and *uvrB* resulted in elongated cells and coccoid-shaped cells respectively
- Confocal analysis revealed a unique sandwich structure for the biofilm of 44D3 (*mgtB* mutant)
- Magnesium reduced biofilm formation for 39G5 at 37°C

7.3 Summary of performed studies

7.3.1 Differences between sporadic and persistent *L. monocytogenes* strains isolated from mussel-processing plants

Through studying a selection of 16 *L. monocytogenes* strains, including eight persistent and eight sporadic isolates for differences in phenotypic behaviour that might help the bacteria to persist and contaminate mussels in the seafood-processing industry, attributes that favour persistence were identified. Biofilm formation measured by cell enumeration was significantly greater for persistent strains compared with sporadic strains after 48 h incubation at 30°C, although differences were small (average of 6.68 $\log_{10}$ CFU/well compared to 6.33; $p = 0.031$). Reduced temperature and incubation time did not result in any significant differences.

It is known that biofilm formation protects cells from environmental threats and this impedes the control and/or eradication of the cells. This study showed that persistence is linked to biofilm formation. Whether biofilm formation causes persistence or whether persistence causes enhanced biofilm formation is yet to be determined. Biofilm formation alone is unlikely to account for persistence, but might be a contributing factor. Regardless, biofilm formation and persistence are probably the main reasons for the problem of *L. monocytogenes* in food-processing plants.

Heat treatment for 5 min at 58°C did not show a significant difference between persistent and sporadic strains, but two of the three most heat-resistant strains were persistent as determined

by plate counts. Interestingly the three most heat-resistant strains had not been directly exposed to heat in the process line. Therefore, heat resistance is independent of the site of isolation.

By evaluating the ability of planktonic and biofilm cells to survive on dry surfaces this study hoped to identify traits that favour persistence. The cell numbers recovered decreased with increased storage time. Biofilm cells survived better than the planktonic cells for persistent isolates after 7 d and 14 d, but no difference was observed for the biofilm and planktonic cells of the sporadic isolates. Biofilm cells showed a steady decline, whereas planktonic had a bigger drop on day 5 and declined faster overall. It is probable that not just one factor alone causes persistence but rather a combination of events.

As persistence was found to be linked to biofilm formation, the *L. monocytogenes* isolate 15G01 was selected for further analysis. Of the 16 isolates, 15G01 showed the greatest biofilm formation in the crystal violet assays at both temperatures tested. Even though cell numbers remained the same at 30°C after 24 and 48 h, the absorbance measured in the crystal violet assay continued to increase, indicating production of an extracellular matrix. Furthermore, this strain was one of the three strains exhibiting high heat resistance.

7.3.2 Optimum conditions for biofilm formation of *L. monocytogenes* 15G01

Biofilm formation of *L. monocytogenes* 15G01 was tested under different environmental conditions to determine the optimum conditions for a transposon mutant library screen. Growth and biofilm formation were investigated at three different temperatures (20°C, 30°C, 37°C) in seven different media, including nutrient-rich, nutrient-deficient and minimal media. Lack of biofilm formation in nutrient-deficient media seemed to be due to poor growth. Nutrient-rich media favoured growth and biofilm formation at 37°C, however, greatest biofilm formation at 30°C was formed in MWB when incubated dynamically rather than statically or anaerobically.

In conclusion, it was found that biofilm formation of *L. monocytogenes* depends on environmental factors with growth medium (nutrients) and temperature having the biggest influence. When grown in nutrient-rich media, the amount of biofilm formed increased with increasing incubation time at 30°C and 37°C. However, when grown in minimal medium, biofilm mass decreased after about 24 h. This highlights the importance of nutrient availability for biofilm formation. When nutrients are exhausted, the biofilm may start to disperse, releasing cells to contaminate new surfaces. In the food-processing environment, nutrient availability is

quite likely scarce, so if strains persist and manage to establish as a biofilm, the risk of food contamination could be higher due to dispersal taking place earlier.

Studying biofilm formation remains a challenge as no universal mechanism but rather a cascade of factors is involved.

7.3.3 Identification of genes that are associated with biofilm formation by screening a mutant library of the persistent strain *L. monocytogenes* 15G01

A transposon mutant library of *L. monocytogenes* 15G01 was screened for altered biofilm formation using the crystal violet assay.

Thirty-six mutants were selected for the identification of the transposon insertion site. Insertions were detected in 27 genes, with some genes hit multiple times and two insertions in intergenic regions. The biofilm-associated genes were found across multiple functional groups across the genome.

Four mutants, including one high biofilm former (34F11) and three low biofilm formers (33E11, 39G5 and 44D3), were selected for SEM and epifluorescence microscopy. Criteria for selecting these genes were that some of them have not been associated with biofilm formation in *L. monocytogenes* before (*clsA*, *mgtB*) or have not been extensively studied (*uvrB* and *mltD*). SEM images showed two different types of biofilm: single cells held together by thread-like structures and cells embedded in extracellular polymeric substance. Changes in the phenotype due to gene disruption were observed for 33E11 and 39G5 with coccoid-shaped cells and elongated cells, respectively. The microscopy analysis confirmed the results observed with the crystal violet assay used for the initial screen, confirming the crystal violet assay as a reliable and economical way to screen large numbers of strains despite its drawbacks.

All four mutants were complemented with the wild-type gene, however, only one of the four mutants showed full restoration (39G5-C) of the biofilm phenotype and one partial restoration (33E11-C). Complementation was carried out with the plasmid (pIMK), which induces the insertion of the introduced gene at the $tRNA^{Arg}$ locus of the *L. monocytogenes* genome. The partial restoration and the inability to restore the other two mutants' biofilm phenotype may be due to differential expression of the genes upon site-specific integration or due to altered regulatory mechanisms. Other possibilities include changes in enzyme levels as well as protein folding or even polar effects upon insertion of the transposon. However, the two genes, whose function could not be complemented, were hit multiple times in the screen and the transposon insertions were identified to be at different spots. In addition, mutants with the insertion in the

same gene showed the same phenotype, further suggesting the involvement of the particular gene in biofilm formation. Although phenotypic restoration could not be demonstrated after complementation, the above-mentioned reasons support the genes' involvement in biofilm formation.

7.3.4 The involvement of the selected genes in biofilm formation

L. monocytogenes 15G01 and five selected mutants (6B4, 34F11, 33E11, 39G5 and 44D3) were subject to three different assays for biofilm formation. The crystal violet assay was used to determine the whole biofilm mass produced, including live and dead cells and the matrix, the number of viable cells of a biofilm was determined by plating; and the metabolic activity was tested with the XTT assay. All three assays showed a significant correlation ($p \leq 0.05$), which again confirms the reliability of the crystal violet assay. Although it has been subject to criticism (Lourenco et al. 2012, Pantanella et al. 2013) as biofilm can only be measured indirectly, it remains a valuable tool to study large numbers of strains. In general, researchers should not rely on one method only – it is always advisable to add at least another method for confirmation. To reinforce the findings of the crystal violet assay, the two other mentioned assays were used, which identified viable cells (plate counts) and metabolically active cells (XTT assay) in the biofilms. Cell surface characteristics were measured by combining three different assays (MATH test, hydrophobic interaction chromatography (HIC) and cytochrome C assay). No correlation with biofilm formation was observed by any of the surface tests. Having performed surface analysis with three different assays strengthens the conclusion that biofilm formation is, in this study, not related to cell surface characteristics.

Moreover, attachment after 30 min could not be linked to cell surface characteristics, although attachment was correlated (0.88; $p \leq 0.05$) with cell enumeration.

The influence of cations on biofilm formation was investigated and it was found that magnesium was able to alter biofilm mass of two mutants. 39G5, a low biofilm former, formed less biofilm in presence of 5mM compared to 1.67 mM Mg^{2+} at 37°C, whereas 44D3, another low biofilm former, produced more biofilm mass in presence of Mg^{2+} (5 mM) compared with 1.67 mM Mg^{2+}. When observed through the CLSM, 44D3 formed a sandwich structure with static monolayers at the top and bottom and fluid movement in between under low Mg^{2+} presence. This biofilm structure has not been reported before. In the presence of 5 mM Mg^{2+}, this phenotype was reversed and 44D3 showed a monolayer comparable to the wild-type, suggesting restoration of the original phenotype through Mg^{2+}. Both mutants affected by magnesium formed cell

aggregates when grown in liquid culture. The findings in this study and the close proximity of the two genes on the genome of *L. monocytogenes* suggest a common regulative mechanism triggered by magnesium, which will need further investigation.

By exposing all mutants and the wild-type for 1 h to heat (55°C), acid (pH 2.5) and an oxidative reagent (60 mM H_2O_2), this study aimed to identify a link between stress response and biofilm formation. Unexpectedly, there was no significant difference between 33E11 (stress response gene) and the wild-type. However, 34F11, the high biofilm former, showed increased sensitivity to oxidative reagents. The cause and mechanism for increased oxidiser sensitivity is unknown.

7.4 Conclusions

There are significant differences in biofilm formation between persistent and sporadic *L. monocytogenes* strains isolated from mussel-processing facilities. Although differences were small, they indicate a relationship between persistence and biofilm formation. The persistent strain 15G01 showed the greatest biofilm formation of all 16 strains tested with increased production of extracellular polymeric substance when incubated for 48 h compared to 24 h.

Temperature and nutrient content have the biggest influence on biofilm formation of *L. monocytogenes* 15G01 compared with other environmental factors.

Two novel genes, *clsA*, a cardiolipin synthetase, and *mgtB*, a Mg^{2+} P-type ATPase, are involved in biofilm formation of *L. monocytogenes*.

A gene disruption of *mgtB* results in a unique biofilm structure producing a sandwich-like biofilm which is restored to the wild-type phenotype upon magnesium addition.

The two mutants with transposon insertions in *mgtB* and *mltD*, a gene encoding for an autolytic enzyme, are both affected by magnesium presence resulting in increased and decreased biofilm production when higher magnesium amounts are present.

A common regulative mechanism triggered by magnesium presence is suspected for both genes due to the observed effects of magnesium and the close proximity of both genes on the *Listeria* genome.

Restricting magnesium availability may be a useful strategy to reduce biofilm formation in food-processing plants.

Calcium reduces biofilm formation of strain 15G01 only at mammalian temperature but increases biofilm formation at 30°C, making this unsuitable for biofilm control.

7.5 Future Work

Based on the findings of this thesis, it is recommended that future research include the following:

- Evaluate the influence of magnesium in different media including mussel juice to identify whether magnesium presence is a medium/nutrient-dependent effect
- Evaluate the influence of increasing magnesium concentration on biofilm formation of 44D3 and the wild-type to identify any further influence on biofilm formation
- Evaluate the influence of magnesium and calcium together on biofilm formation
- Identify the mechanism of biofilm increase for 44D3 in the presence of magnesium by gene expression profile
- Identify the mechanism of biofilm reduction for 39G5 in the presence of magnesium by gene expression profile
- Identify the mechanism and possible co-regulation of biofilm formation for 39G5 and 44D3 in the presence of magnesium by gene expression profile by creating single and double gene deletion mutants
- Identify the regulatory mechanism that triggers 39G5 to produce less biofilm than the wild-type at 30°C and more biofilm at 37°C by gene expression profile
- Identify the attachment and surface characteristics of 39G5 in the presence of Mg^{2+} to determine whether changes in surface charge inhibit biofilm production
- Prepare a gene expression profile of the wild-type and 34F11after exposure to oxidative stress to identify the mechanism of the oxidative stress response
- Identify and compare the membrane composition of the wild-type and 34F11 to help elucidate the mechanism of biofilm formation
- Identify the membrane composition of 34F11 before and after oxidative stress exposure to help elucidate the mechanism of oxidative stress response
- Examine the growth and biofilm formation of the wild-type and the mutants in co-cultures to simulate industrial conditions

7.6 References

Seafood New Zealand (n.d.). Key Facts. Retrieved 16 January 2017 from http://www.seafood.org.nz/industry/key-facts/.

Lourenco, A., F. Rego, L. Brito and J. F. Frank (2012). Evaluation of methods to assess the biofilm-forming ability of *Listeria monocytogenes*. J Food Prot **75**(8): 1411-1417.

Pantanella, F., P. Valenti, T. Natalizi, D. Passeri and F. Berlutti (2013). Analytical techniques to study microbial biofilm on abiotic surfaces: pros and cons of the main techniques currently in use. Ann Ig **25**(1): 31-42.

Rocourt, J., C. Jacquet and A. Reilly (2000). Epidemiology of human listeriosis and seafoods. Int J Food Microbiol **62**(3): 197-209.

The Institute of Environmental Science and Research Ltd. (2016). Notifiable diseases in New Zealand: Annual Report 2015 Porirua, New Zealand. Retrieved from https://surv.esr.cri.nz/PDF_surveillance/AnnualRpt/AnnualSurv/2015/2015AnnualReportFinal.pdf

US Food and Drug Administration (2015). Quantitative assessment of relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eatfoods.Retrieved 16 January 2017 From http://www.fda.gov/Food/FoodScienceResearch/RiskSafetyAssessment/ucm183966.htm.