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The influence of cations on biofilm formation of *Listeria monocytogenes* persistence strains

A thesis presented in partial fulfilment of the requirements for the degree of

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Abstract

Listeria monocytogenes is a Gram-positive pathogen, that possess a considerable risk to the human health with a high mortality rate. The persistence of pathogens through severe environmental conditions could be associated with their biofilm forming abilities. In this study, four different *L. monocytogenes* isolates from the seafood industry, were examined for their biofilm formation ability in the presence of three the cations: magnesium, calcium and sodium that are readily available in the seafood industry. Out of four the two isolates 15G01 and 33H04, were the persistent isolates from different seafood industry in New Zealand. Isolate 15A04 was a low biofilm former and the last isolate 16A01 was associated with a mussel contamination outbreak. The divalent cations, magnesium and calcium had a significantly greater effect on biofilm formation compared to the monovalent cation, sodium, especially at a concentration of 50mM.

To further understand the effect, comparative transcriptomics was used on *L. monocytogenes* isolate 15G01 (a persistent and high biofilm forming isolate) and 15A04 (a low biofilm former). Both the isolates were exposed to 50mM concentrations of magnesium and sodium. In the presence of magnesium, various genes related to the phosphotransferase system, flagellar assembly, chemotaxis and various signal transduction receptors were upregulated. In case of sodium, the results indicated limited effect on gene expression for both the isolates.

As biofilm is a community of bacteria enclosed in a self-induced matrix called EPS (extracellular polymeric substances), understanding the influence of cations on the composition of the EPS and the structural stability of biofilm is important. Magnesium enhanced the polysaccharide content, thus enhancing biofilm formation particularly in 15G01. eDNA concentration increased in the presence of cations however there were no significant

differences among the cations. A unique hexagonal structure with voids were observed for the first time in the presence of magnesium and calcium for isolate 15A04.

These findings contribute insights into the role of cations in biofilm formation, their involvement in regulating the complex network in biofilms and maintaining their structural integrity.

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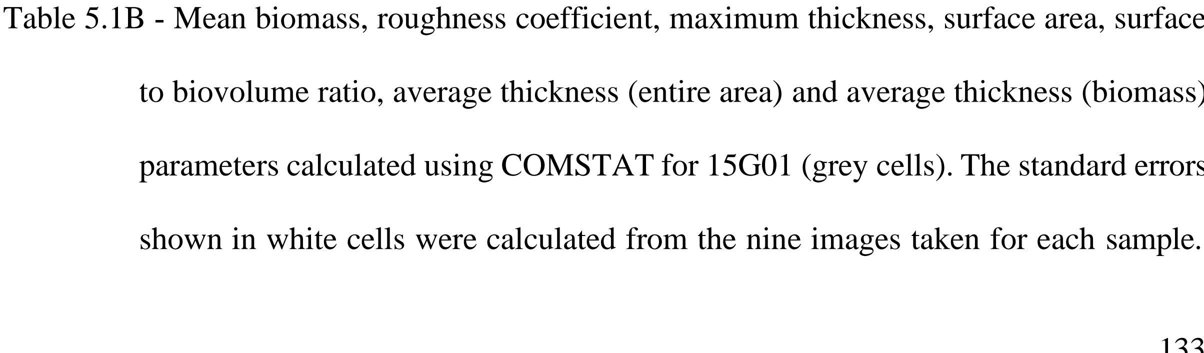

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Chalke, S., Vidovic, S., Fletcher, G. C., Palmer, J., & Flint, S. (2022). Differential effects of magnesium, calcium, and sodium on *Listeria monocytogenes* biofilm formation. *Biofouling*, 38(8), 786–795

Chalke S., Vidovic S., Palmer J., Flint S., Fletcher G.C. (Nov 2022). Understanding the transcriptome profile of *Listeria monocytogenes* biofilms in response to magnesium and sodium. NZMS Conference 2022, Wellington, New Zealand. (Oral presentation)

Chalke S., Vidovic S., Palmer J., Flint S., Fletcher G.C. (July 2021). *Listeria* biofilm in the cationic world – What's happening. NZIFST Conference 2021, Palmerston North, New Zealand (Oral presentation).

Chalke S., Vidovic S., Palmer J., Flint S., Fletcher G.C. (July 2019). Influence of cations on biofilm formation and persistence of *Listeria monocytogenes*. NZFSSRC Annual Symposium 2019, Christchurch, New Zealand (3-minute oral presentation)

Chapter 1: General introduction

Foodborne diseases are a potential threat to public health and the economy. According to World health organization (WHO), worldwide each year foodborne diseases cause 600 million cases and 420,000 deaths (WHO, 2022). *Listeria monocytogenes* is one of the Gram- positive pathogenic bacteria commonly associated with foodborne illness. The pathogen causes a severe bacterial infection known as listeriosis with a high fatality rate in susceptible individuals (Sibanda & Buys, 2022). It has ability to form biofilms, thus possibly leading to persistent contamination in the food industry (Møretrø & Langsrud, 2004). *L. monocytogenes* can contaminate a variety of foods including seafood, meat, vegetables, fruits and ready to eat (RTE) products resulting in product recalls and severe illness in susceptible individuals (Kocot & Olszewska, 2017). The ability to survive and grow in a wide range of conditions and form good biofilm helps the bacterium persist even in harsh conditions.

Biofilm formation is a complex process where the community creates an extracellular matrix to protect the cells from the external environment. The extracellular matrix consists of various components including polysaccharides, eDNA, lipids and proteins. It varies from species to different strains (Colagiorgi, Di Ciccio, Zanardi, Ghidini, & Ianieri, 2016). There are various factors involved in biofilm formation like temperature, pH, nutrients and cations. In the biofilm environment the bacterium becomes more resistant towards external conditions (e.g. high temperature) and disinfectants. Thus, prolonged exposure to these ques might result in persistence in the food processing industry, thereby creating a threat for product contamination and public health.

Biofilm formation by *L. monocytogenes* is a complex process and the exact mechanisms remain unknown. The present study investigates the role of cations in biofilm formation of *L. monocytogenes*.

Sodium, magnesium and calcium are the major cations found in seawater (Riley & Tongudai, 1967). New Zealand exports large amounts of seafood (77,249.1 tonnes in 2023) to various countries like Australia, China, Germany, Japan, Hong Kong, USA (Seafood New Zealand, 2024). As seafood is important for the New Zealand economy, high food safety standards are required to be maintained. Bacteria constantly require cations to maintain their physiology, as they are required for various functions such as regulation of osmotic pressure, cellular pressure and enzymatic functions. Disruption in these might result in bacteriostatic effects, thereby affecting biofilm formation (Niegowski & Eshaghi, 2007).

The first step of this PhD project was to determine the effect of sodium, magnesium and calcium on *L. monocytogenes* isolates from the seafood industry. This included biofilm formation and the planktonic form of *L. monocytogenes* (Chapter 3). The second stage was to understand the effect of cations on gene expression and regulatory pathways that are involved in biofilm formation (Chapter 4). The last stage was studying the effect of cations on the structural integrity of the biofilm (Chapter 6).

1.1 Research questions and hypotheses.

1.1.1 Question

- How do magnesium, calcium and sodium affect the biofilm formation of *L. monocytogenes*? Does it vary between the isolates?
- Is biofilm formation affected by varying cations concentration?
- In presence of certain cations, which specific signalling pathways are upregulated or downregulated?
- Do the cations have any effect on the structural integrity of the biofilm?

- How do the cations affect the composition of extracellular matrix?

1.1.2 **Hypothesis**

- Magnesium, calcium, and sodium each have different effects on *L. monocytogenes* biofilm formation.
- Divalent cations like magnesium and calcium have greater effect compared to monovalent cations (sodium)
- Magnesium and sodium differentially affect signalling pathways involved in biofilm formation, thus demonstrating an exclusive expression profile for each isolate and cation.
- The structural integrity is strongly dependent on the type of cation present and its concentration.

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Chapter 2: Literature review

2.1 *Listeria monocytogenes*

2.1.1 General characteristics

Listeria monocytogenes is a ubiquitous foodborne pathogen that causes listeriosis. It is a Gram-positive, facultative anaerobe, non-spore forming, rod-shaped micro-organism. This bacterium is catalase-positive, oxidase negative, urea negative and indole negative. It has long peritrichous flagella contributing to its characteristic tumbling motility at 20 to 25°C (Jamshidi & Zeinali, 2019; Orsi & Wiedmann, 2016). It has some unique characteristics, like the ability to grow over a wide temperature range (0 to 45°C), with an optimum temperature of 37°C and the ability to survive below -18°C. It can survive over a broad pH range of (4.0 to 9.6), high salt concentration (13–40%), and low water activity (0.97) and is able to form biofilms (Huang, 2004; Osek, Lachtara, & Wieczorek, 2022). In the food industry temperature, pH, salts, oxygen level and water activity are often regulated to control bacterial growth. However. *L. monocytogenes* is highly adaptable and more difficult to control than many pathogens.

2.1.2 Taxonomy and lineages

Listeria monocytogenes belongs to the Firmicutes phylum, Bacilli class, Bacillales order Listeriaceae family and the *Listeria* genus. This family also includes another genus *Bronchothrix*. The *Listeriaceae* family are characterized as Gram-positive bacteria with low G+C-content (Listeriaceae, 2019; Schardt et al., 2017). The genus *L*isteria comprises 23 species and is grouped into two major clades. The first clade, named, as *Listeria* sensu stricto consists of two pathogenic species *L. monocytogenes* and *L. ivanovii* with another four nonpathogenic species *L. innocua*, *L.*

seeligeri, *L. welsimeri*, and *L. marthii* (Redel, 2013).

These species are believed to have mammalian host interactions. The second group, known as *Listeria* sensu lato, are the species that are isolated from food-associated surfaces or environments that are believed to have no mammalian host interaction. This consists of *L. grayi*, *L. rocourtiae*, *L. fleischmannii*, *L. floridensis*, *L. aquatica*, *L. newyorkensis*, *L. cornellensis*, *L. weihenstephanensis*, *L. grandensis*, *L. riparia*, *L. booriae*, *L. costaricensis*, *L. goaensis*, *L. murrayi*, *L. dentrificans*, *L. valentina* and *L. thailandensis*. (Leclercq et al., 2019; Nwaiwu, 2020; Quereda et al., 2020; Schardt et al., 2017).

L. monocytogenes consists of four evolutionary lineages namely, I, II, III and IV, of which lineages I and II are most common (Orsi, den Bakker, & Wiedmann, 2011). Within these four lineages, there are 13 serotypes based on the somatic (O) antigen and flagellar antigen (H) by which the strains are classified. Of these, serotypes 1/2a, 1/2b and 4b are the three most common serotypes that are recognized from the strains involved in human disease. However, a bias is observed within the serovars causing human listeriosis with 64% of serovar 4b, 15% of serovar 1/2a and 10% of serovar 1/2b involved in listeriosis cases (Ferreira, Wiedmann, Teixeira, & Stasiewicz, 2014).

2.1.3 Pathogenicity and epidemiology of *L. monocytogenes*

The bacterium *L. monocytogenes* causes listeriosis in humans, mainly affecting infants, immunosuppressed individuals, the elderly and pregnant women, however, it can also be a threat to healthy individuals (Rocourt, Jacquet, & Reilly, 2000). The most common source of *L. monocytogenes* is through food, however other possible routes of infection are direct contact with infected animals or contaminated environments. Listeriosis causes significant localised and systemic infections, notably in immunocompromised individual with a hospitalisation rate of up to 94% and a high mortality rate of 30% (Rocourt et al., 2000; Scallan et al., 2011). Though it’s difficult to measure the infective dose, it is estimated that 10^4 to 10^7 bacteria can cause illness in

a susceptible individual and more than 10^7 bacteria are required in a healthy person (Osek et al., 2022). On consumption of the contaminated food by the host, *L. monocytogenes* encounters the intestinal epithelium barrier and crosses into the lamina propria. Upon entering the lamina propria it disseminates through the lymph and blood to its target organs like the liver and spleen. It has the ability to cross the blood- brain barrier or fetoplacental barrier in pregnant women (Radoshevich & Cossart, 2018). Figure 1 illustrates this transfer. The most common clinical manifestations observed are the central nervous system (CNS) infections like meningitis as well as sepsis, endocarditis, and gastroenteritis. Pregnant women are ten times more likely to be infected than other groups in the population. *L. monocytogenes* can cause serious illness, miscarriage, stillbirths and preterm labour (Listeriosis, 2016).

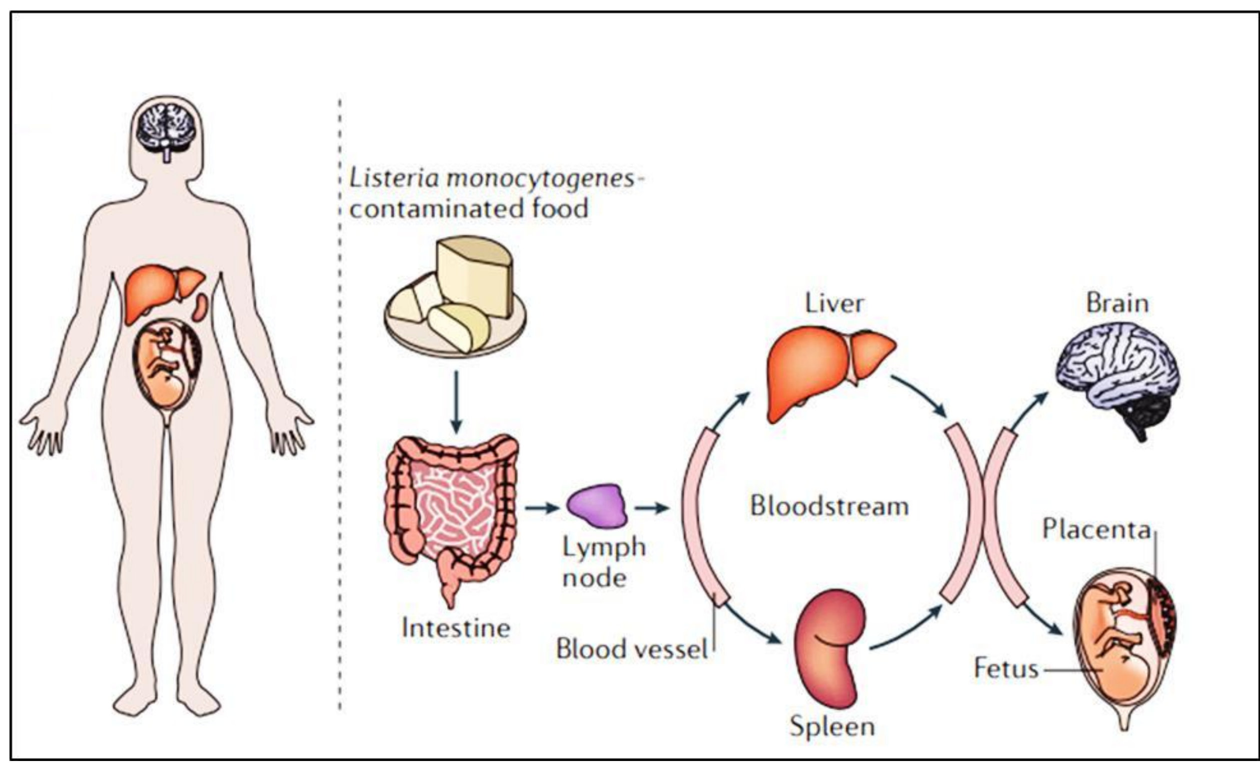

Figure 2.1 Schematic of *Listeria monocytogenes* infection in a human host. Figure adapted from Radoshevich and Cossart (2018).

In the USA, listeriosis is the third leading cause for the foodborne associated death (Orsi &

Wiedmann, 2016). In New Zealand, according to the Annual report concerning Foodborne Diseases in New Zealand 2022, listeriosis is reported at 0.8 cases per 100,000. Among the cases, the hospitalization rate was 95% with 6 deaths (Horn et al., 2023). Figure 2 shows the listeriosis notifications in New Zealand for each year since 2003 for both perinatal and non- perinatal cases.

Figure 2.2 - Listeriosis notification in New Zealand since 2003 (both perinatal and non- perinatal). Figure adapted from Horn et al. (2023)

Most of the listeriosis cases appear to be sporadic and are highly associated with contaminated food as a primary source of infection. The main food types responsible for illness are RTE (Ready to eat) foodstuffs like smoked seafood, deli meats, salads, soft cheese, and long shelf-life refrigerated food. In 2021, the European Union reported 2183 cases with 923 hospitalisations and 196 deaths due to listeriosis (EFSA & ECDC,2022). It was reported as the 5th most common zoonosis. According to the Centers for Disease Control and Prevention, it is estimated 1600 cases of listeriosis, and 260 deaths occur in the USA

annually (CDC, 2021).

2.1.4 Regulation to control *L. monocytogenes*

To control the prevalence of *L. monocytogenes,* stringent regulations are applied. In New Zealand, the Ministry of Agriculture and Forestry (MAF) launched a *L. monocytogenes* Risk Management Strategy 2008-2013 with the objective of "no increase in the reported incidence of foodborne listeriosis after five years". In New Zealand, all food industry operators have either a Risk management program (RMP) or follow the Food Act 2014 with all producers required to comply with the Microbiological Limits for Food contained in Standard 1.6.1 of the Australia New Zealand Food Standards Code (FSC). According to the FSC standard 1.6.1, the limit is 100 (colony forming units) cfu/g for *L. monocytogenes* in RTE foods in which growth will not occur, and the absence of *L. monocytogenes* in 25g of RTE food in which growth can occur (FSANZ, 2019). In the United States of America, for all RTE food, they have a 'zero-tolerance' policy for *L. monocytogenes* (FDA, 2003). According to the European Commission (2005), three main categories for RTE food were established for *L. monocytogenes*, as detailed in Table 2.1.

Food category	**Limits**	**Analytical reference methods**	**Stage where the criterion applies**
Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes	Absence in 25 g	EN/ISO 11290-1	Products placed on the market during their shelf-life
Ready-to-eat foods able to support the growth of *L. monocytogenes,* other than those intended for infants and for special medical purposes	100 cfu/g	EN/ISO 11290-2	Products placed on the market during their shelf-life
	Absence in 25 g	EN/ISO 11290-1	Before the food has left the immediate control of the food business operator, who has produced it
Ready-to-eat foods unable to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes	100 cfu/g	EN/ISO 11290-2	Products placed on the market during their shelf-life

Table 2.1 Food safety criteria for *Listeria monocytogenes* adapted from European Commission, 2005.

2.1.5 L. monocytogenes in the seafood industry

Seafood is an excellent source of omega-3 fatty acids, proteins, vitamin D, iodine and selenium thereby providing essential health benefits required for the brain, vision and cognitive development (Elbashir et al., 2018; M. D. Smith et al., 2010). According to the Food and Agriculture Organization (FAO), global fish production increased on average by 3.1% over a period of 56 years (1961-2017). In the year 2018, a total of USD 401 billion was the value of fisheries and aquaculture production globally. Fish consumption has increased from 9 kg to 20.5 kg per capita from 1961 to 2017 (FAO, 2020). In New Zealand, every year around 600,000 tonnes of seafood are harvested, with $924 million earnings in seafood exports in 2022 (Seafood New Zealand, 2022). New Zealand's marine fisheries waters rank the 4th largest exclusive economic zone in the world with territorial seas comprising 4.4 m km^2 (Seafood New Zealand, 2020).

Many seafood products like processed cold smoked or cold salted fish or oysters and mussels are consumed raw or undercooked. Therefore, it is essential to maintain hygiene and frequently test for microbial pathogens in processing plants. The condition in which fish are packed (moderate salt concentrations, vacuum packaging, and cold storage) may favour the growth of *L. monocytogenes*. There are numerous studies in the literature regarding the prevalence of *L. monocytogenes* in seafood and seafood processing plants. Miettinen, Aarnisalo, Salo, and Sjoberg (2001), examined surface contamination for *Listeria* species in a fish processing factory in Finland. It was found that 12% of the product and raw fish together and 20% of cold salted fish were contaminated with *L. monocytogenes*. Another study conducted in Iceland on 128 samples of raw fish, smoked fish, dried fish, frozen shellfish, frozen shrimps and fish salads observed 46% of samples showing presumptive positive results for *L. monocytogenes* (Hartemink & Georgsson, 1991). A group of researchers in the Republic of Ireland conducted a study to detect the occurrence of *L.*

monocytogenes in eight different seafood processing plants over two years. They found a 2.5% prevalence in the environmental samples and 3.8% in the food samples. All the isolates they found were serotype 1/2a except for one which was serotype 4b/4e. During this study, using pulsed field gel electrophoresis, they found eight pulsotypes, of which T4 was common to four independent processing units. However, it was not regarded as persistent as it was not isolated over an interval of more than 6 months (Leong, Alvarez-Ordonez, Zaouali, & Jordan, 2015). In New Zealand, a survey on RTE hot and cold salmon for *L. monocytogenes* at the retail outlets found 8 out of 1212 samples positive for *L. monocytogenes* of which 1.3% were cold smoked salmon. From the eight samples, three of the samples exceed the 100cfu/g microbiological limits according to the FSC standard 1.6.1. (MPI, 2011). Shineman and Harrison (1994) demonstrated growth of *L. monocytogenes* was faster on raw and cooked catfish and the shrimp than on beef and chicken when stored at 4°C for 11 days. This difference was due to the inherent pH of the fresh tissues which, in the case of shrimp, was 7.6, while that of beef was 5.7. Embarek (1994), in his review, mentioned a 1.5-2% prevalence of *L. monocytogenes* in raw and processed cooked shrimps. These results agree with the results from other groups like Motes (1991); Rahimi, Shakerian, and Raissy (2012). In 2018, a group of researchers screened different seafood contact surfaces including a fishing harbour, landing centre, processing plant, fish market and fish curing yards of the Tuticorin Coast of India. They found a high incidence of *L. monocytogenes* (10.3%) at the fishing harbour followed by 5.9% on landing centres and 4.1% in processing plants (Selvaganapathi et al., 2018). In January 2022, there was an outbreak linked to smoked fish contaminated with *L. monocytogenes* where six people from England and Scotland were infected (Food safety news, 2022). This suggests that *L. monocytogenes* not only poses a health risk to the public but also a risk to the economy.

2.2 Biofilm

Bacteria often do not exist as plankton in the natural environment but grow and persist in an organized community known as biofilms. Biofilms can be defined as a group of microorganisms growing on a solid surface or as suspended aggregates, enclosed in an extracellular matrix, creating a dynamic environment which allows them to reach homeostasis by actively using the available nutrients. Depending on the species, the cells in the biofilm may have different growth rates, and cellular morphology. They may form a monolayer while others may form a multilayer three-dimensional structure. They may also have different physiological responses to the environment. A study of the diffusion of gases and liquids through a biofilm matrix has shown oxygen and nutrient availability is limited in a biofilm, compared with bacteria in planktonic form. This results in bacteria altering their physiology in the biofilm environment resulting in increased resistance to sanitizers enabling them to persist in the food industry. This ability to resist antimicrobials makes it's difficult to eradicate biofilms from the food industry, and they act as a source of contamination, and a risk to public health (Rodríguez-Lozano & McLandsborough, 2009).

The development of biofilm takes place in five steps (Figure 2.3) – (i) the initial attachment of newly adherent cells on the surface. In this step, the cells are loosely attached and can be readily detached from the surface. This stage is called reversible attachment. The initial attachment depends on various factors like bacterial motility, the gravitational force of the planktonic cells, shear forces exerted by the surrounding liquid, physiochemical properties of the bacterium, surface properties of the bacteria and substrate, pH and temperature (Srey, Jahid, & Ha, 2013). The bacterium undergoes various morphological changes that lead to the next step: (ii) irreversible attachment. Pre-existing extracellular polymeric substances (EPS) or films consisting of macromolecules and organic substances enhance the attachment of the bacteria. The shift from reversible to irreversible attachment involves a change from weak interactions between the bacteria and the surface to strong and permeant bonding of the cell with the surface. Depending on

the species this involves many steps. For example, in *Pseudomonas aeruginosa*, this shift is mediated by type IV pilli followed by microcolonization where a group of cells forms and provides strength to attach to the surface (Stoodley, Sauer, Davies, & Costerton, 2002). This is followed by the next step (iii), early development of biofilm structure, which involves the production of EPS. EPS helps to strengthen the bonds between the bacteria and protects the cells from the external environment. EPS is composed of extracellular polysaccharides, proteins and eDNA (Rabin et al., 2015). The next step in biofilm formation (iv), is the maturation of the biofilm structure. This results in complex architectures that can vary from flat to mushroom-shaped structures with pores and channels. This is followed by bacterial redistribution (shedding or bio transfer potential) of some of the bacteria away from the substratum which is the final stage (v). This final stage may be initiated by starvation, reduction in EPS, cells reaching stationary phase, increased fluid shear or internal enzymatic degradation. This stage reverts the shed biofilm cells back again to the planktonic mode and thus the cycle repeats (Armbruster & Parsek, 2018; Rabin et al., 2015; Srey et al., 2013; Stoodley et al., 2002).

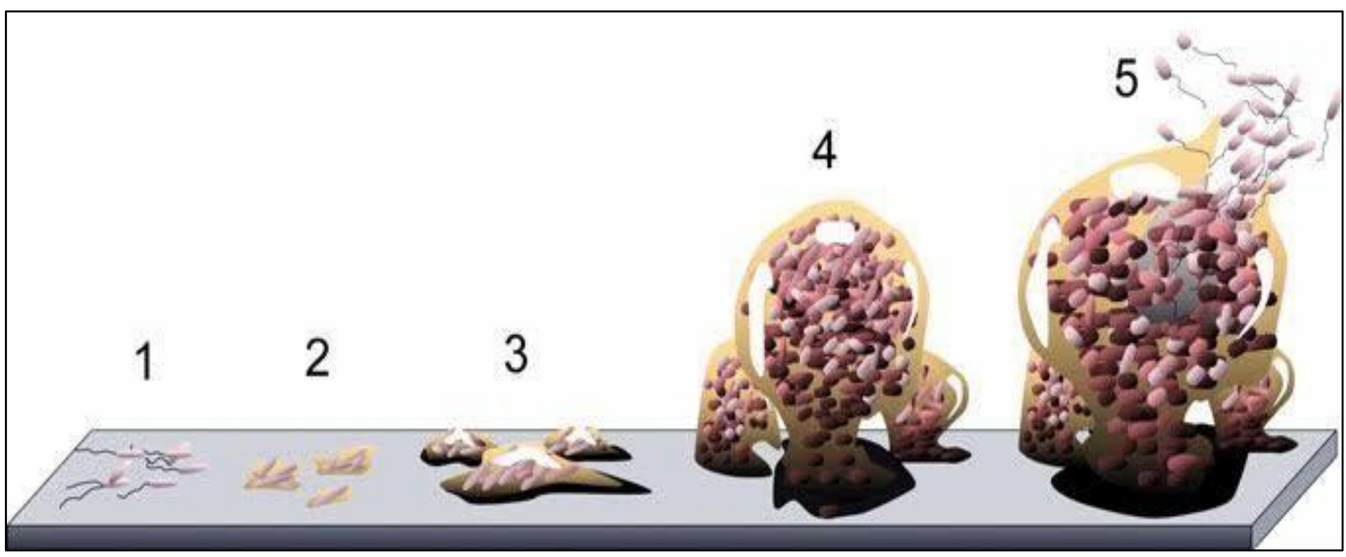

Figure 2.3- The five stages of biofilm development. Stage 1: Planktonic (free-floating) bacteria adhere to the biomaterial surface. Stage 2: Cells aggregate, form microcolonies and excrete extracellular polymeric substances (EPS), i.e., slime. The attachment becomes irreversible. Stage 3: A biofilm is formed. It matures and cells form multi-layered clusters. Stage 4: Three-dimensional growth and further maturation of the biofilm, providing protection against host defence mechanisms and antibiotics. Stage 5: The biofilm reaches a critical mass and disperses planktonic bacteria, ready to colonize other surfaces. Figure adapted from Monroe (2007).

2.3 Biofilm formation of *L. monocytogenes*

As *L. monocytogenes* is ubiquitous in nature and is known to transmit through various foods like seafood, meat, dairy, vegetables, fruits, and cheese, ready to eat products, ice-cream and poultry (CDC, 2019). Most surfaces in the food industry are able to support *Listeria sp*. biofilms. There are many intrinsic and extrinsic factors that affect *L. monocytogenes* biofilm formation. Many reports have tried to correlate serotype, lineage to biofilm formation but this remains a controversial topic. A study suggested that serotype 1/2c strains adhere strongly when compared with 4b and 1/2a (Norwood & Gilmour, 1999). While Kadam et al. (2013), found that 1/2a and 1/2b form better biofilm than 4b in nutrient-rich media and 1/2b is better than 1/2a and 4b in nutrient-poor media. Pan, Breidt, and Kathariou (2009), reported that serotype 1/2a was better than 4b and dominated in mixed biofilm. This difference might be due to different regulation of the biofilm phenotype. In contrast some authors like Doijad et al. (2015) and Lee et al. (2017), demonstrated no relationship between the serotypes and pulsotype in the biofilm-forming ability of *L. monocytogenes*. Lee et al. (2017), reported high variability among strains, suggesting that the strong biofilm-forming ability is not linked with its persistent nature. In brief, *L. monocytogenes* strains may have diverse responses during biofilm formation, however, further research is required to study the mechanism involved.

Other common factors are temperature and nutrient availability. Di Bonaventura et al. (2008) and Kadam et al. (2013) reported that in *L. monocytogenes*, biofilm formation increases with an increase in the temperature (enhanced biofilm formation at 37°C compared to biofilm formation at lower temperatures like 4°C or 20°C) and increases in minimal media rather than rich media.

Various types of surfaces like stainless steel, Teflon, polypropylene, polystyrene, glass, biotic surfaces and PVC (Polyvinyl Chloride) are widely used in food industries. *L.monocytogenes* is capable of forming a biofilm on a wide range of surfaces, however, variation in the surface

composition may influence the rate and extent of biofilm growth (Mafu, Roy, Goulet, & Magny, 1990). Palmer, Flint, and Brooks (2007) identified several factors (surface conditioning and charge, mass transfer, hydrophobicity, surface microtopography and roughness) involved in the initial attachment of the bacterial cell to a surface. Cell hydrophobicity increases with temperature and adhesion increases with an increase in the hydrophobicity of the cell surface (Di Bonaventura et al., 2008; Van Loosdrecht, Norde, & Zehnder, 1990). Varied results have been reported in the literature. For example, a number of researchers, Bonsaglia et al. (2014); Chavant, Martinie, Meylheuc, Bellon-Fontaine, and Hebraud (2002); and Russo et al. (2018), found that *L. monocytogenes* adheres better to hydrophilic surfaces like glass and stainless steel, rather than hydrophobic surfaces such as polystyrene. In contrast, other researchers, Djordjevic et al. (2002), Midelet &Carpentier (2002) and Takahashi et al.(2010), found higher cell attachment to hydrophobic surfaces. However, one also needs to account for the physiochemical properties of the material which may vary according to the different exudates conditioning the surface. For example, the surface might be hydrophobic but the material that is conditioning the surface might be hydrophilic thus affecting the attachment based on hydrophobicity.

Apart from surface properties, bacterial cell surface features like flagella are also important when it comes to the initial attachment to different surfaces. *L. monocytogenes* produces five to six peritrichous flagella (O'Neil & Marquis, 2006). In *L. monocytogenes,* the expression of flagella is temperature-dependent and at 37°C, most of the *L. monocytogenes* strains do not produce flagella and are non-motile because at 37°C the MogR protein represses transcription of the flagellar genes. At 30°C or below the *L. monocytogenes* is motile, as the GmaR antirepressor of MogR is activated, thereby permitting flagellar gene transcription. Flagella can act as an adhesin, promoting direct attachment or provide motility to cells onto the surface to assist colonization. It has been reported earlier that motility plays an important role in biofilm formation (Gorski, Duhe, & Flaherty, 2009; Lemon, Higgins, & Kolter, 2007; Todhanakasem & Young, 2008). There are also

studies reporting that motility impacts biofilm architecture (Guttenplan & Kearns, 2013; Wood, González Barrios, Herzberg, & Lee, 2006). Biofilm formation increases with an increase in the temperature so, although at 37°C flagellin expression is repressed, *L. monocytogenes* cells passively attach to the surface (Tresse, Lebret, Garmyn, & Dussurget, 2009). *L. monocytogenes* is capable of forming biofilms with a wide range of phenotypes. Biofilm grown in static conditions often consist of homogenous layers of microcolonies displaying morphology similar to planktonic cells. In contrast, biofilm grown under continuous flow conditions will exhibit spherical shaped microcolonies surrounded by elongated cells in the form of knitted chains. This elongation of cells is due to the activation of the YneA gene that regulates the SOS response factor (Abee, Kovacs, Kuipers, & van der Veen, 2011).

There are many genes that regulate biofilm formation. For example, the *prfA* gene is an essential transcriptional activator that regulates virulence gene expression in *L. monocytogenes*. This gene affects the homeostasis of *L. monocytogenes* by regulating repression of other genes involved in transport, metabolic enzymes or the *agr* system that is linked with quorum sensing (Gandra et al., 2019; Lemon, Freitag, & Kolter, 2010; Luo et al., 2013; Zhou et al., 2011). Bacteria communicate with each other to induce changes using quorum sensing. *L. monocytogenes* uses a *luxS* and *agr* system for quorum sensing (Garmyn, Gal, Lemaitre, Hartmann, & Piveteau, 2009). The *luxS* gene encodes for an enzyme S- ribosylhomocysteinase which catalyzes the hydrolysis of S-ribosylhomocysteine to homocysteine and 4, 5-dihydroxy-2,3-pentadione (DPD). DPD serves as a precursor of the autoinducer 2 (AI-2) and AI-2 is considered as the most common language for bacterial communication (Xavier & Bassler, 2005). The *luxS* gene is thought to be involved in repressing components that are required for biofilm formation (Sela, Frank, Belausov, & Pinto, 2006). Challan Belval et al. (2006) and Sela et al. (2006) demonstrated that a mutation in the *luxS* gene enhances biofilm formation. The Agr system involves four genes *agrB*, *agrD* *agrC* and *agrD* organized as an operon (Rieu, Weidmann, Garmyn, Piveteau, & Guzzo, 2007). Gandra et al.

(2019), pointed out that the *agr* locus, particularly the *agrB, agrC,* and *agrD* genes, are key regulators in the early adhesion phase of biofilm formation. Also, extrinsic factors like time and temperature strongly influence the expression levels of the *agr* locus and the *prfA* gene. Gandra et al. (2019) reported higher expression of *agr* locus and *prfA* gene at 37°C and lowest at 10°C. in contract another study reported highest expression of prfA gene under acid shock conditions at 10°C (Neuhaus et al., 2013). These findings suggest that while translation could be temperature dependent, transcription of *prfA* can be induced even at lower temperatures under specific stress conditions.

As discussed earlier, EPS helps the bacteria to strengthen the biofilm and protect it from the external environment. There have been many studies involving the importance of eDNA in biofilm formation. eDNA can adsorb to the cell surface and influence the hydrophobicity of the cell surface thereby affecting the initial attachment of the bacteria (Okshevsky, Regina, & Meyer, 2015). Harmsen, Lappann, Knochel, and Molin (2010) identified that eDNA is essential for initial attachment and early biofilm formation. Sulakova, Pazlarova, Meyer, and Demnerova (2019) found that strain and lineage influences eDNA distribution. They found that lineage II had a slightly higher eDNA/biofilm ratio then lineage I. eDNA is a key component that can be used to control biofilm. Okshevsky et al. (2015) mentioned in their review article that by destabilizing the eDNA's interaction with other components it is possible to control biofilms.

In industry, different types of bacteria are present. Some of them provide a synergetic effect that enhance or protect other bacteria. On the other hand, there are also bacterial species that compete with other species. For *L. monocytogenes* the presence of *Flavobacterium* sp. and *Pseudomonas* sp. enhance biofilm formation (Bremer, Monk, & Osborne, 2001; Puga, Dahdouh, SanJose, & Orgaz, 2018; Sasahara & Zottola, 1993). In contrast, bacteria like *Serratia proteamaculans, Janthinobacterium lividum, Lactococcus lactis, Enterococcus durans, Lactobacillus* strains like *Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus rhamnosus,* and *Staphylococcus*

sciuri and *Listeria innocua* compete and this reduces biofilm formation (Daneshvar Alavi & Truelstrup Hansen, 2013; Fox, Solomon, Moore, Wall, & Fanning, 2014; Guerrieri et al., 2009; Koo, Ndahetuye, O'Bryan, Ricke, & Crandall, 2014; Leriche & Carpentier, 2000; Woo & Ahn, 2013; Zhao, Doyle, & Zhao, 2004; Zhao et al., 2013).

2.4 Importance of cations on bacteria

Cations like sodium, magnesium, calcium, and potassium are fundamental for many biological processes. It is important that these ions are maintained at appropriate levels to maintain the homeostasis of cells. They are involved in many processes like membrane stability, signal transduction, hormonal regulation, cellular energy metabolism and DNA stabilization (Niegowski & Eshaghi, 2007). The importance of these cations and their roles are described below.

2.4.1 Magnesium

In living cells, magnesium is the most abundantly available divalent cation (Ahmed & Mohammed, 2019). It is required for many cellular processes like growth and maintenance and the stability of the plasma membrane. Its exclusive properties make it unique among biological cations. It has the largest hydrated radius (400-time) compared to the ionic, dehydrated form helping to strongly bind six water molecules. It also has the highest charge density and the smallest ionic radius, thus binding strongly to the water molecule and creating stronger water-water interactions in bulk solution (Collins, 1997; Maguire & Cowan, 2002). Therefore, most of biological reactions with Mg^{2+} occur through the hydration sphere, instead of direct interaction with the cation. This makes Mg^{2+} unique compared to other cations. Mg^{2+} is a cofactor with ATP that participates in various enzymatic reactions. Furthermore, Mg^{2+} regulates the assembly of ribosomal subunits, several metabolic enzymes and membrane channels of metal-binding sites (Smith & Maguire, 1998). There are three main magnesium transporter systems available in prokaryotes. The CorA, MgtE and MgtA/B are the three types of transport proteins within the

bacterial cell membrane that facilitate Mg^{2+} homeostasis (Smith & Maguire, 1998).

The CorA family is the primary magnesium transporter that is ubiquitous in both Bacterial and Archaea kingdoms. Apart from magnesium, this system can also transport nickel, cobalt, and cadmium (Niegowski & Eshaghi, 2007). It transports Mg^{2+} with a K_m of 15 µM (Groisman et al., 2013; Moncrief & Maguire, 1999). Co^{2+} and Ni^{2+} can also be transported, however, the K_m values are quite high and can cause a rapid toxic condition in cells. Mn^{2+} is poorly transported and competitively inhibits the cation uptake (Moncrief & Maguire, 1999). In *L. monocytogenes*, the *corA* gene encodes an 11.38 KDa membrane protein.

Like CorA, MgtE is also considered a primary magnesium transport system in bacteria, but it is not as ubiquitous as CorA (Moncrief & Maguire, 1999). It transports Mg^{2+} with a Km of 50uM. Along with Mg^{2+}, MgtE can also transport Co^{2+}. Cations like Zn^{2+}, Ca^{2+}, Mn^{2+} and Sr^{2+} can inhibit cation uptake in the MgtE system. To transport the cations across the cytoplasmic membrane, both CorA and MgtE use an electrochemical gradient. Therefore, any change in the external pH or the electron transport chain component activities or concentration variations in other ions will affect the systems (Groisman et al., 2013).

The MgtA/B families are the P-type ATPases Mg^{2+} transporters and are only confined to a certain subset of bacteria (Groisman et al., 2013). MgtA/B mediates the transfer of Mg^{2+} and Ni^{2+}. Zn^{2+} and Ca^{2+} are competitive inhibitors for MgtA, while both are weakly inhibited by Mn^{2+}. However, none of them have an effect on MgtB. Although all these transporters can import or export Mg, the energy required differs and depends on the various conditions.

2.4.2 Calcium

Calcium is one of the most important cations that is required by any microorganism for their routine cellular processes like nutrient transport, motility, cell-division cycle, metabolism and gene expression. Calcium also affects physiological processes like chemotaxis, heterocyst

differentiation, spore formation and virulence (Dominguez, Guragain, & Patrauchan, 2015). In *L. monocytogenes*, listeriolysin is a major virulence factor, which functions in the Ca dependent cellular response to induce infection in the host cell (Repp et al., 2002). A similar effect is observed in *P. aeruginosa* biofilm, where calcium increases the production of a mucoidal extracellular matrix which contains pyocyanin, a virulence factor that further disrupts the host cellular calcium level (Sarkisova, Patrauchan, Berglund, Nivens, & Franklin, 2005). Norris et al. (1991) mentioned in a review that certain calcium concentrations affect enzyme activity. For example, a calcium concentration under 1mM inhibits DNA nuclease activity and below 10^{-6} M inhibits phospholipase A activity in the outer membrane and phosphoglycerol synthesis in *Escherichia coli*. Calcium also plays a crucial role in the structural stability of bacteria for such things as stabilizing the lipopolysaccharide monolayer, phospholipid turnover and synthesis. At high concentrations of calcium and low temperature, the outer membrane of bacteria is disrupted (Norris et al., 1991). In *L. monocytogenes,* calcium is transported by a P-type ATPases at Imo084 called LMCA1 (Faxen et al., 2011). This Ca^{2+} ATPase is upregulated in alkaline conditions at pH 9.5 and it is possible that it is positioned in the plasma membrane to aid the survival of pathogens under stress conditions such as high Ca^{2+} concentration and high pH (Faxen et al., 2011). Thomas and Rice (2014) demonstrated that the cell wall in low ionic strength environments has a strong affinity for metal ions (calcium and magnesium). This acts as a survival mechanism for the bacteria to capture essential divalent ions in scarce conditions and sequester these in the cell wall. Several components of the cell wall, like lipopolysaccharides, polysaccharides and teichoic acid, have a high affinity for the divalent cation, calcium, to bind to these components and thus increase the aggregation among the cells by forming ionic bridges between the matrix and biopolymers (Das et al., 2014).

Cell walls act as a structural layer surrounding the bacteria that help to maintain the structure and protect them against the external environment. The Gram-positive bacterial cell wall consists of a

cytoplasmic membrane made-up of phospholipids. These phospholipids contain two long-chain fatty acids connected to phosphoric acid via glycerol. Divalent cations like magnesium and calcium neutralize the negative charge of this phosphoric acid (Crismaru et al., 2011). The cytoplasmic membrane is surrounded by a cross-linked thick layer of peptidoglycan, teichoic acids, and other surface proteins. These create a polyanionic matrix that helps to maintain cation homeostasis that assists in the preservation of the overall structural properties like elasticity, tensile strength, porosity and electrostatic interactions (Neuhaus & Baddiley, 2003).

2.4.3 Sodium

Bacteria continuously require energy for their growth and metabolism. In most bacteria, this energy is derived from the proton motive force. But under certain conditions or for certain bacteria H^+ can be replaced by Na^+ ions. For example, neutrophilic bacteria do not require the sodium ion for their growth beyond the residual sodium present in the medium (Dimroth, 1987). Marine microorganisms, like halophilic, methanogenic and certain alkalophilic microorganisms, require sodium as an essential component for their growth. These microorganisms require sodium for the activation of certain enzymes, pH homeostasis, sodium-coupled energy conservation and transduction and ion- solute co-transport systems (Corratge-Faillie et al., 2010; Dimroth, 1987; Hase, Fedorova, Galperin, & Dibrov, 2001). The most common primary sodium pumps observed in bacteria are Na+-Transporting Dicarboxylate /Decarboxylases, Na+-Transporting NADH Dehydrogenase, Na+- Transporting ATPases, Na+-Transporting Terminal Oxidases and Na+-Transporting Methyltransferase. Depending on the microorganism, high levels of sodium in the cytosol form a toxic environment (Hase et al., 2001). Therefore, the cytosol concentration of sodium should be maintained below 10-30mM.

Marquis, Mayzel, and Carstensen (1976) determined the series of relative affinities of various cations in Gram-positive bacteria and found H^+ *La^{3+}.> $Cd^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+}$

> K^+ > Na^+ > Li^+.

2.5 Effect of Cations on biofilm formation

The influence of the cations differs in their action on biofilms, depending upon the microorganism, the type of cation and the ion concentration. However, one aspect that cations have a role in biofilm formation and maintenance is the structural stability of the biofilm in flowing systems. Attachment of the cells to the surface is the initial and critical step in the biofilm formation. This attachment to the surface is a two-step process. The first step where the cells attach reversibly, engaging various forces like electrostatic, van der Waals and hydrophobic interactions (Palmer et al., 2007). Divalent cations enhance the attachment by neutralizing the surface charges, regulating the electrostatic repulsive forces and initiating strong bridging interactions. Non-motile *P. aeruginosa* cell adhesion is supported by cation bridging between the high affinity sites on the bacterial surface and the substrate (Kerchove & Elimelech, 2008). The same authors also demonstrated that the adhesion behaviour in the presence of divalent cations is dependent on the type of specific interaction occurring between the two types of surfaces (bacteria and substrate). For example, adhesion of the motile *P. aeruginosa* cells to clean quartz was dependent on the electrostatic interactions, while on alginate-conditioned adhesion quartz, adhesion was reliant on the structure and viscoelastic properties of the alginate film. Song and Leff (2006) reported that for *Pseudomonas fluorescens,* Mg^{2+} enhanced the initial attachment, probably by decreasing the repulsive force between the negatively charged substratum surfaces and negatively charged bacteria. The second important step in attachment is the irreversible attachment that uses exopolysaccharides or ligands like pili or fimbriae. In this process various forces like covalent and hydrogen bonding and hydrophobic interactions are involved. Ceyhan Guvensen, Demir, and Ozdemir (2013), observed divalent cations Mg^{2+} and Ca^{2+} enhanced the adhesion of *Sphingomonas paucimobilis* by altering the hydrophobicity of the cells attaching to the surface. Similar results were reported for *Arthrobacter* sp. SUK 1201 (Dey & Paul, 2018), *Anoxybacillus*

flavithermus and *Geobacillus species* (Somerton, Flint, Palmer, Brooks, & Lindsay, 2013), *Xylella fastidiosa* (Cruz, Cobine, & De La Fuente, 2012), *Bacillus* sp., *Chlorella* and *Phaeodactylum tricornutum* (He, Wang, Abdoli, & Li, 2016). He et al. (2016), reported how Ca^{2+} and Mg^{2+} can vary in their action when present together in the medium. For instance, in *Chlorella*, Ca^{2+} improved adhesion by regulating protein expression while Mg^{2+} mediates adhesion by regulating EPS formation and polysaccharide synthesis.

Cations also play important roles in influencing the cell morphology, growth and surface properties of bacteria. The three-dimensional structure of the *P. fluorescens* is affected by an increase in the magnesium concentration. As the concentration increases (0.1-1mM) the heterogeneity and depth of the biofilm increases. More interstitial voids or water channels are observed with an increase in magnesium concentration and time (Song & Leff, 2006). With the increasing concentration of NaCl (4-10%), the hydrophobicity of the foodborne pathogens *L. monocytogenes*, *Staphylococcus aureus*, *Shigella boydii*, and *Salmonella enterica* Typhimurium decreases (Xu, Zou, Lee, & Ahn, 2010). At 5% NaCl the hydrophilic character of *L. monocytogenes* is enhanced at pH 5.0 (Bereksi, Gavini, Bénézech, & Faille, 2002). At high NaCl concentration, elongated cells are observed for *L. monocytogenes* (7%) and *S. enterica* Enteritidis (5%). According to the authors, these cells are on the edge of division and will divide immediately into single cells if transferred into a favourable environment (Hazeleger, Dalvoorde, & Beumer, 2006). *L. monocytogenes* can grow at high concentrations of NaCl (9%) and KCl (11%). However, the lag phase increases with the increasing concentrations of these salts (Zarei, Khezizadeh, Kazemipour, Hesami, & Bemani, 2012). Increased calcium concentration also increases the cohesiveness of the biofilm of *P. aeruginosa* (Turakhia & Characklis, 1989). Pan, Breidt, and Gorisk (2010) found *L. monocytogenes* serotype 1/2a forms a denser biofilm than 4b in the presence of sodium chloride (2-7%) at 30°C. Similar results were observed by another research group Jensen, Larsen, Ingmer, Vogel, and Gram (2007), (2-5% NaCl). The aggregation of cells

could be one possible factor that might be boosting the ability of *L. monocytogenes* to resist cleaning agents and persist in a food processing environment (Jensen et al., 2007).

In biofilm formation, eDNA binds to several biopolymers and metabolites, in turn, helping to maintain structural integrity. Ca^{2+} being a positively charged molecule, binds to eDNA by electrostatic interactions. This binding facilitates bacterial cell to cell aggregation by promoting ionic cross bridging between the cells. According to another study as most cells are negatively charged and bacteria adsorb calcium onto the cell surface thereby decreasing the surface electrostatic repulsion, enabling adhesion and aggregation (Das et al., 2014). It was also observed that at high calcium concentration, acidic extracellular polysaccharide is produced. Calcium also influences the ability of microorganisms to colonize specific surfaces. At a calcium concentration of 10mM of $CaCl_2$, the biomass was 20-100 times higher on a hydrophilic surface (glass) then on a hydrophobic surface (Teflon) (Patrauchan, Sarkisova, Sauer, & Franklin, 2005).

Cations affect various signal transduction pathways. Calcium influences *Pseudoalteromonas* sp. 1398, by altering the proteomics in a dose-dependent manner. As the calcium concentration increases (0.25 to 10 mM), the protein expression in the biofilm compared to planktonic state increases, demonstrating how calcium concentration influences the regulatory response resulting in a planktonic to biofilm shift (Patrauchan et al., 2005). In *Bacillus* species, high concentrations of magnesium (50mM) have an inhibitory effect on the two matrix operons *epsA-O* and *tapA*. As these are involved in signal transduction for biofilm formation, biofilm formation is inhibited (Oknin, Steinberg, & Shemesh, 2015). Increasing the concentration of sodium influences stress response in *Clostridium ljungdahlii.* Various stress response proteins and chaperons are upregulated while flagella motility and putative type IV pili biosynthesis genes are downregulated with an increase in NaCl concentration (200mM) (Philips, Rabaey, Lovley, & Vargas, 2017). Lee et al. (2019) reported similar upregulation of osmotic related genes and morphological changes in *E. coli*. Caly, Takilt, Lebret, and Tresse (2009) reported that 0-6% NaCl enhances adhesion of *L.*

monocytogenes on polystyrene and stainless-steel surfaces. However, they reported that adhesion decreases at 11% NaCl, possibly due to the repressed function of flagella.

For some bacteria, as cations increase the biofilm formation is enhanced, but for other species biofilm formation decreases. Shukla and Rao (2013) suggested that calcium has a negative effect on *S. aureus* biofilm. Calcium modulated the biofilm architecture in a dose- dependent manner. As the concentration increases the topography of the biofilm differs and thickness reduces. On addition of a chelating agent, EDTA, this inhibition is reversed (Shukla & Rao, 2013). Magnesium along with high alkalinity exhibits anti-biofilm properties for *P. aeruginosa* and *Staphylococcus epidermidis*. High Mg^{2+} concentration and high alkaline pH creates a toxic environment for the microorganisms by damaging cellular enzymes, increasing the osmotic pressure, decreasing the surface hydrophobicity and disruption of the proton-electron chemical gradient due to an increase in H^+ consumption that directly affects ATP synthesis ultimately leading to bacterial death (Qin et al., 2015).

Calcium increases biofilm formation, enhances surface attachment and confers twitching motility to *Xylella fastidiosa*, a plant pathogen. However, biofilm formation is reduced when extracellular and intracellular calcium is removed using 1.5mM EGTA and 75 uM [1,2- bis(o-aminophenoxy) ethane-N, N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA/AM). These chelating agents do not have any effect on the planktonic cells suggesting that calcium is involved in the initial stages of biofilm formation (Cruz et al., 2012). As adherence of the bacteria is important in biofilm formation, detachment of the cell from the biofilm is also critical to study as this is a source of contamination of food and the environment. NaCl concentration influences the attachment and detachment of foodborne pathogens like *L. monocytogenes*, *S. aureus*, *S. boydii*, and *S. enterica* Typhimurium, depending on the concentration of NaCl. At high NaCl concentration (4-10%), no detachment is observed. At low concentration (0-4%), the bacteria grow, resulting in reduced nutrient availability and a reduction in cells ability to adhere. This suggests that the NaCl enhances

biofilm stability (Xu et al., 2010).

2.6 Conclusion

The impact of cations in various stages of biofilm development (e.g., attachment, maturation, detachment), structure, physiology and gene regulation in different bacterial species is well documented (Hazeleger et al., 2006; Patrauchan et al., 2005; Philips et al., 2017; Song & Leff, 2006; Turakhia & Characklis, 1989; Xu et al., 2010). Depending on the bacterial species, different cations enhance or reduce biofilm formation. These contrasting effects might be due to several reasons such as differences in surface hydrophobicity, varied cell surface polymers or external components. Cations are required by bacteria to maintain their homeostasis. Any change in external or internal concentration will affect the bacterial homeostasis, thereby disturbing various factors such as osmotic pressure, enzyme and cellular functions and many other regulatory pathways. This disturbance by cations will create a toxic environment resulting in inhibition of bacterial growth in biofilm. Sodium, magnesium and calcium are the most abundant free cations available in seawater (Riley & Tongudai, 1967). Based on the reviewed literature, it is evident that these three cations play a critical role in microbial physiology and have been investigated for its role in biofilm formation for other organisms. Therefore, these cations are of interest in studying the effect on *L. monocytogenes* biofilm formation in seafood processing environments. To evaluate the impact of these cations, throughout the studies three concentration 1mM, 10mM and 50mM has been used. This range was chosen to balance experimental simplicity with physiological (data from Pilson 1998) and industrial relevance (cation profile in mussels and mussel intravascular juice), ranging from low to high ionic conditions (Appendix's image A1.a and Appendix's table A1.b). In the case of *L. monocytogenes,* the role of cations with respect to virulence is well documented (Dominguez et al., 2015; Marino et al., 2004; Repp et al., 2002; Vadia & Seveau, 2014). However, very few studies are available with respect to the development of *L. monocytogenes* biofilm, mainly studying the effect on adhesion (Caly et al., 2009; Jensen et al.,

2007). More research is required to study the influence of these cations on various steps including initial attachment, the transition from planktonic cells to biofilm formation and dispersal of cells from the biofilm. Few reports are present that correlate cations to their interaction with regulatory pathways and how these may influence biofilm formation.

The purpose of this PhD project was first to investigate the influence of commonly found cations like calcium, magnesium and sodium on biofilm formation and the persistence of *L. monocytogenes*. Secondly, to study the transcriptome profile to better understand the difference between a good biofilm isolate - persistent isolate and a low biofilm isolate and lastly to view the effects of cations on the extracellular matrix of *L. monocytogenes.*

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Chapter 3: Differential effects of magnesium, calcium, and sodium on *Listeria monocytogenes* biofilm formation

3.1 Abstract

Listeria monocytogenes is a Gram-positive foodborne pathogen that causes outbreaks of listeriosis associated with a diverse range of foods. *L. monocytogenes* forms biofilms as a strategy to enhance its survival in the environment. These biofilms then provide a source of contamination in processing plant environments. Cations like magnesium, calcium, and sodium are commonly found in the environment and are important to bacteria to maintain their homeostasis. It is, therefore, valuable to understand the relationship between these cations and biofilm formation. In this study, four isolates of *L. monocytogenes* from seafood processing environments were used to investigate the influence of magnesium, calcium, and sodium (1, 10, and 50 mM) on biofilms. The isolates were selected as a low biofilm former, a high biofilm former, an outbreak isolate, and a persistent isolate from the seafood industry. The study showed that the divalent cations magnesium and calcium increased biofilm formation compared with the monovalent cation, sodium. Fifty mM concentrations of the divalent cations significantly enhanced biofilm formation. The cations did not have a significant effect on the initial stages of biofilm formation but appeared to influence the later stage of biofilm development.

3.2 Introduction

Listeria monocytogenes is a Gram-positive foodborne pathogen that is ubiquitous in the environment. It is a non-fastidious organism that can survive under a broad range of conditions. For instance, it can survive and/or grow at temperatures of 0 to 45°C, pH 4.4 to 9.6, high salt concentration (13%), low water activity, and can form biofilms. The wide range of growth conditions allows this organism to proliferate in food processing environments and products (Lee et al., 2019; Letchumanan et al., 2018). *L. monocytogenes* causes a severe life-threatening infection known as listeriosis. Although listeriosis is rare, it has a 30% fatality rate (M. H. Choi et al., 2018). Usually, the source of infection is through the consumption of contaminated ready-to-eat foods including vegetables, meat, seafood, or unpasteurized milk, ice creams, and soft cheese. In the United States, according to the Centre for Disease Control (CDC) (CDC, 2021), it is estimated that around 1600 people suffer from listeriosis each year, of which 260 die. In 2019, in New Zealand, 0.6 cases per 100,000 population were notified (Pattis, 2020). The presence of *L. monocytogenes* in the food- processing environment poses a significant public health concern and represents a major economic and reputation challenge for the food processing industry. The ability of *L. monocytogenes* to persist in the food processing environment is related to biofilm formation (Shi & Zhu, 2009; Srey, Jahid, & Ha, 2013). It is capable of forming biofilms on a wide range of surfaces including polystyrene, stainless steel, glass, various rubber and polymers, and leafy substrates like lettuce (Beresford, Andrew, & Shama, 2001; Kyere et al., 2020; Rodríguez-Lozano & McLandsborough, 2009).

A biofilm is defined as a community of micro-organisms attached to a surface and protected through a self-produced matrix known as extracellular polymeric substance (EPS). Biofilm formation is a multistage process where a cell attaches to various types of substrates like steel, glass, propylene, or rubber and produces EPS. This EPS is typically composed of polysaccharides, proteins, and DNA that protect the bacterium against the external environment and facilitate growth in

unfavourable conditions (Lasa, 2006; Oknin, Steinberg, & Shemesh, 2015). The biofilm poses a challenge for eradication of micro- organisms from the food processing environment as it provides resistance against sanitizers and cleaning agents used in the industry (Pan, Breidt, & Kathariou, 2006).

The development of biofilms is dependent on various factors like temperature, pH, and availability of nutrients, including cations like magnesium, calcium, and sodium. These ions can be abundant in a food-processing environment (Shen et al., 2018). Previous studies on other microorganisms have suggested that these cations affect various stages of biofilm formation including attachment, structure, and physiology. For instance, they can affect attachment by changing the electrostatic force or by altering the hydrophobicity. They are also involved in regulating many pathways, thereby influencing the physiology and architecture of the biofilm (Das et al., 2014; Dunne Jr & Burd, 1992; Patrauchan, Sarkisova, Sauer, & Franklin, 2005; Sarkisova, Patrauchan, Berglund, Nivens, & Franklin, 2005; Song & Leff, 2006). Any changes in external or internal concentration of these cations can affect the homeostasis of bacteria. No previous study has been published on the effect of cations on *L. monocytogenes* biofilms. The present study aims to investigate the effect of three major cations on the biofilm formation of *L. monocytogenes*.

3.3 Material and methods

3.3.1 Bacterial isolates and growth conditions

Four isolates of *L. monocytogenes* with distinct biofilm-related phenotypes or clinical relevance were selected from a seafood collection of the New Zealand Institute for Plant and Food Research Ltd. Three isolates (15A04, 15G01 and 33H04) originally from three different seafood factories, were continuously present in the facilities over a long period and therefore were identified as persistent isolates (Nowak et al., 2017). Another isolate 16A01 was associated with a mussel *Listeria* contamination outbreak. All the isolates were recovered from −80°C stock and grown on tryptic soy agar with 0.6% yeast extract (TSAYE) (Difco, BD, USA) plates at 37°C for 24 h. For each experiment, a colony was

freshly picked and overnight cultures were prepared in tryptic soy broth enriched with 0.6% yeast extract (TSBYE) (Difco, BD, USA). These cultures were then used for subsequent experiments.

3.3.2 **Media and cation concentration**

For all the experiments 10% tryptic soy broth (TSB) (Difco, BD, USA) was used. Three cations were used in form of: magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$) and sodium chloride (NaCl). The concentrations used for all cations were 1, 10, and 50 mM. 10% TSB without any added cation was used as a control. To understand the concentration of cations in the media, TSB was analysed by inductively coupled plasma - optical emission spectrometry (ICP-OES) at Hill Laboratories, New Zealand.

3.3.3 **Attachment assay**

For attachment studies, each of the *L. monocytogenes* isolates was revived from the TSBYE (as described in 3.3.1) and plated onto TSAYE plates. One colony from each isolate was then inoculated into 10 mL TSBYE and incubated overnight at 37°C. Seed cultures of each *L. monocytogenes* isolate were diluted until they reached an optical density (OD) of 0.4 at 600 nm. The cultures were then centrifuged (12,000 g for 20 min), washed two times, and resuspended in 10% TSB with the following added cation concentrations: Control, 1, 10, and 50 mM of $MgCl_2$, $CaCl_2$ or NaCl. Inoculated 10% TSB, with the different cation concentrations, was incubated in the wells of 96-well polyvinyl chloride microtiter plates (Falcon® 96, 353072, Becton, Dickinson & Company, USA) at 30°C for 15 min. After incubation, the medium was removed, and the wells were washed three times with 250 µL of sterile 0.1% peptone. Subsequently, each well was scraped with a sterile swab and re-suspended in 10 mL of 0.1% peptone water. Viable cell counts were carried out by tenfold serial dilutions in 0.1% peptone. Aliquots of 0.01 mL were plated on TSAYE plates using the drop plate method.

3.3.4 **Cell surface hydrophobicity assay**

Cell surface hydrophobicity of the isolates was determined using hydrophobic interactive chromatography described by N. Y. Choi, Bae, and Lee (2015) with some modifications. Isolates were inoculated in 10 mL TSBYE overnight at 37°C. After incubation, the cultures (100 µL) were inoculated into 9.9 mL 10% TSB containing added cations at control, 1, 10, and 50 mM and incubated for 24 h at 30°C. The cultures were centrifuged at 12,000 g for 20 min and resuspended in phosphate-buffered saline (pH 7.0) diluted to an OD_{600nm} of 0.4. Chromatography columns were prepared using Pasteur pipettes. These were plugged with glass wool, filled with 1 mL of hydrophobic resin (Phenyl-Sepharose Fast Flow™ (Sigma- Aldrich, USA)), and washed three times with phosphate-buffered saline (pH 7.0). Each resuspended culture (300 µL) was loaded into a column and washed with 900 µL of phosphate-buffered saline (pH 7.0). The absorbance of the eluent and a one-quarter dilution of the original culture was determined at 600 nm and the percentage of bacteria retained in the hydrophobic column was calculated as below.

% retained = $(A_0-A_1)/(A_0*100)$, where A_0 is the OD_{600nm} of a one-quarter dilution of bacterial culture resuspended in phosphate-buffered saline (pH 7.0) and A_1 is OD of the eluted bacterial suspension.

3.3.5 **Swarming motility assay**

The swarming motility assay was carried out as described by Knudsen, Olsen, and Dons (2004). Briefly, the swarming motility of *L. monocytogenes* was tested using TSB + 0.25% agar semi-solid agar plates. The overnight cultures were then used to inoculate 10 mL of TSB broth containing $MgCl_2$, $CaCl_2$, or NaCl at control, 1-, 10-, and 50-mM concentrations and were incubated at 30°C for 24 h. The cultures were then inoculated by stabbing on TSB

+ 0.25% agar plates containing $MgCl_2$, $CaCl_2$, or NaCl at Control 1, 10, and 50 mM respectively.

These plates were then incubated at 30°C for 24 h. Motility was measured by measuring the diameter of the visible halo around the inoculum.

3.3.6 **Effect on biomass and cell viability on planktonic cells and biofilms of *L. monocytogenes***

3.3.6.1 Planktonic cells

The effects of $MgCl_2$, $CaCl_2$, and NaCl on planktonic cells were determined by measuring biomass and cell viability. Briefly, 2 μL of each overnight grown culture (as described above) was transferred into 96–well plates (polystyrene) with each well containing 198 μL 10% TSB (Difco, BD, USA) containing $MgCl_2$, $CaCl_2$, or NaCl at control, 1-, 10-, and 50-mM concentration. The inoculated 96-well plates were incubated for 24 h at 30°C. After incubation, the biomass was measured at 595 nm with a microplate reader. The OD of a control (microtiter wells containing uninoculated media) was subtracted from the OD from the wells inoculated with culture. Cell enumeration was performed in parallel using the bacteria grown as above. Serial tenfold dilutions were prepared in 0.1% peptone water and were plated on TSAYE plates using the drop plate method. Colony-forming units (CFU) were enumerated after 24 h of incubation at 37°C. While viability and enumeration of planktonic cells in this study were measured using biomass and CFU counts, exploring addition methos like CFU enumeration using LIVE/DEAD fluorescence staining and flow cytometry would offer more comprehensive view on cell viability.

3.3.6.2 Biofilm formation

The effect of these three cations on the ability of the four *L. monocytogenes* isolates to form biofilms was determined using a previously described (Djordjevic, Wiedmann, & McLandsborough, 2002) method with some modifications. Briefly, 2 μL of each overnight- grown culture was transferred to 96-well plates (polystyrene) with each well containing 198 μL 10% TSB (Difco, BD, USA) and

$MgCl_2$, $CaCl_2$, or NaCl at control, 1-, 10-, and 50-mM concentration. The cultures were incubated for 24 h at 30°C. After incubation, the plates were washed three times with 200 µL sterile water using a microplate strip washer (ELx50, Biotek; Appendix table A3. 1). The plates were air-dried for 30 min and then stained with 150 µL of 0.5% aqueous crystal violet solution (Sigma Aldrich, NZ). After 30 min of incubation at 30°C, the crystal violet solution was removed, and the cultures washed five times with 200 µL of sterile water. The plates were air dried for 30 min in a Class II biosafety cabinet. After drying, 200 µL of 96% ethanol was added and the crystal violet was destained for 1 h. The OD was measured at 595 nm with a microplate reader. The OD of a control (microtiter wells containing uninoculated media) was subtracted from the obtained OD from the wells inoculated with culture. Biofilm formed was expressed in terms of the biofilm formation index (BFI). The BFI was determined by applying the following formula: BFI = (AB-CW)/G. AB is the OD of stained attached biofilm cells, CW is the OD of control wells and G is the initial OD of cell growth in suspended media (Teh et al. 2010). Cell enumeration was performed in parallel as followed. The bacteria were grown as above, and the wells were washed three times using sterile distilled water. To detach the sessile cells each well was scraped using sterile swabs and transferred into 10 mL 0.1% peptone water. Serial tenfold dilutions were prepared in 0.1% peptone water and were plated on TSAYE plates using the drop plate method. CFU were enumerated after 24 h of incubation at 37°C.

3.3.7 **Statistical analysis**

All the experiments were done with three biological replicates, each comprising three technical replicates. An analysis of variance (ANOVA) was performed at a significance level of $p < 0.05$ and post-hoc testing by Tukey's test to determine the significant differences between treatments (Genstat 20th edition, VSN International, Hemel Hempstead, UK).

3.4 Results

3.4.1 **Cation profile in TSB media**

The media used in this study for growing biofilm was 10% TSB. To better understand the effect of cations on biofilm, the baseline concentration of the three cations in the media was determined using ICP-OES. From Table 3.1, it can be seen that the concentration of the three cations intrinsic to the broth is minimal compared to that added. It is important however, to recognize that the experimental controls do contain small amounts of each cation and the actual cation concentration present in the treatments is the sum of the added cations plus those in Table 3.1.

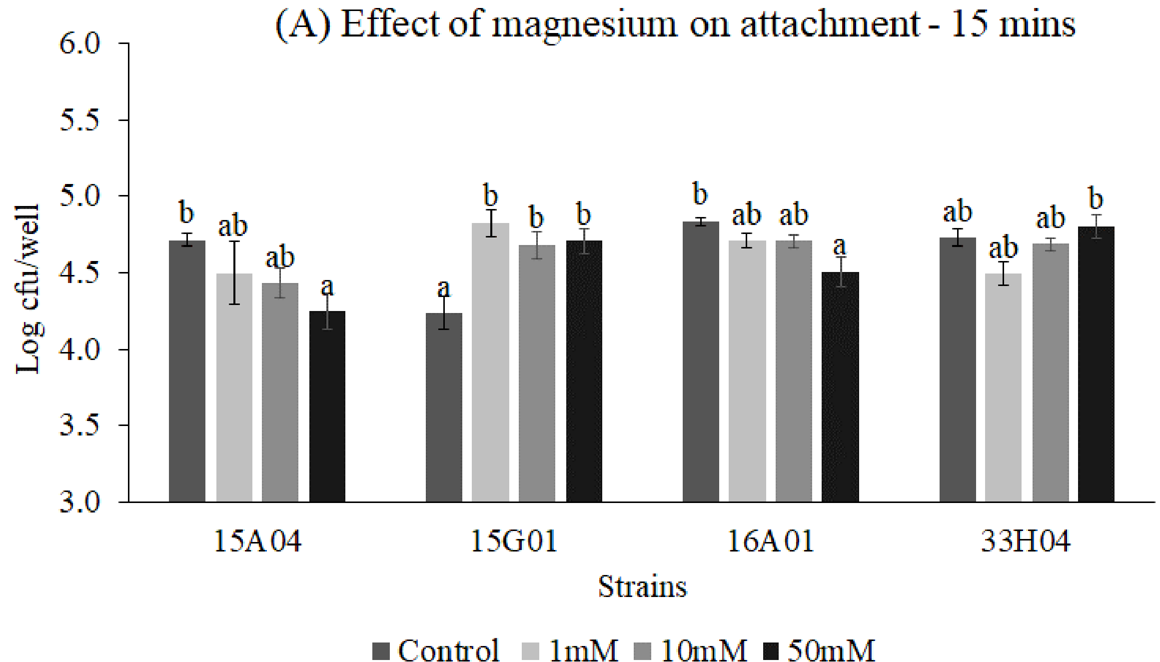
Cation	**Concentration (g/100g)**	**Concentration (mM) in 10% TSB**
Magnesium	0.00141	0.058
Calcium	0.00071	0.017
Sodium	0.024	0.104

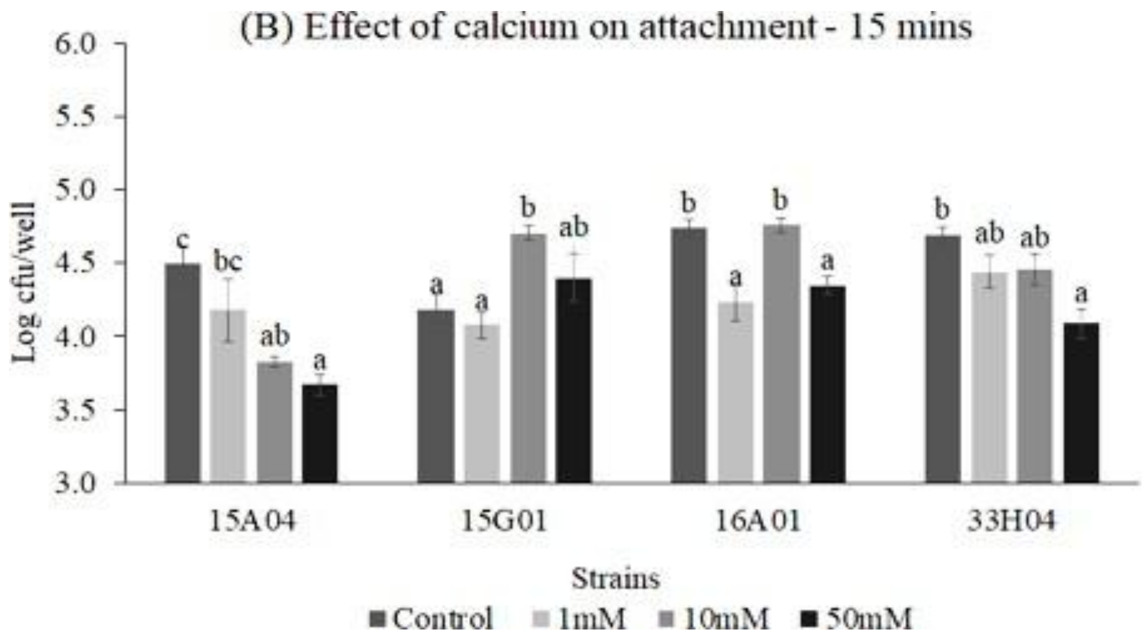
Table 3.1- Cation profile in tryptic soy broth (TSB)

3.4.2 **Effect of cations on attachment**

The effect of cations on the ability of *L. monocytogenes* to attach to a surface is presented in Figure 3.1. The amount of attachment differed between treatments. Averaging across all the isolates, magnesium significantly increased attachment ($p<0.05$) compared to calcium and sodium (Appendix table A3.2 and A3.3). Exposure of the four *L. monocytogenes* isolates to magnesium resulted in three distinct responses. Isolates 15A04 and 16A01 showed decreased cell attachment with increased magnesium concentration (Figure 3.1A). In contrast, isolate 15G01 showed increased attachment in the presence of magnesium, compared to the control. Attachment of isolate

33H04 cells were largely unaffected by magnesium concentration. For calcium and sodium, attachment for all the isolates either reduced or did not change at 50 mM concentration (Figure 3.1B & C) and a significant difference ($p<0.05$) was usually observed between the 50 mM concentration and the control, except in cases of 15G01 and 16A01 for sodium.





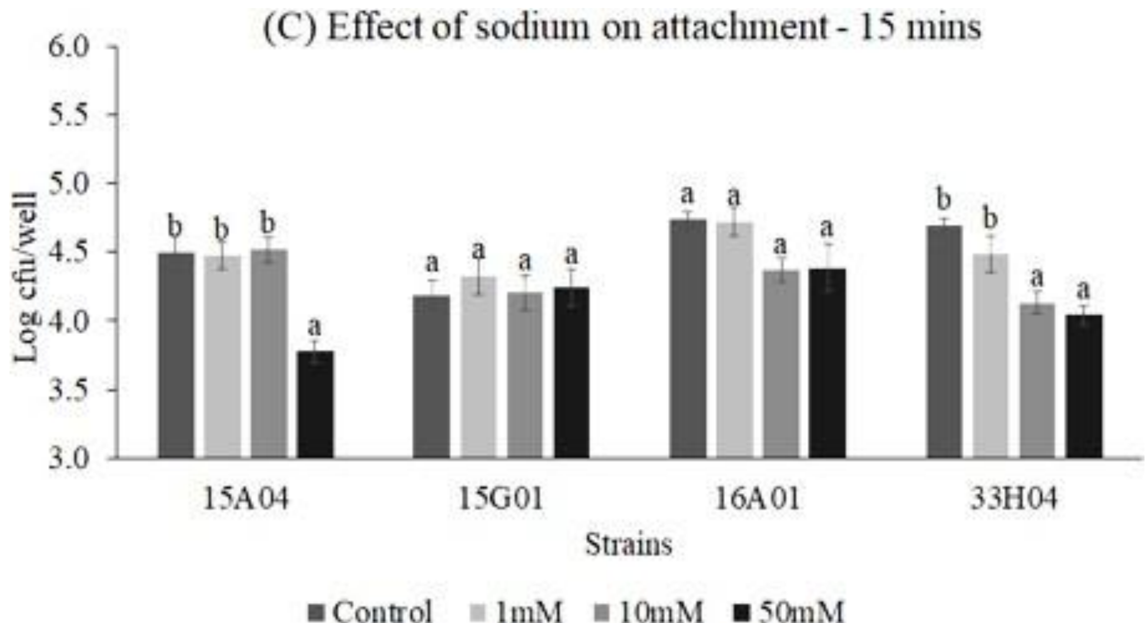
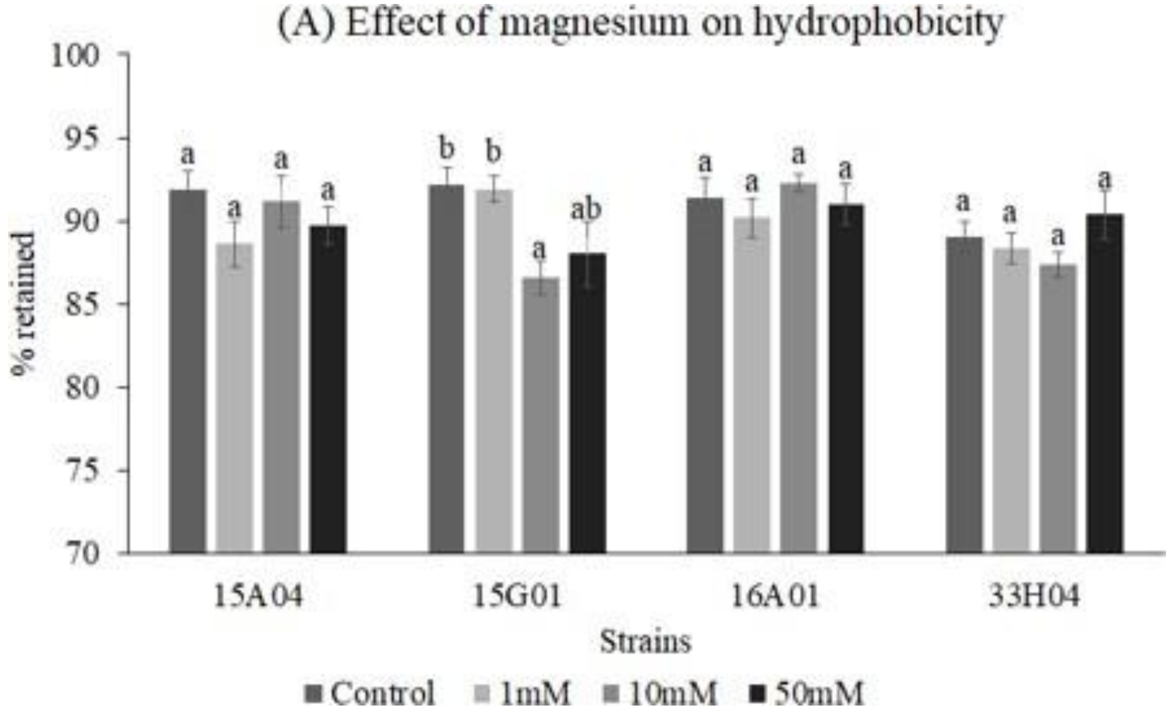
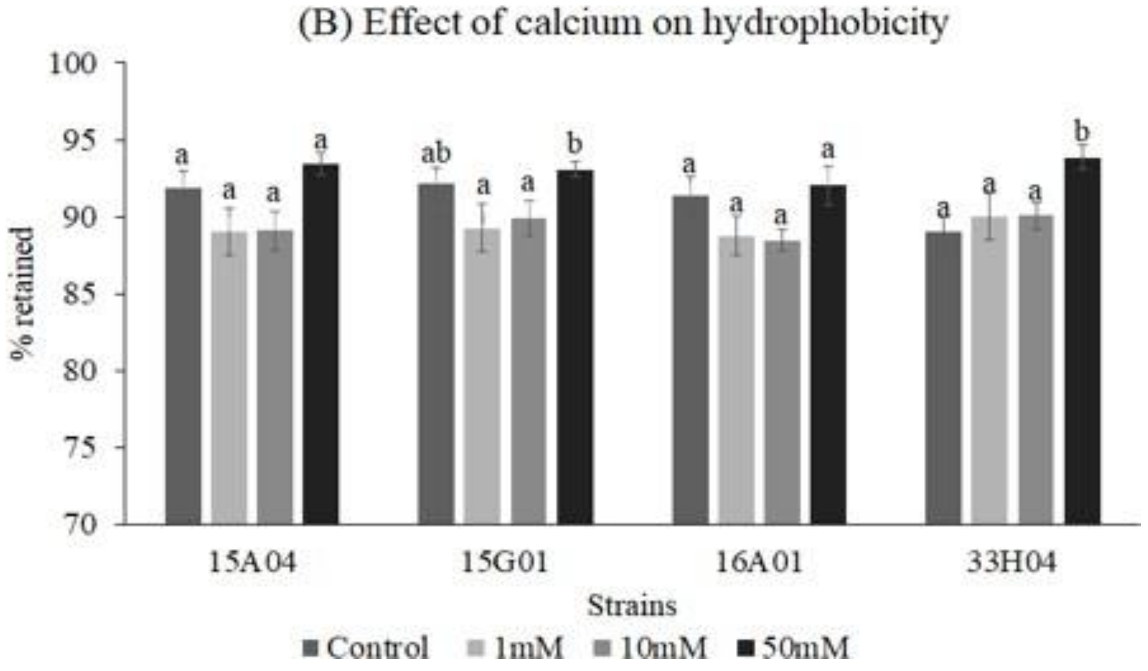


Figure 3.1- Effect on attachment of four *Listeria monocytogenes* isolates in the presence of A) Magnesium, B) Calcium and C) Sodium after 15 mins of incubation at 30°C (error bars represent standard errors of the means for the means of three experiments, different letter represents significant difference ($p < 0.05$) for different concentrations.

3.4.3 **Effect of cations on hydrophobicity**

To better understand the cell surface characteristics in the presence of cations, the hydrophobicity of the cells was studied using hydrophobic interaction chromatography. The affinity of the cations for a phenyl-Sepharose column followed the order calcium > magnesium > sodium. There were minimal changes in hydrophobicity; for all three cations, there were no significant differences in cell hydrophobicity across the cation concentration range for isolates 15A04 and 16A01 (Figure 3.2). For isolate 33H04, 50 mM calcium significantly increased hydrophobicity while 50 mM sodium significantly decreased hydrophobicity (Figure 3.2C).





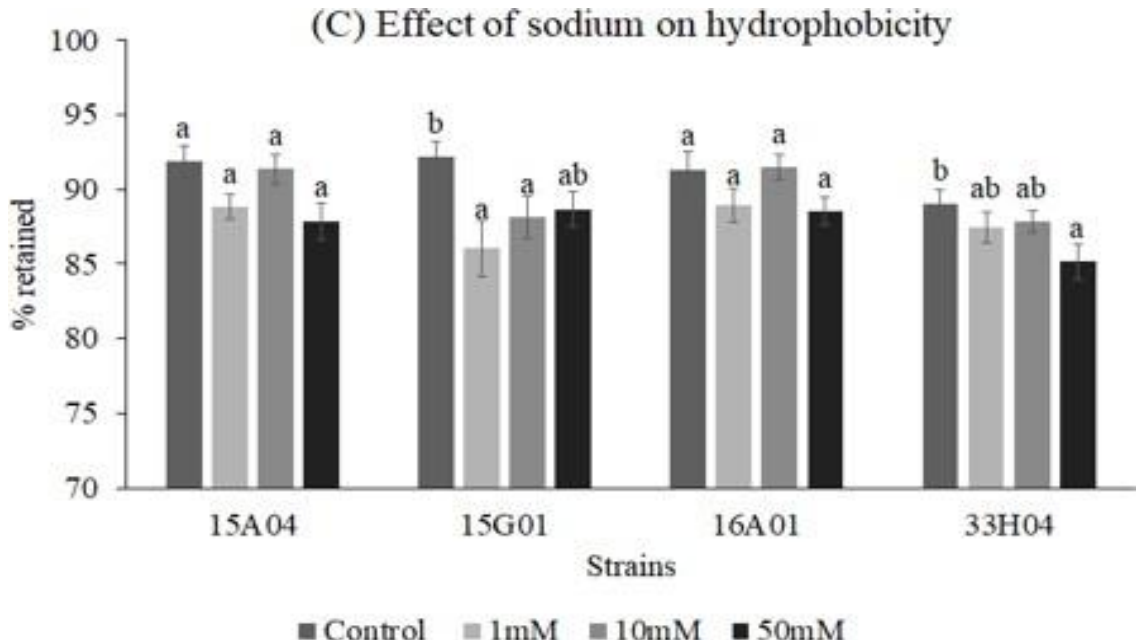
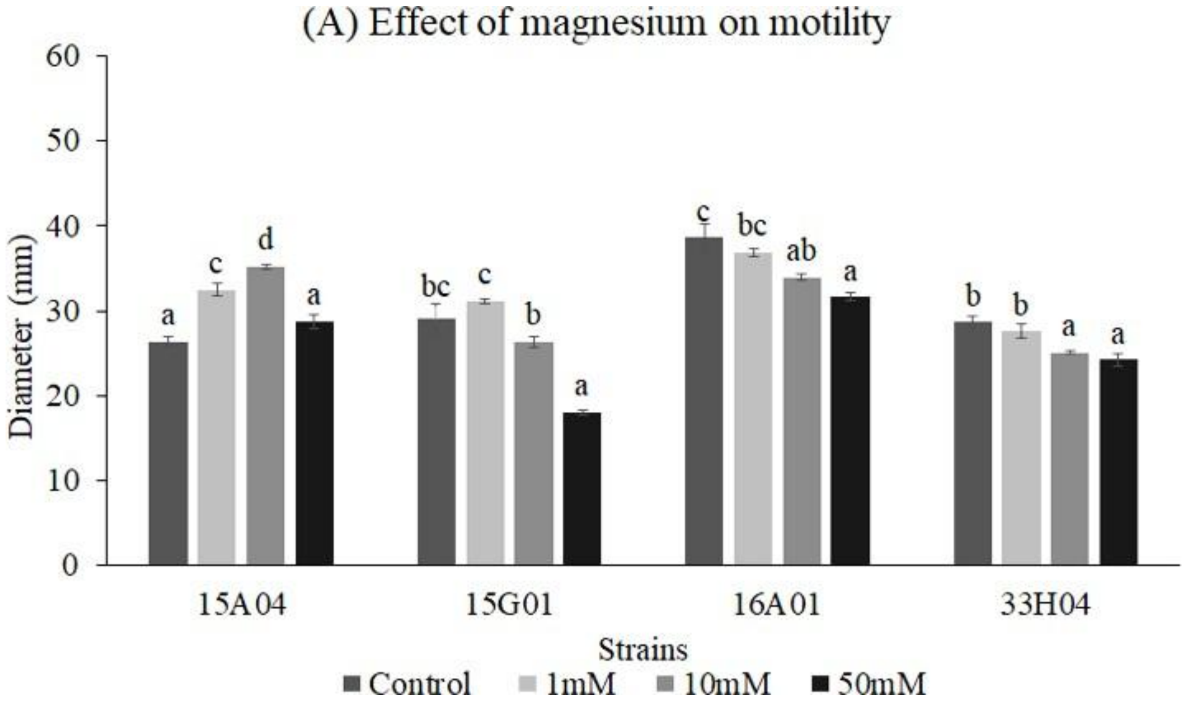
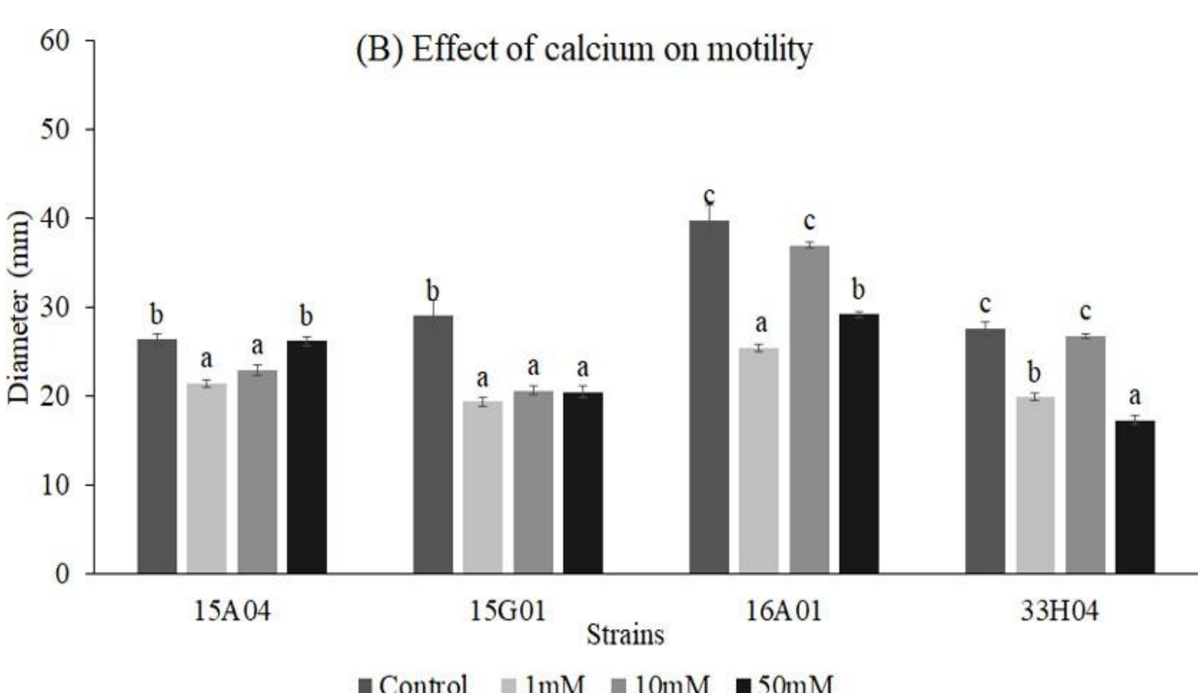


Figure 3.2 - Effect on hydrophobicity of four *Listeria monocytogenes* isolates in the presence of A) Magnesium, B) Calcium and C) Sodium after 15 mins of incubation at 30°C (error bars represent standard errors of the means for the means of three experiments, different letter represents significant difference for different concentrations.

3.4.4 **Effect of cations on motility**

Figure 3.3 shows the effects of cations on the swarming motility of *L. monocytogenes*. Differences in motility for the four *L. monocytogenes* isolates in presence of the three cations were significant ($p<0.05$). Mean motility was substantially higher in the presence of sodium (44.52 mm) compared to magnesium (25.65 mm) and calcium (23.26 mm) at 50 mM concentrations. All isolates, except 15A04, showed decreased motility in the presence of 50 mM magnesium, compared with the control (Figure 3.3A). For isolate 15A04, motility increased significantly ($p<0.05$) with magnesium concentration up to 10 mM magnesium but was similar to the control at 50 mM magnesium. In the case of calcium (Figure 3.3B), all the isolates exhibited a significant decrease in motility at 1 mM concentration. For sodium (Figure 3.3C), isolates 15G01, 16A01, and 33H04 showed decreased motility at 1 mM compared to the control, however, above this concentration, motility significantly increased with increasing concentration and was greater than the control at 50 mM sodium for all isolates except 16A01. In general, the divalent cations like magnesium and calcium reduced the motility across all the isolated (except 15A04 in calcium), while monovalent cation (sodium) increased the motility specifically at 50mM concentration. It was also observed that irrespective of the concentration for all three cations, the persistent isolates (15G01 and 33H04) had lower motility compared to the other two isolates, thus demonstrating strain dependent variability.


(A) Effect of magnesium on motility
Diameter (mm)
60
50
40
30
20
10
0
a
c
d
a
bc
c
b
a
c
bc
ab
a
b
b
a
a
15A04
15G01
16A01
33H04
Strains
Control
1mM
10mM
50mM


(B) Effect of calcium on motility
Diameter (mm)
60
50
40
30
20
10
0
b
a
a
b
b
a
a
a
c
a
c
b
c
b
c
a
15A04
15G01
16A01
33H04
Strains
Control
1mM
10mM
50mM

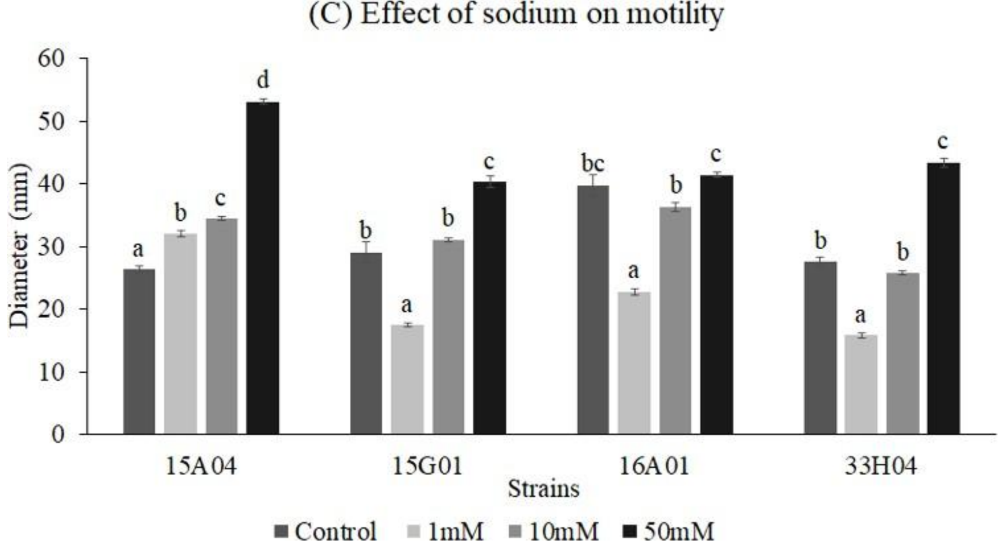


Figure 3.3 - Effect on motility of four *Listeria monocytogenes* isolates in the presence of A) Magnesium, B) Calcium and C) Sodium after 15 mins of incubation at 30°C (error bars represent standard errors of the means for the means of three experiments, different letter represents significant difference for different concentrations

3.4.5 **Effect of cations on planktonic cell and biofilm formation**

Magnesium resulted in a significant ($p < 0.05$) increase in the biomass (OD) of the planktonic cells for all the isolates (Figure 3.4A). Among the four isolates, the persistent isolates 15G01 and 33H04 had greater mean ODs (0.1325 and 0.1411, respectively) compared to 15A04 and 16 A01. For all isolates, except 15G01, 10 mM magnesium resulted in a lower biomass than 1 or 50 mM. Although cell numbers reduced slightly as the magnesium concentration increased, these differences were not significant in the case of culturable planktonic cell counts (Figure 3.4A).

In contrast, both the BFI value and biofilm cell count increased significantly with increasing magnesium concentration for all isolates ($p<0.05$) (Figure 3.4B). There was a significant difference ($p<0.05$) between the mean values at 50 mM and the control for both BFI (10.68 vs 0.38) and cell count (7.085 log CFU/well vs 4.922 log CFU/well). Magnesium had less effect on the biomass of persistent isolates compared to the other isolates.

For calcium, an increase in planktonic biomass and cell count was observed at 1mM concentration for all isolates except 33H04. However, at 50mM calcium, there was a significant reduction in both biomass and planktonic cell numbers for all isolates (Figure 3.5A). However, for biofilm formation, calcium gave similar results to magnesium. The BFI value and cell viability significantly increased at 10- or 50-mM concentrations for all of the isolates (Figure 3.5B).

Sodium had less effect on planktonic cells and biofilm formation than magnesium and calcium (Figure 3.6a and 3.6b). This suggests a role for divalent cations in promoting biofilm growth.

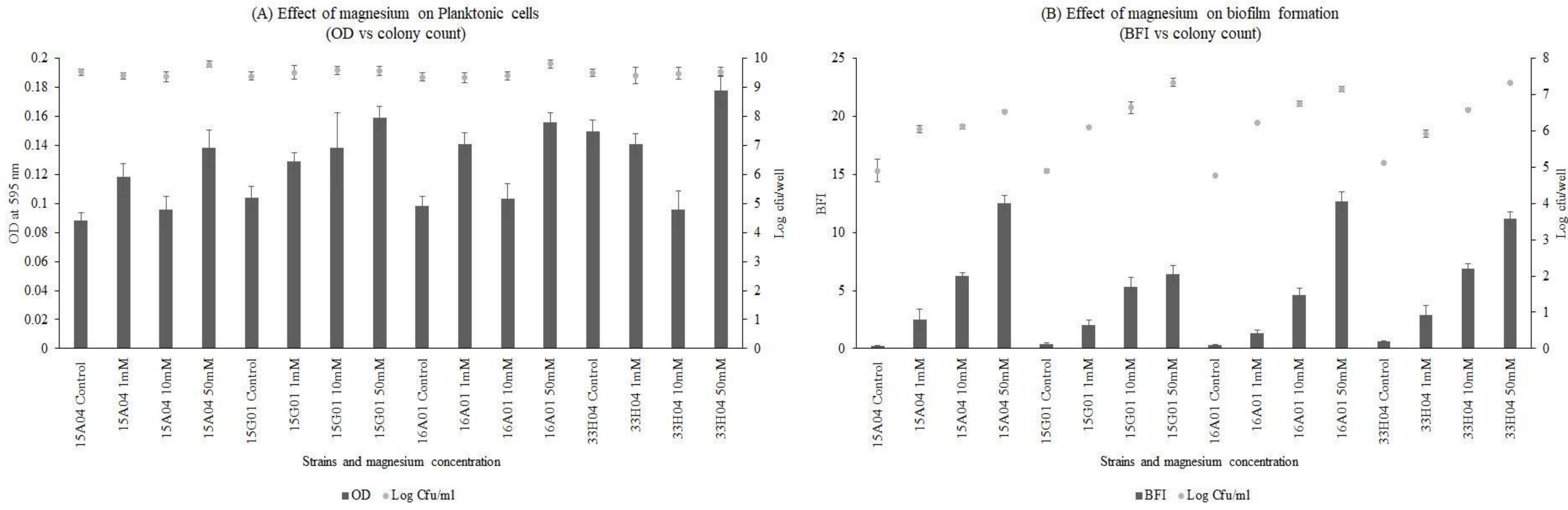


Figure 3.4- Influence of magnesium on A) planktonic cells measured by optical density (bar, strain.concentration LSD (least significant difference) ¼ 0.02822) and plate count cell enumeration (dot, strain.concentration LSD ¼ 0.3777) and B) biofilm formation measured by biofilm formation index (bar, strain.concentration LSD ¼ 1.575) and plate count cell enumeration (dot, strain.concentration LSD ¼ 0.3584) after 24 h of incubation at 30°C (error bars represent standard errors for three experiments)

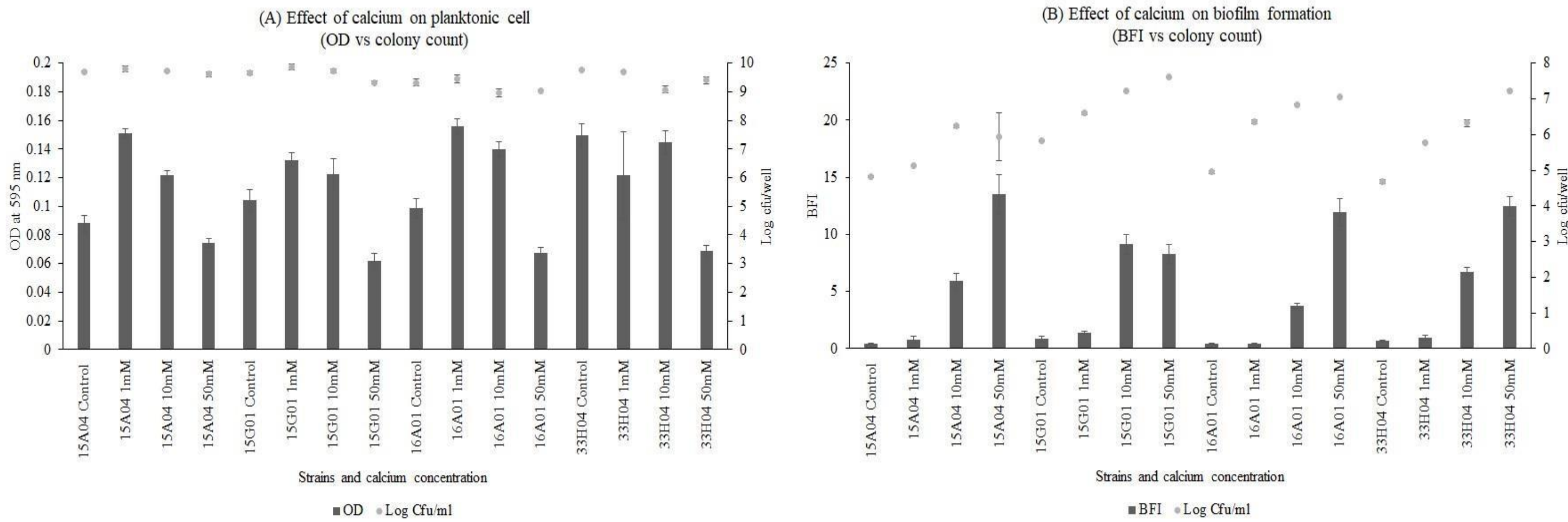


Figure 3.5 - Influence of calcium on A) planktonic cells measured by optical density (bar, strain.concentration LSD (least significant difference) ¼ 0.02822) and plate count cell enumeration (dot, strain.concentration LSD ¼ 0.3777) and B) biofilm formation measured by biofilm formation index (bar, strain. concentration LSD ¼ 1.575) and plate count cell enumeration (dot, strain.concentration LSD ¼ 0.3584) after 24 h of incubation at 30°C (error bars represent standard errors for three experiments)

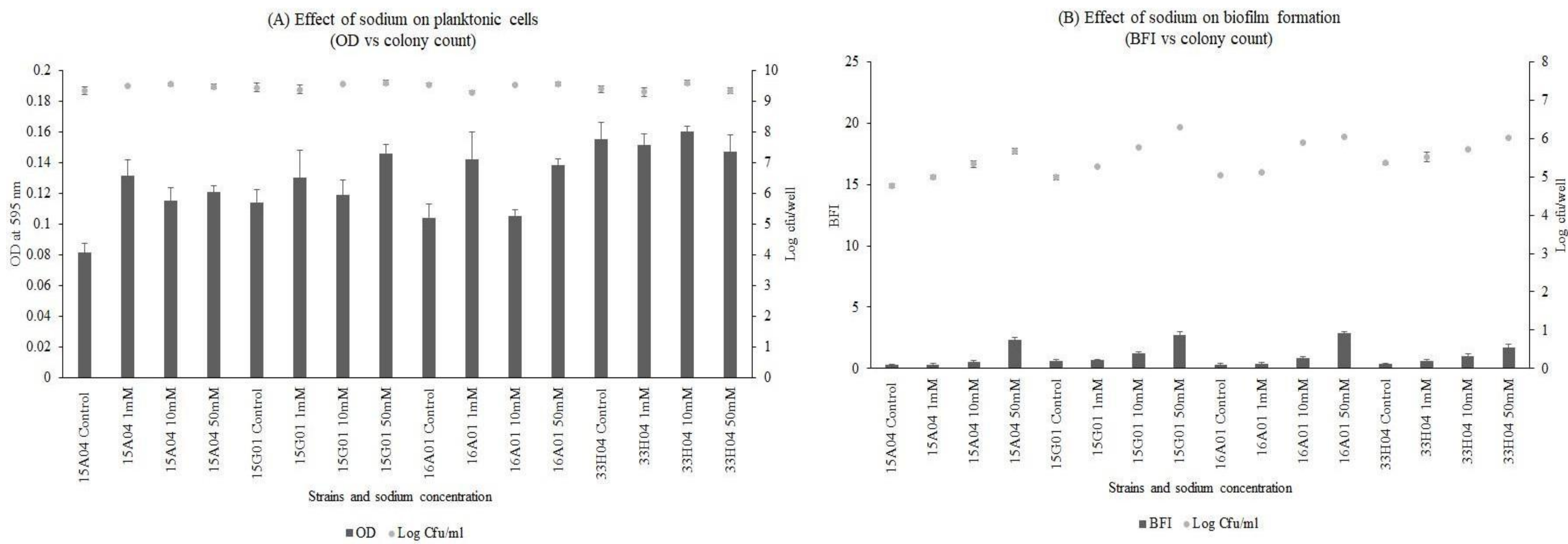


Figure 3.6 - Influence of sodium on A) planktonic cells measured by optical density (bar, strain.concentration LSD (least significant difference) ¼ 0.02822) and plate count cell enumeration (dot, strain.concentration LSD ¼ 0.3777) and B) biofilm formation measured by biofilm formation index (bar, strain. concentration LSD ¼ 1.575) and plate count cell enumeration (dot, strain.concentration LSD ¼ 0.3584) after 24 h of incubation at 30°C (error bars represent standard errors for three experiments)

3.5 Discussion

Listeria monocytogenes poses a serious threat to the food industry and public health. It causes a life-threatening illness known as listeriosis. Almost 99% of listeriosis is caused by contaminated food. The ability of *L. monocytogenes* to persist in food processing facilities and resist cleaning regimes makes it not only difficult to manage but also challenging economically. The persistence of *L. monocytogenes* may be linked to its biofilm-forming capability (Møretrø & Langsrud 2004). Biofilms can form a reservoir of cells able to enter the human food chain so understanding biofilm formation in *L. monocytogenes* is very important. Biofilms are comprised of various components including live and dead cells, and extracellular polymeric substances like carbohydrates, lipids, and eDNA. The composition varies according to the environmental conditions and genetic background of the *L. monocytogenes* strain. In this research project, one environmental factor was studied, cations, examining the effect of magnesium, calcium, and sodium on biofilm formation by *L. monocytogenes*. The effect of these cations on planktonic cells and the initial stages of biofilm formation was also investigated.

Initial adherence to a surface is a crucial step in biofilm formation (Palmer et al. 2007). It involves multiple factors including surface charge, surface roughness, growth medium, hydrophobicity, and motility. In the current study, the effect of magnesium, calcium, and sodium on attachment, hydrophobicity, and motility of *L. monocytogenes* was investigated. In the attachment assay, it was observed that among the three cations, all the isolates were able to attach to a greater extent in the presence of magnesium as opposed to calcium and sodium, which tended to reduce attachment. It is possible that magnesium reduces the repulsive force between the surface and negatively charged groups on the bacterial surface and in the biofilm matrix, thus bringing the cell closer to the surface (Song and Leff 2006). Similar results have been reported by other groups. Dunne and Burd (1992) found that magnesium enhanced the adhesion of *Staphylococcus epidermis* to plastic. Other researchers have also reported that magnesium enhanced the adhesion of *Pseudomonas*

fluorescens and another *Pseudomonas* sp. (Simoni et al. 2000; Song and Leff 2006). Interestingly, the current study found that divalent cations (magnesium and calcium) had differing effects on hydrophobicity and motility compared to the monovalent cation sodium. Cell surface hydrophobicity and motility of the organism play a crucial role in biofilm formation (Lemon et al. 2007; Todhanakasem and Young 2008; Gorski et al. 2009; Krasowska and Sigler 2014). The hydrophobicity of cells will determine their propensity to adhere to a surface. Some studies have reported the impact of change in motility on biofilm architecture (Wood et al. 2006; Guttenplan & Kearns 2013). In the current study it was observed that divalent cations did not have a significant effect while sodium decreased the hydrophobicity. However, for motility it was observed divalent cations decreased the motility of all isolates while the opposite was observed for sodium.

Magnesium, calcium and sodium are involved in many physiological processes. The current study evaluated their effects on planktonic and biofilm cells. The BFI was used to determine biofilm formation using the crystal violet assay. Crystal violet is a basic dye that stains the entire negatively charged biomass and is a good basic screening measure for biofilm formation. Viable cells present in the biofilm are of concern as they are capable of migrating to different surfaces and thus leading to further contamination of surfaces and products. Therefore, the number of viable cells in the biofilm was determined using the drop plate method. Combining these two methods gives an indication of the effect of cations on the growth, viability, and biofilm formation of *L. monocytogenes*. The divalent cations decreased the number of cells in the planktonic state and increased biofilm formation while the monovalent cation sodium had a lesser effect. Similar to our study, other studies have reported the enhancing feature of divalent cations on other organisms. For instance, Cruz et al. (2012) found that calcium enhanced biofilm formation but did not affect the planktonic state of *Xylella fastidiosa*. Similar results were seen in the case of magnesium for *Arthrobacter* sp. SUK 1201, *Sphingomonas paucimobilis*, *Bacillus* sp., *Chlorella*

and *Phaeodactylum tricornutum* (Ceyhan Guvensen et al. 2013; He et al. 2016; Dey & Paul 2018). Two reports found that sodium chloride enhanced biofilm formation by influencing the attachment and stability of the biofilm (Jensen et al. 2007; Xu et al. 2010), which is different to our findings. The influence of the cations appears to differ in their action on the biofilm, depending upon the microorganism, type of cation, and the concentration used.

There are two possible explanations for the different effects of divalent and monovalent cations observed in the current study. The first one uses the Schulze – Hardy rule where divalent cations are more effective than monovalent cations in destabilizing colloidal particles. The theory predicts that, compared with divalent cations, a 4- to 64-fold increase in monovalent cations would be required to completely destabilize a colloidal particle (Kerchove & Elimelech 2008). The second possible explanation is that divalent cations, like magnesium and calcium, enhance cohesiveness, have a higher charge density, and are involved in many functional, structural, and regulatory pathways (Somerton et al. 2015). The bacterial cell wall and the biofilm matrix often contains many negatively charged groups. Divalent cations have a greater capacity to neutralize the negatively charged groups by forming divalent bridges. Divalent cations can bind to regulatory proteins that in return can down- or up-regulate certain pathways, such that there is increased cohesiveness that facilitates formation of the biofilm structure. (Keith Rose & Hogg 1995; Sobeck & Higgins 2002; Jensen et al. 2007; Dobson and O'Shea 2008; Xu et al. 2010; Cruz et al. 2012; Thomas &Rice 2014; Somerton et al. 2015; Choi et al. 2018).

There are several reports on the effect of magnesium, calcium and sodium on the biofilm formation, however none have discussed the effect on *L. monocytogenes* biofilms. This article summarizes how different isolates react to the cations in the external environment at various stages of biofilm formation. These results indicate the importance of ions in *L monocytogenes* biofilm formation. However, the mechanism for the enhancement of biofilm by divalent cations needs further investigation.

3.6 Conclusion

The divalent cations magnesium and calcium enhanced biofilm formation of *L. monocytogenes* substantially more than monovalent sodium. As effects on adhesion and hydrophobicity were minimal, the cations appear to be involved in the later stages of biofilm formation. Since these cations are commonly encountered in seafood processing environments from which the strains were isolated, the current study provides new insight into the effects of these cations on *L. monocytogenes* biofilms and may contribute to developing improved methods to control *L. monocytogenes*. However, further investigation is needed to determine the mechanism for the effect of cations on biofilm formation.

3.7 Acknowledgements

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Chapter 4: Transcriptomic analysis of *Listeria monocytogenes* biofilms in presence of magnesium and sodium

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4.1 Abstract

Listeria monocytogenes is a foodborne pathogen that can adapt to harsh environments and with the ability to form biofilms. This helps *L. monocytogenes* persist in food-processing environments and contaminate food. Cations such as magnesium and sodium are commonly found in seafood-processing environments. Previous studies have demonstrated the differential effects of magnesium and sodium on biofilm formation. However, there is no information available in the literature on how these cations affect the molecular mechanisms underlying *L. monocytogenes* biofilm formation.

As bacteria in a biofilm are constantly adapting to environmental changes. In this study, comparative transcriptomics was used to study the difference between a low biofilm former and a high biofilm former in the presence and absence of magnesium and sodium (50mM). Strains that persist in the mussel industry, one low (15A04) and one high (15G01) biofilm former, were tested for biofilm formation in an in vitro assay. Transcriptomic profiles of the two strains harvested from biofilm with and without cations, were compared. In presence of magnesium various genes relating to the phosphotransferase system, flagellar assembly, chemotaxis and various signal transduction receptors were upregulated. While in the presence of sodium, genes involved in the phosphotransferase system, quorum sensing, and cell wall biosynthesis were affected. These insights into the molecular mechanisms of biofilm formation will provide targets for the development of novel and improved strategies to control *L. monocytogenes* in the food-processing environment.

4.2 Introduction

Listeria monocytogenes is important to the food industry due to its survival and persistence. This psychrotolerant foodborne pathogen causes a potentially severe disease known as listeriosis. It can survive and grow in wide range of conditions and environments thus causing a significant risk to the food industry and consumers. The survivability *of L. monocytogenes* depends on many factors like temperature, pH, salinity, microbiota in the environment and its ability to form biofilm (Mohan et al., 2021).

Biofilms are microorganisms embedded in an extracellular matrix that protects the bacteria from the external environment. This increases the stress tolerance of the microorganisms inside the biofilm, providing greater resistance to antimicrobials compared to those in the planktonic state (Kubota, Senda, Tokuda, Uchiyama, & Nomura, 2009). Biofilm formation is a complex process and the physiology of the bacteria inside the biofilm differs from the planktonic lifestyle. Biofilm changes diversely depending on the physical and chemical environment in which it grows (Folsom et al., 2010). Biofilm formation is a strategy for survival in different environments. This environmental adaptability involves the integration of a wide variety of environmental signals, resulting in profound transcriptional remodelling (Garmyn, Augagneur, Gal, Vivant, & Piveteau, 2012). Bacteria integrate cations as one of these environmental cues to regulate gene expression.

Cations like magnesium and sodium have varied impacts on biofilm formation. For instance, magnesium enhances the adherence to the surfaces by directly influencing the electrostatic interactions or indirectly affecting the physiology-dependent attachment processes of many organisms. For example, attachment of *Pseudomonas sp*. to sand columns (Simoni, Bosma, Harms, & Zehnder, 2000), *S. epidermidis* to plastic (Dunne Jr & Burd, 1992) and *P. fluorescens* to glass (Song & Leff, 2006). However, there are studies that demonstrate that excess magnesium in the environment disrupts biofilm formation. For example, in

Acidithiobacillus ferrooxidans at 0.1 and 0.5M Mg2+, a thinner and looser biofilm is developed and Type IV pilli formation is inhibited, reducing attachment and limiting biofilm growth (Tang et al., 2018). The hydrophobicity of foodborne pathogens like *L. monocytogenes*, *S. aureus*, *S. boydii*, and *S. enterica Typhimurium* decreases with increases in NaCl concentration (4-10%) (Xu, Zou, Lee, & Ahn, 2010). Bereksi, Gavini, Bénézech, and Faille (2002) found at 5% NaCl, the hydrophilic characteristic of *L. monocytogenes* was enhanced. Some authors have also demonstrated denser biofilm formation of *L. monocytogenes* at 2-7% concentrations of NaCl (Jensen, Larsen, Ingmer, Vogel, & Gram, 2007; Pan, Breidt, & Gorisk, 2010).

However, little information is available on the effect of magnesium and sodium on *L. monocytogenes* biofilms. Understanding the changes of *L. monocytogenes* biofilms in the presence of magnesium and sodium remains elusive, as no information is documented in the literature. In our previous studies magnesium increased biofilm formation of *L. monocytogenes* strains, irrespective of their biofilm forming ability. While sodium had a small influence on biofilm formation in contrast to magnesium and calcium at concentrations ranging from 1 mM to 50 mM (Chalke, Vidovic, Fletcher, Palmer, & Flint, 2022). Therefore, the goal of the present study was to understand the mechanisms involved in biofilm formation in the presence of magnesium chloride and sodium chloride at 50mM each. RNA sequencing analysis compared gene expression of a low biofilm former and a high biofilm former with and without the presence of these cations.

4.3 Material and methods

4.3.1 **Bacterial strains and growth condition**

L. monocytogenes PFR15A04 and PFR15G01 isolates from the seafood industry were used in this study. These persistent isolates were selected based on their ability to form biofilms. PFR15A04 (genome sequence accession number: GCA_900162275.1) is a low biofilm former and PFR15G01 (genome sequence accession number: GCA_900162555.1) a high biofilm former from the seafood industry (Chalke et al., 2022; Cruz & Fletcher, 2011). Tryptic Soy Broth (TSB, Difco, BD, USA) with yeast extract (Condalab) (TSBYE) and Tryptic Soy Agar (Difco, BD, USA) with yeast extract (Condalab) (TSAYE) were used as the culture media throughout the study. The isolates were retrieved from the stock culture stored in TSBYE with 20% glycerol at -80°C. The isolates were grown in TSBYE (10mL) overnight at 37°C. These were then streaked on TSAYE plates and incubated overnight at 37°C for 24h. Single colonies from the plates were inoculated into 10% TSB media, incubated overnight at 30°C and used for subsequent experiments.

4.3.2 **Biofilm formation**

The biofilms were grown in 96 well plates (Falcon®, Corning, In vitro technologies) as previously described (Djordjevic et al. 2002) with some modifications. The overnight grown cultures for both the isolates were centrifuged (12,000 g for 20 min) and inoculated into 10% TSB containing 50 mM magnesium and 50mM sodium each in as ratio of 1:100. Briefly, 2 µL aliquots of each overnight-grown culture were transferred to 96-well plates (polystyrene) with each well containing 198 µL media. For this experiment 10% TSB was used as a control and 10% tryptic soy broth (TSB) with magnesium chloride (MgCl2) and 10% tryptic soy broth (TSB) with sodium chloride (NaCl) at 50 mM concentration was used as a treatment.

The cultures were incubated at 30°C for 24 h. After incubation, the plates were washed three times with 200 µL sterile water using a microplate strip washer (ELx50, Biotek). The plates were air-

dried for 30 min. Three biological triplicates of 6 wells for each treatment were used in this study.

4.3.3 **Total RNA isolation**

Total RNA from the biofilm cells was extracted using TRIzol reagent with PureLink RNA Mini kit according to the manufacturer's manual with modification. Briefly, 1 mL of TRIzol reagent was added across the 6 wells and the wells were scraped using sterile scrapers to detach the sessile cells. The content from all the wells was combined in a 1.5 mL microcentrifuge tube. Chloroform (200 µL) was added to the tube. The mixture was shaken vigorously by hand for 15 s and incubated at room temperature for 3 min. After incubation, the protocol was followed as described in the PureLink RNA Mini Kit manual for binding, washing and elution. The total RNA was eluted in 50 µL RNase-free water. The quality and quantity of the RNA was determined using a Nanodrop 2000 spectrophotometer (Thermoscientific) and 2100 Bioanalyzer Instrument (Agilent Technologies). The RNA integrity number (RIN) value was set above 7 for all the samples analysed on the bioanalyzer instrument.

4.3.4 **RNA sequencing**

The RNA samples were sequenced at the University of Auckland genomic facility (Auckland, New Zealand). The libraries were prepared using the Zymo-seq Ribo free total RNA library kit (Zymo research) according to the manufacturer's instructions. Sequencing was preformed using the HiSeq 2500 (Illumina) generating 150 bp paired end sequence data.

4.3.5 **Bioinformatics and statistical analysis**

The sequences were quality checked and filtered using Cutadapt (NBIS (National Bioinformatics Infrastructure Sweden)) (Martin, 2011). The criteria for removal of bases/reads were Phred scores <30. The quality of sequence data was checked using FastQC before and after performing quality checks. The trimmed sequences were mapped to the *L. monocytogenes* genome (GenBank

assembly accession: GCA_900162555.1 – 15G01 and GCA_900162275.1 – 15A04) using HISAT2 (Kim, Langmead, & Salzberg, 2015). FeatureCounts was used to quantify the number of reads mapped to each gene (Liao, Smyth, & Shi, 2013). DESeq2 from the Bioconductor software package was used to analyse differentially expressed genes. The differential expressed genes were determined using criteria of $log_2FC >1$ & $log_2FC > -1$ to be filtered as two-fold upregulated and two-fold down regulated genes respectively.

4.3.6 **Functional annotation**

The functional annotation was performed using DAVID Bioinformatics Resources version 2021 (Sherman et al., 2022). The locus tag for both the strains of the upregulated and downregulated DEGs were submitted to DAVID as a list. All three directs (BP, CC, MF) from gene ontology and the KEGG_PATHWAY were selected. The thresholds were set to a count of 5 and EASE of 0.05. Fisher's exact test, fold enrichment and FDR were applied for statistical purposes (Jiao et al., 2012).

4.4 Results and discussion

4.4.1 **Quantitative analysis of gene expression**

The overall gene expression profile of *L. monocytogenes* biofilms with and without the presence of cations, was examined using RNA-seq. The Hi-seq Illumina platform was used to create raw sequence reads with an average of 11,577,895 for control, 11,998,395 for magnesium treated biofilm and 12,418,234 for sodium treated biofilm. After trimming, and filtering, the clean reads were mapped to the reference genome (*L. monocytogenes* EGD-e – Taxonomy ID:169963) for each sample (Appendix Table A4.1-4.4). The mapping rate was around 94-95%. A total of 2702 genes were assigned to these reads.

4.4.2 **Transcriptional differences between a high and a low biofilm former strain of *L. monocytogenes* in the presence and absence of cations**

The transcriptional difference between the two strains was indicated using a $\log_2$ fold change ($\log_2$FC) throughout the study. In absence of cations (Figure 4.1) a total 407 (15.06%) of 2702 genes were found significantly ($p < 0.05$) expressed in the high biofilm former (15G01) compared to the low biofilm former (15A04). Of these, 191 genes were upregulated ($\log_2$FC > 1, FDR <0.05) and 216 genes were down regulated genes ($\log_2$FC < -1, FDR <0.05) in 15G01 compared to the 15A04 strain.

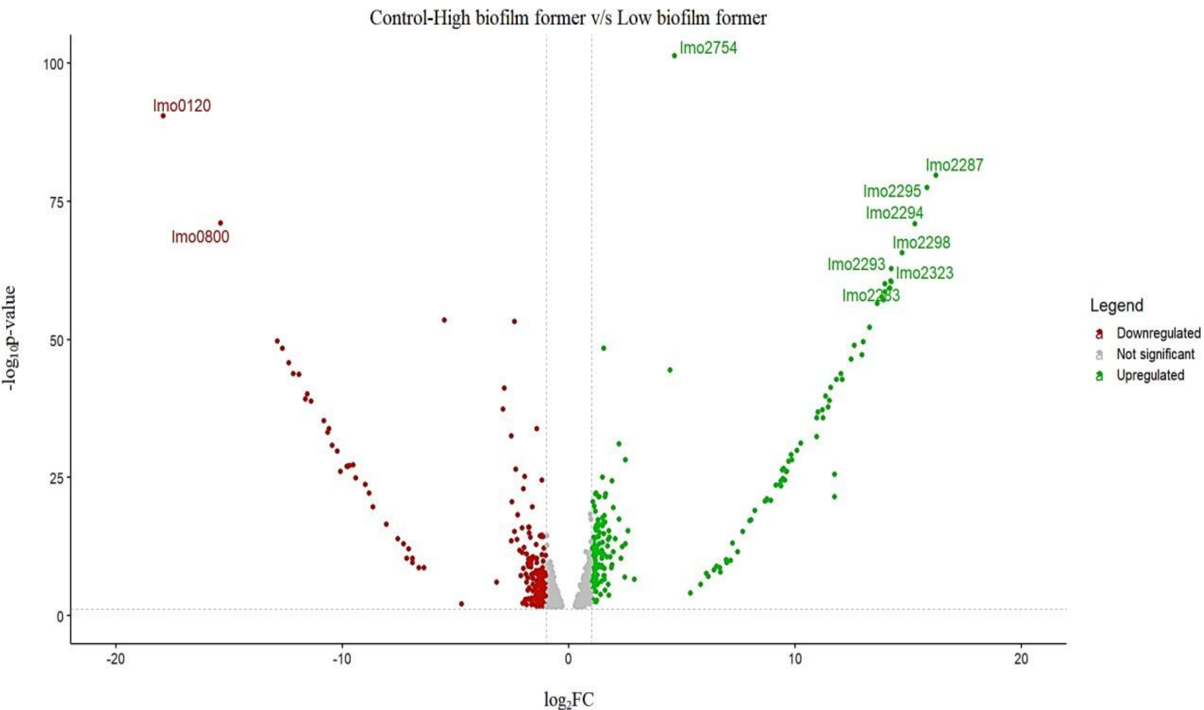

Figure 4.1- Volcano plot for global comparison of transcription profile between high and low biofilm former in absence of cations. The down-regulated genes ($\log_2$FC < -1) are shown as red dots; the up-regulated genes ($\log_2$FC > 1) are shown as green dots; genes that were significantly differentially expressed genes but not identified are shown as grey dots.

In the presence of 50mM magnesium (Figure 4.2), a total of 537 (19.87%) of genes were found to be significantly ($p < 0.05$) expressed. Of these, 253 genes were upregulated (log2FC > 1, FDR <0.05) and 284 genes were down regulated (log2FC < -1, FDR <0.05). In both conditions, the

majority of the genes were downregulated in 15G01, indicating that strain 15G01 is more metabolically stable compared to the low biofilm former.

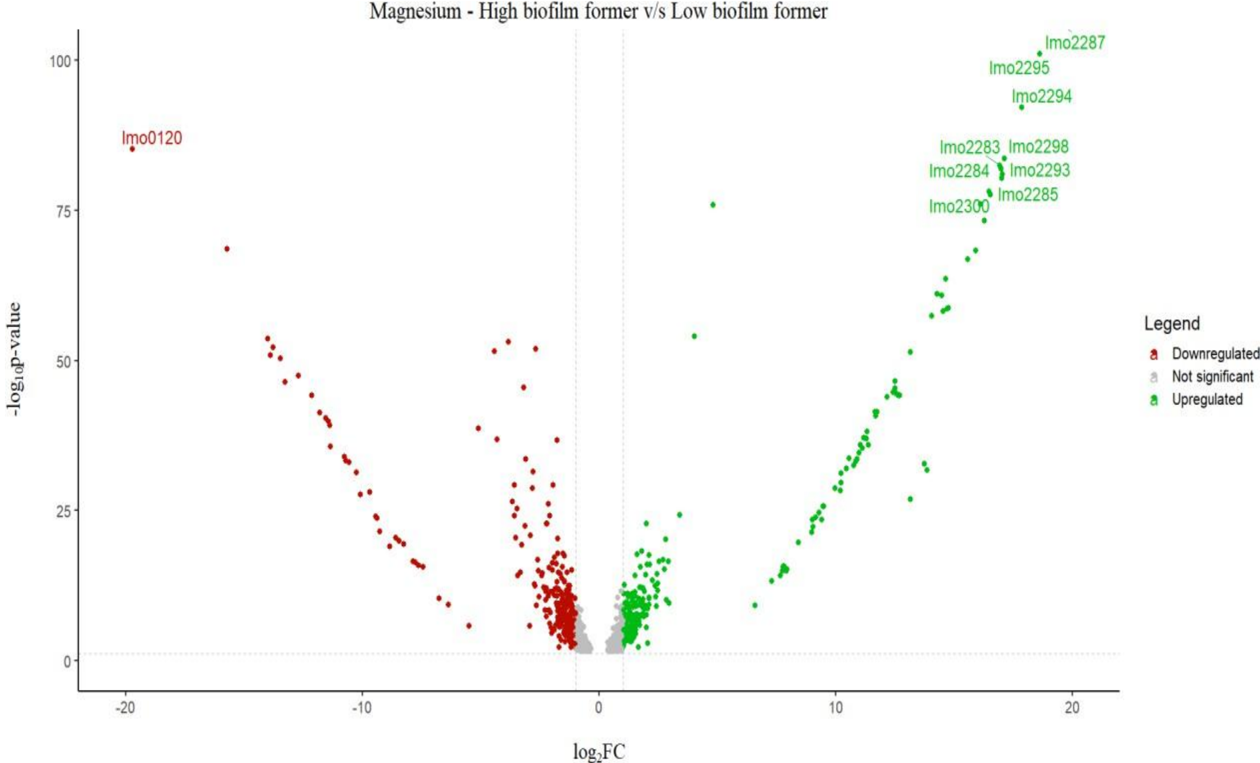

Figure 4.2- Volcano plot for global comparison of transcription profile between high and low biofilm former in presence of magnesium. The down-regulated genes ($log_2FC < -1$) are shown as red dots; the up-regulated genes ($log_2FC > 1$) are shown as green dots; genes that were significantly differentially expressed genes but not identified are shown as grey dots.

In the presence of 50mM sodium (Figure5.2 4.3), a total of 271 (10.02%) of genes were found significantly ($p < 0.05$) expressed. Of these, 178 genes were upregulated (log2FC > 1, FDR <0.05) and 93 genes were down regulated (log2FC < -1, FDR <0.05) in 15G01 compared with 15A04. In the presence of sodium compared to the control, most of the upregulated genes were in 15G01, indicating that strain 15G01 is more metabolically active compared to the low biofilm former.

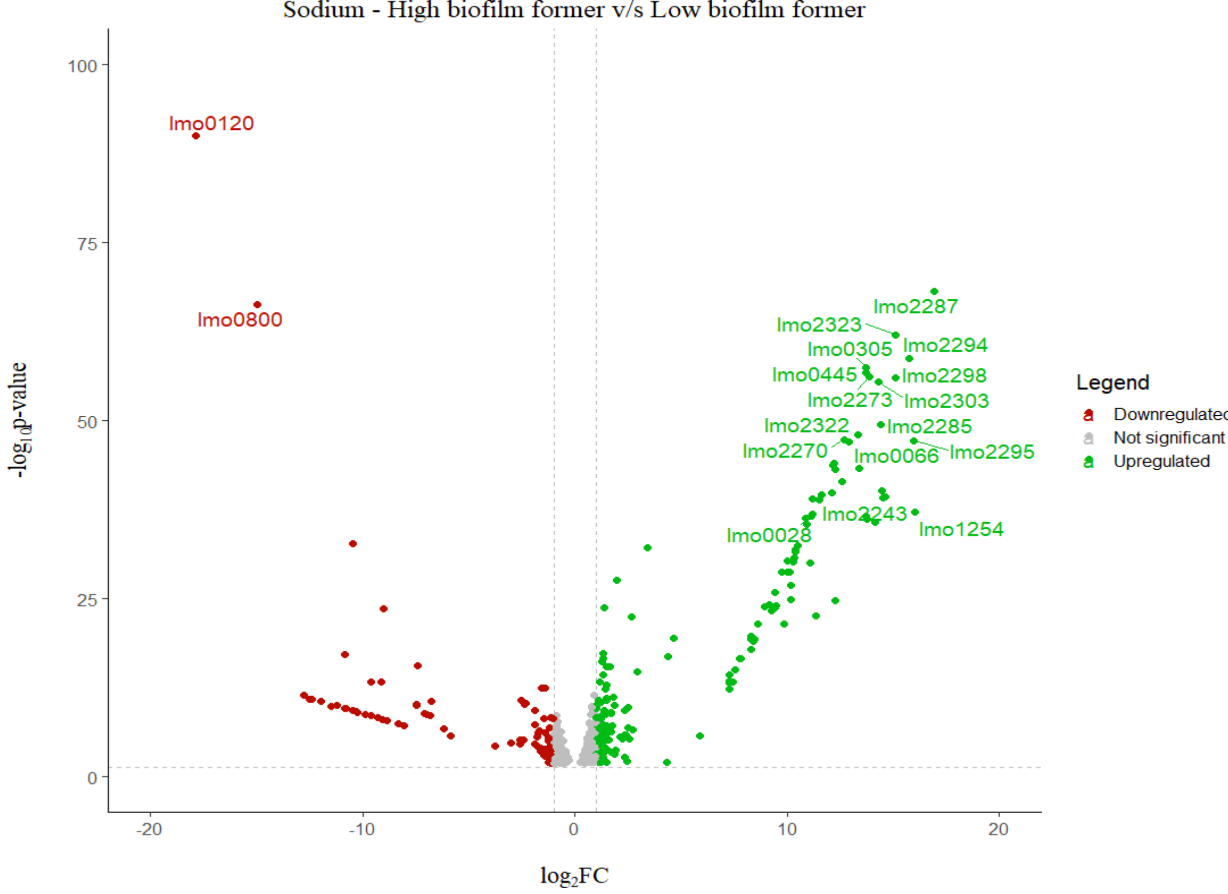

Figure 4.3- Volcano plot for global comparison of transcription profile between high and low biofilm former in the presence of sodium. The down-regulated genes ($log_2FC < -1$) are shown as red dots; the up-regulated genes ($log_2FC > 1$) are shown as green dots; Genes that were significantly differentially expressed genes but not identified are shown as grey dots.

The difference between the up and down regulated genes was further distinguished by examining these groups independently in terms of the GO (Gene Ontology) biological process. In absence of cations (control) - processes related to cobalamin biosynthesis (GO:0009236) and the phosphoenolpyruvate-dependent sugar phosphotransferase system (GO:0009401) were downregulated. While a small percentage of the differentially expressed genes related to the threonine catabolic process (GO:0006567) and the phosphoenolpyruvate-dependent sugar phosphotransferase system (GO:0009401) upregulated in 15G01.

In presence of magnesium, most of the downregulated genes in 15G01, were involved in a wide

range of processes including negative regulation of transcription DNA- template (GO:0045892), SOS response (GO:0009432), nucleotide-excision repair (GO:0006289), protein refolding (GO:0042026) and regulation of transcription, DNA- template (GO:0006355) response. Whereas processes like the 'de novo' IMP biosynthetic process (GO:0006189), the phosphoenolpyruvate-dependent sugar phosphotransferase system (GO:0009401), the Mo- molybdopterin cofactor biosynthetic process (GO:0006777), the glycolytic process (GO:0006096), gluconeogenesis (GO:0006094), the carbohydrate derivative transport (GO:1901264), the glycerol catabolic process (GO:0019563), bacterial-type flagellum- dependent cell motility (GO:0071973) and chemotaxis (GO:0006935) were all upregulated.

In the presence of sodium, processes like phosphoenolpyruvate-dependent sugar phosphotransferase system (GO:0009401), pyridoxal phosphate biosynthetic process (GO:0042823), and quorum sensing (GO:0009372) were upregulated. While processes like carbohydrate derivative transport (GO:1901264), pentose-phosphate shunt, non-oxidative branch (GO:0009052), lipoteichoic acid biosynthetic process (GO:0070395), ATP binding (GO:0005524), metal ion binding (GO:0046872) were downregulated.

4.4.3 **The phosphoenolpyruvate phosphotransferase system (PTS)**

The PTS system (KEGG: ko02060) is a major mechanism utilized by bacteria for carbohydrate uptake and is highly conserved in almost all bacteria. It is responsible for the phosphorylation and translocation of various monosaccharides, disaccharides, polyols, amino sugar and other sugar derivative across the cell membrane (Deutscher, Francke, & Postma, 2006). It plays an important role in biofilm formation of various organisms like *Vibrio cholerae*, *Klebsiella pneumoniae*, *Enterococcus faecium*, *Streptococcus gordonii*, *P. aeruginosa* and *Lactococcus lactis* (Barrière et al., 2005; Cabeen, Leiman, & Losick, 2016; Horng et al., 2018; Houot, Chang, Absalon, & Watnick, 2010; Loo, Mitrakul, Voss, Hughes, & Ganeshkumar, 2003; Paganelli et al., 2016; M.-

C. Wu, Chen, Lin, Hsieh, & Wang, 2012). The system consists of histidine protein (HPr) and phosphoenolpyruvate-protein phosphotransferase or enzyme I (EI) that are non-substrate specific proteins and a substrate specific (EII) transporter (Houot et al., 2010; J. Wu, McAuliffe, & O'Byrne, 2023). The substrate specific transporter consists of three or four functional groups - EIIA, EIIB, EIIC, and EIID and is classified into seven protein families - PTSGlc, PTSFru, PTSAsc PTSGut, PTSGat, PTSMan and PTSLac (Suo, Gao, Baranzoni, Xie, & Liu, 2018). The phosphate enters through the EI in the PTS system and passes through Hpr. The Hpr then transfers it to EII and carries translocation and phosphorylation to specific PTS substrates. The transport of these substrates rapidly decreases the phosphorylated intermediates; thus, the phosphorylation state of PTS components acts as cytoplasmic reporters that indicate the availability of environmental nutrients. These reporters transform cellular processes like chemotaxis, catabolism of carbohydrate and glycogen breakdown (Houot et al., 2010).

The *L. monocytogenes* genome comprises a large number (n = 86) of PTS genes representing 29 complete PTS's components and numerous orphan PTS components (Wu et al., 2023). In the present study, the expression patterns of the various PTS genes were cation and strain specific. In absence of any cations (control) strain 15G01 exhibited upregulation of genes related to mannose, beta-glucoside and galacticol subunits while 15A04 displayed downregulation of numerous genes related to cellobiose, trehalose, beta-glucoside, and fructose transporter along with *bglG* (transcriptional antiterminator). While in presence of magnesium, wide spectrum of PTS genes had enhanced upregulations compared to control for cellobiose, mannose and beta-glucoside. Additionally, MFS transporters, ascorbate transporters and lichenan transporters were also activated. Thus, suggesting presence of magnesium might positively regulate the PTS gene expression enhancing serval pathways important for carbohydrate metabolism and biofilm matrix. In contrast, in presence of sodium strain 15G01 displayed a mixed response. Although mannose (lmo2000, lmo2001, lmo2002) and cellobiose (lmo2762, lmo2782, lmo2783) remained

upregulated, an extensive range of downregulation was observed for sugar transporters like galacticol and especially lmo0027 (beta-glucoside) with a log_2FC of -4.05. for 15A04 strain in presence of sodium most of the genes demonstrated downregulation. Thus, it suggests that sodium appears to repress PTS system across both the strains particularly affecting the galacticol, beta-glucoside, and cellobiose systems (Table 4.3). Similar sets of genes related to PTS were reported to be upregulated in *L. monocytogenes* biofilm in response to Lactocin AL705 at 10°C (Melian, Castellano, Segli, Mendoza, & Vignolo, 2021). These then feed into other pathways like glycolysis, amino sugar metabolism or biosynthesis of secondary metabolites, thus serving as a specific precursor for the other functional groups.

PTS components also affect the expression of virulence genes. According to Li et al. (2022), PTS affects the *prfA*, virulence gene regulator by altering the carbon utilization of bacteria influencing *prfA* expression. PrfA activity is much higher in the presence of non-PTS carbon substrate like glycerol, thus PTS are considered to be inactive during the intracellular growth and expression of virulence (Stoll et al., 2008; Stoll & Goebel, 2010; Freeman et al., 2025). In the present study it was observed that the expression of the *prfA* gene (lmo0200) was upregulated by 1.13 log_2FC in 15A04 in the presence of magnesium. However, no PTS related genes were observed in 15A04 in presence of magnesium, thereby supporting the previous reports. Another study demonstrated that deletion in *bglG* gene leads to reduced virulence in *L. monocytogenes*, in the present work *bglG* gene was found to be downregulated in control conditions (Abdelhamed et al., 2019). PTS involvement in virulence of other bacterial species is also well documented (Kok, Bron, Erni, & Mukhija, 2003; Larsen, Kallipolitis, Christiansen, Olsen, & Ingmer, 2006; Poncet et al., 2009; Stülke, 2007; M.-C. Wu et al., 2012).

The EII_t^{Man} permease is a part of phosphotransferase system (PTS) encoded by mannose permease two (mpt) operon. It is mainly responsible for uptake of glucose but it also plays an important role

in glucose-mediated carbon catabolite repression (CCR) and downregulation of virulence gene expression. The mpt operon contains three genes, which encode the $IIAB^{Man}$ (*mptA*), IIC^{Man} (*mptC*), and IID^{Man} (*mptD*) subunits of the transporter. The *prfA* controls cluster of virulence genes such as *hly*, encoding listeriolysin O, and *plcA* and *plcB*, which encode two phospholipase C enzymes which are essential for assisting escape of internalized bacteria from the phagocytic vacuole of the host cell. In addition, PrfA positively regulates its own expression (*prfA*) as well as *actA*, which encodes the ActA protein required for actin-based intracellular motility and cell-to-cell spread (Scortti et al., 2007; Milohanic et al., 2003). Vu-Khac and Miller (2009) reported that in *mpt* deletion mutant, mRNA levels were two to four-fold higher in the five genes (*prfA, plcA, hly, actA,* and *plcB*) in the prfA gene clusters compared to EGD-e strain. In the present study, for strain 15G01 in presence of magnesium, the EII_t^{Man} .i.e. PTS mannose transporter subunit IIAB(lmo0096), PTS mannose transporter subunit IIC(lmo0097) and PTS mannose transporter subunit IID(lmo0098) were upregulated by log_2FC 1.93, 1.67 and 1.67 respectively. It was also observed that the similar genes reported in Vu-Khac and Miller (2009) i.e. *hly* (-2.01), *plcA* (-2.27), *plcB* (-1.13) and *actA* (-1.49) were downregulated in presence of magnesium for 15G01. Thus, suggesting EII_t^{Man} assisted in reducing the activity of these virulence-related genes. These findings suggest a strain-specific regulatory response under similar conditions, wherein strain 15A04 appears to activate virulence gene expression potentially due to the absence of PTS gene expression, while strain 15G01 expresses specific PTS components—particularly the mannose-specific permease, which may contribute to the repression of virulence gene transcription.

Strain	Locus tag	Product	Log2FC
15G01	LMO0374	PTS beta-glucoside transporter subunit IIB(lmo0374)	2.904814
	LMO2001	PTS mannose transporter subunit IIC(lmo2001)	1.004635
	LMO0097	PTS mannose transporter subunit IIC(lmo0097)	1.42874
	LMO0098	PTS mannose transporter subunit IID(lmo0098)	1.688728
	LMO0373	PTS beta-glucoside transporter subunit IIC(lmo0373)	2.468139
	LMO2099	transcriptional antiterminator(lmo2099)	2.220089
	LMO2097	PTS galacticol transporter subunit IIB(lmo2097)	1.91253
	LMO0096	PTS mannose transporter subunit IIAB(lmo0096)	1.565666
	LMO2096	PTS galacticol transporter subunit IIC(lmo2096)	1.815617
15A04	LMO1997	PTS mannose transporter subunit IIA(lmo1997)	1.252159
	LMO2762	PTS cellbiose transporter subunit IIB(lmo2762)	-2.10466
	LMO2685	PTS cellbiose transporter subunit IIA(lmo2685)	-1.42634
	LMO1255	PTS trehalose transporter subunit IIBC(lmo1255)	-2.86046
	LMO2783	PTS cellbiose transporter subunit IIC(lmo2783)	-1.43342
	LMO2684	PTS cellbiose transporter subunit IIC(lmo2684)	-1.44294
	LMO2683	PTS cellbiose transporter subunit IIB(lmo2683)	-1.15669
	LMO2668	transcriptional antiterminator BglG(lmo2668)	-1.09765
	LMO0301	PTS beta-glucoside transporter subunit IIA(lmo0301)	-1.65082
	LMO2666	PTS galacticol transporter subunit IIB(lmo2666)	-1.11948
	LMO2765	PTS cellbiose transporter subunit IIA(lmo2765)	-1.12962
	LMO0399	PTS fructose transporter subunit IIB(lmo0399)	-1.90607
	LMO2763	PTS cellbiose transporter subunit IIC(lmo2763)	-1.67249

Table 4.1- PTS (phosphoenolpyruvate phosphotransferase system) associated genes in 15A04 and 15G01 in absence of cations

Strain	**Locus tag**	**Product**	**Log2FC**
15G01	LMO0374	PTS beta-glucoside transporter subunit IIB(lmo0374)	2.055348
	LMO0022	PTS fructose transporter subunit IIB(lmo0022)	1.052247
	LMO2684	PTS cellbiose transporter subunit IIC(lmo2684)	1.474121
	LMO2783	PTS cellbiose transporter subunit IIC(lmo2783)	2.051083
	LMO0782	PTS mannose transporter subunit IIC(lmo0782)	1.059951
	LMO2782	PTS cellbiose transporter subunit IIB(lmo2782)	2.948986
	LMO2683	PTS cellbiose transporter subunit IIB(lmo2683)	1.318598
	LMO2650	MFS transporter(lmo2650)	2.047734
	LMO0097	PTS mannose transporter subunit IIC(lmo0097)	1.677497
	LMO0373	PTS beta-glucoside transporter subunit IIC(lmo0373)	1.367022
	LMO0098	PTS mannose transporter subunit IID(lmo0098)	1.676074
	LMO0096	PTS mannose transporter subunit IIAB(lmo0096)	1.931987
	LMO1719	PTS lichenan transporter subunit IIA(lmo1719)	1.895755
	LMO0507	PTS galactitol transporter subunit IIB(lmo0507)	1.027408
	LMO2708	PTS cellbiose transporter subunit IIC(lmo2708)	2.540315
	LMO2649	PTS system ascorbate transporter subunit IIC(ulaA)	2.486761
	LMO0301	PTS beta-glucoside transporter subunit IIA(lmo0301)	1.231687
	LMO1720	PTS lichenan transporter subunit IIB(lmo1720)	1.89361
	LMO0027	PTS beta-glucoside transporter subunit IIABC(lmo0027)	1.361014
	LMO0398	PTS sugar transporter subunit IIA(lmo0398)	1.942094
	LMO0299	PTS beta-glucoside transporter subunit IIB(lmo0299)	1.041993
	LMO0783	PTS mannose transporter subunit IIB(lmo0783)	1.023607
	LMO0784	PTS mannose transporter subunit IIB(lmo0784)	1.042283

Table 4.2- PTS (phosphoenolpyruvate phosphotransferase system) associated genes in 15A04 and 15G01 in presence of magnesium

Strain	Locus tag	Product	Log2FC
15G01	LMO2762	PTS cellbiose transporter subunit IIB(lmo2762)	2.231943
	LMO1255	PTS trehalose transporter subunit IIBC(lmo1255)	2.776487
	LMO2002	PTS mannose transporter subunit IIB(lmo2002)	1.197788
	LMO2783	PTS cellbiose transporter subunit IIC(lmo2783)	1.585796
	LMO2001	PTS mannose transporter subunit IIC(lmo2001)	1.395229
	LMO2782	PTS cellbiose transporter subunit IIB(lmo2782)	1.013289
	LMO2000	PTS mannose transporter subunit IID(lmo2000)	1.43529
	LMO2708	PTS cellbiose transporter subunit IIC(lmo2708)	1.140964
	LMO1997	PTS mannose transporter subunit IIA(lmo1997)	1.320438
	LMO0301	PTS beta-glucoside transporter subunit IIA(lmo0301)	1.701631
	LMO2763	PTS cellbiose transporter subunit IIC(lmo2763)	1.411986
	LMO0916	PTS sugar transporter subunit IIA(lmo0916)	-1.47933
	LMO0098	PTS mannose transporter subunit IID(lmo0098)	-1.24926
	LMO2098	PTS galacticol transporter subunit IIA(lmo2098)	-1.35572
	LMO0914	PTS sugar transporter subunit IIB(lmo0914)	-1.10379
	LMO2097	PTS galacticol transporter subunit IIB(lmo2097)	-1.37328
	LMO0915	PTS sugar transporter subunit IIC(lmo0915)	-1.51737
	LMO1095	PTS cellbiose transporter subunit IIB(lmo1095)	-1.12103
	LMO2095	phosphofructokinase(lmo2095)	-1.13706
	LMO2667	PTS galacticol transporter subunit IIA(lmo2667)	-2.19463
	LMO2666	PTS galacticol transporter subunit IIB(lmo2666)	-2.11567
	LMO0027	PTS beta-glucoside transporter subunit IIABC(lmo0027)	-4.05307
	LMO2665	PTS galacticol transporter subunit IIC(lmo2665)	-2.76927
15A04	LMO2685	PTS cellbiose transporter subunit IIA(lmo2685)	-2.25417
	LMO2684	PTS cellbiose transporter subunit IIC(lmo2684)	-2.19832

Strain	Locus tag	Product	Log2FC
	LMO2683	PTS cellbiose transporter subunit IIB(lmo2683)	-1.93409
	LMO2098	PTS galacticol transporter subunit IIA(lmo2098)	-1.0352
	LMO0916	PTS sugar transporter subunit IIA(lmo0916)	-1.73698
	LMO0915	PTS sugar transporter subunit IIC(lmo0915)	-1.69387
	LMO0901	PTS cellbiose transporter subunit IIC(lmo0901)	-1.05557
	LMO2667	PTS galacticol transporter subunit IIA(lmo2667)	-3.1316
	LMO2666	PTS galacticol transporter subunit IIB(lmo2666)	-3.26266
	LMO0027	PTS beta-glucoside transporter subunit IIABC(lmo0027)	-4.5099
	LMO0398	PTS sugar transporter subunit IIA(lmo0398)	-1.57786
	LMO2665	PTS galacticol transporter subunit IIC(lmo2665)	-3.97571
	LMO0399	PTS fructose transporter subunit IIB(lmo0399)	-2.55378

Table 4.3- PTS (phosphoenolpyruvate phosphotransferase system) associated genes in 15A04 and 15G01 in presence of sodium

4.4.4 **Two-component signalling systems (TCSs)**

Two-component signalling systems (TCSs) are the key mechanisms that help bacteria adapt to various stressful environments that they experience in nature or food processing environments. The TCS system consists of a transmembrane sensor histidine kinase (HK) and a cytoplasmic response regulator (RR). From the external environment, HK permits the bacteria to sense the signals and then transfer that signal to the RR through the phosphorylation cascade inside the cell. In the presence of specific stimulus or stress factors, the HK gets autophosphorylated in the HK dimerization domain. In response to the regulator, the phosphoryl group is then transferred from kinase to a conserved aspartic acid residue. This response modifies the DNA- binding capability, causing upregulation or downregulation of genes (Kallipolitis, Ingmer, Gahan, Hill, & Søgaard-Andersen, 2003;

Pöntinen, Markkula, Lindström, & Korkeala, 2015). In *E. coli* the sensor kinase regulator system *kdpD/kdpE* regulates the *kdpFABC* operon that is a part of the TSC system (Suo et al., 2018). In this study *kdpA* (lmo0682), *kdpB* (lmo0681) and *kdpC* (lmo0680) were upregulated in the presence of magnesium in 15A04 (2.09, 2.17, 1.35 log_2FC) and in 15G01 (1.06, 1.35, 0.74 log_2FC) respectively. Another histidine kinase sensor *agrB* was upregulated by 1.11 log_2FC in 15A04 in the control. The agrB is one of the four components of the agr system (agrA, agrB, agrC and agrD) that are involved in quorum sensing (Zhang, Gray, Novick, & Ji, 2002). Another important two component system that *L. monocytogenes* uses is DegU, a pleiotropic regulator that is required for biofilm formation, motility, chemotaxis, heat resistance and virulence (Cheng et al., 2021; Gueriri et al., 2008). In the present study, *degU* was upregulated significantly in 15A04 by 1.03-fold in the presence of magnesium. In this study, upregulation of genes associated with TCS's was seen in both of the strains. This suggests that magnesium maintains the TCS in *L. monocytogenes*, causing effective signalling between the cells, thus enhancing biofilm formation.

4.4.5 **Cobalamin-dependent gene cluster**

The most notable downregulated gene expression in the absence of cations in 15G01 compared to 15A04 was the Cobalamin-Dependent Gene Cluster (CDGC). The CDGC is well conserved in *L. monocytogenes* and is involved in catabolism of ethanolamine (*eut* genes), 1,2-propanediol (*pdu* genes) and the biosynthesis and import of cobalamin (*cbi/cob* genes). The *eut* operon is a two-component response regulator (EutV/EutW) regulated by cobalamin-dependent riboswitch (Rli55). The second *pdu* operon is centrally regulated by PocR, which is finely tuned by another cobalamin-dependent riboswitch (Rli39) (J. M. Anast & Schmitz-Esser, 2020; S. Tang et al., 2015). In this study, 26 genes, mainly related to *pdu* genes, and the cobalamin operon, were downregulated in the absence of cations in

15G01 (Table 4.3). Previous studies have demonstrated utilization of CDGC in stress conditions helps survival of *L. monocytogenes* (J. M. Anast & Schmitz-Esser, 2020; Fox, Leonard, & Jordan, 2011; S. Tang et al., 2015). Limited information is available on the role of CDGC on biofilm formation. CDGC has previously been reported to play a role in virulence in other organisms like *E. coli* (Kendall, Gruber, Parker, & Sperandio, 2012; Luzader, Clark, Gonyar, & Kendall, 2013), *Salmonella* (Anderson, Clark, Adli, & Kendall, 2015), *Citrobacter rodentium* (Rowley, Sauder, & Kendall, 2020) and *L. monocytogenes* (Justin M. Anast, Bobik, & Schmitz-Esser, 2020; Vásquez et al., 2022). In this study, it was observed that addition of magnesium upregulated an acetate kinase gene (*pduW),* by 11.05, log_2FC. The final product of the *eut* operon is acetate. Acetate is shown to modulate the immune system by supressing IgA production in the gastrointestinal tract, thus disrupting the host immune response. However, this needs to be experimentally validated (J. M. Anast & Schmitz-Esser, 2020; Kaval & Garsin, 2018).

Locus_Tag	**Product**	**Gene name**	**Log₂FC**
lmo1142	Cob(III)alamin reductase @ Cob(II)alamin reductase	*pduS*	-1.2239
lmo1143	Propanediol utilization polyhedral body protein	*pduT*	-1.0109
lmo1145	Propanediol utilization protein	*pduV*	-1.1511
lmo1146	Predicted alpha-ribazole-5-phosphate synthase CblS for cobalamin biosynthesis		-1.3972
lmo1147	Adenosylcobinamide-phosphate guanylyltransferase	*cobU/copB*	-1.0362
lmo1149	Alpha-ribazole-5'-phosphate phosphatase		-1.3141
lmo1150	Propanediol utilization transcriptional activator	*pocR*	-1.1284
lmo1151	Propanediol utilization polyhedral body protein	*pduA*	-1.4211
lmo1153	Propanediol dehydratase large subunit	*pduC*	-1.9121
lmo1154	Propanediol dehydratase medium subunit	*pduD*	-1.8563
lmo1155	Propanediol dehydratase small subunit	*pduE*	-2.0371

Locus_Tag	**Product**	**Gene name**	**Log_2FC**
lmo1156	Propanediol dehydratase reactivation factor large subunit	*pduG*	-1.4633
lmo1157	Propanediol dehydratase reactivation factor small subunit	*pduH*	-1.6388
lmo1158	Propanediol utilization polyhedral body protein	*pduK*	-1.6467
lmo1160	Propanediol utilization protein	*pduL*	-1.4562
lmo1161	Ethanolamine utilization protein	*eutL*	-1.489
lmo1164	Cob(I)alamin adenosyltransferase	*pduO*	-1.3635
lmo1165	CoA-acylating propionaldehyde dehydrogenase	*pduP*	-1.0723
lmo1171	Alcohol dehydrogenase	*pduQ*	-1.1412
lmo1192	Adenosylcobinamide-phosphate synthase	*cobD*	-1.3609
lmo1193	Cobalt-precorrin-8x methylmutase	*cbiC*	-1.2916
lmo1194	Cobalt-precorrin-6 synthase, anaerobic	*cbiD*	-1.2386
lmo1195	Cobalt-precorrin-6y C5-methyltransferase	*cbiE*	-1.392
lmo1196	Cobalt-precorrin-6y C15-methyltransferase [decarboxylating]	*cbiT*	-1.2921
lmo1198	Cobalamin biosynthesis protein CbiG	*cbiG*	-1.2585
lmo1199	Cobalt-precorrin-3b C17-methyltransferase	*cbiH*	-1.2971

Table 4.4- Genes associated to Cobalamine dependent cluster genes in 15G01 in absence of cations.

4.4.6 **Flagellar assembly and bacterial chemotaxis**

Motility is a crucial aspect of biofilm formation. In Gram-positive bacteria like *L. monocytogenes,* the flagellum is composed of three major parts: the flagella filament, the hook complex, and the basal body. This assembly intersects with the bacterial cell membrane and the flagella-associated cytoplasmic ring. Flagella-related proteins are transported through the basal body to the outside of the cell, where they are assembled into the helical hook and filament complexes. Bacterial motility is regulated by flagella in accordance with

the external environment (Cheng et al. 2018). Chemotaxis is the movement of the bacteria in response to certain chemicals in the environment (Suo et al., 2018). Several genes involved in the signalling cascade of bacterial chemotaxis and flagella assembly were upregulated in 15G01 biofilm in the presence of magnesium (Table 4.4). Attached to the basal body are the stator complexes made up of MotA and MotB. MotA is a membrane protein that interacts with FliG and MotB is a membrane protein that attaches to peptidoglycan. MotA and MotB combine to form an ion channel and the flow of ions causes a conformational change in MotA, which interacts with FliG to produce a torque. In the present study we found that the *motA* and *fliG* were upregulated (1.33 and 1.28 log_2FC), thus affecting the swimming capability of the bacterium. Another gene responsible for flagellar biosynthesis, *flaA*, that encodes flagellin, a primary component of bacterial flagellum, increased by 1.03 log_2FC in the presence of magnesium. These align with data reported for *L. monocytogenes* in the literature (Casey et al., 2014; Fox et al., 2011; Siderakou et al., 2022). Directly downstream of *flaA* are the two genes encoding products for chemotaxis, *cheA* and *cheY*. CheA is a two-component sensor histidine kinase and CheY is a chemotaxis response regulator involved in the transmission of sensory signals from the chemoreceptors to the flagellar motors (Dons et al. 2004). In the present study both the genes were upregulated by 1.13 and 1.29 log_2FC respectively. The genes *flaA, motB* and *cheY* are very important for biofilm formation (Fan et al., 2020; Lemon, Higgins, & Kolter, 2007) and a substantial up regulation of a combination of genes associated with chemotaxis and motility, indicates that the bacteria are in a stressful environment where they move away from the unfavorable conditions or are at the dispersion state of biofilm formation. The notable difference in upregulation of these genes compared to the control condition might suggest that the cells are interacting constantly with the environment and changing to adapt to unfavorable conditions to survive.

Locus_tag	**Product**	**Gene name**	**Log2FC**
lmo0696	Flagellar basal-body rod modification protein FlgD		1.983918
lmo0698	Flagellar motor switch protein FliN		1.736676
lmo0697	Flagellar hook protein FlgE	*flgE*	1.532029
lmo0712	Flagellar hook-basal body complex protein FliE		1.499762
lmo0693	Flagellar motor switch protein FliN		1.443117
lmo0700	Flagellar motor switch protein FliN		1.420247
lmo0699	Flagellar motor switch protein FliM	*fliM*	1.40573
lmo0685	Flagellar motor rotation protein MotA	*motA*	1.330473
lmo0711	Flagellar basal-body rod protein FlgC	*flgC*	1.316153
lmo0706	Flagellar hook-associated protein FlgL	*flgL*	1.292543
lmo0714	Flagellar motor switch protein FliG	*fliG*	1.286681
lmo0716	Flagellum-specific ATP synthase FliI	*fliI*	1.27247
lmo0713	Flagellar M-ring protein FliF	*fliF*	1.235301
lmo0715	Flagellar assembly protein FliH	*fliH*	1.14086
lmo0707	Flagellar hook-associated protein FliD	*fliD*	1.114024
lmo0682	Flagellar basal-body rod protein FlgG	*flgG*	1.087028
lmo0705	Flagellar hook-associated protein FlgK	*flgK*	1.045118
lmo0690	Flagellin protein FlaA	*flaA*	1.034143
lmo0698	Flagellar motor switch protein FliN		1.736676
lmo0693	Flagellar motor switch protein FliN		1.443117
lmo0700	Flagellar motor switch protein FliN		1.420247
lmo0699	Flagellar motor switch protein FliM	*fliM*	1.40573
lmo0685	Flagellar motor rotation protein MotA		1.330473
lmo0691	Chemotaxis regulator-transmits chemoreceptor signals to flagelllar motor components CheY	*cheY*	1.290411
lmo0714	Flagellar motor switch protein FliG	*fliG*	1.286681

Locus_tag	Product	Gene name	Log2FC
lmo0692	Signal transduction histidine kinase CheA	*cheA*	1.132078

Table 4.5- Upregulated genes involved in the signalling cascade of bacterial chemotaxis and flagella assembly in presence of magnesium for 15G01.

4.4.7 **Quorum sensing**

Quorum sensing in biofilm formation of *L. monocytogenes* has been discussed by many authors (Ali, 2011; Kannan, Balakrishnan, & Govindasamy, 2020; Riedel et al., 2009). In this study, the glutamate decarboxylase (GAD) system was upregulated. *L. monocytogenes* frequently encounters low pH environments and requires a robust system to deal with this. To overcome the acidic stress present in the external environment or during the pathogenic life cycle, *L. monocytogenes* activates the GAD system (Cotter, Ryan, Gahan, & Hill, 2005). There are two proteins involved in the GAD system: cytoplasmic glutamate decarboxylase (GadA or GadB) and a glutamate to γ-aminobutyrate (GABA) antiporter. This system was only activated in the presence of sodium for the 15A04 strain. Two genes, *lmo2363* encoding for glutamate decarboxylase (1.60) and *lmo2362* encoding for GABA antiporter (1.91), were upregulated. Feehily et al. (2014), demonstrated the relation of GAD to virulence to survive under acidic conditions to maintain pH homeostasis. The authors demonstrated *L. monocytogenes* double mutant (EGDm *∆gadD1D3)* with deletion of both *gadD1* and *gadD3* displayed greater sensitivity to low pH exhibiting significant virulence in the mouse infection model. Cationic antimicrobial peptide (CAMP) resistance.

In *L. monocytogenes* in all stress conditions, a gene encoding for D-alanine-D-alanyl carrier protein ligase (*dltA*) is expressed (Abachin et al., 2002). This enzyme acts on the cell wall net charge during the formation of lipoteichoic acid (LTA) to maintain cation homeostasis

and absorption. LTA is a major part of the Gram-positive bacteria cell wall. This enzyme also plays key role in resistance to cationic antimicrobial peptides. These peptides are generated by various microorganisms as a component of their innate immune systems. Thus, they are important as a defence against bacterial infection to prevent disruption of the cell membrane. *dltA* specifically transfers D-alanine-D-alanine dipeptides to the carrier protein *dltC*, which subsequently transfers the D-alanine-D-alanine to LTA. These modifications decrease the cell wall net negative charge, thus making it less susceptible to CAMPs. Also, *dltA* has been associated with *L. monocytogenes* pathogenicity, specifically through modifying and controlling virulence genes like *prfA* (D'Onofrio et al., 2023; Golberg, Rae, & Rubinsky, 2012; Silhavy, Kahne, & Walker, 2010). The present study observed that in the presence of sodium chloride, the entire cascade of genes related to the dlt operon – *dltA* (-1.23), *dltB* (-1.63*), dltC* (-2.01) and *dltD* (-1.71) was down regulated in 15G01 only.

4.4.8 ABC transporters

ATP-binding cassette (ABC) transporters characterize one of the major and most functionally diverse protein families in most bacteria. These transport systems facilitate the translocation of a wide range of substrates across cellular membranes, thereby playing essential roles in nutrient and trace elements uptake, extracellular toxin secretion, and drug resistance. ABC transporters naturally consist of two key components: (i) a transmembrane domain that forms the substrate-specific channel, and (ii) a cytoplasmic ATP-binding domain responsible for binding and hydrolyzing ATP to enable substrate transport (Dassa & Bouige, 2001). There are more than 30 copies of ABC transporters in genome of *L. monocytogenes* (Liu et al., 2012). A study identified lm.G_1771 a part of novel ABC transporter which negatively regulates biofilm formation in *L. monocytogenes*. A mutant LM-49 was created by inserting a Tn917 transposon into the genome of the bacterium. This insertion disrupted a gene called lm.G_1771, that encodes a putative ABC

transporter permease. Disruption of this gene resulted in stronger biofilms compared to the wild type strain. Bioinformatics analysis presented lm.G_1771 and its neighboring gene lm.G_1772 were organized into an operon, working together as part of an ABC transporter system (Zhu et al., 2008). In the present study, in presence of magnesium a set of genes of this operon were observed to be downregulated in 15G01 such as lmo1771 (-1.37) and lmo01772 (-1.20) (Table 4.6).

Locus tag	**Product**	**Log_2FC**
lmo2507	cell division protein FtsE(ftsE)	-1.8373
lmo1771	phosphoribosylformylglycinamidine synthase subunit PurS(lmo1771)	-1.37138
lmo2681	potassium-transporting ATPase subunit B(kdpB)	-1.35261
lmo2240	ABC transporter ATP-binding protein(lmo2240)	-1.34863
lmo1767	phosphoribosylaminoimidazole synthetase(purM)	-1.31704
lmo0716	flagellum-specific ATP synthase(fliI)	-1.27247
lmo1769	phosphoribosylformylglycinamidine synthase II(purQ)	-1.21799
lmo1770	phosphoribosylformylglycinamidine synthase I(purL)	-1.20892
lmo1772	phosphoribosylaminoimidazole-succinocarboxamide synthase(purC)	-1.20574
lmo1431	ABC transporter ATP-binding protein(lmo1431)	-1.09885
lmo0919	antibiotic ABC transporter ATP-binding protein(lmo0919)	-1.08752
lmo1764	phosphoribosylamine--glycine ligase(purD)	-1.0649
lmo2679	histidine kinase(lmo2679)	-1.06354
lmo2090	argininosuccinate synthase(argG)	-1.05182
lmo2069	co-chaperonin GroES(groES)	-1.01324
lmo1835	carbamoyl-phosphate synthetase(carB)	-1.01175

Table 4.6- Downregulated genes of the ABC transporter in presence of magnesium for 15G01

Furthermore, it is suggested that lmo1771 genes is involved in regulation of several cell surface

proteins that are involved in biofilm formation. Transcriptomics studies of lm.G_1771 gene deletant (Δ1771) strain revealed that in mutant Δ1771, *dlt* operon was downregulated, thus demonstrating its role in down regulation of biofilm formation (Zhu et al., 2011). In present study, it was observed that, in presence of magnesium the *dlt* operon genes were upregulated compared to sodium (Table 4.7). Thus, these findings suggest that in presence of magnesium, where lmo1771 expression was downregulated and the simultaneous upregulation of the *dlt* operon may contribute to enhanced biofilm formation, potentially expaling the observed increased biofilm biomass in presence of magnesium compared to sodium (Chalke et al., 2022).

Locus tag	**Product**	**Log_2FC in magnesium**	**Log_2FC in sodium**
lmo0974	D-alanine--poly(phosphoribitol) ligase subunit 1(dltA)	3.94	-1.23
lmo0973	D-alanine esterification of lipoteichoic acid and wall teichoic acid(dltB)	3.99	-1.63
lmo0972	D-alanine--poly(phosphoribitol) ligase subunit 2(dltC)	3.69	-2.01
lmo0971	D-alanine esterification of lipoteichoic acid and wall teichoic acid(dltD)	3.73	-1.71

Table 4.7- Dlt operon in presence of magnesium and sodium for strain 15G01

4.5 Conclusion

Cations plays crucial role in the regulation of metabolic processes in *L. monocytogenes*. In its absence, genes involved in cellular functions such as cobalamin biosynthesis (GO:0009236) and the phosphoenolpyruvate-dependent sugar phosphotransferase system (GO:0009401) were downregulated, indicating a decrease in metabolic activity or nutrient transport. However, in presence of magnesium not only restored but also enhanced these pathways, with significant upregulation of processes like glycolysis (GO:0006096), gluconeogenesis (GO:0006094), and carbohydrate derivative transport (GO:1901264), highlighting magnesium's role as an important cofactor in energy metabolism. Possible pathways affected by magnesium that led to biofilm formation are detailed in Figure 4.4. Additionally, in presence of magnesium genes associated

with bacterial motility, such as those involved in flagellum-dependent motility (GO:0071973) and chemotaxis (GO:0006935), which are key to biofilm formation and survival in environments like food processing facilities were also observed. Furthermore, magnesium activated membrane-associated signal transduction genes (e.g., *kdp, cheA,* and *degU*), triggering downstream genetic responses. This study was the first to investigate the impact of magnesium on *L. monocytogenes* biofilm formation, revealing 13 distinct pathways that were affected in its presence (Figure 4.5), thus suggesting new insights into *L. monocytogenes* biofilm formation.

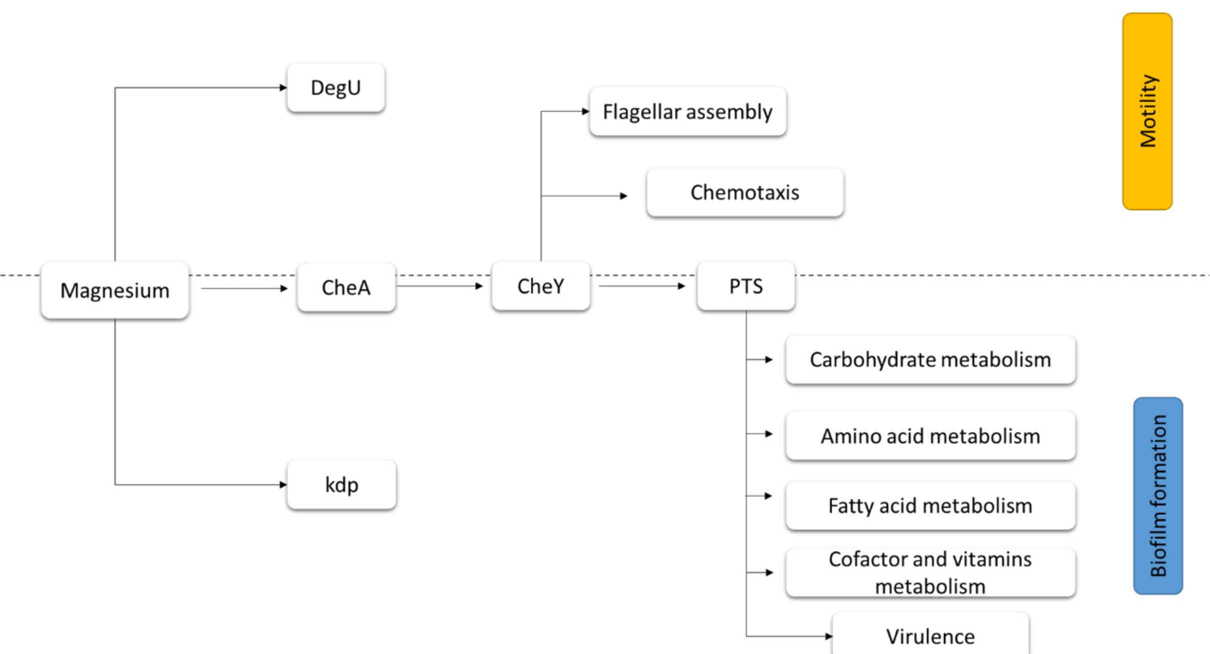

Figure 4.4- Possible pathways affected in presence of magnesium for *L. monocytogenes* biofilm formation

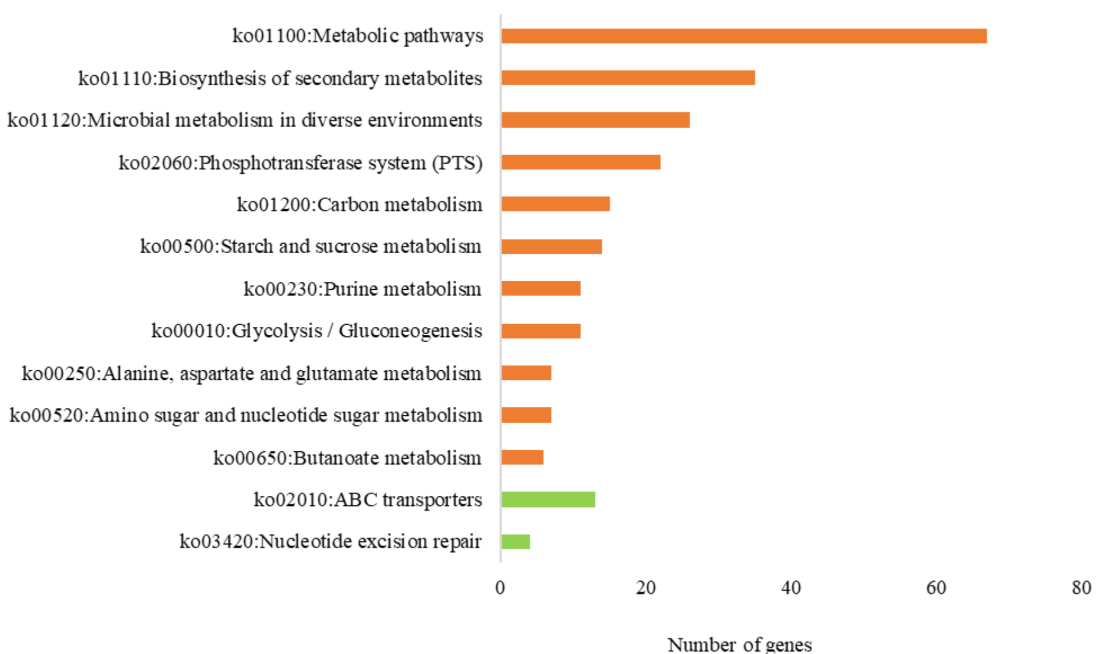

Figure 4.5- KEGG pathways in presence of magnesium: a, KEGG pathway classification of genes (orange, up-regulated; green, down-regulated, 15G01 v/s 15A04)

In contrast, in presence of sodium downregulation of several signal transduction pathways were observed (Figure 4.6). This included the *dlt* operon, whose downregulation might reduce the bacterium's ability to resist antimicrobial peptides or modify cell wall composition thus affecting adherence and biofilm integrity. Interestingly, the phosphotransferase system (PTS) genes exhibited both up- and downregulation (Table 4.3), indicating selective sugar utilization during biofilm development. Sodium also led to the downregulation of genes related to amino acid biosynthesis and the pentose phosphate pathway, indicating a shift in energy by the bacterium towards maintaining the biofilm. These findings emphasize the complex and differential regulatory effects of magnesium and sodium chloride on *L. monocytogenes*, revealing potential targets to alter biofilm formation and enhance food safety. However, further research is necessary to fully unravel the complicated network of responses, particularly in the presence of sodium chloride.

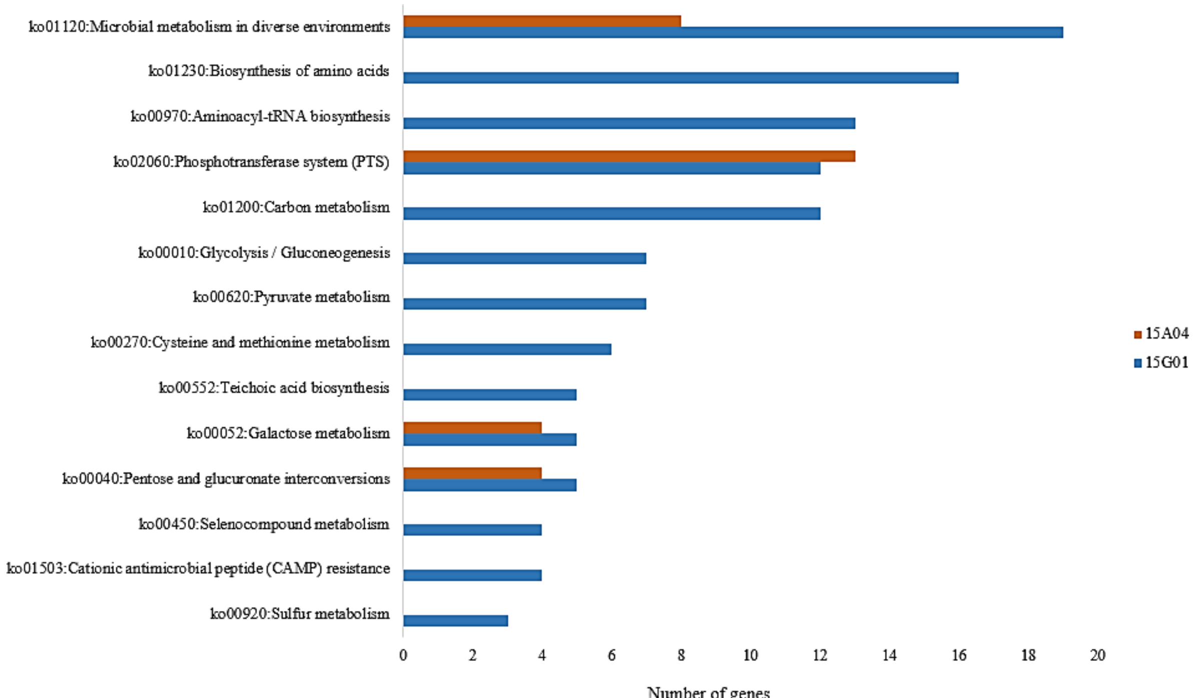

Figure 4.6- KEGG pathways downregulated in presence of sodium: KEGG pathway classification of genes (orange, 15A04; Blue, 15G01, 15G01 v/s 15A04)

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STATEMENT OF CONTRIBUTION
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Name and title of main supervisor:	Steve Flint
In which chapter is the manuscript/published work?	Chapter 5

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

The candidate carried out the laboratory work and analysis, prepared the manuscript with input and guidance of co authors and supervisors. Ria Rebstock and Ian Hallett helped the candidate with confocal laser scanning microscopy set up and data analysis. Lindy Guo reviewed the statistical analysis.

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Chapter 5: Visualizing and understanding the effect of cations on extra polymeric substance in *Listeria monocytogenes* biofilms.

5.1 Abstract

Listeria monocytogenes is a foodborne pathogen that is ubiquitous in nature. It is also found in the food processing environment. It possesses some unique characteristics that help to enhance survival and persistence in the food processing environment. Biofilms are a group of organisms attached to a surface and covered by an extracellular matrix known as the extracellular polymeric substance (EPS). EPS is a major component of the biomass of the biofilm and is made up of protein, carbohydrate, eDNA and other extracellular substances from the microbial community living in the biofilm. It contributes to the distinctive characteristics of the biofilm structure and the virulence of the pathogenic organisms residing in it. EPS is a complex set of arrays dependent on various factors. It protects the cells from the external environment providing resistance towards the sanitizers commonly used in the industry.

This study looks at the effect of magnesium, calcium and sodium on biomass activity, morphology and functional groups in EPS (eDNA and polysaccharides). The biofilm formation ability varied among strains, and cations had different effects on two strains, a high (15G01) and a low biofilm former (15A04). This study found that the cations increased eDNA secretion in both strains. In the presence of magnesium, the extracellular polysaccharides that are important for biofilm structure increased significantly compared to other cations for strain 15G01. Unique hexagonal structures with voids were observed for the low biofilm former (strain 15A04) in the confocal laser scanning microscopy studies, possibly transporting nutrients between the biofilm and the external medium. Biofilms are

thought to be a source of cross-contamination in the processing environment. This study provides new insights into the formation of the EPS in *L. monocytogenes* biofilms that can be used to help design reliable approaches for preventing or eradicating these bacterial communities.

5.2 Introduction

In both developed and developing nations, foodborne illnesses are major causes of disease and mortality. *Listeria monocytogenes* is a foodborne pathogen that causes listeriosis. Although listeriosis is rare it has a high mortality (20-30%) and hospitalization rate (over 90%) (Vidovic, Paturi, Gupta, & Fletcher, 2022). It remains a significant public health risk, especially for pregnant women, the elderly and individuals with impaired immune systems. The primary source of infection is through contaminated food. It can tolerate and survive at extreme conditions thus impeding control strategies used in food industries (Carpentier & Cerf, 2011). The pathogen's persistence and subsequent product contamination have been linked to the ability of *L. monocytogenes* to form biofilms on food contact surfaces (Carpentier & Cerf, 2011; Mazaheri, Cervantes-Huamán, Bermúdez-Capdevila, Ripolles- Avila, & Rodríguez-Jerez, 2021; Nowak et al., 2017).

A biofilm is a well-organized three-dimensional structure that contains various microorganisms that adheres to biotic and abiotic surfaces (Maggio et al., 2021; J. Wu & Xi, 2009). There are many factors that affect biofilm formation including genetic makeup of a bacterial strain (Borucki, Peppin, White, Loge, & Call, 2003; Orsi, den Bakker, & Wiedmann, 2011; Pirone-Davies et al., 2018), temperature, pH, different surfaces, nutrients, hydraulic shear force, and cations (Bonsaglia et al., 2014; Chalke, Vidovic, Fletcher, Palmer, & Flint, 2022; Chavant, Martinie, Meylheuc, Bellon-Fontaine, & Hebraud, 2002; Cole, Jones, & Holyoak, 1990; Di Bonaventura et al., 2008; Kim & Frank, 1995; Mai & Conner, 2007).

Microbial cells are embedded in a self-produced matrix called extra polymeric substance (EPS), a complex mixture that consists of various components including exopolysaccharides, proteins, lipids and eDNA (Colagiorgi, Di Ciccio, Zanardi, Ghidini, &

Ianieri, 2016). The most important and abundant component in a biofilm matrix is the exopolysaccharide (Sutherland, 2001). Jordan et al. (2008), found proteins like BapL are important for the attachment of *L. monocytogenes* to surfaces thus affecting biofilm formation. The importance of eDNA at initial attachment and biofilm formation was demonstrated by Harmsen et al., (2010) and Sulakova et al.(2019). The matrix helps in maintaining the biofilm architecture, holding the cells together and maintaining its stability. EPS plays an important role in biofilm formation, protection against the harsh environment and chemicals, establishing resistance to various sanitizing agents (Kirby et al., 2012) and in eventual biofilm detachment (Kirby, Garner, & Levin, 2012; Sutherland, 2001). Pan, Breidt, and Kathariou (2006) suggested that the composition of the matrix affects the resistance to biocides and the penetration of some antimicrobials as the matrix grows.

Understanding the EPS structure and its function in the presence of different conditions is critical for designing rational strategies aiming to disrupt biofilms. The purpose of this study was to quantify the effect of cations on the components of the extracellular matrix. This is the first study, quantifying the effect of cations on polysaccharide and eDNA content in the biofilm matrix.

5.3 Material and methods

5.3.1 Bacterial strains and culture conditions

Two *L. monocytogenes* isolates were selected from the seafood culture collection of the New Zealand Institute for Plant and Food Research Ltd, based on their biofilm forming abilities. Isolate 15G01, is a persistent isolate and is a strong biofilm former while 15A04 is characterised as low biofilm former. The isolates were recovered from −80°C stock and grown on tryptic soy agar with 0.6% yeast extract (TSAYE) (Difco, BD, USA) plates at 37°C for 24 h. For each experiment, a colony was freshly picked, and overnight cultures were prepared in tryptic soy broth (TSB) enriched with 0.6% yeast extract (TSBYE) (Difco, BD, USA).

5.3.2 Media and cation concentration

For all the experiments, 10% TSB (Difco, BD, USA) was used. Three cation compounds were used: magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$) and sodium chloride (NaCl), each at a concentration of 50 mM. For the control, 10% TSB without any added cation was used.

5.3.3 Biofilm formation

Biofilm formation experiments were performed as described previously with minor modifications (Chalke et al., 2022; Djordjevic, Wiedmann, & McLandsborough, 2002). An overnight bacterial culture was inoculated into freshly prepared 10% TSB with or without added cations in a ratio of 1:100. 1mL per well of the culture was transferred to 24 well microtiter plates (µ-Plate 24 Well, ibidi) and incubated for 48 h at 30°C. The processing of the plates is described with each specific experiment.

5.3.4 Quantification of biomass by crystal violet staining

After incubation, the plates were washed three times with 1 mL sterile water. The plates were air-dried for 30 min and then stained with 1mL of 0.5% aqueous crystal violet solution (Sigma Aldrich, NZ). After 30 min of incubation at 30°C, the crystal violet solution was removed, and the cultures washed five times with 1 mL of sterile water. The plates were air dried for 30 min in a Class II biosafety cabinet. After drying, 1 mL of 96% ethanol was added, and the crystal violet was destained for 1 h. The OD of each well was measured at

595 nm with a microplate reader. The OD of a control (microtiter wells containing uninoculated media) was subtracted from the obtained OD from the wells inoculated with culture.

5.3.5 eDNA quantification by spectrophotometry

The eDNA was quantified by using a Quant-iT™ Pico-Green® dsDNA Assay Kit (Thermo Fisher Scientific, USA) (Sulakova, Pazlarova, Meyer, & Demnerova, 2019; Tang, Schramm, Neu, Revsbech, & Meyer, 2013). After incubation the wells were washed twice with 1ml PBS (1X, pH 7.4). After washing, 100 µL TE buffer (Invitrogen™) was added, followed by 100 µL of freshly made Pico-Green® solution (1 µL Pico-Green® dye in 199 µL TE buffer). The TE buffer and the Pico-Green® solution were mixed thoroughly in the wells by pipetting up and down 10 times. The wells were incubated for four minutes at room temperature, before measuring the fluorescence intensity (excitation 485 nm/emission 535 nm, 0.1 s) using a microplate reader. Lambda DNA (Invitrogen™ Molecular Probes®) was used to generate a standard curve.

5.3.6 Polysaccharide quantification

After incubation of the inoculum at 30°C for 48 h the 96-well plates were washed three times with sterile distilled water. The biofilm was disrupted, and polysaccharide content was measured using a method described by Dubois et al. (1956) and Wu & Xi (2009) with slight modification. Briefly, 200 µL of N-Glycosidase solution (1U/mL) was added to each well and then incubated at 37°C for 30 min. Next, 20 µL of Proteinase K was added in a final concentration of 5 µg/mL and incubated at 37°C for 30 min. The biofilm was then disrupted by vigorous pipetting. Five wells per condition were prepared for each strain and pooled together then filtered through a 0.2 µm membrane (cellulose acetate).

The 200 µL solution was used to quantify extracellular polysaccharides present in the biofilms using the phenol-sulphuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). A 200 µL aqueous 5% phenol solution was added to the 200 µL sample, then 1 mL of concentrated H_2SO_4 (98%) 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.

5.3.7 Confocal laser scanning microscopy (CLSM)

The biofilm for both the strains was formed using the same protocol mentioned above with a few amendments. The biofilm was grown in a 24 well plate (µ-Plate 24 Well Black ID 14 mm (ibidi)) for 48 h at 30°C. These plates are high optical quality, ideal for high resolution imaging. After incubation the plates were washed thrice in 150 mM NaCl to remove any non-adherent cells. The Live/Dead BacLight^{TM} Bacterial Viability Kit was used to stain the biofilm according to the manufacturer's instructions. Wavelengths for the SYTO9 stain were excitation at 488 nm and emission at 498 to 550 nm, and for the propidium iodide stain, were excitation at 561 nm and emission at 571 to 700 nm. The CLSM image acquisition was performed using an Olympus FV3000 with FV31S-SW viewer software. Visualization was performed using 60 X objectives under oil immersion. Three horizontal plane image stacks with Z step of 0.30 µm, were acquired for each well at three different random areas in a well. Three independent experiments were performed containing three replicates. Thus, a total of 54 images per condition were obtained (2 strains by 3 random areas by 3 wells by 3 replicates). For image analysis, ImageJ software was used. The quantitative parameters (biomass, roughness coefficient, maximum thickness, surface area, surface to biovolume ratio and average thickness (biomass)) were calculated using COMSTAT (Heydorn et al., 2000).

5.3.8 Statistical analysis

Analysis of variance (ANOVA) was performed at a significance level of $p<0.05$ and post-hoc testing by Tukey's test to determine significant differences between treatments (Genstat 20th edition, VSN International, Hemel Hempstead, UK).

5.4 Results

5.4.1 Effect on biofilm formation

The biofilm formation abilities in the presence of magnesium, calcium and sodium of both the strains are shown in Figure 5.1. In general, strain 15G01 showed higher biomass production compared to 15A04 under the same experimental conditions. This difference between the two strains indicates variability in their metabolic activity, growth characteristics or specific response to the external conditions. For 15A04, highest ($p<0.05$) biomass was observed in calcium (2.74 ± 0.26) followed by magnesium (2.48 ± 0.22) and sodium (0.60 ± 0.06) compared to the control (0.18 ± 0.04). In contrast, strain 15G01 demonstrated higher biomass in magnesium (3.47 ± 0.21) and sodium (1.38 ± 0.20) and indicated the ability to establish a stronger growth response to all conditions compared with 15A04. Divalent cations like magnesium and calcium resulted in higher biomass compared to sodium and the control.

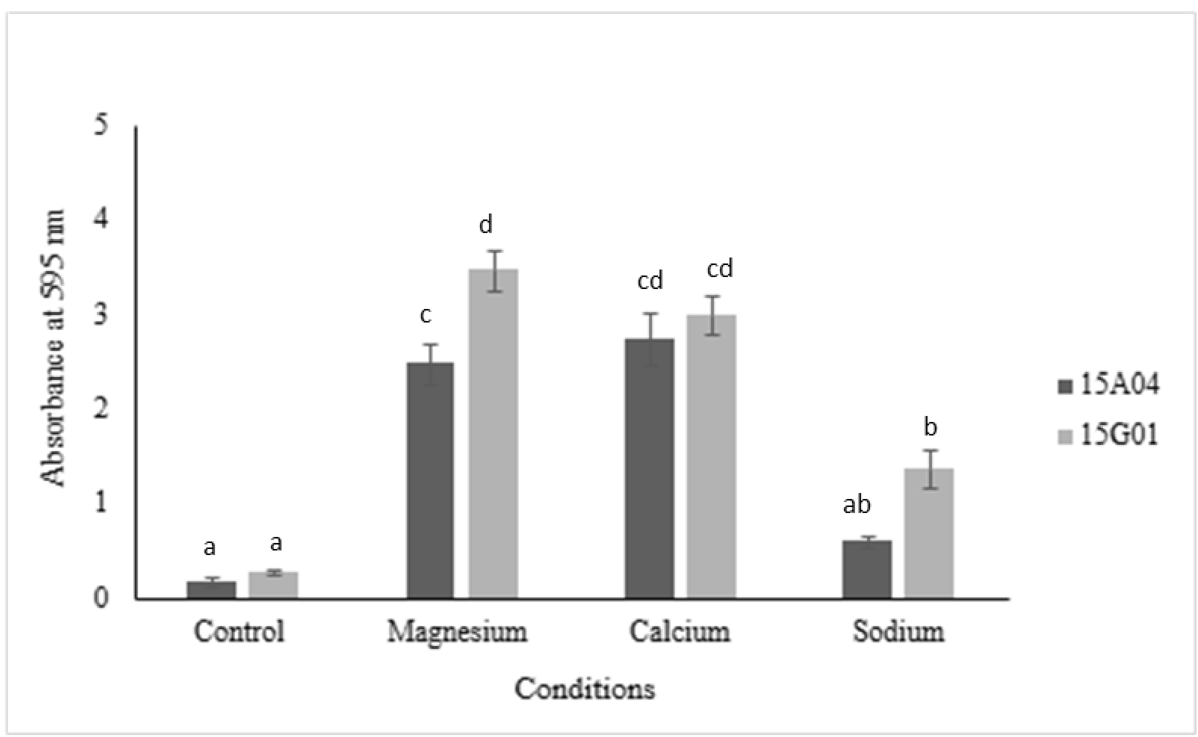

Figure 5.1- Effect of cations (50 mM) using crystal violet assay on the biomass of *Listeria monocytogenes* strains after 48h of incubation at 30°C. Error bars represent the standard

error of the mean (n=9)

5.4.2 Effect on polysaccharide content

Quantification of polysaccharide content extracted from the *L. monocytogenes* biofilm in the presence of cations is shown in Figure 5.2. The total amount of polysaccharides in the biofilms was affected by the cations and the strain involved in biofilm formation. The polysaccharide content for both strains 15A04 and 15G01 varied across the different cations, however, was higher in 15G01 biofilm compared to 15A04 biofilm. In the presence of magnesium, both strains demonstrated a significant increase in the polysaccharide content compared to the control. Especially for 15G01, the polysaccharide content was about 11.40 $\pm$ 0.01 compared to 15A04 which was 8.27 $\pm$ 0.09. This suggests that magnesium might play an important role in promoting polysaccharide synthesis in biofilm formation. With 50mM calcium, there were only minor impacts on the polysaccharide content for either strain. While for sodium, both strains exhibited a moderate increase in polysaccharide compared to the control indicating that sodium might contribute to enhancing polysaccharide pathways, although not as significantly as magnesium.

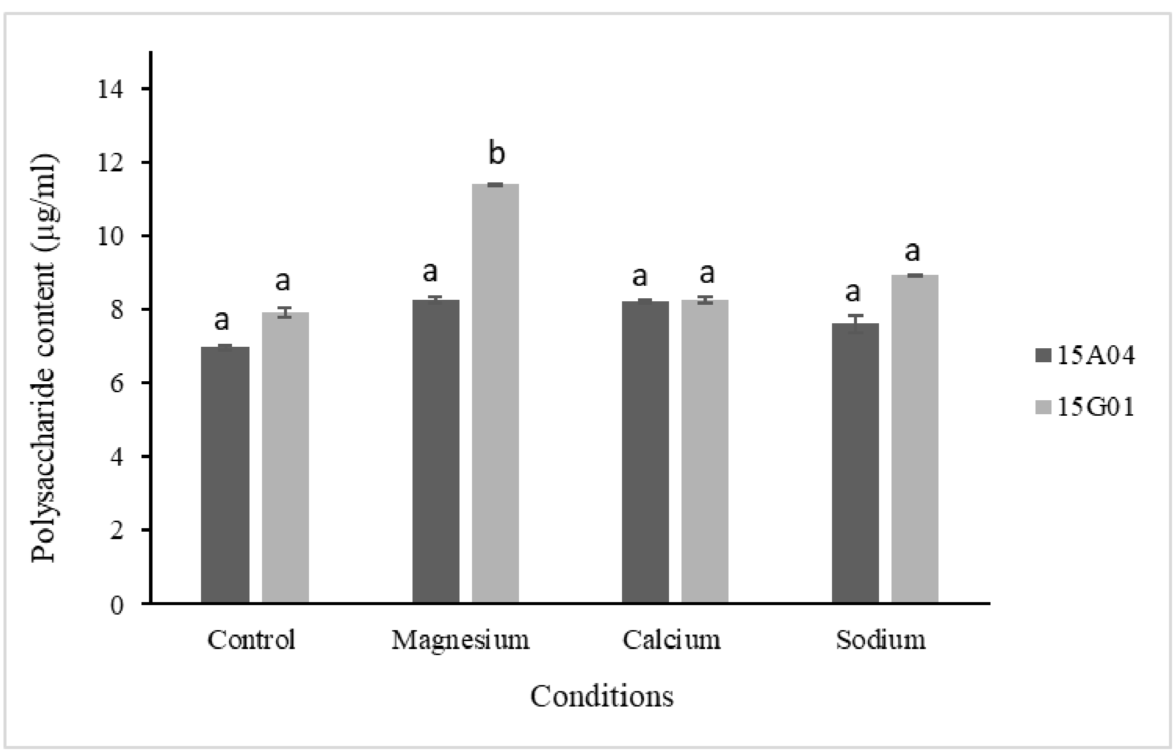

Figure 5.2 - Effect of cations on polysaccharide content in *Listeria monocytogenes* biofilms. Error bars represent the standard error of mean (n= 9)

5.4.3 Effect on eDNA

The eDNA concentration increased significantly ($p < 0.05$) in the presence of cations compared to the control for both strains (Figure 5.3). Overall, the eDNA concentration appeared to be slightly higher in 15A04 compared to 15G01, however there was no significant difference between the strains ($P = 0.123$). However, all the cations had a significant effect compared to control. This positive influence of calcium, magnesium, and sodium on eDNA concentration demonstrates the potential role of these nutrients in influencing microbial eDNA concentration in the external environment.

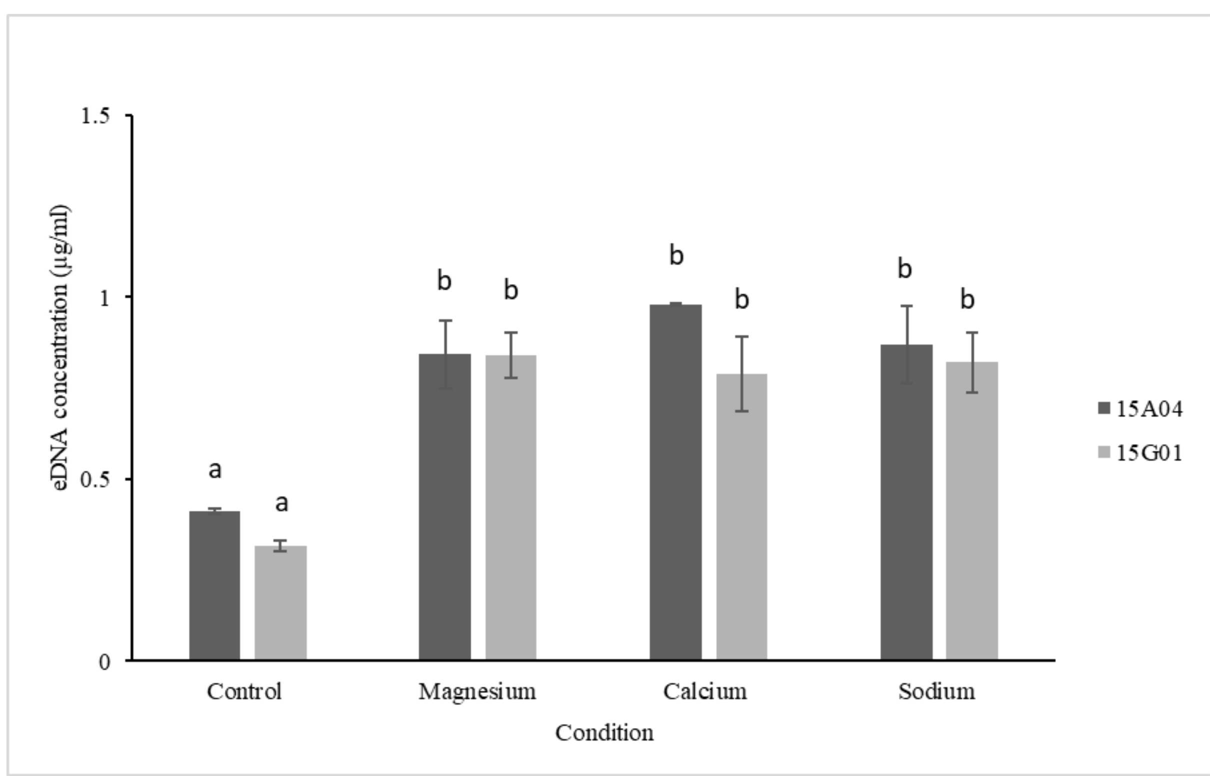

Figure 5.3 - Effect of cations on eDNA content in *Listeria monocytogenes* biofilms. Error bars represent the standard error of mean (n=9)

5.4.4 Effect on structure

Seven parameters were considered for this study - biomass, roughness coefficient, maximum thickness, surface area, surface-to-biovolume ratio, average thickness (entire area) and average thickness (biomass area). Each parameter was measured for the control, magnesium, calcium, and sodium growth conditions (Tables 5.1A and 5.1B).

Overall, for both strains the divalent cations (magnesium and calcium) had a greater effect on the biofilm structure compared to the control and sodium. The Maximum thickness is defined as the thickest piece of the biofilm taking holes and cavities in the structure into account. The divalent cations increased the biomass and thickness (maximum thickness and average thicknesses) of the biofilm for both of the strains. However, sodium had a significantly lesser effect. A hexagonal structure was observed in presence of magnesium and calcium especially in 15A04 (Figure 6.4b and 6.4c). To our best knowledge this structure has not been reported previously. Surprisingly

these cations had a more significant structural effect on strain 15A04 compared to 15G01. For instance, in contrast to CV (crystal violet) staining, the biomass observed with CLSM for 15A04 (8.15 ± 0.41) was much higher than 15G01 (2.50 ± 0.17) in the presence of magnesium (Figure 6.4B and 6.4f). This difference could be due to various reasons including the difference in the methods, CV staining measures biomass by staining both cells and extracellular matrix but lacks spatial resolution. In contrast, confocal microscopy offers detailed structural information such as biofilm thickness and architecture, but may not reflect total biomass, especially if the biofilm is unevenly distributed. Therefore, both techniques were used to provide more comprehensive understanding of biofilm formation while countering its limitation. Another reason could be use of different substratum on which the biofilms were developed, that could lead to variation in the surface properties, such as hydrophobicity, roughness thus affecting adhesion and biofilm formation. The surface area was significantly ($p<0.05$) increased in the presence of calcium compared to other cations whereas sodium significantly reduced the surface area compared to the control. The roughness coefficient reflects the variability in height across the biofilm. In the presence of cations, the roughness coefficient was slightly decreased, though there was no significant difference. However, the roughness coefficient reduced for 15A04 in presence of sodium and for 15G01 in presence of calcium, these differences could possibly be due to a change of cation composition and its interaction with the bacteria’s surface properties. The surface-to-biovolume ratio specifies an understanding of the surface coverage relative to the volume of the biofilm. The ratios indicate that all three cations produced a higher surface-to-biovolume ratio than the control, suggesting a potentially increased or complex surface structure.

A) 15A04				
	Control	**Mg**	**Ca**	**Na**
Biomass (µm^3/µm^2):	1.88	8.52	7.49	3.9
	0.45	0.42	0.98	0.21
Roughness Coefficient (Ra*):	1.31	1.23	1.17	0.77
	0.18	0.06	0.13	0.16
Maximum thickness (µm):	4.44	18.11	13.66	8.55
	0.4	1.16	1.26	1.11
Surface Area (µm^2):	556115	786411.7	1160110	1051.44
	37446.76	38980.02	220474	341.37
Surface to biovolume ratio (µm^2/µm^3):	1.62	3.16	2.09	3.57
	0.38	0.06	0.69	0.08
Average thickness (Entire area) (µm):	3.85	22.18	15.94	4.16
	0.35	2.01	1.13	0.31
Average thickness (Biomass) (µm):	4.8	22.75	16.61	1.29
	0.66	1.64	0.64	0.48

Table 5.1A - Mean biomass, roughness coefficient, maximum thickness, surface area, surface to biovolume ratio, average thickness (entire area) and average thickness (biomass) parameters calculated using COMSTAT for 15A04 (grey cells). The standard errors shown in white cells were calculated from the nine images taken for each sample.

B) 15G01				
	Control	**Mg**	**Ca**	**Na**
Biomass (µm^3/µm^2):	0.62	2.51	2.86	0.07
	0.35	0.17	0.5	0.04
Roughness Coefficient (Ra*):	1.73	1.45	0.59	1.44
	0.05	0.28	0.37	0.3
Maximum thickness (µm):	4.33	5.89	4.7	2.85
	1.29	0.95	0.04	0.43
Surface Area (µm^2):	103371.9	12959.56	310183.8	6918.67
	49641.83	1613.88	18000.19	1137.36
Surface to biovolume ratio (µm^2/µm^3):	2.52	3.32	3.18	3.09
	0.12	0.25	0.47	0.18
Average thickness (Entire area) (µm):	0.06	4.17	3.75	0.01
	0.05	0.14	0.44	0
Average thickness (Biomass) (µm):	1.1	1.47	4.58	1.7
	0.39	0.21	0.14	0.18

Table 5.1B - Mean biomass, roughness coefficient, maximum thickness, surface area, surface to biovolume ratio, average thickness (entire area) and average thickness (biomass) parameters calculated using COMSTAT for 15G01 (grey cells). The standard errors shown in white cells were calculated from the nine images taken for each sample.

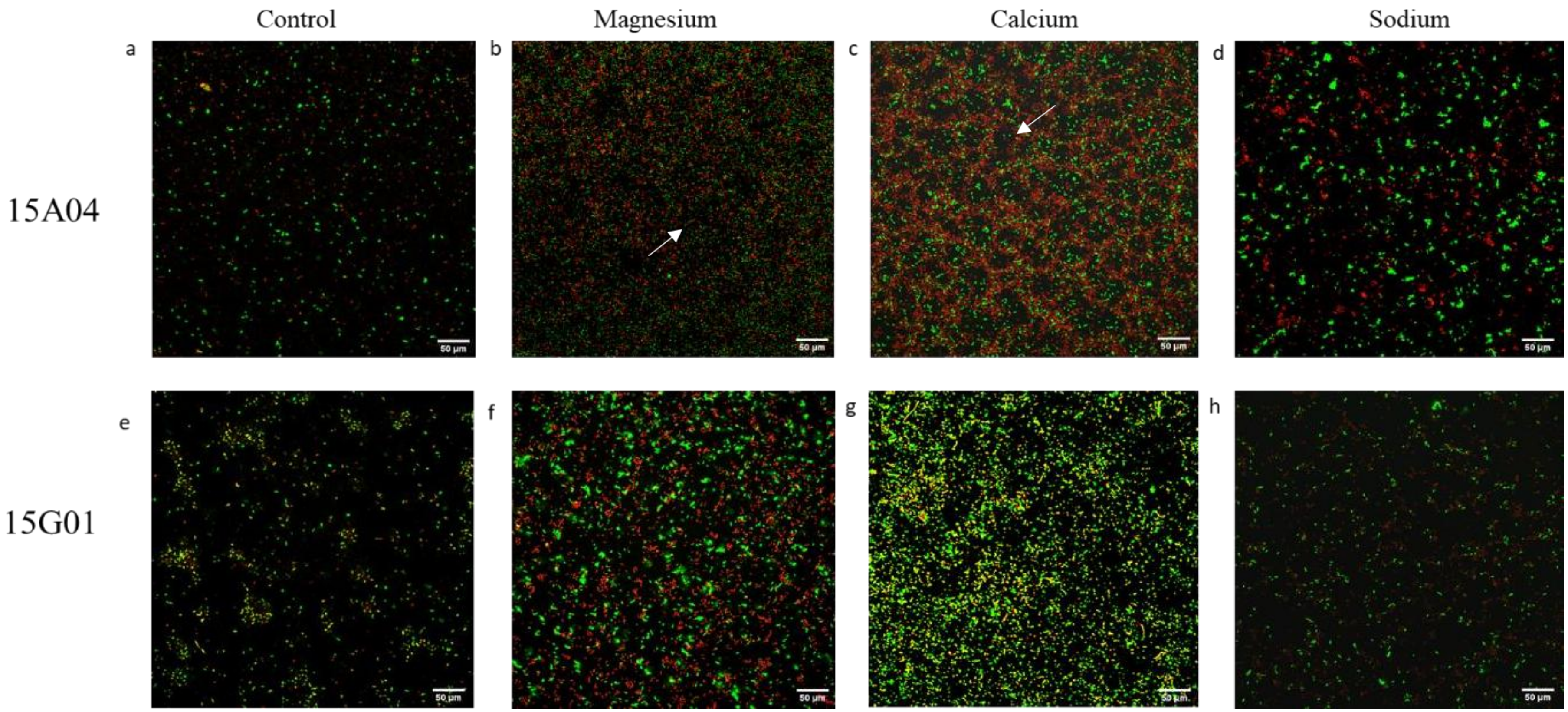

Figure 5.4- Confocal laser scanning microscope analysis of strain 15A04 a) control, b) 50mM magnesium, c) 50mM calcium, d) sodium and for strain 15G01 e) control, f) 50mM magnesium, g) 50 mM calcium and h) 50 mM sodium. The biofilm was stained with SYTO9 stain (green colour) and propidium iodide stain (red colour). Hexagonal structure observed for 15A04 (Figure b and c). Dispersed biofilm structure observed for control and sodium for both the strains (figure a, e, d and h). For 15G01 more live cells were observed in presence of magnesium and calcium compared to 15A04 (Figure b,c,f and g)

5.5 Discussion

The objective of this study was to understand the effect of cations (magnesium, calcium and sodium) on the EPS of *L. monocytogenes* biofilm. Biofilm formation capability varies among strains in *L. monocytogenes* (Borucki et al., 2003; Djordjevic et al., 2002; Norwood & Gilmour, 1999). In the present study, the cations had a different effect on two different strains. This difference could possibly be due to the different region in the seafood industry where they originated (different surfaces and different manufacturing conditions). In the present study, strain 15G01 had an enhanced effect on biofilm formation compared to 15A04 in presence of cations. The cation effect might be due its ionized form, making the cation biologically active so it interacts with the bacterium and/or the substrate. An alternative explanation might be the numerous negatively charged components in the EPS, like amines, phosphates and carboxyl groups that could interact with the positively charged cations. The specificity of the interaction with the cations, may mean that some polymers have greater affinity to interact than others. (Dobson & O'Shea, 2008; Rose & Hogg, 1995; Somerton, Lindsay, Palmer, Brooks, & Flint, 2015; Steiger, Muelli, Braissant, Waltimo, & Astasov-Frauenhoffer, 2020). The bacterial outer membrane contains various cations including magnesium, sodium and calcium. These cations increase the stability of the outer membrane and protect it from damage by antibiotics by inhibiting their activity (Sahalan, Aziz, Hing, & Abd Ghani, 2013).

As biofilm formation is mediated by a self-produced matrix known as EPS that not only protects the biofilm from the external environment but also plays an important role in the adhesion of the cells to the surface, cell to cell adhesion and most importantly in biofilm maturation. This matrix is made up of various components like proteins, carbohydrates, eDNA and lipids. eDNA enhances biofilm formation and stability by providing structural integrity by binding to the various structures present in the EPS (polysaccharides and

proteins). The cations, being positively charged, bind to eDNA that is negatively charged through electrostatic interactions. Harmsen, Lappann, Knochel, and Molin (2010) showed the importance of eDNA at the adhesion and early steps of the biofilm formation. Cations like calcium were found to enhance biofilm formation by eDNA promoting adhesion and to increase bacterial aggregation, possibly by facilitating formation of cationic bridging (Das et al., 2014). In the present study cations increased the eDNA secretion, however there was no significant difference ($p>0.05$) among the cations. Similar results were observed by another group showing no significant difference between calcium/magnesium on eDNA (R. X. Wu, Zhang, Guo, Zhao, & Guo, 2022).

Extracellular polysaccharides facilitate surface adhesion and sustain the biofilm architecture. Polysaccharide can provide chemical or immune protection to the cells and biofilm and is associated with water retention and the absorption of inorganic and organic compounds, promoting the development of biofilms (Arciola, Campoccia, Ravaioli, & Montanaro, 2015). In the presence of 10 mM magnesium, the *Pseudomonas stutzeri* strain XL-2 increased production of EPS polysaccharides by 52.9% compared to control and calcium (R. X. Wu et al., 2022). In the present study, similar results were observed, especially for 15G01 (a good biofilm former) with an increase of 69.9% compared to the control and strain 15A04. This might indicate the role of polysaccharides in enhancing biofilm formation. Magnesium promoted the secretion of polysaccharides compared to the control, calcium and sodium. Surprisingly calcium and sodium had almost a similar effect on the polysaccharide levels of the EPS. Similar results were observed for *Bacillus* sp. under the influence of 10 mM magnesium and calcium. Both of the cations enhance polysaccharide secretion (He, Wang, Abdoli, & Li, 2016). The action of magnesium having better affinity to the polysaccharide secretion may be due to its smaller size than calcium and sodium, thereby preventing steric hinderance while bonding with the hydroxyl group.

CLSM studies were carried out to examine the effect of cations on *L. monocytogenes* biofilm surface topography and architecture. Various structures have been reported for static *L. monocytogenes*. Shukla and Rao (2013) found that high concentrations of calcium affected the architecture of the biofilm. As the concentration of calcium increased from 1 to 10 mM, structural changes like flattening of the biofilm with a reduction in the pillar size and formation of voids. Figure 6.4 showed the effect of cations on both the strains. For both strains, magnesium and calcium resulted in denser biofilms compared to the control and sodium. This is probably due to the cell-to-cell interaction enhanced in the presence of these divalent cations. Another reason could be due to the ability of calcium to cross-link polyanionic polymers in the matrix of the biofilm. Magnesium is hypothesized to increase the adhesion of the cells by reducing the repulsive force between the cells and surface, thereby enhancing colonization (Chen & Stewart, 2002; Song & Leff, 2006). Similar results were reported for *Cronobacter sakazakii* where 1.5% magnesium and 0.25% calcium contributed more towards biofilm formation compared to 0.5% sodium observed under CLSM at 28°C after 24 h incubation. Interestingly for 15A04 in the present study, hexagonal structures with voids were seen, possibly providing the means for nutrient transport between the biofilm and bulk medium (Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995). To our best knowledge this hasn't been reported in the past literature.

5.6 Conclusion

The presented data indicates that the EPS content of strains 15A04 and 15G01 is influenced by the presence of magnesium, calcium, and sodium. The 15G01 strain, which is a good biofilm former, exhibited significantly higher polysaccharide content compared to the low biofilm former 15A04. This leads to the hypothesis that these physiological characteristics might contribute to enhanced biofilm development. The variations observed between the strains and under different conditions highlight the need for further research to unravel the

precise molecular mechanisms that govern the development of the EPS matrix in these strains. While this study provides valuable insights for strains 15A04 and 15G01 under different conditions, further investigations are needed to understand the underlying mechanisms responsible for the observed changes. Additional studies, such as Fourier transform infrared spectroscopy (FTIR) and GC-MS could be used to understand the change in functional group and composition of polysaccharide in presence of cations in the EPS matrix.

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Chapter 6: Final discussion and future work

6.1 Summary

Listeriosis is a severe bacterial infection caused a food borne pathogen *L. monocytogenes.* It is a life-threatening disease especially for immunosuppressed people, including elderly, pregnant women and neonates (Ferreira, Wiedmann, Teixeira, & Stasiewicz, 2014). Most of the cases are highly associated with contaminated food as a primary source of infection. According to the Centers for Disease Control and Prevention, it is estimated 1600 cases of listeriosis, and 260 deaths occur in the USA annually (CDC, 2021). *L. monocytogenes* has an ability to form good biofilm and survive and grow in wide range of conditions, thus enabling the bacterium to persist even in harsh conditions. The literature review (Chapter 2) highlights characteristics of *L. monocytogenes*, it's tolerance in food industry across the globe, biofilm formation and how cations play important roles in biofilm formation. So far there have been reports of cations influencing other organism biofilms, but limited information is present in case of *L. monocytogenes.*

To investigate the influence of cations on biofilm formation of *L. monocytogenes* in this study, three cations that are readily available in the seafood industry were used, magnesium, calcium and sodium. Three concentrations 1mM, 10mM and 50mM has been used, based on the physiological (data from Pilson 1998) and industrial relevance (cation profile in mussels and mussel intravascular juice), spanning from low to high ionic conditions (Appendix's image A1.a and Appendix's table A1.b). However future studies should be aimed to investigate the spatial distribution and accumulation of these cations within specific areas of seafood processing areas prone to biofilm formation. Four strains from seafood industries were chosen, based on their biofilm forming ability. PFR15A04 demonstrated low biofilm forming ability, PFR15G01 was a persistent isolate, which showed great biofilm

forming ability, PFR16A01 was associated with a mussel *Listeria* contamination outbreak and PFR33H04 was another persistent isolate collected from a different seafood factory.

To study the influence of these cations in chapter 3, I investigated their effect on various stages of biofilm formation – the planktonic state, attachment, motility, hydrophobicity, and biofilm formation – were investigated. Irrespective of the strain type, the differential effect of the monovalent cation sodium and divalent cations like magnesium and calcium highlighted the complexity of the bacterial response to the cations.

The attachment studies data revealed across the different isolates that magnesium enhanced the attachment of *L. monocytogenes*, emphasizing its possible role in initial surface colonization. However, sodium and calcium had a contrasting effect, especially at high concentration. In the case of hydrophobicity, most isolates showed minimal changes with significant changes at high cation concentration. This suggests that cation induced attachment of the bacteria might not be due to the change in hydrophobicity but still affects how the bacteria interact with surfaces in presence of cations.

The difference in the effect between the monovalent and divalent cations was highlighted in the motility studies. The monovalent cation, sodium, increased motility compared to the divalent cations, magnesium and calcium. The reduction in motility in the presence of the divalent cations is likely to promote biofilm formation by localizing the bacteria for attachment leading to biofilm growth.

The effect of cations on planktonic cells and biofilm formation was determined. The planktonic cell biomass and biofilm formation increased with the increase in magnesium concentration. Variable results were observed for planktonic cells in the presence of calcium, however biofilm formation increased with increasing calcium concentration. Sodium had less effect on planktonic cells and biofilm formation than the divalent cations. These results

suggest that divalent cations tend to facilitate biofilm formation, possibly due to stronger ionic interactions, stabilizing the EPS within the biofilm matrix.

To further understand the effect of cations on *L. monocytogenes* biofilm, the gene expression profile of low (15A04) and high (15G01) biofilm formers in the presence of magnesium and sodium were studied.

In the absence of cations, 15.06% of genes were significantly expressed in 15G01 compared with 15A04. The percentage increased to 19.87% in the presence of magnesium, indicating that magnesium has an impact on gene expression. Interestingly, in the presence of magnesium, 15G01 demonstrated a higher number of downregulated genes compared with 15A04 suggesting a more metabolically stable state for 15A04. In the presence of sodium, the percentage of differentially expressed genes was 10.02%, suggesting sodium had less effect on biofilm formation compared to magnesium. More genes were upregulated in presence of sodium, indicating the possibility of isolates assimilating to the external environment in the presence of sodium.

The gene ontology analysis discovered distinct differences in biological processes in the presence and absence of magnesium and sodium. Carbohydrate transportation pathways like the PTS system were the major group affected. In the absence of cations, large numbers of genes were up and downregulated (Table 4.1). In case of magnesium, all the genes specifically related to galacticol, mannose and fructose were upregulated in 15G01 (Table 4.2). This utilization of specific carbohydrates suggests that the bacteria are focusing on biofilm growth and stability by ensuring a steady supply of the nutrients and energy needed to maintain the biofilm structural integrity and survival of the bacteria. PTS affects the *prfA* virulence gene regulator by modulating carbon utilization by the bacteria (Li et al., 2022). In the presence of magnesium, the *prfA* gene in 15A04 was upregulated by 1.13 log_2FC.

This aligns with the past studies related to PTS involvement in virulence of other bacteria.

In the presence of magnesium, a total of 13 pathways were affected. Figure 4.4 depicts the possible mechanism. In the presence of magnesium, genes encoding for DegU, CheA and Kdp are upregulated. These genes then activate a cascade of genes that are related to flagellar assembly, chemotaxis, carbohydrate metabolism, amino acid metabolism, fatty acid metabolism, cofactor and vitamins metabolism and virulence. This establishes an appropriate environment for the survival of bacteria in the biofilm environment and protects from the external environment.

In the case of sodium, the majority of the genes related to PTS were downregulated in both 15A04 and 15G01. As carbohydrates are the main source of energy, downregulation in PTS genes results in lower energy, thus affecting the growth and metabolic activity inside the biofilm. Thus, these conditions create a stressful environment thereby up-regulating stress related genes in the presence of sodium. Two genes - *lmo2363* encoding for glutamate decarboxylase (1.60) and *lmo2362* encoding for GABA antiporter (1.91) related to the GAD system were upregulated in 15A04. The GAD system in *L. monocytogenes* is often activated in low pH environments (Cotter, Ryan, Gahan, & Hill, 2005). However, in the case of 15G01, a set of genes related to the *dlt* operon that are identified in stress conditions and are related indirectly to virulence were downregulated. This demonstrates how two different isolates exhibit gene expression in similar conditions. More studies are needed to better understand the complexity of biofilm formation in the presence of sodium.

Lastly the effect of cations on the structural integrity of the biofilm was studied. To study the effect on the EPS, the crystal violet assay, eDNA and polysaccharide content were measured. The architecture of the biofilm under the influence of cations was studied using confocal laser scanning microscopy. The results from this study demonstrate the effect of

magnesium, calcium and sodium on various aspects of *L. monocytogenes* biofilm development. In the presence of cations, 15G01 exhibited significantly higher biomass compared with 15A04 under all conditions. Magnesium and calcium had a significantly greater effect compared to sodium.

The polysaccharide content, that is important for biofilm structure and stability, was also influenced by the cations. Magnesium increased polysaccharide content compared to other conditions for both of the strains. Isolate 15G01 consistently exhibited greater polysaccharide content compared with 15A04 across all conditions, emphasizing the potential differences in metabolic pathways and regulatory mechanisms in the synthesis of polysaccharide. Another important component of biofilm formation is eDNA. In this study, the concentration of eDNA was significantly increased in the presence of cations compared to the control condition, irrespective of the strain type.

The topography and architecture of *L. monocytogenes* biofilm under the influence of cations were studied using CLSM. Divalent cations significantly increased the biofilm thickness and biomass for both isolates. A distinct hexagonal structure in the presence of magnesium and calcium was observed for 15A04. This structure has not been reported in past studies. Thus, the structural diversity among the cations inducing specific architectural changes, suggest potential influence on the stability and resistance to the external environment.

CSLM examination also revealed that isolate 15G01 had more live cells in the presence of magnesium and calcium suggesting that these cations not only stabilize the biofilm structure but also enhance its cellular and metabolic stability. Furthermore, transcriptomic data from Chapter 4 supports this observation. In the presence of magnesium, genes involved in essential pathways such as the phosphoenolpyruvate-dependent sugar phosphotransferase system (GO:0009401), glycolysis (GO:0006096), and the de novo IMP biosynthetic process

(GO:0006189 were upregulated. These pathways are important for energy production and nucleotide biosynthesis, which are important for supporting cell viability and proliferation within biofilms. Furthermore, genes involved in nucleotide excision repair and protein refolding were also observed to be upregulated. However, it is important to observe that even though the expression of genes supporting cell viability were increased, several key virulence genes *hly*, *plcA*, *plcB* and *actA* were also observed to be downregulated in 15G01 in presence of magnesium. This suggests that under specific environmental condition (non-host environment) the bacterium may shift from invasive to persistence focused phenotype, however additional studies are necessary to confirm this adaptive response.

In conclusion, this study highlights the strain-dependent influence of cations on biofilm formation in *L. monocytogenes*. The two strains investigated, 15G01 and 15A04, exhibited different responses to cation exposure, in terms of biofilm-forming ability, gene expression and architectural. For instances, the polysaccharide content observed in presence of magnesium was significantly higher compared to 15A04 but there was no difference observed in both the strains in presence of calcium or sodium or no cations. Another example was in presence of magnesium, 15A04 expressed virulence gene (prfA) however in same condition, 15G01 suppressed the expression of virulence genes. These differences could likely be due to their distinct environmental origins, as the isolates were obtained from different seafood processing facilities with different locations. Therefore, the differences might be due to distinct conditions, sanitation practices, or ecological pressures, enabling specific strains to persist and thrive under particular condition with distinct genetic and phenotypic modifications. These findings highlight the importance of considering strain variability along with effect of cations on biofilm formation of *L. monocytogenes* for developing targeted strategies to control biofilm formation in food processing environments.

6.2 Highlights or key findings

- Divalent cations – magnesium and calcium had a greater influence on biofilm formation compared to the control and sodium, irrespective of the isolate origin.
- Magnesium enhanced the attachment of the bacteria, while calcium and sodium had an opposite effect especially at 50mM concentration.
- The differential gene expression was greater in the presence of magnesium (19.87%) compared to the control (15.06%) and sodium (10.02%).
- A unique hexagonal structure within the biofilms was observed for isolate 15A04 in the presence of 50mM magnesium and calcium.

6.3 Future work

This PhD study has provided a new understanding of the influence of cations on *L. monocytogenes* biofilm formation. It has also generated new directions for future studies as follows.

- Evaluate the effect of magnesium, calcium and sodium at higher concentrations on planktonic and biofilm formation to determine whether the higher concentrations of these cations enhance or inhibit biofilm formation.
- Evaluate the effect of cations on different substrate such as stainless steel and it's effect on biofilm grown on flow conditions.
- Investigate the effect of combinations of cations likely to be found in the environment on biofilm formation, to understand potential synergistic or antagonistic interactions.
- Replicate industrial conditions by adjusting the cation concentrations to those commonly found in the seafood industry, to examine the effect on growth and biofilm formation of *L. monocytogenes.*
- The data presented in the current study represents a comprehensive baseline to understand

the cation interaction during biofilm formation at 30°C. However, as the seafood industry processing plant have varied temperature depending on the product or process, effect of cations on biofilm formation at wide range of temperature would be of interest.

- Evaluate metabolomes of *L. monocytogenes* in the presence of magnesium and sodium to study the differences in metabolic profiles.
- Use gene knockouts or overexpression studies of key pathways (PTS, TCS, Quorum sensing, flagellar assembly and chemotaxis) to provide a deeper insight of their roles.
- Investigate how *L. monocytogenes* biofilm formation is affected in the presence of cations in response to different environmental stresses like pH, temperature and other species.

6.4 Reference

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Appendix

Appendix for chapter 3

Appendix table A 3. 1- Parameters for microplate strip washer (ELx50, Biotek)

		Dispense						Aspirate				
Method	# cycles	Volume (µL)	Flow Rate (µL/well/sec)	Height (mm)	Prime Before start	Prime Volume (mL)	Prime Flow Rate (µL/well/sec)	Height (mm)	Rate (µL/well/sec)	Delay (sec)	Crosswise	Final aspiration
Initial wash	3	250	5	120	yes	5	1	40	1	0	No	No
After staining wash	5	250	5	120	yes	5	1	40	1	0	No	No

Appendix table A3. 2- Descriptive statistics for effect of cations on attachment

Strains		15A04	15G01	16A01	33H04
Cations		**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**
Magnesium	**Control**	4.714 ± 0.042	4.233 ± 0.108	4.833 ± 0.029	4.729 ± 0.053
	1 mM	4.498 ± 0.205	4.823 ± 0.084	4.708 ± 0.048	4.491 ± 0.077
	10 mM	4.429 ± 0.095	4.682 ± 0.088	4.707 ± 0.043	4.684 ± 0.041
	50 mM	4.245 ± 0.117	4.704 ± 0.08	4.505 ± 0.098	4.804 ± 0.079
Calcium	**Control**	4.493 ± 0.123	4.186 ± 0.103	4.736 ± 0.055	4.692 ± 0.055
	1 mM	4.177 ± 0.212	4.075 ± 0.089	4.234 ± 0.126	4.439 ± 0.109
	10 mM	3.829 ± 0.034	4.704 ± 0.054	4.757± 0.049	4.456 ± 0.109
	50 mM	3.671 ± 0.073	4.397 ± 0.161	4.348 ± 0.057	4.086 ± 0.102
Sodium	**Control**	4.493 ± 0.123	4.186 ± 0.103	4.736 ± 0.055	4.692 ± 0.055
	1 mM	4.474 ± 0.094	4.329 ± 0.132	4.721 ± 0.104	4.488 ± 0.13
	10 mM	4.517 ± 0.093	4.205 ± 0.128	4.365 ± 0.086	4.130 ± 0.078
	50 mM	3.774 ± 0.081	4.245 ± 0.132	4.387 ± 0.174	4.040 ± 0.066

Appendix table A3. 3- Tukey's 95% confidence interval comparison to test the effect of cations on attachment. (Ca- Calcium; Na- Sodium; Mg – Magnesium). The column with the letter denotes the significant difference.

	Mean	Letter
Mg	4.612	b
Na	4.362	a
Ca	4.331	a

Appendix table A3. 4 - ANOVA summary table to test the effect of cations on attachment.

Source of variation	**d.f.**	**F value**	**p**
Cations	2	38.14	<.001
Concentration	3	17.91	<.001
Strain	3	20.65	<.001
Cation.Concentration	6	5.6	<.001
Cation.Strain	6	3.71	0.001
Concentration.Strain	9	8.93	<.001
Cation.Concentration.Strain	18	3.3	<.001

Appendix table A3. 5- Descriptive statistics for effect of cations on hydrophobicity

Strains		**15A04**	**15G01**	**16A01**	**33H04**
Cations		**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**
Control	**Control**	91.854 ± 1.118	92.157 ± 1.062	91.346 ± 1.244	89 ± 0.956
Magnesium	**1 mM**	88.627 ± 1.388	91.919 ± 0.809	90.2 ± 1.162	88.355 ± 0.94
	10 mM	91.158 ± 1.573	86.588 ± 1.051	92.259 ± 0.557	87.384 ± 0.746
	50 mM	89.73 ± 1.126	88.047 ± 1.968	91.012 ± 1.242	90.408 ± 1.461
Calcium	**1 mM**	89.051 ± 1.523	89.256 ± 1.575	88.757 ± 1.27	90.0371 ± 1.509
	10 mM	89.102 ± 1.293	89.907 ± 1.137	88.493 ± 0.685	90.081 ± 0.867
	50 mM	93.42 ± 0.721	93.093 ± 0.451	92.024 ± 1.277	93.842 ± 0.773
Sodium	**1 mM**	88.853 ± 0.856	86.064 ± 1.868	88.926 ± 1.141	87.443 ± 1.032
	10 mM	91.362 ± 0.962	88.139 ± 1.45	91.498 ± 0.813	87.818 ± 0.744
	50 mM	87.887 ± 1.221	88.673 ± 1.157	88.557 ± 0.936	85.146 ± 1.161

Appendix table A3. 6- ANOVA summary table to test the effect of cations on hydrophobicity.

Source of variation	**df**	**F value**	**P**
Cation	3	10.89	<.001
Concentration	3	2.43	0.065
Strain	3	3.36	0.019
Cation.Concentration	3	11.42	<.001
Cation.Strain	9	2.49	0.009
Concentration.Strain	9	0.76	0.658
Cation.Concentration.Strain	9	2.44	0.011

Appendix table A3. 7- Tukey's 95% confidence interval comparison to test the effect of cations on hydrophobicity. (Ca- Calcium; Na- Sodium; Mg – Magnesium)

Comparison	**Difference**	**Lower 95%**	**Upper 95%**	**Significant**
Na vs Mg	-1.277	-2.42	-0.13	yes
Na vs Ca	-2.225	-3.37	-1.078	yes
Na vs Control	-2.725	-4.35	-1.1036	yes
Mg vs Ca	-0.948	-2.1	0.1986	no
Mg vs Control	-1.449	-3.07	0.173	no
Ca vs Control	-0.501	-2.12	1.121	no

Appendix table A3. 8 - Descriptive statistics for effect of cations on motility

Strains		15A04	15G01	16A01	33H04
Cations		**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**
Control	**Control**	26.356 ± 0.525	29.024 ± 1.813	39.771 ± 1.728	27.580 ± 0.764
Magnesium	**1 mM**	32.417 ± 0.746	31.18 ± 0.269	36.842 ± 0.486	27.58 ± 0.764
	10 mM	35.201 ± 0.298	26.304 ± 0.559	33.963 ± 0.336	25.06 ± 0.23
	50 mM	28.728 ± 0.867	18.037 ± 0.242	31.627 ± 0.412	24.212 ± 0.728
Calcium	**1 mM**	21.402 ± 0.454	19.333 ± 0.481	25.39 ± 0.379	19.865 ± 0.425
	10 mM	22.916 ± 0.545	20.555 ± 0.496	36.928 ± 0.37	26.721± 0.265
	50 mM	26.168 ± 0.506	20.432 ± 0.62	29.144 ± 0.393	17.28 ± 0.526
Sodium	**1 mM**	32.012 ± 0.547	17.494 ± 0.384	22.808 ± 0.557	15.817 ± 0.393
	10 mM	34.433 ± 0.336	31.071 ± 0.348	36.368± 0.686	25.708 ± 0.308
	50 mM	53.081 ± 0.423	40.263 ± 0.884	41.378 ± 0.387	43.367 ± 0.615

Appendix table A3. 9- ANOVA summary table to test the effect of cations on motility.

Source of variation	df	F value	p
Strains	3	331.7	<.001
Cation	2	301.79	<.001
Concentration	3	146.52	<.001
Strains.Cation	6	29.46	<.001
Strains.Concentration	9	43.11	<.001
Cation.Concentration	6	288.04	<.001
Strains.Cation.Concentration	18	17.34	<.001

Appendix table A3. 10 - Tukey's 95% confidence interval comparison to test the effect of cations on motility. (Ca- Calcium; Na- Sodium; Mg – Magnesium)

Comparison	Difference	Lower 95%	Upper 95%	Significant
Ca vs Mg	-4.064	-4.713	-3.415	yes
Ca vs Na	-6.729	-7.378	-6.08	yes
Mg vs Na	-2.665	-3.315	-2.016	yes

Appendix table A3. 11- ANOVA summary table to test the effect of cations on Biofilm formation and planktonic cells.

Nature	Cations	Source of variation	d.f.	F value	P
Biofilm	Magnesium	Strains	3	9.53	<.001
		Concentration	3	261.36	<.001
		Strains.Concentration	9	7.44	<.001
	Calcium	Strains	3	2.7	0.048
		Concentration	3	280.97	<.001
		Strains.Concentration	9	8.03	<.001
	Sodium	Strains	3	16.39	<.001
		Concentration	3	334.43	<.001
		Strains.Concentration	9	9.01	<.001
Planktonic cells	Magnesium	Strains	3	6.76	<.001
		Concentration	3	205.6	<.001
		Strains.Concentration	9	2.88	0.004
	Calcium	Strains	3	27.78	<.001
		Concentration	3	67.87	<.001
		Strains.Concentration	9	2.17	0.028
	Sodium	Strains	3	13.22	<.001
		Concentration	3	60.2	<.001
		Strains.Concentration	9	1.76	0.082

Appendix for chapter 4

Appendix table A4. 1- RNA sequencing general statistics for 15G01 in presence of magnesium (Raw reads: the total amount of reads of raw data. For paired-end sequencing, it equals the amount of read1 and read2. All the samples have sequences of 150 bp).

15G01					
Sample	**Raw reads**	**Total base count (Q30)**	**GC content**	**Unique reads**	**Duplicate reads**
Magnesium_1_1	11,100,080	95.19	41%	1,067,525	10032555
Magnesium_1_2			42%	5,179,132	5920948
Magnesium_2_1	12,593,020	95.5	42%	1,017,413	11575607
Magnesium_2_2			43%	4,870,609	7722411
Magnesium_3_1	11,055,360	95.08	40%	1,115,469	9939891
Magnesium_3_2			41%	5,065,443	5989917
Magnesium_4_1	12,808,587	94.89	44%	1,166,290	11642297
Magnesium_4_2			45%	5,822,651	6985936
Magnesium_5_1	12,502,425	95.41	42%	1,193,635	11308790
Magnesium_5_2			43%	5,811,828	6690597
Magnesium_6_1	11,930,896	94.77	40%	1,496,194	10434702
Magnesium_6_2			40%	6,814,321	5116575
Control_1_1	18,702,289	95.98	51%	949,887	12353335
Control_1_2			51%	5,037,275	8265947
Control_2_1	13,303,222	94.84	48%	495,877	18206412
Control_2_2			48%	3,014,938	15687351
Control_3_1	10,117,994	95.52	43%	923,295	9194699
Control_3_2			44%	4,027,680	6090314
Control_4_1	10,922,888	94.91	41%	1,323,529	9599359
Control_4_2			42%	5,804,375	5118513
Control_5_1	11,250,324	94.91	42%	1,214,683	10035641
Control_5_2			43%	5,749,602	5500722
Control_6_1	10,167,079	95.32	42%	1,075,490	9091589
Control_6_2			42%	4,234,450	5932629

Appendix table A4. 2- RNA sequencing general statistics for 15A04 in presence of magnesium (Raw reads: the total amount of reads of raw data. For paired-end sequencing, it equals the amount of read1 and read2. All the samples have sequences of 150 bp).

15A04					
Sample	**Raw reads**	**Total base count (Q30)**	**GC content**	**Unique reads**	**Duplicate reads**
Magnesium_1_1	12,704,986	95.21	**39%**	1230751	11474235
Magnesium_1_2			41%	6913410	5791576
Magnesium_2_1	10,995,951	95.65	42%	976925	10019026
Magnesium_2_2			43%	4637155	6358796
Magnesium_3_1	10,903,668	95.05	40%	1227792	9675876
Magnesium_3_2			41%	4645320	6258348
Magnesium_4_1	12,967,248	94.96	41%	1630187	11337061
Magnesium_4_2			42%	6819690	6147558
Magnesium_5_1	12,807,118	95.72	41%	1191979	11615139
Magnesium_5_2			42%	5821926	6985192
Magnesium_6_1	11,206,046	94.84	44%	1411693	9794353
Magnesium_6_2			44%	5793487	5412559
Control_1_1	12,179,660	95.27	49%	559272	11620388
Control_1_2			49%	3798762	8380898
Control_2_1	13,534,205	95.05	47%	1200068	12334137
Control_2_2			47%	5503058	8031147
Control_3_1	8,260,135	95.91	45%	713101	7547034
Control_3_2			46%	3023480	5236655
Control_4_1	10,923,471	94.42	47%	937168	9986303
Control_4_2			48%	4644398	6279073
Control_5_1	11,017,006	95.21	41%	1233807	9783199
Control_5_2			43%	5680743	5336263
Control_6_1	8,556,467	95.04	43%	762126	7794341
Control_6_2			44%	3717188	4839279

Appendix table A4.3- RNA sequencing general statistics for 15G01 in presence of sodium. Raw reads: the total amount of reads of raw data. For paired-end sequencing, it equals the amount of read1 and read2. All the samples have sequences of 150 bp.

15G01					
Sample	**Raw reads**	**Total base count (Q30)**	**GC content**	**Unique reads**	**Duplicate reads**
Control_1_1	18,702,289	95.98	51%	949,887	12353335
Control_1_2			51%	5,037,275	8265947
Control_2_1	13,303,222	94.84	48%	495,877	18206412
Control_2_2			48%	3,014,938	15687351
Control_3_1	10,117,994	95.52	43%	923,295	9194699
Control_3_2			44%	4,027,680	6090314
Control_4_1	10,922,888	94.91	41%	1,323,529	9599359
Control_4_2			42%	5,804,375	5118513
Control_5_1	11,250,324	94.91	42%	1,214,683	10035641
Control_5_2			43%	5,749,602	5500722
Control_6_1	10,167,079	95.32	42%	1,075,490	9091589
Control_6_2			42%	4,234,450	5932629
Sodium_1_1	12,537,876	95.05	45%	829,508	11708368
Sodium_1_2			46%	4,173,754	8364122
Sodium_2_1	15,254,868	94.81	50%	484,677	14770191
Sodium_2_2			50%	5,395,064	9859804
Sodium_3_1	17,351,022	94.7	43%	1,541,640	15809382
Sodium_3_2			44%	7,626,923	9724099
Sodium_4_1	12,103,286	94.62	41%	1,285,435	10817851
Sodium_4_2			42%	5,300,352	6802934
Sodium_5_1	12,928,540	94.69	46%	828,616	12099924
Sodium_5_2			47%	4,404,999	8523541
Sodium_6_1	11,509,368	94.33	41%	1,190,224	10319144
Sodium_6_2			42%	5,737,403	5771965

Appendix table A4.4 - RNA sequencing general statistics for 15A04 in presence of sodium. Raw reads: the total amount of reads of raw data. For paired-end sequencing, it equals the amount of read1 and read2. All the samples have sequences of 150 bp.

15A04					
Sample	**Raw reads**	**Total base count (Q30)**	**GC content**	**Unique reads**	**Duplicate reads**
Control_1_1	12,179,660	95.27	49%	559272	11620388
Control_1_2			49%	3798762	8380898
Control_2_1	13,534,205	95.05	47%	1200068	12334137
Control_2_2			47%	5503058	8031147
Control_3_1	8,260,135	95.91	45%	713101	7547034
Control_3_2			46%	3023480	5236655
Control_4_1	10,923,471	94.42	47%	937168	9986303
Control_4_2			48%	4644398	6279073
Control_5_1	11,017,006	95.21	41%	1233807	9783199
Control_5_2			43%	5680743	5336263
Control_6_1	8,556,467	95.04	43%	762126	7794341
Control_6_2			44%	3717188	4839279
Sodium_1_1	14,518,815	95.19	46%	854195	13664620
Sodium_1_2			46%	5240625	9278190
Sodium_2_1	10,726,378	95.18	45%	854946	9871432
Sodium_2_2			46%	3650239	7076139
Sodium_3_1	9,752,421	94.86	40%	1129541	8622880
Sodium_3_2			42%	4113197	5639224
Sodium_4_1	8,204,892	94.54	40%	1344432	6860460
Sodium_4_2			41%	4222600	3982292
Sodium_5_1	12,994,197	95.32	42%	1172905	11821292
Sodium_5_2			43%	5449695	7544502
Sodium_6_1	11,137,148	94.99	44%	1089132	10048016
Sodium_6_2			44%	4831903	6305245

Appendix for chapter 5

Appendix table A5.1- ANOVA summary table to test the effect of cation on biofilm formation.

Source of variation	**d.f.**	**F**	**p**
Strains	1	17.07	0.001
Cation	3	114.52	<.001
Strains.Cation	3	2.71	0.085

Appendix table A5.2- ANOVA summary table to test the effect of cation on polysaccharide content in EPS.

Source of variation	**d.f.**	**F**	**p**
Strains	1	18.78	<.001
Cation	3	10.17	<.001
Strains.Cation	3	4.26	0.025

Appendix table A5.3- ANOVA summary table to test the effect of cation on eDNA content in EPS.

Source of variation	**d.f.**	**F**	**p**
Strains	1	2.69	0.123
Cation	3	23.36	<.001
Strains.Cation	3	0.61	0.62

Appendix table A5.4- Tukey's 95% confidence interval comparison to test the effect of cations on biofilm formation, eDNA and polysaccharides. The column denotes the significant difference between the two cations.

	Significant		
Comparison	**Biomass**	**eDNA**	**Polysaccharide**
Control vs Sodium	yes	yes	no
Control vs Calcium	yes	yes	no
Control vs Magnesium	yes	yes	yes
Sodium vs Calcium	yes	no	no
Sodium vs Magnesium	yes	no	yes
Calcium vs Magnesium	no	no	yes

Appendix for overall study

Appendix Image A1.a- Concentration of major ions in seawater (Pilson, 1998, Chpt. 4)

Table 4.1 *Concentrations of the major constituents in surface seawater*

		At salinity (PSS 1978): S = 35.000%		
	$mg\ kg^{-1}\ S^{-1}$	g/kg	mmol/kg	mM
Na^+	308.0	10.781	468.96	480.57
K^+	11.40	0.399	10.21	10.46
Mg^{++}	36.69	1.284	52.83	54.14
$*Ca^{++}$	11.77	0.4119	10.28	10.53
$*Sr^{++}$	0.227	0.00794	0.0906	0.0928
Cl^-	552.94	19.353	545.88	559.40
$SO_4^=$	77.49	2.712	28.23	28.93
$*HCO_3^-$	3.60	0.126	2.06	2.11
Br^-	1.923	0.0673	0.844	0.865
$B(OH)_3$	0.735	0.0257	0.416	0.426
F^-	0.037	0.00130	0.068	0.070
Totals	1004.81	35.169	1119.87	1147.59
*Alkalinity	—.—	—.—	2.32	2.38
Everything else	—.—	~0.03	—.—	—.—
Water	—.—	~964.80	~53,555.	~54,881.

Appendix Table A1.b – Baseline concentration of the three cations in the mussel meat and mussel intravalvular juice was determined using ICP-OES

	Mussel meat (mM)	Mussel intravalvular juice (mM)
Calcium	7.49	15.22
Magnesium	15.22	37.03
Sodium	121.85	326.37