The Efficacy of Bacteriocins to Control *Listeria monocytogenes*

by

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Abstract

Listeria monocytogenes is a problem in ready-to-eat meat products and new methods of food preservation, such as bacteriocins, are being investigated to control this organism. Leucocin A and carnocyclin A, are class IIa and class IIc bacteriocins that have antilisterial activity. The aims of this research were to: a) improve the purification protocols for leucocin A and carnocyclin A to increase the yield of peptide obtained for downstream experiments and potential commercialization purposes; and b) evaluate the efficacy of leucocin A and an analogue of leucocin A to inhibit the growth of *L. monocytogenes* on wieners in the presence of spoilage organisms. The strains of *L. monocytogenes* used in this research were associated with foodborne illness and developed to use in challenge studies. The strains of *L. monocytogenes* were also grown in media supplemented with different carbohydrates (glucose, sucrose, fructose, mannose, cellobiose) with or without the addition of leucocin A or carnocyclin A and the growth curves were modelled to determine the growth kinetics. Lastly, RNA-Sequencing was employed to investigate the transcriptome of *L. monocytogenes* FSL C1-056 when cells were grown in mannose or cellobiose and treated with carnocyclin A. Additionally, the genome of *L. monocytogenes* FSL C1-056 was sequenced, annotated and used to assess the expressed genes and potential ncRNA regions that may relate to *L. monocytogenes* stress response to carnocyclin A. Minimum inhibitory concentrations could not be determined for *L. monocytogenes* exposed to leucocin A in broth; however, on agar the minimum inhibitory concentrations ranged from 11.7–62.5 μM and 62.5–>500 μM for leucocin A and leucocin A analogue, respectively. The inactivation profiles of *L. monocytogenes* in broth in the presence of leucocin A suggested each isolate had different

levels of resistance to the bacteriocin as determined by the initial bactericidal effect. The formation of spontaneously resistance subpopulations were also observed for each strain of *L. monocytogenes*. *In situ*, leucocin A reduced the counts *L. monocytogenes* on wieners during storage, regardless of the presence of *C. divergens. B. thermosphacta* was unaffected by the presence of leucocin A on wieners over the duration of storage. Growth in media containing glucose, mannose, and fructose increased the sensitivity of some strains of *L. monocytogenes* to leucocin A and carnocyclin A, while growth in cellobiose and sucrose increased the resistance of *L. monocytogenes* to leucocin A and carnocyclin A, as evidenced by a shorter lag phase. Strains of *L. monocytogenes* developed resistance to both leucocin A and carnocyclin A, but the time to develop resistance was longer when strains were exposed to carnocyclin A. *L. monocytogenes* transcriptome revealed the expression of genes involved in the mannose phosphotransferase system were down-regulated when *L. monocytogenes* was grown with carnocyclin A in either mannose or cellobiose by 6.87 to 7.42- $\log_2$ fold and 2.14 to 2.22- $\log_2$ fold, respectively, as compared to cultures grown without carnocyclin A. The expression of genes involved in β-glucoside and cellobiose specific phosphotransferase systems was upregulated by 1.64 to 1.77- $\log_2$ fold when *L. monocytogenes* was grown in cellobiose, as compared to cultures grown without carnocyclin A. There were a total of 366 ncRNAs predicted from the genome, yet only 7 of them were differentially expressed for all the treatment group comparisons, including 4 T-box riboswitches, Rli47, Rli53, and Rli24. These studies suggest that leucocin A may be beneficial to the industry as a surface application on wieners to help reduce *L. monocytogenes* counts due to post-processing contamination even in the presence of spoilage organisms. Carbohydrates influence the growth kinetics

and stress response of *L. monocytogenes* strains in the presence of leucocin A and carnocyclin A. The stress response and the development of resistance to carnocyclin A in *L. monocytogenes* FSL C1-056 may be influenced by regulatory ncRNAs. The functional genes and ncRNAs expressed are informative in understanding the impact of bacteriocins on *L. monocytogenes* growth and resistance. All together the efficacy of bacteriocins to control *L. monocytogenes* can be influenced by application methods, product composition, and strain individuality. The findings presented in this thesis will allow for a more complete understanding of the broad stress response to bacteriocins in *L. monocytogenes,* which can lead to the development of resistance, and how the industry can mitigate this risk for effective control of *L. monocytogenes* in ready-to-eat meat products.

Supplemental material related to this thesis is available at https://era.library.ualberta.ca/collections/7p88ck40x

Preface

Chapter 1 of this thesis has been submitted as D.R. Balay and L.M. McMullen, "A review of *Listeria monocytogenes* stress response and development of resistance to bacteriocins," *Frontiers in Microbiology*. I was responsible for concept formation, data collection, and the manuscript composition. L.M. McMullen was the supervisory author and was involved with concept formation and manuscript composition.

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Chapter 1

1.0 Introduction

In recent years, consumers have demanded more readily available, fresh, minimally processed foods that do not contain traditional chemical preservatives (Hartmann et al., 2011; Sullivan et al., 2012; Zink, 1997). In response to consumer demands the food processing industry has responded by investigating natural food preservatives. Natural food preservatives can include naturally sourced chemical preservatives such as celery powder in replace of nitrites, essential oils and essences (ex. rosemary, clove, thyme), and biopreservatives, that include microorganisms and their metabolites (Gálvez et al., 2010). These natural food preservation methods align with consumer demands for more minimally processed, RTE foods. Some biopreservatives are generally recognized as safe (GRAS) making them attractive alternatives to traditional preservatives (Castellano et al., 2017; Chen and Hoover, 2003; Parada et al., 2007).

Bacteriocins are a promising class of biopreservative to control pathogens and spoilage organisms on perishable foods. Bacteriocins are ribosomally synthesised, cationic, antimicrobial peptides that have a narrow spectrum of activity (Cotter et al., 2005; Héchard and Sahl, 2002; Klaenhammer, 1993). Class I bacteriocins undergo extensive post-translational modification that results in non-standard amino acids, such as lanthionine. Class I has three subsets, the most studied is class Ia (lantibiotics), and the remainder are class Ib (labyrinthopeptins) and class Ic (sanctibiotics). Class II bacteriocins are small, heat stable peptides that undergo minimal post-translational modification. The four subsets of class II bacteriocins include class IIa (pediocin-like bacteriocins), class IIb (two-peptides unmodified bacteriocins), class IIc (circular bacteriocins) and class IId (unmodified, linear, non-pediocin-like bacteriocins). Class IIa are the most defined and studied bacteriocins of this class. Class III bacteriocins are large, heat liable peptides and have received limited attention (Kumariya et al., 2019).

Most bacteriocins are synthesized as precursors, transported across the cell membrane and a leader sequence is cleaved to produce an active bacteriocin (Chen et al., 2001; Cotter et al., 2005; Neis et al., 1997; Nes et al., 2013; van der Meer et al., 1994). The

positively charged bacteriocins electrostatically interact with the negatively charged target cell membrane before it binds to the cell membranes and forms ion selective pores (Chen et al., 1997; Johnsen et al., 2005; Rashid et al., 2016; Tiwari et al., 2009). These pores ultimately lead to cell death through dissipation of the proton motive force and depletion of intracellular ATP, and leakage of intracellular substrates (Bruno and Montville, 1993; Héchard and Sahl, 2002; Thibaut Jacquet et al., 2012; Montville and Bruno, 1994; Waite et al., 1998).

Bacteriocins are an attractive alternative method of food preservation for RTE foods, and many have potent antilisterial activity (Bruno and Montville, 1993; Castellano et al., 2017; Silva et al., 2018). Unfortunately, cells can adapt to their environments and become resistant to antimicrobial treatments. When cells become resistant to a bacteriocin, a common cellular response are changes to the fatty acid composition of the cell membrane (Mazzotta and Montville, 1997; Ming and Daeschel, 1993; Ming and Daeschel, 1995; Vadyvaloo et al., 2002). These changes result in a more positively charged cell surface, which repels the bacteriocins (Vadyvaloo et al., 2004a; Wu et al., 2018). Another common resistance mechanism is the downregulation of genes responsible for enzymes that are used as bacteriocin docking molecules. In this case, the cell surface does not have the receptor for bacteriocin docking. *Listeria monocytogenes* can form resistance to class I and class II bacteriocins (Kaur et al., 2011). This may be a concern for application of bacteriocins as preservatives to control the growth of *L. monocytogenes* in RTE food products.

Listeria monocytogenes are a gram positive, facultative anaerobic, rods, that can cause listeriosis, which has a high fatality rate of 20 to 40 % (Farber and Peterkin, 1991). Immunocompromised, elderly and/or pregnant women are at increased risk of being infected by *L. monocytogenes* (Drevets and Bronze, 2008; Farber and Peterkin, 1991; Gálvez et al., 2008). On average there are 1,600 individuals infected with *L. monocytogenes*, resulting in 260 deaths each year in the United States of America (CDC, 2014; Fuchs et al., 2012). In Canada, 7.2 cases per million population was reported in 2009 (Health Canada, 2011). The European Union summary report on trends and sources of zoonoses, zoonotic agents and foodborne outbreaks (2017) reported a trend of

increasing confirmed cases of listeriosis during 2008-2017 (Lanciotti et al., 2019). *L. monocytogenes* may not have the highest case frequency rate compared to other foodborne pathogens, but it has serious implications if a susceptible individual is infected (Thomas et al., 2015; Thomas et al., 2013). *L. monocytogenes* is often associated with ready-to-eat (RTE) food products and there are strict guidelines in place to control the growth of *L. monocytogenes* in RTE food products (European Food Safety Authority, 2014; FDA, 2007; Health Canada, 2011). *L. monocytogenes* is most commonly associated with post-processing contamination, which is a serious concern because most RTE products are not cooked before they are consumed (Health Canada, 2011). To further increase the risk of infection, *L. monocytogenes* can grow at a wide temperature range (-0.4 to 45°C), pH ≧ 4.4, a_w ≧ 0.92, and up to 10% NaCl (Health Canada, 2011).

The current research focuses on two bacteriocins, leucocin A and carnocyclin A, both of which are reported to have antilisterial activity (Hastings et al., 1991; Martin-Visscher et al., 2008). Both bacteriocins are defined as class II bacteriocins (Kumariya et al., 2019). Leucocin A is a class IIa bacteriocin that is produced by *Leuconostoc gelidum* UAL187 and carnocyclin A is a class IIc bacteriocin that is produced by *Carnobacterium maltaromaticum* ATCC PTA-5313 (Hastings and Stiles, 1991; Martin-Visscher et al., 2008). Leucocin A is a 37-residue bacteriocin with a molecular mass of 3,903 Da, it contains a conserved, charged, hydrophilic N-terminus motif of YGNGV(X)C(X)4C(X)V(X)4A (X denotes any amino acid residue) that forms a β-sheet structure (Bodapati et al., 2013; Fregeau Gallagher et al., 1997). The charged terminus allows for electrostatic interactions between the target cell surface and the bacteriocin (Fimland et al., 2005). There are cysteine residues (located at positions 9 and 14 in leucocin A) that form a disulphide bridge, which is important for proper conformation of the bacteriocin and subsequent antibacterial activity (Fregeau Gallagher et al., 1997; Sit et al., 2011). Leucocin A uses the cell bound enzymes of the mannose phosphotransferase system (Man-PTS) system as a docking station on the target cell and pore formation (Drider et al., 2006; Hastings and Stiles, 1991).

Carnocyclin A is a 60-residue circular bacteriocin as the N- and C- termini connect via an amide bond (Martin-Visscher et al., 2008; van Belkum et al., 2011). Circular bacteriocins

have increased stability against proteases due to their unique circular conformation (van Belkum et al., 2011). Carnocyclin A is thought not to require a docking molecule to form pores in the cell membrane as a study by Gong et al., (2009), demonstrated it forms anion selective channels in the lipid membrane; however more research is needed to ensure that there are no other modes of action for carnocyclin A.

Resistance to class IIa bacteriocins, such as leucocin A, has been correlated with the down regulation of genes associated with the Man-PTS, which affects the transcription of enzyme II subunits (Dalet et al., 2001; Diep et al., 2007; Gravesen et al., 2002). *L. monocytogenes* can also develop resistance to leucocin A by increasing the fluidity of the cell membrane and increasing the overall positive charge of the cell wall (Vadyvaloo et al., 2004b, 2004a, 2002). *L. monocytogenes* forms resistance to carnocyclin A though the underlying mechanism(s) is unknown at this time (Balay et al., 2018).

Additionally, the product composition and natural microflora on the RTE food product can affect the efficacy of the bacteriocin against *L. monocytogenes*. Certain microorganisms can produce proteases that have the potential to cleave bacteriocins, resulting in an inactive peptide (Balay et al., 2017; Casaburi et al., 2014; Hastings and Stiles, 1991; Nowak and Piotrowska, 2012). There is also a concern that bacteriocins may disrupt the natural balance of microflora on a product and allow for the outgrowth of potential pathogens due to the lack of a competitive microflora (Pothakos et al., 2015; Sullivan et al., 2012). The composition of the product is also an important aspect to consider because certain carbohydrates can influence the effect of some bacteriocins on *L. monocytogenes* (Arous et al., 2004; Balay et al., 2018; Bucur et al., 2018; Nilsson et al., 2004). These factors need to be investigated to ensure optimal efficacy of bacteriocins.

The overall hypothesis for this research was that bacteriocins (leucocin A and carnocyclin A) will control the growth of *L. monocytogenes* in RTE meats. More specifically, leucocin A and an analogue control the growth of *L. monocytogenes* on RTE meats in the presence of an autochthonous microbiota. Moreover, the carbohydrates available for metabolism will determine the growth kinetics and development of resistance to bacteriocins. Additionally, availability of specific carbohydrates will change the

expression of genes in response to exposure to bacteriocins. As such, the objectives of the current research were to:

1. investigate the efficacy and application of leucocin A and a leucocin A analogue on *L. monocytogenes* when applied to RTE-meat products in the presence of natural spoilage organisms;
2. assess the effect different carbohydrates have on the growth of *L. monocytogenes* in the presence of leucocin A and carnocyclin A; and
3. examine the effect of carnocyclin A on the transcriptome (mRNA and ncRNA) of *L. monocytogenes* grown in the presence of mannose or cellobiose.

The following review of the literature focuses on the stress response of *L. monocytogenes* to bacteriocins.

1.1 Literature Review

1.1.1 Introduction

Listeria monocytogenes is a foodborne pathogen that causes an estimated 178 cases of foodborne listeriosis annually in Canada, with a fatality rate of 20–30 % (Health Canada, 2011; Thomas et al., 2013). The European Union summary report on trends and sources of zoonoses, zoonotic agents and foodborne outbreaks (2017) reported a trend of increasing confirmed cases of listeriosis during 2008-2017 (Lanciotti et al., 2019). *L. monocytogenes* may not have the highest case frequency rate compared to other foodborne pathogens, but it has serious implications if a susceptible individual is infected (Thomas et al., 2015; Thomas et al., 2013). *L. monocytogenes* is typically associated with ready-to-eat (RTE) products as a result of post-processing contamination (Health Canada, 2011). *L. monocytogenes* can grow at refrigeration temperatures, which poses serious concern in RTE products, as they typically do not have an additional heating step (Zhu et al., 2005). In recent years consumers are looking for more RTE meals that are fresh and preserved with minimal addition of chemical (traditional) preservatives (Gálvez et al., 2010, 2008). Therefore, novel methods of biopreservation, such as bacteriocins, are being investigated as they have the potential to control a diverse group of foodborne pathogens, including *L. monocytogenes* (Gálvez et al., 2010).

Bacteriocins are antimicrobial peptides, that are ribosomally synthesized by lactic acid bacteria, and usually are active against gram positive bacteria (Cotter et al., 2005; Perez et al., 2014; Tessema et al., 2009). The bacteriocins are grouped into different classes; however classification systems vary (Alvarez-Sieiro et al., 2016; Cotter et al., 2005; Klaenhammer, 1993; Kumariya et al., 2019), for the purposes of this review the classification system devised by Kumariya et al., (2019), will be used. Bacteriocins form ion selective pores in the target cell membrane, that disrupts the proton motive force and ultimately leads to cell death (Alvarez-Sieiro et al., 2016; Cotter et al., 2005; Klaenhammer, 1993; Kumariya et al., 2019). However, there is concern for use of bacteriocins as biopreservatives (Vignolo et al., 2000), as *L. monocytogenes* isolates can be naturally resistant (Ennahar et al., 2000; Rasch and Knøchel, 1998) or acquire resistance to bacteriocins (Gravesen et al., 2002; Katla et al., 2003).

RNA sequencing (RNAseq) creates a snapshot of the transcriptional profile of an organism under specific conditions (Brantl and Brückner, 2014; Conesa et al., 2016; Croucher and Thomson, 2010; Mellin and Cossart, 2012). The technology provides both qualitative and quantitative information in terms of present transcripts under given conditions and relative gene expression of each transcript (Conesa et al., 2016). It allows for the examination of changes in gene expression for a given circumstance or treatment as well as the discovery of novel transcripts, such a non-coding RNA (ncRNA) (Brantl and Brückner, 2014). An analysis of *L. monocytogenes* transcriptome can identify key changes in gene expression related to the development of resistance to bacteriocins. It will help identify if there is narrow transcriptional regulation or a more global stress response that leads to bacteriocin resistance. Transcriptomics can provide information about pathogens present in food at the molecular level. The technology can predict which genes may be involved in different physiological states and identify shifts in the molecular response to a specific environment (Stasiewicz et al., 2015), which would allow the food industry to target strategies that will limit the development of resistance of *L. monocytogenes* to bacteriocins in foods.

Bacteria can sense and orchestrate a cascade of altered gene expression and protein activity in response to stress, such as bacteriocins (Citartan et al., 2016; Mentz et al., 2013), which ncRNA may regulate some of these changes in stress responses. To date, this has not received attention in the research literature. The ncRNAs are not translated into proteins, but do play a role in regulating transcription and translation in cells (Mentz et al., 2013). There is a more rapid connection between quorum sensing and direct destabilization of mRNA for ncRNAs than with proteins, and allows for fine tuning of the response at a post-transcriptional level (Ahmed et al., 2018). The majority of ncRNAs regulate translation or affect RNA stability; most inhibit translation and very few activate translation (Brantl and Brückner, 2014). Inhibition of translation can directly block the ribosomal binding site (RBS), induce structural alterations downstream of the RBS, or block a ribosome standby site required for efficient translation (though this is typically only detected in *Escherichia coli*) (Brantl and Brückner, 2014). In *L. monocytogenes* there are between 100 - 200 regulatory ncRNAs (Mellin and Cossart, 2012; Sievers et al.,

2015). These ncRNAs can be divided into 3 groups based on their mode of action for regulation:

1) Cis-acting RNAs reside in 5' untranslated regions (UTRs) of mRNA and affect transcription or translation (Citartan et al., 2016; Dar and Sorek, 2017; Mellin and Cossart, 2012). These ncRNA sense the surrounding environment and affect the stability and turnover of mRNA by altering the RNA structure of the regulator and turn expression "on" for the downstream open reading frame (ORF) (Dar and Sorek, 2017). This type of ncRNA is often associated with resistance mechanisms in bacteria, thus allowing *L. monocytogenes* to circumvent the fitness burden associated with the long term maintenance of genes related to resistance (Dar and Sorek, 2017; Dersch, et al., 2017).

2) Antisense RNAs (asRNAs) template strand runs 3' – 5' and are the least studied group of ncRNA (Citartan et al., 2016; Dar and Sorek, 2017; Mellin and Cossart, 2012). Transcription and translation of the sense encoded gene are mediated by asRNAs by interrupting the RNA polymerase activity on the opposite (template) strand (Citartan et al., 2016; Dar and Sorek, 2017; Mellin and Cossart, 2012).

3) Trans-acting small RNAs (sRNAs) base pair with target mRNA and modulate translational outcomes, but can also directly bind to proteins to change activity (Stasiewicz et al., 2015). Often asRNAs require a Hfq chaperone protein as a cofactor to allow interaction between the ncRNA and the target mRNA (Ahmed et al., 2018; Citartan et al., 2016). A common subset of sRNAs known as riboswitches (riboregulators), act as sensors for substrates and metabolites, and possess aptamers (RNA regions that are specific for a metabolite) (Ahmed et al., 2018). Once a riboswitch structure is changed due to a specific substrate or metabolite, it terminates transcription of the downstream gene (Ahmed et al., 2018). Riboswitches can be trans- or cis- acting (Ahmed et al., 2018).

RNA-Seq analysis of both mRNA and ncRNA can shed light on the global stress response and development of resistance in *L. monocytogenes* in the presence of bacteriocins, without the need for prior sequence knowledge as is the case with microarrays (Behrens et al., 2014). This review focuses on the effect of bacteriocins on

the stress response of *L. monocytogenes* and any potential ncRNAs that may play a regulatory role in the formation of resistance to bacteriocin exposure.

The most prominent class I bacteriocin that is active against *L. monocytogenes* is nisin, a class Ia bacteriocin produced by *Lactococcus lactis* (Kaur et al., 2011). Nisin acts upon the target cell membrane by binding to the anionic lipid II, displacing it and then inserting itself into the membrane to form a pore to ultimately cause cell death (Alvarez-Sieiro et al., 2016; Crandall and Montville, 1998). In addition to nisin there are other lantibiotcs and nonlantibiotics such as, lactococcin 972, which also use lipid II, as a docking molecule (Morett and Segovia, 1993; Shingler, 1996). The development of resistance to these bacteriocins is likely similar to that of nisin, though more research is needed on the formation of resistance to other bacteriocins within class I. There are a number of class II bacteriocins with activity against *L. monocytogenes* including pediocin, sakacin P, leucocins, enterococin, mesentericin Y105, garvicin, linocin M18, lacctoccins, and others (Eppert et al., 1997; Ovchinnikov et al., 2016; Perez et al., 2014; Ríos Colombo et al., 2018; Tosukhowong et al., 2012). Many class II bacteriocins require docking molecules (e.g. Man-PTS, sugar ABC transporters, lipid II), and *L. monocytogenes* can develop resistance through the regulation of gene expression of genes related to the cell wall and docking molecules (Gravesen et al., 2002; Ramnath et al., 2004, 2000, Vadyvaloo et al., 2004b, 2004a, 2002). There are no class III bacteriocins with documented activity against *L. monocytogenes* reported in the literature.

1.1.2 Cell Well Modifications

Modulation to the cell membrane in response to nisin stress is thought to be the main response in the development of resistance (Kaur et al., 2011). Changes in the cell membrane result in a more positive, rigid, surface making it harder for the bacteriocin to penetrate the membrane. When there are more positively charged D-alanine residues incorporated into the cell membrane, nisin is not able to bind to the target cell, thus increasing the resistance of the cell (Giaouris et al., 2008). Similarly, *Streptococcus bovis* has a more positively charged cell wall and has increased resistance to nisin (Mantovani

and Russell, 2001). However, the downregulation of *dltA* and *dltB* in nisin resistant persister cells of *L. monocytogenes* suggests that D-alanine residues are not always involved in the elevated resistance to nisin (Wu et al., 2018). This report also emphasized significant changes in expression of two cell-wall associated genes (*lmo2714*, *lmo2522*), which encode a peptidoglycan anchored protein, and a cell wall-binding protein, respectively. These proteins are perhaps responsible for changes in the cell wall that increase the tolerance of *L. monocytogenes* to nisin. Other changes to the cell wall that increase nisin resistance are changes in the β-1,4 glyosidic bonds between N-acetylmuramicglucosamine and N-acetylglucosamine of the cell wall peptidoglycan (Crandall and Montville, 1998), which makes the membrane more rigid and harder for the nisin to penetrate. The fatty acid and phospholipid composition of the cell membrane is another way *L. monocytogenes* can increase its resistance to nisin (Mazzotta and Montville, 1997; Ming and Daeschel, 1995, 1993). *L. monocytogenes* with stable nisin resistance contains a greater proportion of straight chain fatty acids compared to branched chain fatty acids (Ming and Daeschel, 1995), which aligns with the thought that a more rigid cell membrane allows for increased resistance to nisin. Other changes to the fatty acid composition include increased long chain fatty acids versus short chain fatty acids making it hard for nisin to penetrate all the way through the membrane (Mazzotta and Montville, 1997). *L. monocytogenes* with phospholipid heads that contain less diphosphatidylglycerol (DPG) are more resistant to nisin (Demel et al., 1996). Furthermore, *L. monocytogenes* cells that produced more phosphatidylglycerol than DPG were more resistant to nisin, which affects the ability of nisin to penetrate through the cell wall (Verheul et al., 1997). In addition to regulation of cell wall components, divalent cations are also thought to play a role in membrane stabilization, which could in turn impact the resistance to bacteriocins (Crandall and Montville, 1998). The divalent cations interact with the more negatively charged regions of the cell membrane to prevent binding of bacteriocins to these anionic areas (Crandall and Montville, 1998). Divalent cations also cause the acyl chains to pack more tightly together to form a more rigid cell membrane that could allow the cell to withstand the inhibitory effects of nisin (Kaur et al., 2011). Ultimately, all the changes to the cell wall result in a more rigid and overall positively charged cell wall that hinders the interactions between nisin and *L.*

monocytogenes anionic phospholipids (Driessen et al., 1995; Garcia et al., 1993; Martin et al., 1996).

Class II bacteriocins also cause changes to the cell wall of *L. monocytogenes* that result in resistance. However, the main difference is that class II bacteriocins do not make the cell membrane more rigid as in the case of class I bacteriocin resistance, but in fact makes the cell membrane more fluid. *L. monocytogenes* that are resistant to class II bacteriocins have a greater concentration of unsaturated fatty acyl chains and short acyl chains of phosphoglycerids (Vadyvaloo et al., 2002), and these changes result in a more fluid membrane. In addition, Vadyvaloo et al., (2004a), reported that resistant *L. monocytogenes* also has a more positive cell surface, which is due to the incorporation of more D-alanine into the techoic acids, which is similar to that of nisin resistance in *L. monocytogenes*. The charge of the cell membrane was also influenced by an increase in the lysinylation of phospholipids, as when more L-lysine is incorporated into the phospholipids the cell membrane becomes more positive and cationic bacteriocins have difficulty binding to the membrane and form pores (Vadyvaloo et al., 2004a). A functioning MprF protein is necessary for the biosynthesis of lysylphosphatidylglycerols (Peschel et al., 2001), which contribute to a more positive membrane. In *mprF* gene mutants, the incorporation of lysine into the membrane phospholipids was dysfunctional and increased the sensitivity to bacteriocins (Peschel et al., 2001; Samant et al., 2009; Thedieck et al., 2006). Class IIa bacteriocin stress induces changes in *L. monocytogenes* membrane allowing it to overcome pore formation and evade cell death. This is similar to that seen in the presence of nisin; however one main difference is how the cell becomes more fluid in the presence of class IIa bacteriocins rather than rigid to prevent pore formation.

Cyclical bacteriocins, which are now classified as class IIc bacteriocins (Kumariya et al., 2019) also have activity against *L. monocytogenes* (Potter et al., 2014; van Belkum et al., 2011). The first cyclic bacteriocin to be reported was enterocin AS-48, a bacteriocin produced by *Enterococcus faecalis* (Gálvez et al., 1986). The mechanism of resistance to cyclic bacteriocins is similar to that reported for class IIa bacteriocins. A higher proportion of branched chain fatty acids in a mutant of *L. monocytogenes*, increases

tolerance to enterocin AS-48 (Mendoza et al., 1999), which is similar to one of the resistance mechanisms observed with class IIa bacteriocins. Interestingly, the mutant with increased tolerance to enterocin AS-48 also had a higher proportion of C15:C17 fatty acids than wildtype cells and an increased cell wall thickness, which is similar to the mechanism of resistance of *L. monocytogenes* to nisin (Mendoza et al., 1999). Changes in cell envelope structure have been identified in *Bacillus cereus* that are resistant to enterocin AS-48. Transcriptomic analysis of resistant *B. cereus* exposed to enterocin AS-48 revealed that membrane associated or secreted proteins and a gene involved in a PadR-type transcriptional regulator were upregulated (Grande Burgos et al., 2009). However, the same was not observed when cells were exposed to nisin. *Bacillus subtilis* 168, which lacks the PadR-type operon, is sensitive to enterocin AS-48 and nisin (Grande Burgos et al., 2009), revealing the importance of the PadR-type operon in resistance. Furthermore, when the gene encoding the membrane protein (BC4207) was introduced and expressed in *B. subtilis* 168, the transformant had increased resistance to enterocin AS-48, but not to nisin (Grande Burgos et al., 2009). This indicates that the membrane protein BC4207 is one of the resistant mechanisms that *B. cereus* has against enterocin AS-48, which could also be a candidate for the formation of resistance to enterocin AS-48 and other cyclic bacteriocins in *L. monocytogenes*.

As all bacteriocins have to come into contact with the target cell membrane before they can bind and disrupt the membrane to form a pore, which allows for cellular leakage and death, it makes sense that the first line of defence against bacteriocins would be to modulate the cell surface. *L. monocytogenes* can change the charge of its cell membrane to prevent electrostatic interaction, increase the thickness and complexity of the cell wall to hinder penetration, as well as change the fluid dynamics of the cell membrane depending on the bacteriocin present. All of these stress responses work together and play a role in the prevention of pore formation and lead to increased resistance to the presence of bacteriocins.

1.1.3 Two Component Systems

Two component systems are an important part of regulating the stress response and formation of resistance to bacteriocins in *L. monocytogenes* (Gandhi and Chikindas,

2007; Kaur et al., 2011). The two component system LisRK is linked to multiple stress responses, including exposure to nisin (Soni et al., 2011) and both components play a role in the resistance of *L. monocytogenes* to nisin. In nisin resistant persister cells, *lisR* is downregulated (4.12-fold change) which indicates a role in resistance (Wu et al., 2018). A mutant strain of *L. monocytogenes* Δ*lisK*, which lacks the histidine kinase sensor, displays enhanced resistance to nisin (Cotter et al., 2002). The LisRK two component system regulates a three component system, known as $LiaFSR_{Lm}$, which responds to environmental stresses (Fritsch et al., 2011). The $LiaFSR_{Lm}$ is induced in the presence of antibiotics or cyclic adenosine monophosphate and results in changes to proteins and the cytoplasmic composition of a cell, making *L. monocytogenes* sensitive to antimicrobials such as nisin (Collins et al., 2012; Fritsch et al., 2011; Helmann and Eiamphungporn, 2008; Mascher et al., 2004). When *L. monocytogenes* is exposed to nisin the expression of the gene *lmo1021* (LiaS, which encodes another histidine kinase) is significantly down regulated and the *L. monocytogenes* Δ*liaS* exhibits increased resistance to nisin (Collins et al., 2012), further confirming its role in the response of *L. monocytogenes* to nisin. LisRK is also a known regulator for a penicillin binding protein (*lmo2229/pbp2229*), which has increased transcription in strains of *L. monocytogenes* that are resistant to nisin (Collins et al., 2012). A double knockout Δ*liaS* Δ*lisK* disrupted *pbp2229* function enough to cause dramatic sensitization to nisin (Collins et al., 2012). Other studies have also found that *pbp2229* is upregulated in nisin resistant strains of *L. monocytogenes* (Calvez et al., 2008; Gravesen et al., 2001).

Other important genes related to nisin resistance under the regulation of LiaR include *lmo1746* and *lmo1967*, which encode an ABC transporter permease and are predicted to be part of an antimicrobial peptide detoxification module and a toxic ion resistant protein (TelA), respectively (Bergholz et al., 2013; Collins et al., 2010). *L. monocytogenes* with a *lmo1746* knockout is more sensitive to nisin compared to the wildtype strain; however, more research is needed to determine the role of Lmo1746 in the detoxification process (Bergholz et al., 2013). TelA deletion mutants in *L. monocytogenes* are more susceptible to nisin than their complements, suggesting a role in the ability of *L. monocytogenes* to develop resistance to nisin, although there is no proposed mechanism for its role in

resistance (Collins et al., 2010). Other genes, such as *lmo0287* (a two component response regulator) have recently been implicated as having a role in the resistance of persister cells of *L. monocytogenes* to nisin (Wu et al., 2018). The two component system LisRK and its regulon are involved in *L. monocytogenes* ability to develop resistance to nisin. However, more research is needed in all the downstream genes that are affected by the changes in regulation to LisRK.

Another important two component system, VirRS, and its response regulator, VirR, protect against cell envelope stress by increasing the cell surface charge by affecting the *dltABCD* operon, MprF, and AnrAB (an ABC transporter thought to be involved in the detoxification of antimicrobials; (Abachin et al., 2002; Collins et al., 2010; Grubaugh et al., 2018; Kang et al., 2015; Thedieck et al., 2006). VirRS regulates genes involved in cell wall modification that are linked to the development of resistance in *L. monocytogenes*. In a *L. monocytogenes* mutant, Δ*virR*, there was a 70 -fold downregulation in transcription of a regulatory ncRNA, Rli32 (*lmos24*; Grubaugh et al., 2018). The regulatory ncRNA is of interest due to the strong correlation of impaired transcription related to the loss of VirR. Rli32 could affect the response of *L. monocytogenes* to class I bacteriocins as the regulatory ncRNA affects the VirR regulon, which provides *L. monocytogenes* defense against bacteriocins. Together the altered gene expression of two component systems and the possible regulatory function of ncRNA, may play an important role in modulating the stress response and formation of resistance to class I bacteriocins. However, there has been no established link between two component systems and the stress response in *L. monocytogenes* when exposed to class II bacteriocins.

1.1.4 ABC Transporters

Various ABC transporters in *L. monocytogenes* are known to participate in bacteriocin resistance (Collins et al., 2010; Grubaugh et al., 2018). The VirRAB ABC transporter is required for nisin resistance in *L. monocytogenes*, which was confirmed by a knockout, *L. monocytogenes* ΔVirAB, that lost its resistance to nisin (Grubaugh et al., 2018). Sugar ABC transport systems are key for energy metabolism in cells and the transport of sugar. ABC transporter genes (*tcsA, lmo1730 and lmo0278*) are linked to the decreased

metabolic function of bacteriocin resistant cells (Wu et al., 2017). ABC transporters related to efflux systems (*zurA, lmo0919, lmo0152,* and *lmo0135*) are upregulated in nisin persister cells (Wu et al., 2018). Efflux systems specifically related to metal ion transporters undergo significant changes in gene expression in nisin resistant persister cells of *L. monocytogenes,* as compared to non-persister cells, indicating their importance in nisin resistance. The antibiotic ABC transporter ATP-binding protein gene (*lmo0919*) and the peptide ABC transporter substrate-binding protein genes (*lmo0152* and *lmo0135*) are distinctly upregulated in nisin resistant persister cells of *L. monocytogenes*. The antibiotic regulated riboregulator, *lmo0919*, is capable of mediating expression of mRNA that encodes for antibiotic resistance genes, specifically for lincomycin, but transcription regulated by *lmo0919* is terminated in the absence of the antibiotic (Dar and Sorek, 2017). The *lmo0919* gene is likely to be a major contributor to the formation of nisin resistant persister cells of *L. monocytogenes.* When nisin is removed from the environment, the persister cells become sensitive to nisin, which is similar to how cells behave in the presence of lincomycin (Wu et al., 2018). However, the phenotypic resistance of persisters should be confirmed by the knock down of the riboregulator. However, this may not be possible as the riboregulator may be essential for other functions in the cell. In addition to the riboregulator, two ABC transporter binding protein (*lmo0152*, *lmo0135*) play a role in nisin resistance (Wu et al., 2018). These proteins are structurally related to OppA, which is required for bacterial survival under a variety of stresses (Borezee et al., 2000). OppA mediates the transport of oligopeptides across the cytoplasmic membrane for use in quorum sensing (Bierne and Cossart, 2007). Therefore, it is thought that Lmo0152 and Lmo0135 are involved in quorum sensing in *L. monocytogenes* in the presence of nisin, which assists in the formation of resistance. Another example of an ABC transporter involved in bacteriocin resistance is AnrAB, which protects *L. monocytogenes* against bacitracin and β-lactam antibiotics (Collins et al., 2010). AnrAB is under the control of the two component system VirRS, which has increased expression in the presence of nisin (Wu et al., 2018), suggesting that AnrAB plays a role in detoxification of antimicrobials, including nisin. *L. monocytogenes* stress response to class I bacteriocins affect the expression of ABC transporters, which are related to various general stress response pathways. These include efflux systems, cell

membrane proteins, cell signalling pathways and metabolism, all of which are for the cell to react to the stress of the bacteriocin and become resistant. Some ABC transporters are regulated by two component systems that are also affected in the presence of bacteriocins further indicating the breadth of the two component regulons and their importance to the downstream affected genes.

ABC transporters are also involved in resistance to circular bacteriocins. A spontaneous mutant of *Lactococcus lactis* IL1403 had increased resistance to garvicin ML (Gabrielsen et al. 2012), a cyclic bacteriocin (Bastos et al., 2015). The increased resistance to garvicin ML was linked to a deleted ABC transporter for maltose (*malEFG* operon). When the mutants were complemented with the *malEFG* operon they became sensitive to garvicin ML. This could indicate that garvicin ML uses the maltose ABC transporter as a receptor on target organisms.

Exposure of *L. monocytogenes* to carnocyclin A, a circular bacteriocin, resulted in changes in the transcriptome of *L. monocytogenes* related to a regulatory ncRNA, *rli53* (Chapter 4, this thesis). Rli53 is flanked by the gene *lmo0919* that is a primary candidate for resistance to nisin (Lebreton and Cossart, 2017). Rli53 does not function properly in the presence of a sublethal dose of lincomycin; however, it typically acts as a riboswitch for a downstream ABC transporter that is involved in lincomycin resistance (Lebreton and Cossart, 2017). Although no cross-resistance has been documented between nisin and circular bacteriocins, there is overlap in the effect of bacteriocins on expression of some of the genes involved in the development of resistance in *L. monocytogenes*, which suggests a more broad response in the formation of resistance to bacteriocins.

1.1.5 Sugar Phosphotransferase Systems

Resistance of *L. monocytogenes* to nisin is linked to reduced expression of the Man-PTS (Crandall and Montville, 1998; Wu et al., 2018). In addition, cellobiose PTS genes were also downregulated, ultimately slowing sugar metabolism in response to exposure to nisin. Together sugar PTS and sugar ABC transporters downregulate *L. monocytogenes* metabolism in response to nisin. It could also indicate that the proteins involved in sugar

PTS could be an alternate docking station for nisin and the downregulation of the genes impedes the ability of nisin to bind to the cell, thus increasing resistance (Wu et al., 2018).

Resistance of *L. monocytogenes* to class IIa bacteriocins such as pediocin PA-1, leucocin A, sackacin P, and class IIb bacteriocins lactococcin A and lactococcin 972 has been linked to the down regulation of the Man-PTS (Gravesen et al., 2002; Kjos et al., 2011; Opsata et al., 2010; Ramnath et al., 2004, 2000; Tessema et al., 2011). Study of *L. monocytogenes* that are resistant to divergicin V41 lead to the discovery that resistance to class IIa bacteriocins is through the regulation and expression of the EII_t^{man} (Gravesen et al., 2002). Others have confirmed that resistance to class IIa bacteriocins involves the enzymes of the Man-PTS, specifically the enzyme II subunits A/B,C,D, with the membrane bound subunits EIIC and EIID used as docking stations for these bacteriocins (Gravesen et al., 2002; Kjos et al., 2011; Opsata et al., 2010; Ramnath et al., 2004, 2000). Pediocin PA-1 resistant *L. monocytogenes* mutants upregulate fragments associated with a β-glucoside-specific PTS (Gravesen et al., 2000). This is considered a general mechanism of *L. monocytogenes* to compensate for downregulated genes related to the Man-PTS (Dalet et al., 2001; Gravesen et al., 2002). This is similar to the resistance mechanism associated with class I bacteriocins, indicating that the Man-PTS is an important aspect of the response of *L. monocytogenes* to bacteriocins.

There is a fitness cost associated with resistance to class IIa bacteriocins, which aligns with changes to ABC transporters and sugar PTS in nisin resistance. *L. monocytogenes* has a decreased growth rate due to the energy expensive metabolic pathways in resistant strains (Balay et al., 2018; Dykes and Hastings, 1998). The reduction in specific growth rate and the reduced consumption rate of glucose or mannose is due to the downregulation of the Man-PTS, which is responsible for the transport of glucose and mannose across the membrane (Balay et al., 2018; Glaser et al., 2001). The two main Man-PTS operons responsible for the stress response of *L. monocytogenes* to bacteriocins and resistance are the mannose permease two (*mpt*-operon) and the mannose permease one (*mpo*-operon) (Arous et al., 2004; Dalet et al., 2001). Both operons are under the control of the alternative sigma/transcription factor σ^{54}/σ^{L} encoded by *rpoN* (Xue and

Miller, 2007). ManR, an associated activator for σ^{54}, is responsible for *L. monocytogenes* resistance to class IIa bacteriocins specifically mesentericin Y105 (Dalet et al., 2001). Overall, the *rpoN* gene is associated with resistance to class IIa bacteriocins such as divergicin V41, mesentercin Y105, and pediocin PA 1/Ach.

A similar effect on the downregulation of the Man-PTS system was observed in *L. monocytogenes* in the presence of carnocyclin A (class IIc; Chapter 4, this thesis). *L. monocytogenes* Man-PTS genes (EII_t^{man}) were downregulated regardless of availability of a single carbohydrate source, either mannose or cellobiose, suggesting that the Man-PTS system plays a role in resistance to nisin, class IIa and class IIc bacteriocins (Chapter 4, this thesis). It also suggests an additional mode of action for carnocyclin A that targets the Man-PTS on cell membranes as a docking receptor, in addition to forming anion selective pores (Gong et al., 2009).

Sugar PTS are a common site for bacteriocins to interact with on *L. monocytogenes* membrane. The most frequently affected system documented in the literature is the Man-PTS, which is involved in the development of resistance in *L. monocytogenes* when exposed to three different subsets of bacteriocins. These results could indicate that when *L. monocytogenes* is exposed to bacteriocins there is a more general stress response that takes place to resist the killing effects of bacteriocins than specific mechanism of resistance for each bacteriocin.

1.1.7 General Stress Response

In addition to downregulation of genes related to sugar transport and metabolism, *L. monocytogenes* slows protein metabolism under nisin stress through the downregulation of the *clp* gene (Bucur et al., 2018). In response to stress *L. monocytogenes* SOS response and DNA repair coding genes are activated. The SOS response initiates the DNA repair functions that are important for resistance formation to stress (Harms et al., 2016), including nisin exposure (Wu et al., 2018, 2017). Activators of the SOS response (*recF* and *recR*) were induced along with DNA polymerases *lmo0162* and *lmo1975* in the presence of nisin, which coincided with the downregulation of *lmo1302*, a SOS repressor

(Wu et al., 2018). All these seemingly unconnected stress responses *L. monocytogenes* undergoes in the presence of bacteriocins further suggests that the development of resistance to bacteriocins is part of a more general stress response than initially anticipated.

The *sigB* gene, an alternative sigma factor (σ^B) involved in general stress response, is an important mediator for bacterial stress response and is involved in the resistance of *L. monocytogenes* to bacteriocins (Chaturongakul et al., 2011, 2008; Chaturongakul and Boor, 2006; Kazmierczak et al., 2003; Palmer et al., 2009). The SigB regulon consists of putative efflux pumps, penicillin binding proteins, autolysins and proteins involved in the modification of the bacterial cell envelope; however, the exact role of SigB in bacteriocin resistance has not yet been determined. A mutation in *sigB*, reduced *L. monocytogenes* resistance to nisin and lacticin 3147 in broth (Begley et al., 2006). It is thought that SigB may control membrane change or lipid composition, which ultimately will affect bacteriocin pore formation (Begley et al., 2006). Transporters involved in bacteriocin efflux may also be under SigB control; though, *L. monocytogenes ΔsigB* mutant has increased nisin resistance, which is contradictory to the thought that SigB is involved in resistance (Palmer et al., 2009), and suggests that nisin resistance does not rely solely on SigB involvement. For a class IIa bacteriocin, Laursen et al., (2015) reported that *L. monocytogenes* SigB dependent genes participate in a more general defence mechanism, whose role in bacteriocin sensitivity has not yet been elucidated, while SigL dependent genes participate in protein synthesis, carbohydrate transport and motility and is involved in *L. monocytogenes* development of resistance to class IIa bacteriocins such as pediocin (Laursen et al., 2015). *L. monocytogenes ΔsigL* became more resistant to pediocin than the wildtype cells (Laursen et al., 2015), which aligns with the fact that SigL controls the transcription of the Man-PTS in *L. monocytogenes* and when the transcription of the Man-PTS is downregulated *L. monocytogenes* displays increased resistance to different class subsets of bacteriocins (Kjos et al., 2010; Xue et al., 2005). SigL has been documented to be part of SigB regulon (Hain et al., 2008; Mujahid et al., 2013b; Palmer et al., 2009), which suggests that if SigB is affected in response to the exposure of bacteriocins any genes that are under the regulation of SigB may consequently affect its downstream regulon.

A transcriptional regulator, Rli47, is a ncRNA that is SigB dependent and displayed differential expression between *L. monocytogenes* treated with and without carnocyclin A (Chapter 4, this thesis). Rli47 (*sbrE*) is a trans-acting ncRNA that affects the expression of the virulence regulator PfrA (Izar et al., 2011; Mellin and Cossart, 2012; Mujahid et al., 2013a; Vivant et al., 2017). SigB is related to the stress response of *L. monocytogenes* to class I and class II bacteriocins (Kazmierczak et al., 2003; Palmer et al., 2009) making it possible that Rli47 is involved in the formation of resistance to bacteriocins. A less studied ncRNA, Rli24 was significantly upregulated in this transcriptional study in the presence of carnocyclin A, and has been associated with *L. monocytogenes* stress response when it grows in macrophages (Kuenne et al., 2013). T-boxes, which represent the largest class of riboswitches in *L. monocytogenes* act as cis-regulators that control gene expression by binding to tRNA (Izar et al., 2011; Mraheil et al., 2011a) were significantly both upregulated and downregulated in *L. monocytogenes* exposed to carnocyclin A (Chapter 4, this thesis). Riboswitches may also play a role in adaptation and resulting physiological changes seen when *L. monocytogenes* is exposed to carnocyclin A (Balay et al., 2018). When *L. monocytogenes* is under bacteriocin stress regulatory ncRNA likely plays a role in regulating the cellular response of *L. monocytogenes* to bacteriocins and assist in the pathways which may lead to resistance.

1.1.8 Phage

Phages remnants or prophages can be present in *Listeria* strains and can be influenced by the stress response caused by bacteriocin exposure. Prophage DNA present within *L. monocytogenes* can interact with infecting phage and result in interactions that drive bacterial evolution via lateral gene transfer (Brüssow et al., 2004; Canchaya et al., 2003; Hatfull, 2008; Hendrix, 2003; Juhas et al., 2009). In *Escherichia coli* K-12 defective prophages enhance resistance to environmental stresses (Wang et al., 2010), and therefore can be suspected to play a similar role in *L. monocytogenes*. Food processing plants place bacteria under environmental stress due to the regular antimicrobial interventions, and Verghese et al., (2011), found *comK*-prophages in *L. monocytogenes* that were isolated from the plants. Similarly, when *L. monocytogenes* was exposed to nisin, phage-encoding

genes are downregulated (Wu et al., 2018). However, when *L. monocytogenes* was grown in cellobiose and treated with carnocyclin A, phage remnants were upregulated in response to the exposure of the bacteriocin (Chapter 4, this thesis). There is a mosaicism of prophages present in many prokaryotes (Morgan and Pitts, 2008) and though the exact nature of the role they play in *L. monocytogenes* stress response is unclear, they are present and can be affected by bacteriocins of different classes. Further research needs to be done on the role phage and phage remnants have on *L. monocytogenes* ability to form resistance to bacteriocins.

1.1.9 Cross Resistance

It is suggested to use bacteriocins of different classes, specifically with different modes of action, on target organism to prevent the formation of resistance to bacteriocins (Gravesen et al., 2002; Vignolo et al., 2000). Therefore, it is important to determine if cross-resistance occurs when an organism becomes resistance to one of the two or more types of bacteriocins used (Gravesen et al., 2004). There are instances where cross-resistance can form between two bacteriocins; however, there are contradictions to these findings where using two bacteriocins from different classes did not result in cross-resistance. There are reported cases of cross resistance between class I and class IIa bacteriocins, specifically nisin and pediocin PA-1 (Bouttefroy and Milliere, 2000; Crandall and Montville, 1998), nisin, pediocin PA-1, leuconocin S (Bruno and Montville, 1993), and nisin, pediocin PA-1, leucocin A (Gravesen et al., 2004). Conversely, though there was no cross-resistance was found between nisin and pediocin PA-1 by Rasch and Knøchel (1998) in *L. monocytogenes*. Between different classes of bacteriocins the research shows discrepancy about the development of cross-resistance indicating that there is more than cellular response that results in resistance; however, cross-resistance between the same subclass of bacteriocin seems to be common and well established. Mutants of *L. monocytogenes* that were resistant to one of three class II bacteriocins (mesenterocin 52B, curvaticin 13, plantaricin C19) exhibited some degree of cross-resistance to the other two class II bacteriocins, but not to nisin (Rekhif et al., 1994). Cross-resistance was established between lantibiotcs (lacticin 3147, nisin and lacticin

481) but not to the class II bacteriocin, lactococcin ABM (Guinane et al., 2006; Schaik et al., 1999). Complete cross-resistance was observed for class IIa bacteriocins (pediocin PA-1, leucocin A, carnobacteriocin B2), and is frequently reported (Dykes and Hastings, 1998; Gravesen et al., 2002; Ramnath et al., 2004; Rasch and Knøchel, 1998)). Cross-resistance was noted between class IIa (leucocin A) and class IIc (carnocyclin A) in *L. monocytogenes* grown in different carbohydrates (Balay et al., 2018). Cross-resistance is more consistently observed in bacteriocins that use the same mode of action to inhibit target organism, though it is clear there are some overlapping stress responses that lead to the formation of resistance despite the class of bacteriocin.

1.2 Conclusions

L. monocytogenes can be sensitive to bacteriocins, which would theoretically allow for the control of *L. monocytogenes* in RTE food products; however, the development of resistance still occurs. Upon the formation of resistance to bacteriocins in *L. monocytogenes* the risk for consuming contaminated food products increases, which is unacceptable, and can result in morbidity and/or mortality. Therefore it is important to understand *L. monocytogenes* global response to bacteriocin exposure to mitigate possible conditions, ingredients, and/or interventions that may allow for or accelerate the development of resistance to the bacteriocin being applied. The formation of resistance would be easily identified and dealt with if there was one specific mechanism of resistance for each bacteriocin mode of action. However, when *L. monocytogenes* is exposed to bacteriocins from different classes it elucidates a stress response that is not entirely unique to the class of bacteriocin being used for treatment (Figure 1).

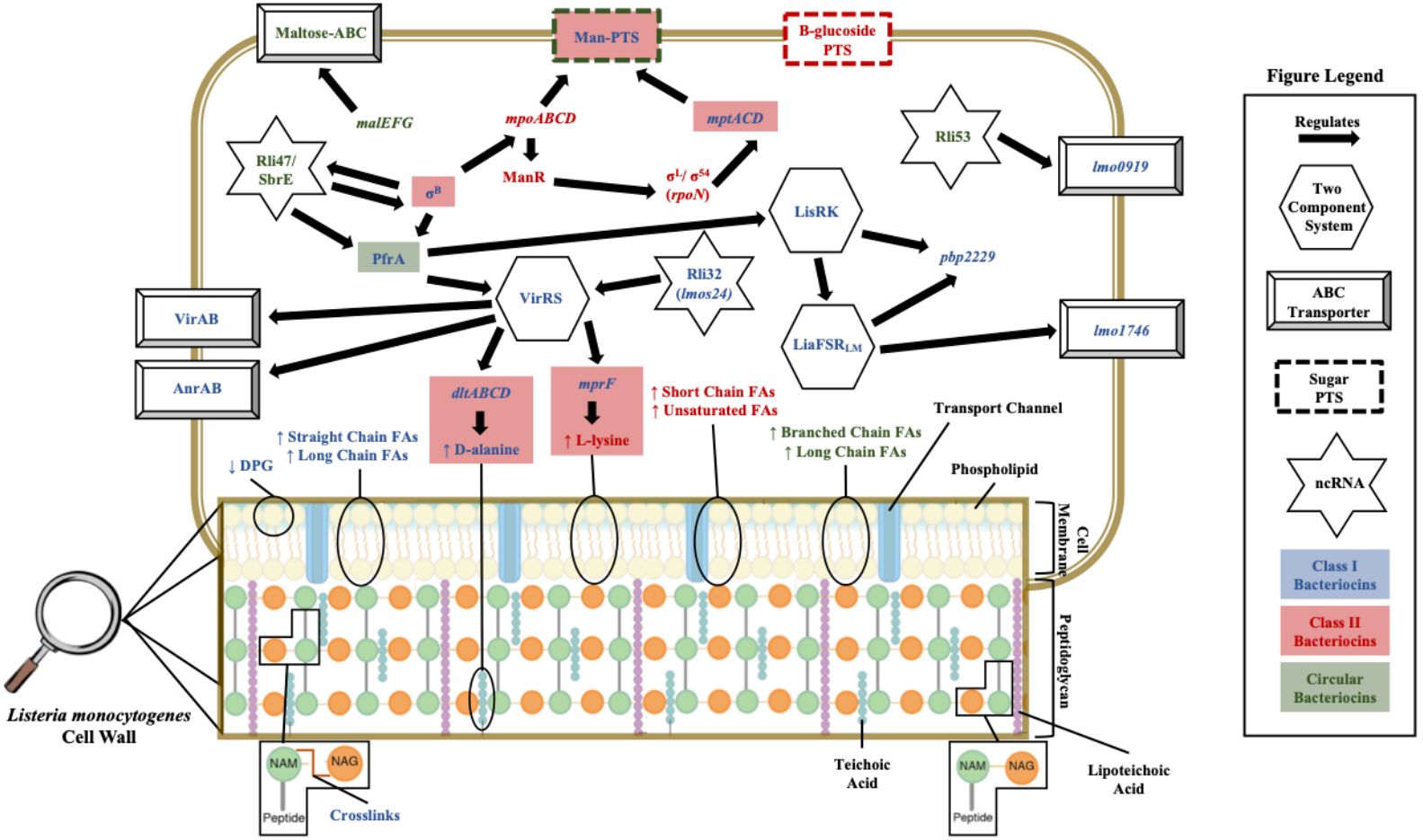

Figure 1.1 Potential mechanisms for the development of resistance in *L. monocytogenes* in response to exposure to different classes of bacteriocins. The top portion of the figure illustrates the genetic pathways that regulate stress responses to bacteriocins; the bottom portion illustrates the changes in the cell wall in response to bacteriocins. Responses that are illustrated in multiple colors correspond to the same effect observed with exposure to different classes of bacteriocins. Class I bacteriocins (blue) affect *L. monocytogenes* cell

membranes by decreasing diphosphatidylglycerol (DPG), and increasing straight and long chain fatty acids (FAs) in the phospholipids in the cell membrane. The cell wall becomes more rigid and positively charged with crosslinks formed between N-acetylmuramicglucosamine (NAM) and N-acetylglucosamine (NAG) and the incorporation of D-alanine, respectively. The increase in D-alanine residues are produced by the *dltABCD* operon, which is under the control of a two component system (hexagon), virulence regulator (VirRS), which is also involved in *L. monocytogenes* response to class II bacteriocins (red). VirRS regulates (black arrow) two ABC transporters (gem shape box) that are affected by class I bacteriocin stress; VirAB and an ABC transporter involved in nisin resistance (AnrAB). Additionally, VirRS regulates the multiple peptide resistance factor gene (*mprF*), which increases incorporation of L-lysine into the phospholipids when exposed to class II bacteriocins. A regulatory non-coding RNA (ncRNA; star), Rli32, encoded by *lmos24*, regulates VirRS in the presence of class I bacteriocins. LisRK, another two component system affected in the presence of class I bacteriocins regulates a three component system LiaFSR (hexagon), both of which can upregulate the penicillin binding protein gene (*pbp2229*) that plays a role in the development of resistance to class I bacteriocins. LiaRS regulates an ABC transporter permease, encoded by the gene *lmo1746,* which is upregulated in the presence of class I bacteriocins. The antibiotic ABC transporter for lincomycin encoded by the gene *lmo0919* is upregulated in the presence of class I bacteriocins, and is flanked by the ncRNA, Rli53. Rli53 is upregulated in the presence of circular bacteriocins (green). The general stress response alternative sigma factor B (σ^B) responds to class I and class II bacteriocins and regulates the pleiotropic virulence regulator (PfrA), which in turn regulates VirRS and LisRK. Cell membrane modifications to the phospholipids in the presence of class II bacteriocins are increased unsaturated and short chain FAs; and in the presence of circular bacteriocins are increased branched and long chain FAs. Class II bacteriocins affect the σ^B regulon of the mannose permease one operon (*mpoABCD*), which affects (ManR) the associated activator for the alternative sigma factor L ($\sigma^{L/54}$). $\sigma^{L/54}$ regulates the mannose permease two operon (*mptACD*), which is downregulated in the presence of class I, class II, and circular bacteriocins. Sugar phosphotransferase systems (PTS; dashed box) for mannose are encoded by these two mannose operons and are downregulated and result in the development of resistance to class I and II bacteriocins. β-glucoside PTS are upregulated in the presence of class II bacteriocins and play a role in the development of resistance. The maltose ABC transporter is affected by the presence of circular bacteriocins and the development of resistance is associated with the downregulation of *malEFG*, which encodes the ABC transporter. In the presence of circular bacteriocins, the ncRNA SbrE/Rli47 regulates σ^B and PfrA, and is regulated by σ^B.

Although there are some uniquely identifying mechanisms that *L. monocytogenes* responds with to certain bacteriocins, there is no "one shoe fits all" approach when dealing with the resistance.

L. monocytogenes undergoes cell wall modifications to resist the lethal effects of class I bacteriocins, which are in turn regulated by key two component systems and downstream ABC transporters. Though there is some overlap between the stress response to class I bacteriocins and class II bacteriocins. *L. monocytogenes* also adapts the cell wall to hinder the interaction between class II bacteriocins and the cell membrane. Some adaptions are similar to those seen when dealing with class I bacteriocin exposure, but some are unique to class II bacteriocin exposure. Namely, membrane fluidity, which becomes more rigid in the presence of class I bacteriocins and conversely more fluid in the presence of class II bacteriocins. This could relate to the different modes of action each class of bacteriocin has against *L. monocytogenes*. Though distinct modes of action between bacteriocin classes, a well-recognized mechanism of resistance would be the downregulation of gene expression related to the Man-PTS. *L. monocytogenes* responds this way to class I, class IIa, and class IIc bacteriocin exposure, making it a significant pathway in the bacteriocin stress regulon. Much the same for the pathways that increase the charge on the cell wall to hinder electrostatic interactions between bacteriocins and the target cell membranes. However, more research is needed on the global effects each class of bacteriocin has on *L. monocytogenes* to establish all the overlapping mechanisms of resistance.

This is why RNA-seq technology is so powerful to analyze the whole transcriptome of a bacteriocin in a specific condition without any prior sequence knowledge. The technology allows for a complete picture of how broad or specific the stress response is to bacteriocins. Being able to analyze all the nuances of gene expression in relation to bacteriocin stress overlapping mechanisms can be determined and any possible origins of altered gene regulation that may result in resistance. It also allows for the identification of regulatory ncRNAs that may play a role in modulating the stress response of bacteriocins. The transcriptional response of ncRNAs to bacteriocin exposure is not something that has been extensively researched, but would be worthwhile to link suspected regulatory

ncRNAs to stress response pathways that lead to the development of bacteriocin resistance in *L. monocytogenes.*

To use such a global perspective allows for the dismissal of the idea that there are specific resistance mechanisms for bacteriocins and the prospect of a stress response regulon for bacteriocins. This would allow for the optimization of a hurdle system in RTE food products that would balance the interventions strengths and weaknesses. If *L. monocytogenes* has an established stress response to one bacteriocin it could be balanced with another bacteriocin or intervention that does not share regulation of resistant pathways to ensure a safe food product. To control the risk of *L. monocytogenes* in RTE food products effectively with the use of a naturally derived preservatives such as, bacteriocins, a complete understanding of how *L. monocytogenes* forms resistance and/or develops cross resistance to bacteriocins is necessary to ensure safe foods for consumers. By developing a greater understanding of *L. monocytogenes* stress regulon related to bacteriocin intervention will allow for the seamless transition of traditional or naturally derived preservatives into the food industry without the risk of increased exposure to *L. monocytogenes.*

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Chapter 2

2.0 Introduction

Listeria monocytogenes is associated with ready-to-eat (RTE) products due to post-processing contamination (Flint et al., 2005). *L. monocytogenes* typically affects immunocompromised, elderly or pregnant individuals (Health Canada, 2011) and has a fatality rate of 20-40% (Drevets and Bronze, 2008; Farber and Peterkin, 1991; Gálvez et al., 2008).. *L. monocytogenes* can grow over a wide range of temperatures, including refrigeration temperatures (Health Canada, 2011). This poses a concern for the safety of RTE meat products, as they typically are not subjected to an additional heating step prior to consumption (Zhu et al., 2005). Strict guidelines have been developed to control *L. monocytogenes* in RTE products (Health Canada, 2011). With consumer demands for the food industry to increase availability of fresh, RTE and minimally processed products, research on novel methods of bio-preservation is needed. The potential of biopreservatives to meet consumer demands and maintain product integrity have been demonstrated against a diverse group of foodborne pathogens, including *L. monocytogenes* (Gálvez et al., 2010).

Biopreservatives are antimicrobial products of living organisms (Gálvez et al., 2010) and include bacteriocins, which are ribosomally synthesized either with or without posttranslational modification, are excreted out of the cell and have a narrow-spectrum of activity (Cotter et al., 2005). Nisin has been approved for use in foods in over 50 countries (Gálvez et al., 2008). However, nisin cannot be used with raw meat as it reacts with glutathione, which inactivates the peptide (Rose et al., 1999). Class IIa bacteriocins have antilisterial activity on meats and can be applied to RTE meat products (Zhu et al., 2005). However, suspected proteolytic degradation of leucocin A on wieners indicated that it may be cleaved at residue 17 between asparagine and tryptophan and neither portion had activity against *L. monocytogenes* (unpublished data, Kaur). To prevent fragmentation, a synthetic analogue (leucocin N17L) was synthesized where the asparagine was replaced by a leucine.

Leucocin A is a plasmid mediated, 37 amino acid residue Class IIa bacteriocin with a molecular mass of 3930 Da that is produced by *Leuconostoc gelidum* UAL187 (Hastings and Stiles, 1991). *Leuc. gelidum* UAL187 was isolated from chilled, processed meat stored in an atmosphere containing CO_2 (Hastings and Stiles, 1991) making it an ideal candidate for application on RTE meat products. Leucocin A is a good candidate for use on RTE meat products due to its stability under extreme conditions (100°C for 20 min at pH 2–3); however, it is degraded by various proteases (Hastings and Stiles, 1991). Class IIa peptides have a conserved, charged, hydrophilic N-terminus motif of YGNGV(X)C(X)$_4$C(X)V(X)$_4$A (X denotes any amino acid residue) that forms a β-sheet structure (Bodapati et al., 2013; Fregeau Gallagher et al., 1997), which allows for electrostatic interactions association between the target cell surface and the bacteriocin (Fimland et al., 2005). The cysteine residues (located at positions 9 and 14 in leucocin A) form a disulphide bridge that is important for proper conformation and antibacterial activity (Fregeau Gallagher et al., 1997; Sit et al., 2011). The more hydrophobic C-terminus residues are suspected to play a role in receptor protein interactions allowing for receptor mediated recognition mechanisms to elucidate antibacterial activity (Kaur et al., 2004). The mode of action is based on pore formation and ion disruption of the target cell (Kaur et al., 2004). The antilisterial activity and inhibitory action against a range of other lactic acid bacteria, including *Enterococcus faecalis* (Hastings and Stiles, 1991) make leucocin A a candidate for further investigation for preservation use in RTE meat products.

The objectives of this research were to purify sufficient quantities of leucocin A and to use the purified leucocin A in experiments to determine the efficacy of leucocin A to inhibit the growth of *L. monocytogenes* in the presence of natural spoilage microbiota on vacuum packaged RTE meat. The spoilage organisms of interest were *Brochothrix thermosphacta* and *Carnobacterium divergens*. A variant of leucocin A was synthesized to determine if activity against *L. monocytogenes* could be increased.

2.1 Materials & Methods

2.1.1 Bacterial Strains and Culture Conditions

Table 2.1 Strains of *L. monocytogenes* (Fugett, 2006).

L. monocytogenes	**Lineage**	**Serotype**	**Origin**
FSL C1-056	II	1/2a	Isolated from human sporadic case '98
FSL J1-177	I	1/2b	Isolated from human sporadic case '97
FSL N3-013	I	4b	Isolated human listeriosis epidemic '88-'90
FSL R2-499	II	1/2a	Human isolate associated with US outbreak '00
FSL N1-227	I	4b	Food isolate associated with US outbreak (Bilmar, wiener outbreak '98-'99)

Strains of *Listeria monocytogenes* (Table 2.1) developed as a cocktail for challenge tests were obtained from Cornell University (Fugett, 2006). *Carnobacterium divergens* UAL9, a sensitive indicator for leucocin A, was obtained from the University of Alberta Lactic Acid Bacteria culture collection. *Brochothrix thermosphacta* strains A401, B401, P104, P107 were previously isolated from RTE meat products (Miller et al., 2014). All culture strains were stored in 30% glycerol in media at -80°C until needed.

Prior to use in experiments, cultures were streaked onto appropriate culture media, and a single colony was inoculated into broth media. *Leuc. gelidum* UAL187 was grown anaerobically at 25°C in semi-defined casamino acids (CAA) media (Hastings et al., 1991) . Strains of *L. monocytogenes* were grown aerobically at 37°C in tryptic soy broth (TSB; Becton, Dickinson and Company, New Jersey, USA). Strains of *C. divergens* and *B. thermosphacta* were grown anaerobically and aerobically, respectively, at 25°C in All Purpose Tween (APT; Becton, Dickinson and Company).

2.1.2 Production and Purification of Leucocin A

Leucocin A was purified using a modified protocol of Hastings et al. (1991). To produce leucocin A, *Leuc. gelidum* UAL187 was grown in CAA media without Tween® 80 (initial pH of 6.5; 2% v/v inoculum), at 25°C for 24 h under anaerobic conditions. For purification, the spent culture broth was not centrifuged after fermentation and the entire

cell suspension was loaded onto a column packed with 60 g/L Amberlite® XAD®-2 (Supelco, Bellefonte, PA, USA) resin equilibrated with trifluoroacetic acid (TFA; 0.1% v/v) at 5 mL/min. The column was washed sequentially with 500 mL each of sterile distilled water (dH_2O) 10 mL/min, ethanol (EtOH) (20% v/v) 10 mL/min and EtOH (40% v/v) 10 mL/min. Leucocin A was eluted with 800 mL EtOH (75% v/v) with TFA (0.1% v/v) at 5 mL/min. The eluent was concentrated on a Buchi® rotary evaporator (Brinkman Instruments, Westbury, NY, USA) at 30°C under vacuum and lyophilized (Labconco® FreezeZone, Kansas City, MO, USA). Lyophilized samples were washed with 1 mL of 100% acetonitrile (ACN) two times to remove residual colour compounds. Washed samples were solubilized in TFA (0.1% v/v) for further purification using reversed-phase high performance liquid chromatography (RP-HPLC; Prostar 210, Varian Inc., Palo Alto, CA, USA). Samples were injected onto a semi-preparative C18 column (Grace™ Vydac™, Fisher Scientific, Edmonton; 10 mm x 250 mm, particle size 5 µm) and eluted with a gradient of 15% to 85% ACN-TFA 0.1% (v/v) dH20 with a flow rate of 1 mL/min, leucocin A was eluted at ~28.5 min. Leucocin A identity was confirmed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF). Pure leucocin A was lyophilized and stored at -20°C until needed for applied experiments on meat, at which point it was suspended in TFA (0.1% v/v) to the desired concentration.

Peptides were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a UHPLC system (Waters nanoAquity Waters, Milford, MA) coupled to a QTOF analyser (Water Premier, Waters). The sample (5 µL) was loaded onto a nano trap cartridge (PepMap C18, 300 µm x 1 mm, Thermo Scientific, Sunnyvale, CA) coupled to a nano analytical column (75 µm × 150 mm, 100 Å, PepMap C18 column, Thermo Scientific). Desalting on the peptide trap was achieved by flushing the trap with 99% eluent A (0.1% formic acid in water) and 1% eluent B (0.1% formic acid in acetonitrile) at a flow rate of 10 µL/min for 3 min. Peptides were separated at a flow rate of 350 nL/min with 2% eluent B for 3 min, a gradient of 2 to 10% eluent B over 3 min, a gradient of 10 to 98% eluent B over 42 min, 98% eluent B for 10 min and 2% eluent B for 5 min. Analysis was done manually using the MassLynx software supplied by Waters. Pure leucocin A was lyophilized and stored at -20°C until needed, at which point it was suspended in TFA (0.1% v/v) to the desired concentration.

2.1.3 Synthesis of Leucocin N17L

Leucocin N17L (Fig. 2.1) was synthesized as a 37 amino acid peptide using previously standardized solid phase method (Bodapati et al., 2013) with 0.2 mM equivalent of Wang resin. During synthesis, a leucine was substituted for an asparagine at residue 17. The synthesis was carried out using an automatic peptide synthesizer following double coupling procedures. The pure peptide was subjected to oxidative folding (disulphide formation) by air oxidation in freshly degassed Tris buffer (50 mM, pH 8.3) solution, with gentle stirring in an open-air flask for 48 h. The reaction was monitored by using an Ellman test and mass spectrometry. Oxidized peptide was subjected to RP-HPLC to obtain pure leucocin N17L. MALDI-TOF mass analysis was done to confirm the molecular weight of the desired product.

leucocin A

H_2N KYYGNGVHCTKSGCSV**N**WGEAFSAGVHRLANGGNGFW-COOH

leucocin N17L

H_2N KYYGNGVHCTKSGCSV**L**WGEAFSAGVHRLANGGNGFW-COOH

Figure 2.1 Amino acid sequences of leucocin A and leucocin N17L. Underlined residue in leucocin N17L indicates the change from the native peptide.

2.1.4 Determination of Minimum Inhibitory Concentrations

Minimum inhibition concentrations (MIC) of leucocin A and leucocin N17L were determined to assess the varying degree of sensitivity among strains of *L. monocytogenes* in broth versus on agar. The MIC was determined using an adapted protocol by Weigand et al. (2008) and the Kirby-Bauer disc diffusion method (Bauer et al., 1966). To determine MICs in broth, strains of *L. monocytogenes* were grown overnight in APT at 25°C. An aliquot (100 µL) of APT was added to the wells of a Costar® sterile 96-well cell culture flat bottom microtiter plate with a low evaporation lid (Corning Incorporated, Corning, NY, USA), and 100 µL of leucocin A or leucocin N17L was added to the first well and two-fold dilutions were done across the plate. An aliquot (50 µL) of each

overnight culture was added to the wells to obtain 10^5 CFU per well. Plates were incubated at 25°C for 18 h. Determination of the MIC was done by observing turbidity in the wells. The MIC was determined as the concentration in the well before the first turbid well. To assess the MIC on agar, sterile blank paper discs (6 mm diameter; Becton, Dickinson and Company) were loaded with 20 µL of two-fold serial dilutions of leucocin A. The discs were placed onto APT agar and incubated at 25°C for 72 h. The MIC was the lowest concentration with a clear zone of inhibition. The determination of MICs was replicated three times.

2.1.5 Growth of *L. monocytogenes* in the Presence of Leucocin A

To determine the effect of leucocin A on the growth of each strain of *L. monocytogenes*, strains of *L. monocytogenes* were grown in APT broth (25°C, 16 h) and diluted in 0.1% (w/v) peptone to 10^5 CFU/mL. Strains were combined with APT broth and leucocin A for a final bacteriocin concentration of 0.33 mM. Cultures were incubated at 25°C and cell counts were determined every 2 h for 16 h and again at 24 h by plating onto APT agar. Three independent replicates were performed.

2.1.6 Stability of Leucocin A in the Presence of *B. thermosphacta in vitro*

To investigate if there were any negative effects of *B. thermosphacta* on the activity of leucocin A against *L. monocytogenes,* strains of *B. thermosphacta* and *C. divergens* UAL9 were grown overnight in APT broth at 25°C. Overnight cultures were subcultured (1% v/v inoculum) into 9 or 9.9 mL of fresh APT broth containing either 1 mL of *Leuc. gelidum* UAL187 cell-free-supernatant (2.6×10^4 activity units per mL of leucocin A) or 100 µL of 1mM leucocin A for a final leucocin A concentration of 0.01mM. Cultures were incubated aerobically for 48 h at 25°C. Cultures were heated at 70°C for 30 min to kill live cells before use in spot-on-lawn assays (Ahn and Stiles, 1990) against *C. divergens* UAL9. A 1% (v/v) inoculum of *C. divergens* UAL9 was added to tempered (~50°C) soft APT agar (0.75% w/v agar), mixed, poured into a sterile petri dish, and allowed to dry for 1 h. After drying, aliquots (10 µL) of heat-treated supernatant

containing leucocin A, were spotted onto plates and allowed to dry, and plates were incubated at 25°C for 24 h. Spent, cell-free supernatants of spoilage organisms combined with leucocin A were analysed by RP-HPLC and MALDI-TOF to determine if there was any degradation of leucocin A. Three independent assays were performed.

2.1.7 *In situ* Inhibition of *L. monocytogenes* by Leucocin A in the Presence of Spoilage Organisms

The efficacy of leucocin A against *L. monocytogenes* in the presence of spoilage organisms was determined on the surface of vacuum packaged wieners stored at refrigeration temperatures. Overnight cultures of each strain of *B. thermosphacta* and *C. divergens* UAL9 were pelleted, washed two times and suspended in 0.85% (w/v) saline. Strains of *B. thermosphacta* were diluted in 0.85% saline and combined to achieve a cell density of 10^5 CFU/mL. *C. divergens* UAL9 was diluted to 10^5 CFU/mL. The cocktail of *L. monocytogenes* was prepared as described above for the *B. thermosphacta* cocktail.

Smoked wieners (Griffith Foods, Toronto, ON) were made from a mixture of mechanically deboned chicken, pork trim and beef trim, modified corn starch and wheat flour as binders and sodium tripolyphosphate, sodium nitrite and sodium erythorbate. Wieners were aseptically cut into half cylinders with a surface area of 13 cm^2. Each piece was immersed in the *B. thermosphacta* cocktail or *C. divergens* culture two times (10 s each time), and allowed to dry. Leucocin A was re-suspended in TFA (0.1% v/v) and applied with a sterile loop to the wieners to a final concentration of 0.01mM leucocin A per cm^2. Wieners were placed in 76.2 µm thick, nylon and polypropylene coextruded vacuum bags (Unipac Packaging Products Ltd., Edmonton, Canada) bags and vacuum packaged (Model C200; Multivac, Kansas City, MO) immediately following leucocin A application and stored at 7°C for 48 h. This was done to allow spoilage organisms to grow and potentially produce enzymes that might degrade leucocin A. After the 48 h of storage, wieners were removed from bags and immersed in a cocktail of *L. monocytogenes* (10^4 CFU/mL) two times (10 s each). Immediately following the inoculation with *L. monocytogenes,* wieners were vacuum packaged as described above

and stored for an additional 14 d at 7°C. Cell counts were determined by aseptically opening each bag, adding 3 mL of 0.85% saline solution and hand massaging each piece of wiener for 1 min to remove cells. The saline was diluted and plated on PALCAM (Oxoid, Nepean, ON, Canada), STAA (Oxoid), and cresol red thallium acetate sucrose inulin agar (CTSI) (Wasney et al., 2001) to enumerate *L. monocytogenes*, *B. thermosphacta* and *Carnobacterium* spp., respectively. Total aerobic plate counts were determined on Plate count agar (PCA) (Becton, Dickinson and Company). The wash from each sample was filter-sterilized (Fisherbrand™ 33 mm, 0.22 μm filter; Fisher Scientific) and stored at -20°C. The presence of leucocin A or its degradation products in the wash was determined by RP-HPLC, MALDI-TOF, and LCMS/MS using procedures described above. Four independent replicates were done. The wash was lyophilized and suspended in 0.1% TFA prior to use in spot-on-lawn assays using *C. divergens* as an indicator organism.

2.1.8 Statistical analysis

Statistical analysis was done using analysis of variance and Tukey Test with a 95% confidence interval to determine statistical differences among treatments ($P \leq 0.05$).

2.2 Results

2.2.1 Enhanced Purification of Leucocin A

A new method to purify leucocin A was developed to increase the yield obtained for *in vitro* and *in situ* experiments. Leucocin A was purified from the culture without separation of cells. This method resulted in purification of a total of 2.33 mg of leucocin A per litre of culture. MALDI-TOF confirmed the purified product had a mass of the documented leucocin A, 3930.03 Da. LCMS/MS confirmed the amino acid sequence of active leucocin A (Hastings et al., 1991).

2.2.2 Sensitivity of *L. monocytogenes* to Leucocin A

The sensitivity of the strains of *L. monocytogenes* to leucocin A varied among strains as well as between liquid- and solid-phase determinations of the MIC. A MIC based on turbidity was not determined in broth due to the lack of inhibition of any of the strains of *L. monocytogenes* after 18 h incubation for both leucocin A and the analogue (Table 2.2). On agar, MICs ranged from 11.7-62.5 μM and 62.5 ->500.0 μM for leucocin A and leucocin N17L, respectively (Table 2.2). *L. monocytogenes* FSL J1-177 was the most sensitive strain, and FSL R2-499 and FSL N1-227 were the least sensitive strains to both leucocin A and the analogue (Table 2.2). Experimentation with leucocin N17L was discontinued because it was not as active against the *L. monocytogenes* strains as native leucocin A.

Table 2.2 MIC of leucocin A and leucocin N17L for strains of *L. monocytogenes* and *C. divergens* UAL9 determined in APT broth or on APT agar incubated at 25 °C for 18 h. Results are the mean of three replicates.

	Minimum inhibitory concentration (μM)			
	Broth		**Agar**	
	leucocin A	**leucocin N17L**	**leucocin A**	**leucocin N17L**
L. monocytogenes				
FSL C1-056	>2200.0	>2200.0	23.5	125.0
FSL J1-177	>2200.0	>2200.0	11.7	62.5
FSL N3-013	>2200.0	>2200.0	62.5	>500.0
FSL R2-499	>2200.0	>2200.0	39.1	125.0
FSL N1-227	>2200.0	>2200.0	62.5	250.0
***C. divergens* UAL9**	1.7	170.0	31.3	500.0

Leucocin A had an initial bactericidal effect on *L. monocytogenes* FSL C1-056, FSL J1-177, and FSL N3-013 with an immediate reduction in cell counts by > 2 $\log_{10}$ CFU/mL (Fig. 2.2). *L. monocytogenes* FSL R2-499 and FSL N1-227 were only reduced by ~ 1 $\log_{10}$ CFU/mL. A similar pattern of growth for each sensitive strain of *L. monocytogenes*

was observed. Cultures treated with leucocin A did not reach a mean maximum cell density after 24 h incubation (Fig. 2.2).

The MIC determinations in broth are in alignment with the results of the inactivation profiles. Each strain had a subpopulation that was able to recover and grow within 1.0 to 2.9 $\log_{10}$ CFU/mL of the original cell density within a 24 h timeframe (Fig. 2.2).

2.2.3 Leucocin A Stability *in vitro* in the Presence of *B. thermosphacta*

Leucocin A (1 mM) did not inhibit the growth of *B. thermosphacta* in broth. A spot-on-lawn assay using the supernatant obtained from filtering the overnight culture of *B. thermosphacta* combined with the leucocin A as the spot against *C. divergens* UAL9 did not produce a zone of inhibition, which may indicate that the bacteriocin was degraded by the *B. thermosphacta*. To determine if this was the case, the cell-free supernatant of *Leuc. gelidum* UAL187 was combined with strains of *B. thermosphacta* or *C. divergens* UAL9 and resulting supernatant did not produce clear zones of inhibition. RP-HPLC and MALDI-TOF analyses were not sufficiently sensitive to determine if any fragmentation of leucocin A had occurred.

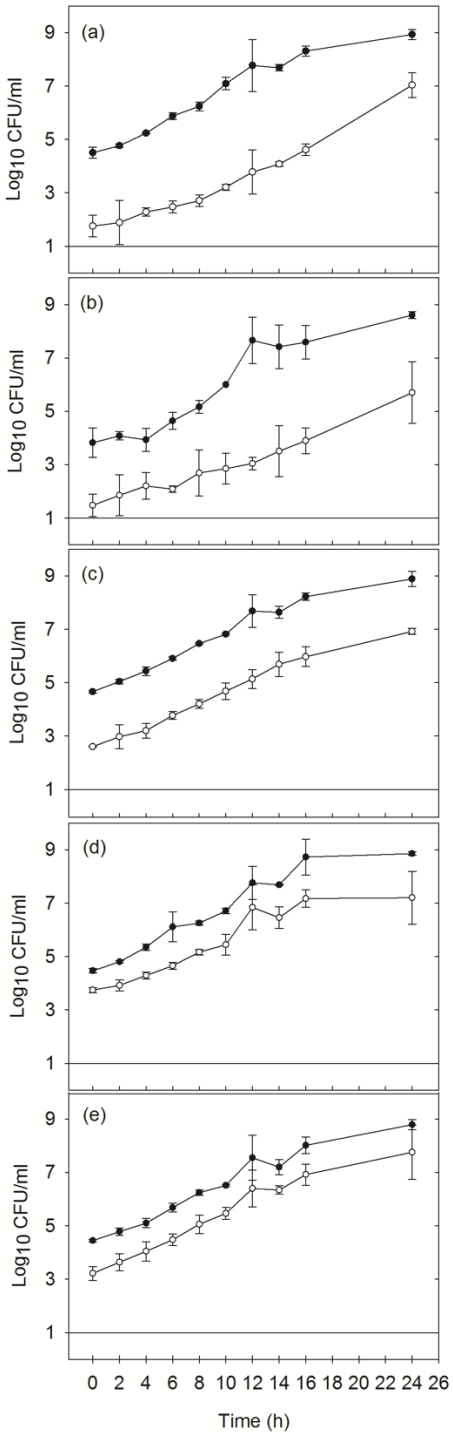

Figure 2.2 Growth of *L. monocytogenes* FSL C1-056 (a); FSL J1-177 (b); FSL N3-013 (c); FSL R2-499 (d); FSL N1-227 (e) alone (●) or in the presence of leucocin A (0.33 mM) (○). Cultures were incubated at 25°C for 24 h. Data are means ± standard deviation (n=3).

2.2.4 Ability of Leucocin A to Inhibit the Growth of *L. monocytogenes* and Spoilage Organisms on Wieners

The addition of leucocin A to the surface of wieners significantly reduced the growth of *L. monocytogenes* during storage (Fig. 2.3a). After 16 d storage, samples with leucocin A and *C. divergens* UAL9 had lower counts ($P < 0.05$) of *L. monocytogenes* than samples with only *L. monocytogenes* and *C. divergens* UAL9. The presence of *B. thermosphacta* had no effect on counts of *L. monocytogenes* (Fig. 2.3a). During storage, total plate counts were reduced by the addition of leucocin A and were significantly increased in samples inoculated with spoilage organisms (Fig. 2.3b). In the presence of spoilage organisms leucocin A had no effect on total plate counts (Fig. 2.3b). Counts for uninoculated control samples were below detection limits (data not shown). After 16 d storage, counts on CTSI from samples inoculated with *C. divergens* UAL9 and leucocin A were not significantly different from the control (Fig. 2.4a); however, total counts were significantly reduced after 16 d of storage on samples inoculated with *C. divergens* UAL9 and treated with leucocin A (Fig. 2.4b). When *L. monocytogenes* was added to the samples, there were no significant effects on the counts obtained from CTSI or PCA (Fig. 2.4). In all treatments, *Carnobacterium* and total plate counts (determined on CTSI and PCA, respectively) obtained after 16 d of storage were significantly higher than counts obtained after 24 h, except for *Carnobacterium* counts on wieners inoculated with *C. divergens* and treated with leucocin A (Fig. 2.4). The presence of leucocin A or *L. monocytogenes* had no effect on the growth of *B. thermosphacta* (Fig. 2.5).

Attempts were made to detect leucocin A or its degradation products in the wash from the wieners that had been stored for 16 d. No leucocin A or any of its degradation products were detected with analytical methods; however, zones of inhibition were observed in spot-on-lawn assays with the wash (data not shown).

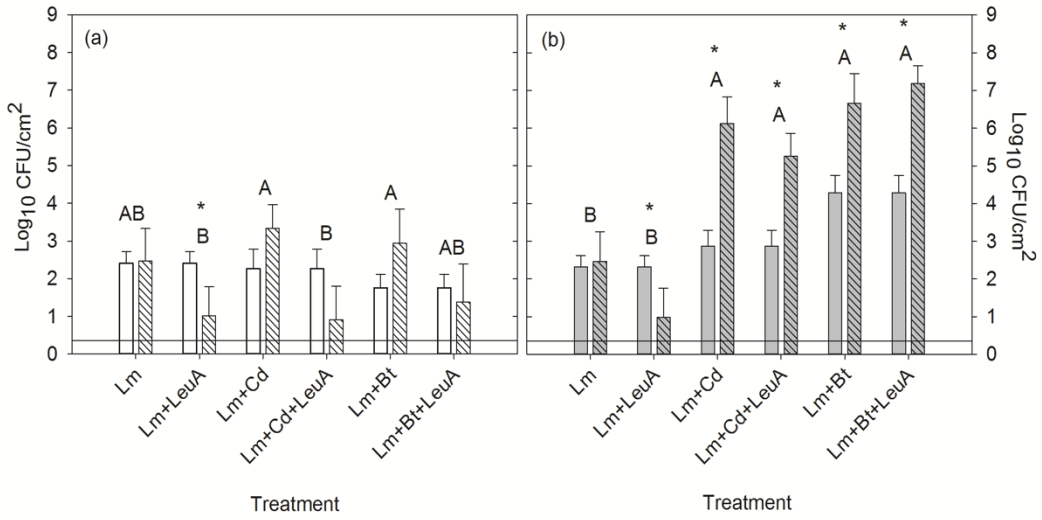

Figure 2.3 Mean cell counts of *L. monocytogenes* (Lm) from the surface of vacuum packaged wieners treated with and without leucocin A (LeuA) and inoculated with *L. monocytogenes* cocktail and different combinations of spoilage organisms, *C. divergens*, (Cd) and *B. thermosphacta* (Bt). Panel a shows Lm on PALCAM agar at 24 h (□) and 16 d (▨) of storage. Panel b shows the mean total plate counts to assess sub-lethal injury of leucocin A (LeuA) on the surface of vacuum packaged wieners inoculated with a cocktail of *L. monocytogenes* and different combinations of spoilage organisms, C. divergens, (Cd) and B. thermosphacta (Bt), determined on PCA at 24 h (■) and 16 d (▨) of storage. All samples were stored at 7°C for 16 d. The x axis intercepts the y axis at the detection limit (0.36 log10 CFU/cm2). Data are means ± standard deviation (n=4). Treatment means at 16 d storage within each panel with different letters are significantly different ($P<0.05$); * indicates that the mean at 16 d storage is significantly different ($P<0.05$) from the mean at 24 h.

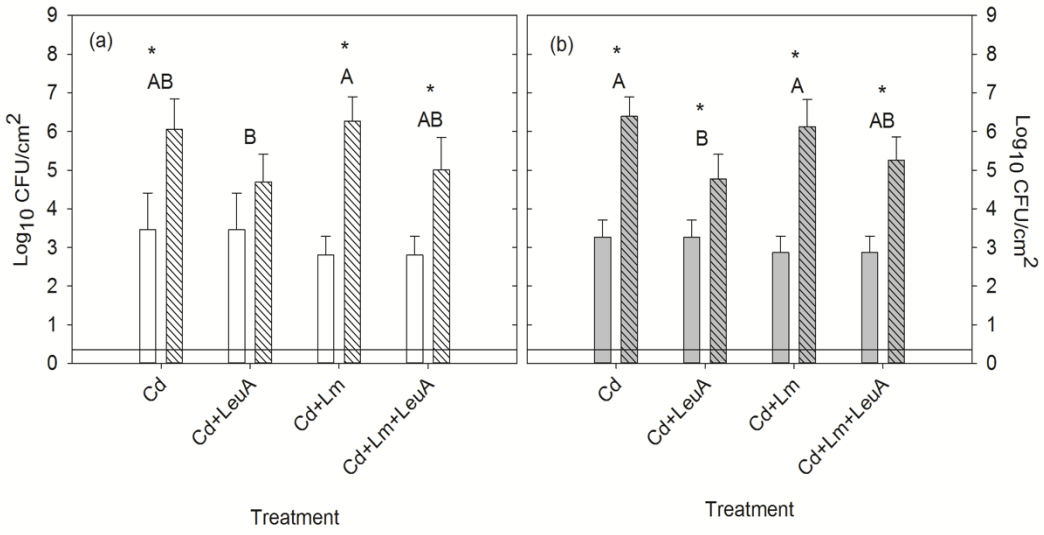

Figure 2.4 Mean cell counts of *C. divergens* (Cd) on the surface of vacuum packaged wieners in the presence of leucocin A (LeuA) inoculated with a cocktail of *L. monocytogenes* (Lm). Panel a shows Cd counts on CTSI agar after 24 h (□) and 16 d (▨) of storage. Panel b shows the mean total plate counts of cells to assess sub-lethal injury of leucocin A (LeuA) on the surface of vacuum packaged wieners inoculated with a cocktail of *L. monocytogenes* (Lm) and *C. divergens,* (Cd) determined on PCA at 24 h (■) and 16 d (▨) of storage. All samples were stored at 7°C for 16 d. The x axis intercepts the y axis at the detection limit (0.36 $\log_{10}$ CFU/cm^2). Data are means ± standard deviation (n=4). Treatment means at 16 d storage within each panel with different letters are significantly different ($P<0.05$); * indicates that the mean at 16 d storage is significantly different ($P<0.05$) from the mean at 24 h.

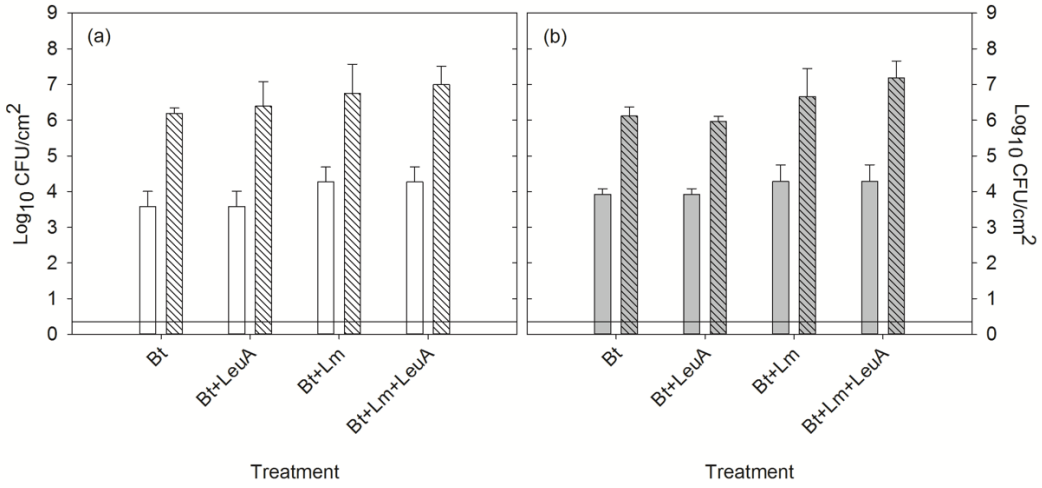

Figure 2.5 Mean counts of *B. thermosphacta* (Bt) on the surface of vacuum packaged wieners in the presence of leucocin A (LeuA) inoculated with a cocktail of *L. monocytogenes* (Lm). Panel a shows Bt on STAA agar at 24 h (□) and 16 d (▨) of storage. Panel b shows the mean total plate counts of cells to assess sub-lethal injury of leucocin A (LeuA) on the surface of vacuum packaged wieners inoculated with a cocktail of *L. monocytogenes* (Lm) and *B. thermosphacta* (Bt) determined on PCA after 24 h (■) and 16 d (▨) of storage. All samples were stored at 7°C for 16 d. The x axis intercepts the y axis at the detection limit (0.36 $\log_{10}$ CFU/cm^2). Data are means ± standard deviation (n=4). * indicates that the mean at 16 d storage is significantly different ($P<0.05$) from the mean at 24 h.

2.3 Discussion

L. monocytogenes is a serious foodborne pathogen that is associated with RTE meat products due to the long storage life where the organism can grow (Zhu et al., 2005). Although there are many proposed preservation techniques to control *L. monocytogenes* growth such as chemical preservatives or irradiation (Zhu et al., 2005) consumers demand a more natural approach to control potential pathogens in their food (Gálvez et al., 2008; Zink, 1997). Bacteriocins, such as leucocin A (Hastings and Stiles, 1991), have antilisterial activity (Chen and Hoover, 2003; Perez et al., 2014) and they are active in the nano-to-micro molar range (Perez et al., 2014).

To apply bacteriocins to meat products, the producing culture can be used or the bacteriocins of interest can be partly purified for application. Traditional methods of purification are tedious and only recover very small amounts of peptide for application in food (Garsa et al., 2014). As part of the current research, a new method for purification of bacteriocins was developed. This was based on previously published methods but the protocol was simplified. The surfactant, Tween 80, was removed from the media as it interfered with purification and MALDI-TOF analysis. Others have demonstrated that Tween 80 increases yield of bacteriocins as it decreases bacteriocin adhesion to cells and adsorption to surfaces (Huot et al., 1996; Joosten and Nuñez, 1995); however, some hydrophobic bacteriocins do not benefit from the addition of Tween 80 (Garneau et al., 2003). To obtain more peptide, centrifugation to separate the cells from the spent supernatant was not done as pure leucocin A is hydrophobic (Fregeau Gallagher et al., 1997) and centrifugation could remove some of the free peptide that adhered to cells. The ammonium sulphate precipitation step was eliminated which also removed the need for subsequent desalting. The greatest loss of activity during purification using the original protocol was during the precipitation and desalting steps (Hastings et al., 1991). The revised purification protocol had a five-fold increase in the amount purified leucocin A compared to the original protocol. This substantial increase in yield makes application of purified hydrophobic bacteriocins in foods feasible.

The efficacy of leucocin A against *L. monocytogenes* differed between solid and liquid-phase systems. This may indicate that a subpopulation of *L. monocytogenes* either was innately or spontaneously resistant, allowing the outgrowth of the subpopulation. Similarly, Jacquet et al. (2012) reported that the regrowth of *Carnobacterium maltaromaticum* DSM20730 in the presence of carnobacteriocin BM1 was due to a resistant subpopulation. In the current experiment, the variability in the initial bactericidal effect on the strains of *L. monocytogenes* and outgrowth of subpopulations indicates that each strain has different innate resistance and each develops a subpopulation of spontaneously resistant cells. The initial kill of the sensitive population could be observed on agar as a zone of inhibition, while the spontaneously resistant subpopulations were better identified in broth where the strains grew to almost maximum population. Innate resistance of *L. monocytogenes* against class IIa bacteriocins may be attributed to a more

positive cell surface, a more fluid membrane not allowing for efficient binding of bacteriocin to the cell surface, and/or a decrease in the expression of the mannose permease operon leading to interference with the bacteriocin efficacy (Gravesen et al., 2002; Jacquet et al., 2012; Vadyvaloo et al., 2004).

The application of leucocin A to the surface of wieners may have potential to control the growth of *L. monocytogenes* during storage. The antimicrobial effects were not impacted by the presence of potential spoilage organisms. In contrast, when tested in broth, *B. thermosphacta* was able to cause inactivation of the bacteriocin, resulting in growth of *L. monocytogenes*. This may have been in part due to the production of enzymes by *B. thermosphacta*, such as α-chymotrypsin (Casaburi et al., 2014; Nowak and Piotrowska, 2012). It has been documented that α-chymotrypsin, as well as other proteases have the ability to degrade leucocin A and cause inactivation (Hastings and Stiles, 1991). The difference in results between the activity of the pure leucocin A on meat versus in broth could be due to the fact enzymes are synthesized in greatest concentration during an extended stationary phase (Braun and Sutherland, 2003) and the cocktail of *B. thermosphacta* may not have been able to produce enough proteolytic enzyme to degrade leucocin A *in situ*. In addition, spoilage organisms are slow to produce enzymes below 6°C (Braun et al., 1999). This may explain why *B. thermosphacta* did not impact the ability of leucocin A to inhibit the growth of *L. monocytogenes* on the surface of wieners.

It was not possible to determine if leucocin A fragmentation occurred *in vitro* or *in situ* due to inconclusive results obtained from LC-MS/MS analysis. This may have been due to absorption of leucocin A to the proteins on the surface of the wiener through ionic or hydrophobic associations making it difficult to recover detectable amounts of leucocin A with the wash step (Aasen et al., 2003). Residual activity was detected when the concentrated wash from wieners that had been stored for 16 d was tested against the sensitive indicator (data not shown). If partial fragmentation had occurred, the remaining peptide remained active to inhibit the growth of *L. monocytogenes*.

The cocktail of *L. monocytogenes* used in this study is recommended for use in challenge studies (Fugett, 2006). However, as all strains have varying resistance to leucocin A, the use of this cocktail may bias results of challenge studies. This knowledge is valuable for

the food industry, as it appears that even though leucocin A is active against food isolates of *L. monocytogenes,* it may be better suited to use in conjunction with other interventions as part of a hurdle system (Allen et al., 2014; Chen and Hoover, 2003; Gálvez et al., 2008; Parada et al., 2007; Perez et al., 2014; Tiwari et al., 2009; Zhu et al., 2005). On solid-phase systems leucocin A can control *L. monocytogenes* even in the presence of naturally occurring spoilage organisms such as *C. divergens.* Further experimentation is needed with longer storage times to ensure the outgrowth of *L. monocytogenes* does not occur as some strains have resistance to leucocin A.

Bacteriocins have the potential to enhance food safety and reduce the economic impact of foodborne illness on healthcare systems. Leucocin A can control the growth of *L. monocytogenes* in the presence of spoilage organisms; however, there is concern that some spoilage organisms may result in less inhibition. During extended storage of RTE meat products, it is possible that subpopulations of *L. monocytogenes* may be or become resistant to bacteriocins. To optimize class IIa bacteriocins for use as food additives, more research is needed to determine efficacy in the presence of a larger range of spoilage organisms. Additionally, it will be advantageous to understand if strains of *L. monocytogenes* suggested for use in challenge studies are innately resistant to class II bacteriocins and if they can develop spontaneous resistance upon exposure.

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Chapter 3

3.0 Introduction

Listeria monocytogenes is a foodborne pathogen that causes infections with a fatality rate of 20–30% (Farber and Peterkin, 1991; Thomas et al., 2013). *L. monocytogenes* poses a food safety risk in relation to ready-to-eat (RTE) products as *L. monocytogenes* grows over a wide range of temperatures, including refrigeration temperatures (Zhu et al., 2005; Health Canada, 2011). According to the Centers for Disease Control and Prevention, the case frequency of listeriosis remained unchanged over the past 3 years (CDC, 2014) even though strict guidelines have been developed to control *L. monocytogenes* in RTE products (USDA-FSIS, 2014). With consumer demands for the food industry to increase availability of fresh, RTE and minimally processed products, novel methods of bio-preservation, such as bacteriocins, are needed. Bacteriocins are ribosomally synthesized antimicrobial peptides that inhibit the growth of foodborne pathogens and are an attractive alternative to traditional preservatives to improve food safety (Perez et al., 2014). However, *L. monocytogenes* can develop resistance to class IIa bacteriocins (Ramnath et al., 2000; Gravesen et al., 2002; Kaur et al., 2011;Vadyvaloo et al., 2002; Vadyvaloo et al., 2004a), but little is known about resistance to circular bacteriocins.

Class IIa bacteriocins are small, hydrophobic, heat stable peptides that undergo minimal post-translational modification, and are degraded by various proteases (Cotter et al., 2005; Alvarez-Sieiro et al., 2016). Their mode of action is based on pore formation and disruption of ion gradients of the target cell (Héchard and Sahl, 2002). Previous work in our lab demonstrated that the 5 strains of *L. monocytogenes* that are recommended for use in challenge studies (Fugett et al., 2006) are resistant to 3.3 mM leucocin A in broth and will grow on vacuum packaged wieners that had 0.01 mM/cm^2 leucocin A on the surface (Balay et al., 2017).

Resistance to class IIa bacteriocins has been linked to the mannose phosphotransferase system (Man-PTS; Dalet et al., 2001, Diep et al., 2007) and changes in cell membrane properties. Changes in the membrane can block pore formation by bacteriocins (Ramnath et al., 2004) due to increased membrane fluidity and a more positively charged cell wall

(Vadyvaloo et al., 2002, 2004a, 2004b). Resistance has also been correlated with the down regulation of the genes associated with the Man-PTS, which effects the transcription of enzyme II subunits (Gravesen et al., 2002). The membrane bound subunits are thought to provide a docking station for bacteriocins *(*Kjos et al., 2011, 2010). There are many sugar-PTS that are dedicated to specific carbohydrates that are transported across the membrane. It may be possible that a culture does not become resistant to a bacteriocin if the specific PTS involved in bacteriocin docking is required for carbohydrate transport to support growth. However, a study by Tessema et al., 2009, showed spontaneous mutants of *L. monocytogenes* that were highly-resistant (IC_{50}, >104 ng/ml) to sakacin P were able to grow significantly faster in media supplemented with mannose and glucose than low-level resistant (IC_{50}, <104 ng/ml) spontaneous mutant strains of *L. monocytogenes*. Additionally, there was upregulation of the *mptA* in some stains of *L. monocytogenes* that were highly-resistant to Class IIa bacteriocins compared to the sensitive, wild type strains (Gravesen et al., 2004; Tessema et al., 2009), which indicates there could be additional mechanisms for cells to form resistance to bacteriocins that are not fully understood yet.

Leucocin A produced by *Leuconostoc gelidum* UAL187 is a 37-residue class IIa bacteriocin with a molecular mass of 3,903 Da, which docks with the Man-PTS (Hastings and Stiles, 1991, Drider et al., 2006). Carnocyclin A, a 60-residue circular bacteriocin with potent antilisterial activity, is produced by *Carnobacterium maltaromaticum* ATCC PTA-5313 (Martin-Visscher et al., 2008; van Belkum et al., 2011). Circular bacteriocins have some distinguishing features including an amide bond that connects the N- and C-termini, and increased stability against proteases (van Belkum et al., 2011). Carnocyclin A forms anion selective channels in the lipid membrane, therefore carnocyclin A does not require a docking molecule to form a pore (Gong et al., 2009). However, more research is needed to fully understand if ion selective pores are the only mode of action for carnocyclin A (Drider et al., 2006; Gong et al., 2009). As cyclic bacteriocins interact differently with the membrane compared to Class IIa bacteriocins, there may be different mechanisms for bacteriocin resistance in *L. monocytogenes*.

The objective of this research was to determine the effect of different carbohydrates on the growth kinetics of *L. monocytogenes* strains associated with food borne illness in the presence of two bacteriocins that differ in class and mode of action, leucocin A and carnocyclin A.

3.1 Materials & Methods

3.1.1 Bacterial Strains and Preparation

Outbreak strains of *Listeria monocytogenes* (FSL N1-227, FSL R2-499, FSL N3-013, FSL J1-177, FSL C1-056) developed for use in challenge studies were obtained from Cornell University (Fugett et al., 2006). *Carnobacterium maltaromaticum* ATCC-PTA 5313, carnocyclin A producer, and *Leuconostoc gelidum* UAL187, leucocin A producer, were obtained from the University of Alberta Lactic Acid Bacteria culture collection. All strains were stored in 30% glycerol in media at -80°C until needed.

Prior to use in experiments, cultures were streaked onto All Purpose Tween agar (APT; Becton, Dickinson and Company, New Jersey, USA), and a single colony was inoculated into broth media. *C. maltaromaticum* ATCC-PTA 5313 and *Leuc. gelidum* UAL187 were grown anaerobically at 25°C in APT broth and semi-defined Casamino Acids (CAA) broth without Tween® 80 (Hastings et al., 1991; Balay et al., 2017), respectively. Strains of *L. monocytogenes* were grown at 32°C in APT broth.

3.1.2 Production and Purification of Bacteriocins

Leucocin A was purified using a protocol described in (Balay et al., 2017). Carnocyclin A was purified using a modified protocol of Martin-Visscher et al., (2008). *C. maltaromaticum* ATCC PTA-5313 was grown in 1 L APT broth at 25°C for 24 h. For purification, the culture, including the cells, was loaded onto an Amberlite® XAD-16N column (100 g/L; 2.5 cm x 50 cm; 5 ml/min; Sigma-Aldrich®, St. Louis, MO, USA) at 6°C. Resin was pre-conditioned with 100% isopropanol (IPA) and rinsed with sterile

distilled water (dH_2O). The column was washed sequentially at a flow rate of 10 ml/min with 500 ml of dH_2O, 500 ml of 30% (v/v) ethanol (EtOH), 500 ml of 40% (v/v) IPA, 500 ml 70% (v/v) IPA, and carnocyclin A was eluted with 500 ml 70% (v/v) IPA, pH 2. The fraction was concentrated using a Buchi® rotary evaporator (Brinkman Instruments, Westbury, NY, USA) at 30°C under vacuum to 10 ml. The concentrated fraction was loaded onto a Sep-Pak® Vac 12cc (2 g) C18 cartridge (Waters, Milford, MA, USA), which had been pre-washed with 50 ml methanol followed by 50 ml dH_2O. The column was washed consecutively with 50 ml of dH_2O, 50 ml of 30% (v/v) EtOH, 50 ml of 30% (v/v) acetonitrile (ACN), 50 ml 40% (v/v) IPA, 50 ml 70% (v/v) IPA, and carnocyclin A was eluted with 100 ml 70% (v/v) IPA, pH 2. This fraction was concentrated to 2 ml using a rotary evaporator and lyophilized (Labconco® FreezeZone, Kansas City, MO, USA). The lyophilized sample was suspended in 5 ml of dH_2O for reverse-phase high performance liquid chromatography (RP-HPLC; Beckman System Gold, Beckman Coulter Inc., Mississauga, Ontario, Canada). Samples of 1 ml were injected onto a C8 column (5 μm particle size, 10 x 250 mm, Vydac 208TP510, Hichrom, Berkshire, UK). The RP-HPLC mobile phase consisted of (A) IPA and (B) H_2O, each containing 0.1% trifluoroacetic acid. A gradient RP-HPLC method was used, consisting of a 8 min hold at 20% A; 30 min increase of A from 20 – 86%; 5 min hold at 86% A; and a 3 min decrease from 86 – 20% A, with a flow rate of 1 ml/min and ultraviolet detection set at 220 nm. Carnocyclin A eluted as a sharp peak (retention time 23 to 24 min). After purification, all carnocyclin A fractions were combined, lyophilized and stored at -20°C until needed. For experimental use, carnocyclin A was suspended in dH_2O to the desired concentration.
The purified samples were subjected to matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) to confirm the presence of leucocin A or carnocyclin A.

3.1.3 Growth Kinetics of *L. monocytogenes* Grown in Different Carbohydrates Treated With Leucocin A or Carnocyclin A

For growth experiments, basal media supplemented with individual carbohydrates was used. For 1 L of basal medium the following formulation was used: 10 g Proteose

Peptone No. 3 (Becton, Dickinson and Company), 10 g Beef Extract (Becton, Dickinson and Company), 5 g Yeast Extract (Becton, Dickinson and Company), 1 g Tween® 80, 2 g disodium phosphate, 1 ml sterile basal media salt solution [10 g magnesium sulfate, 5 g manganese sulfate dissolved in 100 ml dH_2O], and for each litre of basal medium, 20 g of a carbohydrate (glucose, sucrose, fructose, mannose, or cellobiose) was added.

To determine growth kinetics, 5 series of 4 wells in separate 96 well microtiter plates(s) for each strain were filled with 50 µl of each of the 5 basal media supplemented with individual carbohydrates. An additional 100 µl of each basal media was added to the first well of each series as a negative control. For each microtiter plate, an overnight culture of one strain of *L. monocytogenes* was centrifuged, suspended in sterile 0.1% (w/v) peptone water (Becton, Dickinson and Company) and serially diluted with peptone water with the final dilution made in basal media. To the second well of each series, 50 µl of basal media and 50 µl of one strain of *L. monocytogenes* was added. The third series of wells contained basal media, *L. monocytogenes* and leucocin A; and the fourth series included *L. monocytogenes* and carnocyclin A. The final concentration of bacteriocins was 3.3 mM and cell density was ~10^4 CFU per well, confirmed by plate count on APT agar. The concentration of bacteriocin was chosen as it is equal or higher than the MIC against *L. monocytogenes* determined on a critical dilution assay on agar after incubation for 24 h (data not shown; Balay et al., 2017) but did not prevent growth of resistant derivatives upon prolonged incubation. Preliminary experiments indicated that the response of the 5 strains of *L. monocytogenes* to challenge with bacteriocins was similar when pre-cultures were prepared with APT (glucose) or supplemented basal media containing carbohydrates other than glucose.

Each microtiter plates was covered with a clear optical adhesive film (MicroAmp, Applied Biosystems, Thermo Fischer Scientific, Burlington, Ontario, Canada) to prevent evaporation, loss of culture or contamination and placed in a spectrophotometer (Multiskan Ascent, Thermo Electron Corporation, Shanghai, China) and incubated at 25°C for 72 h. Each plate was shaken every 2 h for 10 s at 700 RPM, and the optical density (630_{nm}) of each well was recorded. Growth curves were generated using

Mulitskan Ascent Software (Version 2.6, Thermo Fischer Scientific). The experiment was replicated independently three times.

3.1.4 Identify Phenotypic Differences of the Subpopulation to Bacteriocins

To characterize phenotypical differences of the subpopulation that grew in the presence of either bacteriocin from the growth experiments (Materials & Methods 2.3), two strains were selected for further testing; FSL R2-499 and FSL C1-056. Both strains of *L. monocytogenes* were grown in basal media supplemented with either mannose or cellobiose and treated with 3.3 mM leucocin A or carnocyclin A.
To compare growth kinetics of the original cultures compared to that each subpopulation first a growth experiment under similar conditions as outlined in Materials & Methods 2.3, followed by isolation of individual colonies from the subpopulation that grew, which then were used in a second growth experiment with the same conditions as the first growth experiment.

To determine the growth kinetics for the original cultures FSL R2-499 and FSL C1-056, 2 series (one for each carbohydrate) of 4 wells was used in separate 96 well microtiter plates(s) for each strain. The total well volume used was 200 µl. The first well of each series was filled with 200 µl of each basal media supplemented with individual carbohydrates (mannose or cellobiose) as a negative control. For each microtiter plate, an overnight culture of one strain of *L. monocytogenes* was centrifuged, suspended in sterile 0.1% (w/v) peptone water (Becton, Dickinson and Company) and serially diluted with peptone water with the final dilution made in basal media. To the second well of each series, 150 µl of basal media and 50 µl of one strain of *L. monocytogenes* was added. The third series of wells contained basal media, *L. monocytogenes* and leucocin A; and the fourth series included *L. monocytogenes* and carnocyclin A. The final concentration of bacteriocins was 3.3 mM and cell density was ~10^6 CFU per well, confirmed by plate count on APT agar. Each microtiter plates was covered with a clear optical adhesive film (MicroAmp, Applied Biosystems, Thermo Fischer Scientific, Burlington, Ontario, Canada) to prevent evaporation, loss of culture or contamination and placed in a

spectrophotometer (Multiskan Ascent, Thermo Electron Corporation, Shanghai, China) and incubated at 25°C for 40 h. Each plate was shaken every 2 h for 10 s at 700 RPM, and the optical density (630_{nm}) of each well was recorded. Growth curves were generated using Mulitskan Ascent Software (Version 2.6, Thermo Fischer Scientific).

Following the first growth experiment seals were removed from microtiter plates and a loopful of culture was obtained from each well of the both series. The culture was then streaked on APT agar to obtain single colonies. Plates were incubated at 32°C for 24 h before individual colonies were picked and cultured into APT broth at 32°C for 24 h. The same growth experiment then followed for the isolated subpopulations as outlined above with the original cultures. The experiment was replicated independently four times.

3.1.5 Predictive Modelling of Growth Curves

Growth curves were fitted to a modified logistic model (Zwietering et al., 1990) to assess how maximal growth rates (μ_m) and lag phase (λ) were affected by the presence of leucocin A or carnocyclin A when *L. monocytogenes* was grown in different carbohydrates. SigmaPlot 12.5 was used for all curve fit procedures.

3.1.6 Statistical Analysis

Data were analyzed using the PROC ANOVA of SAS Studio (Release 3.5 University Edition, SAS Institute Inc., Cary NC, USA) with strain, carbohydrate and bacteriocins as categorical variables and lag phase and growth rate as dependent variables. To determine significant differences among means, Tukey Test with a 95% confidence interval was used to determine significant interactions. To determine the specific impact of the main effects of strain, carbohydrate or bacteriocin, one-way ANOVA was done with differences among means determined with a Tukey Test.

3.2 Results

3.2.1 Purification

The novel protocol for purification of leucocin A and carnocyclin A recovered 2.3 mg/L and 4.8 mg/L of leucocin A and carnocyclin A, respectively. Purification yields of leucocin A and carnocyclin A were 5-fold and 2.5-fold higher, respectively, than the yields reported by Hastings et al., (1991) and Martin-Visscher et al., (2008). The molecular mass of the purified leucocin A and carnocyclin A was 3,930.03 Da and 5,863.50 Da, respectively, matching prior reports (Hastings et al., 1991; Martin-Visscher et al., 2008).

3.2.2 Predictive Modelled Growth Curves

Individual growth curves were fitted to a modified logistic model with an $R^2 \geq 0.99$ with an overall fit of $R^2 = 0.995$ as shown in the unity plot (Fig. 3.1). Each of the experimental growth curves with the modeled curve for each growth condition is shown in Appendix A (Fig. S3.1-7). *L. monocytogenes* FSL N1-227 grown in fructose with carnocyclin A and FSL J1-177 grown in glucose with carnocyclin A gave a growth curve that did not precisely fit the sigmoidal shape of the logistic model (Appendix A, Fig. S3.1, S3.4), but the R^2 was >0.99. The modelled growth curves for each strain of *L. monocytogenes* grown in the presence of each carbohydrate and leucocin A or carnocyclin A are shown in Fig. 3.2. Leucocin A at a concentration of 3.3 mM did not inhibit the growth of any of the strains of *L. monocytogenes* in the presence of any of the tested carbohydrates (Fig. 3.2a), demonstrating that all strains developed resistance against leucocin A. Only two of the five strains shows an extended lag-phase in presence of leucocin A in presence of some but not all carbohydrates (Table 3.1), indicating that the development of resistance is dependent on the strain and the carbohydrate source. Carnocyclin A extended the lag-phase and reduced the growth rate of all strains; the extent of inhibition again depended on individual strains and/or carbohydrates (Fig. 3.2b).

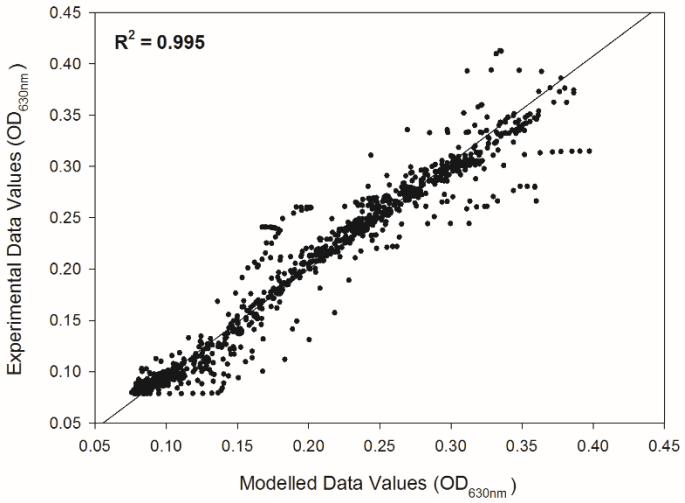

Figure 3.1 Unity plot of experimental OD_{630nm} data values versus the fitted (modelled) OD_{630nm} data values of all 5 *L. monocytogenes* (FSL N1-227, FSL R2-499, FSL N3-013, FSL J1-177, FSL C1-056) grown in each of the carbohydrates (glucose, sucrose, fructose, mannose, cellobiose) for the control, or treated with 3.3 mM leucocin A or carnocyclin A (n=3).

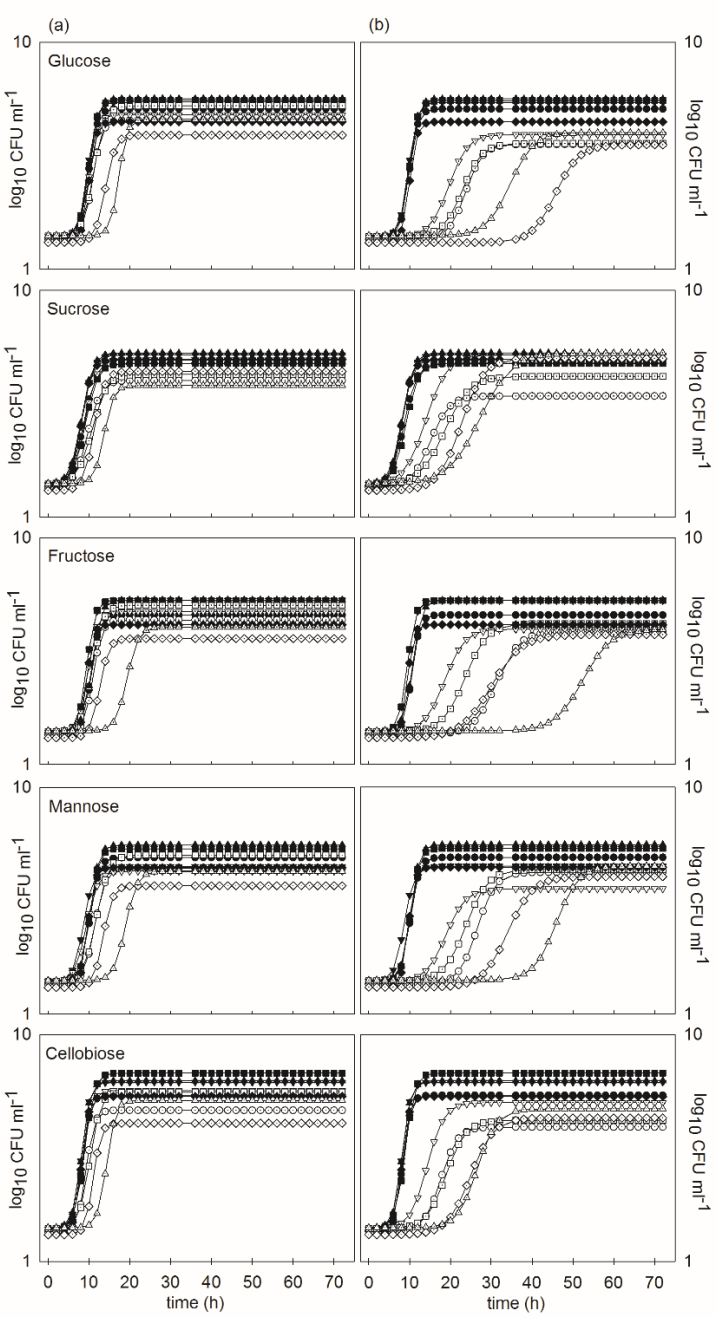

Figure 3.2 Modelled growth curves of *L. monocytogenes* FSL N1-227 (●,○); FSL R2-499 (▼,▽); FSL N3-013 (■,□), FSL J1-177 (◆,◇), FSL C1-056 (▲,△), with the addition of bacteriocins (open symbols) or without bacteriocin (closed symbols) in media supplemented with different carbohydrates glucose (A), sucrose (B), fructose (C), mannose (D), cellobiose (E) and measured at 630 nm every 2 h for 72 h at 25°C. Plots in the left column were treated with leucocin A and those in the right column were treated with carnocyclin A (n=3).

Table 3.1 Least squared means of maximal growth rate and lag phase of *L. monocytogenes* determined at 25 °C in basal medium supplemented with different carbohydrates (glucose, sucrose, fructose, mannose, cellobiose) in the presence of leucocin A or carnocyclin A (n=3).

		control		leucocin A		carnocyclin A	
		α_m *	λ †	α_m	λ	α_m	λ
FSL N1-227	glu	0.31 ± 0.03^{a}	7.69 ± 0.92^{b}	0.26 ± 0.02^{bxy}	8.52 ± 1.35^{by}	0.12 ± 0.01^{c}	19.56 ± 6.49^{a}
	suc	0.20 ± 0.01^{ay}	5.47 ± 0.88^{b}	0.17 ± 0.01^{ay}	6.55 ± 0.60^{aby}	0.10 ± 0.01^{b}	10.42 ± 2.79^{ayz}
	fru	0.31 ± 0.02^{ay}	8.55 ± 0.97^{b}	0.26 ± 0.01^{bx}	8.73 ± 1.15^{by}	0.10 ± 0.01^{cxy}	25.64 ± 2.45^{ay}
	man	0.28 ± 0.03^{ay}	7.79 ± 0.85^{b}	0.22 ± 0.01^{axy}	8.72 ± 1.30^{by}	0.13 ± 0.05^{b}	22.13 ± 6.88^{axy}
	cel	0.36 ± 0.01^{a}	6.38 ± 0.59^{b}	0.30 ± 0.01^{bxy}	7.22 ± 0.86^{by}	0.13 ± 0.01^{cxy}	13.55 ± 2.40^{axy}
FSL R2-499	glu	0.33 ± 0.02^{a}	7.60 ± 0.26^{b}	0.29 ± 0.03^{ax}	7.91 ± 0.64^{by}	0.12 ± 0.03^{b}	14.99 ± 4.35^{a}
	suc	0.25 ± 0.01^{ax}	5.60 ± 0.17	0.22 ± 0.01^{ax}	6.12 ± 0.41^{y}	0.13 ± 0.02^{b}	8.76 ± 2.47^{z}
	fru	0.32 ± 0.02^{axy}	7.41 ± 0.43^{b}	0.27 ± 0.02^{ax}	7.52 ± 0.81^{by}	0.11 ± 0.01^{bx}	13.61 ± 2.54^{ay}
	man	0.24 ± 0.11^{ay}	6.26 ± 2.77^{b}	0.24 ± 0.10^{bx}	7.69 ± 2.88^{by}	0.11 ± 0.04^{c}	14.07 ± 5.55^{ay}
	cel	0.38 ± 0.03^{a}	6.18 ± 0.15^{b}	0.34 ± 0.02^{ax}	6.52 ± 0.19^{by}	0.15 ± 0.01^{bx}	10.13 ± 0.64^{ay}
FSL N3-013	glu	0.33 ± 0.00^{a}	7.83 ± 0.46^{b}	0.24 ± 0.02^{bxy}	8.44 ± 0.69^{by}	0.10 ± 0.02^{c}	18.18 ± 5.94^{a}
	suc	0.20 ± 0.01^{ay}	6.13 ± 0.28^{b}	0.15 ± 0.00^{bz}	7.11 ± 0.53^{by}	0.09 ± 0.00^{c}	12.60 ± 2.50^{axyz}
	fru	0.33 ± 0.00^{axy}	7.46 ± 0.50^{b}	0.23 ± 0.02^{bxy}	8.07 ± 0.82^{by}	0.10 ± 0.01^{cxy}	18.13 ± 5.01^{ay}
	man	0.31 ± 0.02^{axy}	8.07 ± 0.36^{b}	0.21 ± 0.02^{bxy}	8.77 ± 0.58^{by}	0.11 ± 0.02^{c}	18.45 ± 4.99^{ay}
	cel	0.34 ± 0.03^{a}	6.61 ± 0.29^{b}	0.27 ± 0.02^{by}	7.61 ± 0.52^{by}	0.11 ± 0.01^{cxy}	14.17 ± 3.41^{axy}
FSL J1-177	glu	0.34 ± 0.02^{a}	8.17 ± 0.65^{b}	0.22 ± 0.02^{by}	11.54 ± 2.52^{abxy}	0.09 ± 0.01^{c}	39.89 ± 21.17^{a}
	suc	0.25 ± 0.01^{ax}	5.42 ± 0.40^{b}	0.23 ± 0.01^{ax}	8.60 ± 1.53^{bxy}	0.12 ± 0.05^{b}	17.05 ± 4.58^{axy}
	fru	0.36 ± 0.01^{ax}	7.86 ± 0.73^{b}	0.22 ± 0.04^{bxy}	10.31 ± 1.93^{axy}	0.07 ± 0.01^{cy}	22.51 ± 9.83^{ay}
	man	0.36 ± 0.02^{ax}	7.97 ± 0.79	0.22 ± 0.02^{bxy}	11.19 ± 2.61^{y}	0.09 ± 0.02^{c}	27.87 ± 14.51^{xy}
	cel	0.39 ± 0.01^{a}	6.29 ± 0.37^{b}	0.30 ± 0.02^{by}	9.12 ± 1.77^{abxy}	0.10 ± 0.03^{cy}	19.19 ± 7.04^{axy}
FSL C1-056	glu	0.33 ± 0.02^{a}	7.87 ± 0.88^{b}	0.29 ± 0.00^{bx}	15.27 ± 1.70^{bx}	0.09 ± 0.01^{c}	28.82 ± 6.94^{a}
	suc	0.25 ± 0.02^{ax}	5.50 ± 1.07^{c}	0.22 ± 0.00^{bx}	11.43 ± 1.99^{bx}	0.08 ± 0.01^{c}	19.43 ± 2.21^{ax}
	fru	0.29 ± 0.02^{ay}	8.45 ± 1.45^{b}	0.19 ± 0.01^{by}	16.57 ± 2.57^{bx}	0.07 ± 0.00^{cy}	45.54 ± 9.33^{ax}
	man	0.32 ± 0.02^{axy}	8.09 ± 1.04^{c}	0.19 ± 0.01^{by}	16.15 ± 2.16^{bx}	0.10 ± 0.01^{c}	40.39 ± 4.93^{ax}
	cel	0.36 ± 0.02^{a}	6.14 ± 0.91^{b}	0.29 ± 0.01^{by}	12.09 ± 1.75^{bx}	0.13 ± 0.01^{cxy}	21.83 ± 4.28^{ax}

* maximal growth rate (h^{-1}), † lag phase (h); $^{a, b, c}$ across rows indicate significantly different ($P \leq 0.05$) μ_m or λ values based on the presence (or lack thereof) of bacteriocin for each strain of *L. monocytogenes* grown in each different carbohydrates; $^{x, y, z}$ down columns indicate significantly different ($P \leq 0.05$) μ_m or λ values among strains grown in the same carbohydrate for each bacteriocin and the control

3.2.3 Growth Rate as a Function of Lag Phase

Growth rate was plotted as a function of lag phase duration for each strain grown in each carbohydrate based on bacteriocin treatment to assess trends in the data (Fig. 3.3). In the absence of either bacteriocin, the lag phases of all strains of *L. monocytogenes* were of similar duration, although there was greater variation among their subsequent growth rates (Fig. 3.3). In the presence of carnocyclin A this situation was reversed, in that the lag phase of *L. monocytogenes* varied considerably, but their subsequent growth rates were broadly similar. In the presence of leucocin A there was less variation among lag phases, and among subsequent growth rates, than in the absence of bacteriocin, or in the presence of carnocyclin A (Fig. 3.3).

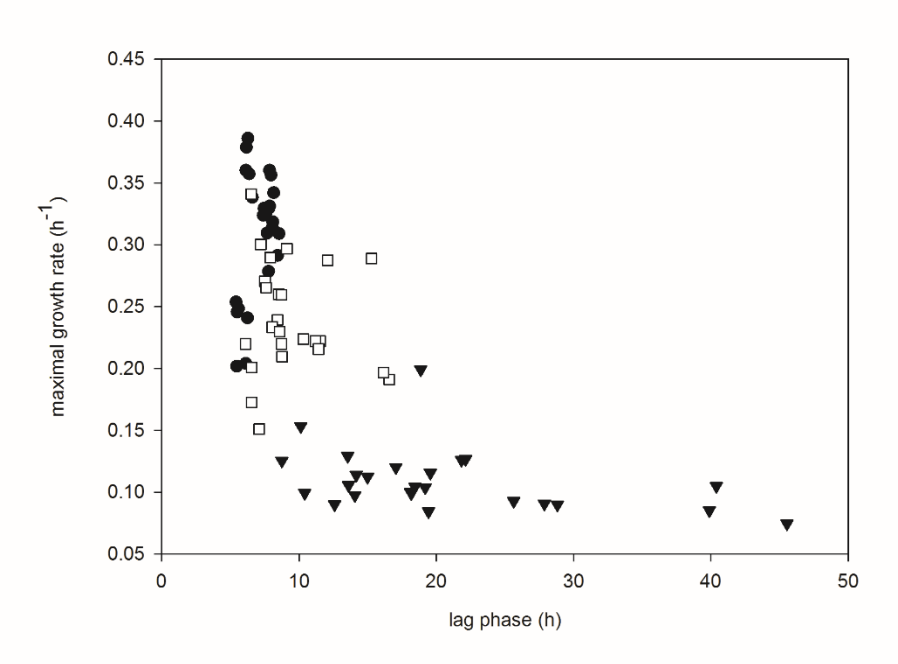

Figure 3.3 Growth rate plotted as a function of lag phase for all strains of *L. monocytogenes* grown in all carbohydrates for the control (●) or in the presence of leucocin A (□) or carnocyclin A (▼) (n=3).

3.2.4 Impact of Carbohydrate on Growth of *L. monocytogenes*

The two-way interaction means for the growth rate and lag phase of cultures grown in media with individual carbohydrates, across all bacteriocin treatments are shown in

Figure 3.4. There were significant interactions between strain and carbohydrate that resulted in differences in growth rate and lag phase for individual carbohydrates (Fig. 3.4a, b). For FSL N1-227 and FSL N3-013, the maximal growth rate was significantly lower when strains were grown in sucrose, whereas growth in cellobiose resulted in a significantly higher growth rate for all strains (Fig. 3.4a). The lag phases of *L. monocytogenes* FSL C1-056 and FSL J1-177 were significantly longer when grown in mannose, glucose and fructose (Fig. 3.4b).

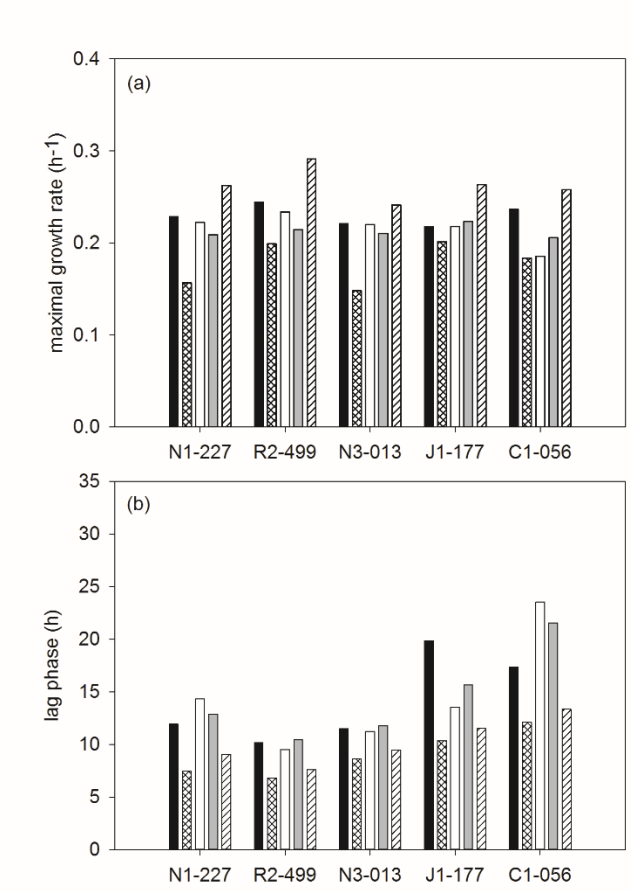

Figure 3.4 Least squared means of significant two-way interactions for the maximal growth rate (a) and lag phase (b) of each strain of *L. monocytogenes* (FSL N1-227, FSL R2-499, FSL N3-013, FSL J1-177, FSL C1-056) grown in different carbohydrates. *L. monocytogenes* was grown in glucose (■), sucrose (▨), fructose (□), mannose (■), cellobiose (▨), regardless of bacteriocin treatment (n=3).

3.2.5 Impact of Bacteriocin and Carbohydrate on the Growth of *L. monocytogenes*

There were significant two-way interactions between strain and bacteriocin treatment, and carbohydrate and bacteriocin treatment (Fig. 3.5 and 3.6, respectively).

For each strain, regardless of carbohydrate, leucocin A resulted in a significantly lower growth rate than the untreated control, while carnocyclin A resulted in a significantly lower growth rate than strains treated with leucocin A or the untreated control (Fig. 3.5a). When treated with leucocin A, FSL R2-499 had the fastest growth rate ($P<0.05$) and FSL N3-013 had the slowest growth rate ($P<0.05$; Fig. 3.5a). All strains had the same lag phase when not treated with bacteriocin, but treatment of strains FSL C1-056 and FSL J1-177 with leucocin A resulted in a significantly longer lag phase than that observed for the untreated control (Fig. 3.5b). Treatment with carnocyclin A significantly increased the lag phase for all cultures compared to the untreated control (Fig. 3.5b). The lag phase of strains FSL N1-227, FSL C1-056 and FSL J1-177 were significantly longer when treated with carnocyclin A compared to those treated with leucocin A.

Regardless of strain, growth in cellobiose resulted in the highest growth rate ($P<0.05$) within each bacteriocin treatment (Fig. 3.6a). Growth in media with carnocyclin A and any of the carbohydrates significantly reduced growth rates (Fig. 3.6a). Sucrose resulted in the slowest growth rate for the untreated control and cells treated with leucocin A, whereas fructose resulted in the slowest growth rate for all strains treated with carnocyclin A (Fig. 3.6a). Compared to the untreated control, there was no difference ($P>0.05$) in lag phase of cultures treated with leucocin A regardless of the carbohydrate, but the lag phase increased ($P<0.05$) when cultures were treated with carnocyclin A (Fig. 3.6b). Growth in glucose, fructose or mannose increased ($P<0.05$) the lag phase in media with carnocyclin A compared to cultures grown in the presence of leucocin A (Fig. 3.6b).

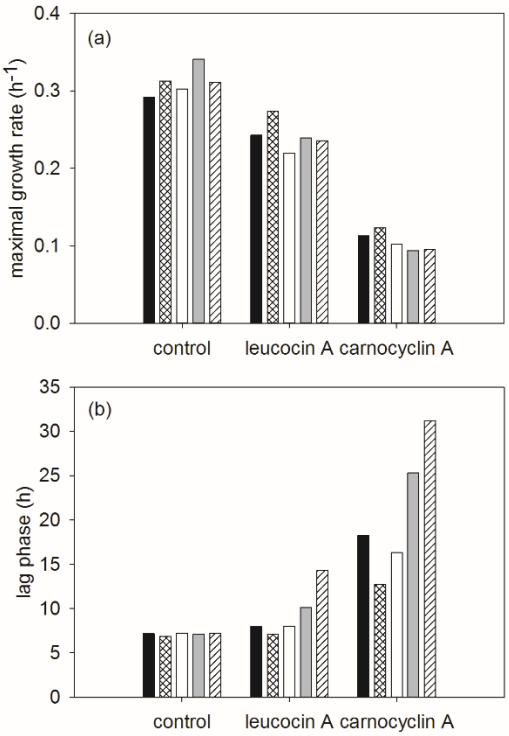

Figure 3.5 Least squared means of significant two-way interactions for the maximal growth rate (a) and lag phase (b) of each strain of *L. monocytogenes* by control or bacteriocin treatment (leucocin A, carnocyclin A). The strains of *L. monocytogenes* FSL N1-227 (■), FSL R2-499 (▨), FSL N3-013 (□), FSL J1-177 (■), FSL C1-056 (▨), regardless of carbohydrate (n=3).

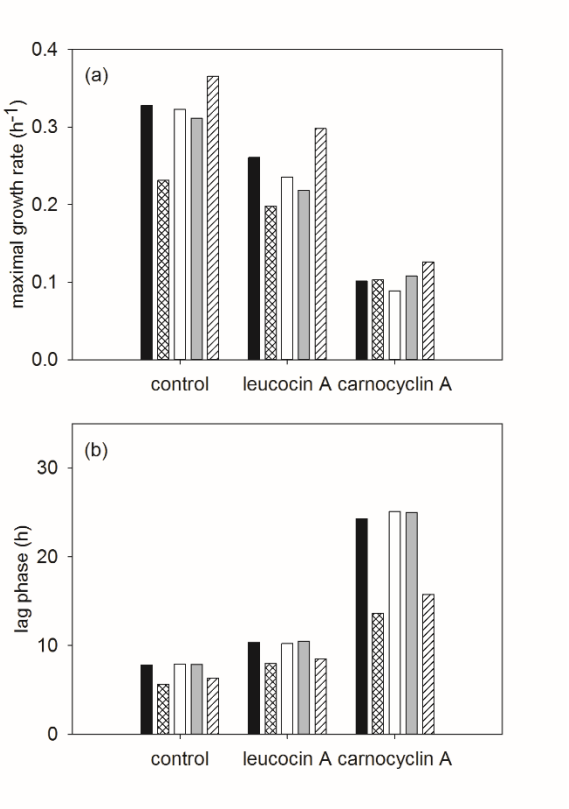

Figure 3.6 Least squared means of significant two-way interactions for the maximal growth rate (a) and lag phase (b) of each carbohydrate by control or bacteriocin treatment (leucocin A, carnocyclin A), regardless of strain of *L. monocytogenes*. The carbohydrates used were glucose (■), sucrose (▩), fructose (□), mannose (■), cellobiose (▨) (n=3).

3.2.6 Impact of Individual Factors on Growth Rate and Lag Phase of *L. monocytogenes*

The impact of strain, bacteriocin or carbohydrate was determined by one-way ANOVA (Table 3.1). Strain did not affect growth rate when cultures were grown in glucose or cellobiose without bacteriocin (Table 3.1). Growth rate was significantly reduced for strain FSL N1-227 when grown in sucrose, fructose and mannose compared to the untreated control group (Table 3.1). Strain had no effect on the lag phase for the untreated control group regardless of the carbohydrate (Table 3.1).

Leucocin A significantly decreased the growth rates of all strains when grown in glucose, fructose, or cellobiose, except for FSL R2-499 (Table 3.1). Strain FSL R2-499 was not

affected by leucocin A except when grown in mannose, which significantly decreased the growth rate compared to the untreated control (Table 3.1). When FSL N1-227 was grown in mannose it was resistant to leucocin A as there was no effect on growth rate compared to the untreated control, which was the exact opposite for FSL R2-499 (Table 3.1). Strain FSL C1-056 had the slowest growth rate when grown in fructose, mannose, or cellobiose, and treated with leucocin A (Table 3.1). However, when FSL C1-056 was grown in glucose or sucrose it was one of the fastest growing strains despite having a significantly increased lag phase compared to the untreated control in sucrose and mannose (Table 3.1). In all carbohydrates, when FSL C1-056 was treated with leucocin A there was a significant increase in lag phase (Table 3.1).

Carnocyclin A significantly decreased the growth rates of all strains compared to the untreated control, but fructose and cellobiose caused variation in growth rates among strains (Table 3.1). Carnocyclin A significantly increased the lag phase for all strains when grown in glucose, fructose or cellobiose compared to the untreated control. There was no difference in lag phase across strains when grown in glucose and treated with carnocyclin A. When grown in sucrose FSL R2-499 had increased resistance to carnocyclin A as the lag phase was not affected by bacteriocin treatment. Strain FSL C1-056 had an increase in lag phase when grown in sucrose in the presence of carnocyclin A compared to strains FSL N1-227 and FSL R2-499. Similarly, FSL C1-056 had the longest lag phase compared to all other strains when grown in fructose and treated with carnocyclin A. Strain FSL C1-056 had significantly longer lag phase when treated with carnocyclin A and grown in mannose when compared to strains FSL R2-499and FSL N3-013 (Table 3.1).

3.2.7 Phenotypical Characteristics of the Growth Kinetics of the Subpopulation

The growth kinetics of cultures was analyzed after growth in the presence of both bacteriocins to compare the maximal growth rate and lag phase to that of original cultures.

The original cultures prepared in absence of bacteriocin grew in both sugars (Fig. 3.7a,b and Appendix A, Table S3.1); growth parameters were slightly modified, which reflects the different culture conditions for the inoculum of the respective cultures. *L. monocytogenes* FSL C1-056 had longer lag phases in both carbohydrates when treated with either bacteriocin compared to FSL R2-499 (Figure 3.7 and Appendix A, Table S3.1). Carnocyclin A reduced the maximal growth rate for both strains grown in both carbohydrates compared to that of the control (Figure 3.7 and Appendix A, Table S3.1).

After pre-adaptation of *L. monocytogenes* to bacteriocins, neither carnocyclin A nor leucocin A increased the lag-phase of the cultures (Fig. 3.7 and Appendix A, Table S3.1). This indicates that adaptation to the bacteriocins eliminates or reduces the time for development of resistance upon sub-culture in presence of bacteriocins. One noticeable exception was *L. monocytogenes* FSL C1-056. The lag-phase of this strain in mannose or mannose-carnocyclin A did not differ after pre-adaptation to carnocyclin A; however, the strain still showed an extended lag phase to carnocyclin A after adaptation in presence of cellobiose (Appendix A, Table S3.1).

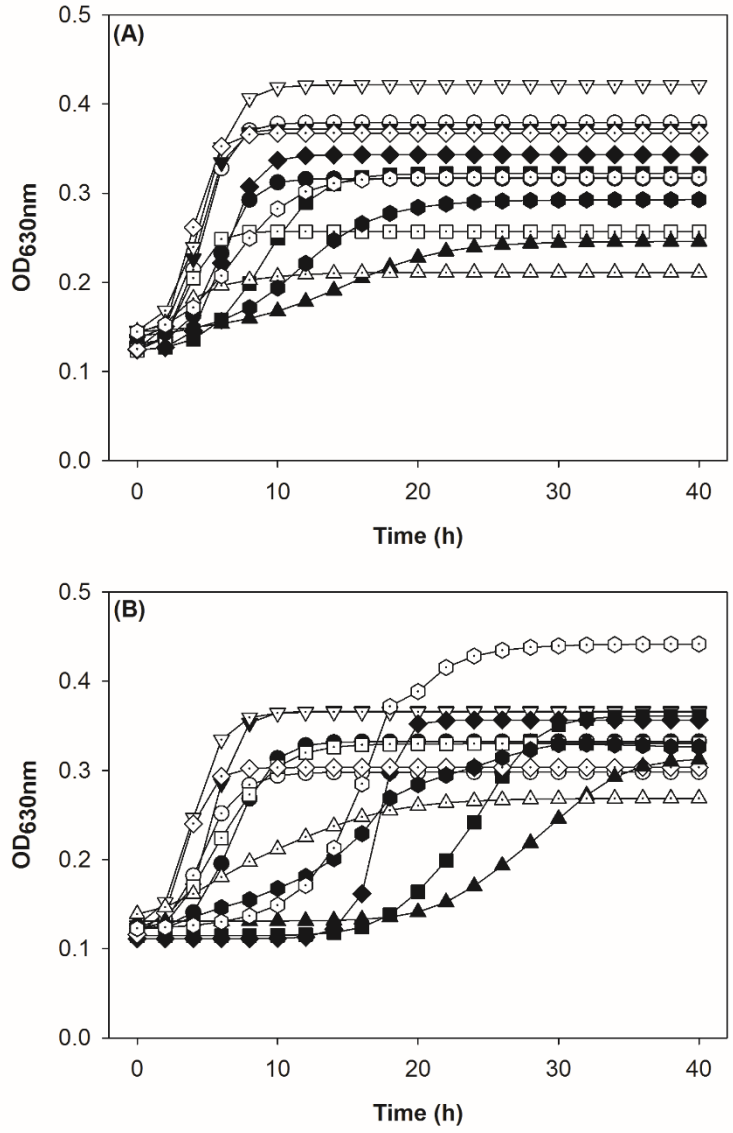

Figure 3.7 Modelled growth curve data of *L. monocytogenes* strains FSL R2-499 (A) and FSL C1-056 (B). Both panels compare the growth of after inoculation with adapted pre-cultures that grown in presence of 3.3 mM bacteriocins (open symbols) to the growth of after inoculation with "naïve" pre-cultures that were not exposed to bacteriocins (closed symbols). Cultures were grown in presence of mannose (●,○); mannose and leucocin A (■,□); mannose and carnocyclin A (▲,△); cellobiose (▼,▽); cellobiose and leucocin A (◆,◇); cellobiose and carnocyclin A (□,□) at 25 °C for 40 h. Optical density was measured at 630 nm and data is displayed as the average of four independent replicates.

3.3 Discussion

The application of bacteriocins in food to inhibit the growth of *L. monocytogenes* requires a better understanding of how carbohydrates affect the efficacy of bacteriocins and development of resistance.

Leucocin A decreased the growth rate of some strains of *L. monocytogenes* in most carbohydrates, but extended the lag phase only for a few strains. When the growth of the *L. monocytogenes* is delayed due to the presence of leucocin A, development of resistance to the bacteriocin occurs allowing the growth of the culture after an initial reduction cell counts (Balay et al., 2017). The observation that leucocin A concentrations exceeding the MIC on agar-assays did not significantly alter growth parameters in liquid culture indicates that resistance develops in a relatively large proportion of the population, and that fitness cost of resistance are marginal. Results from this study also confirm that development of resistance to bacteriocins results in a stable phenotype. Bacteriocin resistance as stable phenotype is supported by the observation that bacteriocins increased the lag-phase in the original cultures but not in cultures that were adapted to the respective bacteriocins.

Our results showed that growth in different carbohydrates supported development of resistance to bacteriocins, but the effect depended on the strain and the carbon source. For class IIa bacteriocins, concentrations exceeding the MIC 5- to 1,000-fold can induce resistance in *L. monocytogenes* (Duffes et al., 2000; Kaur et al., 2011). Although others have reported inhibition of *L. monocytogenes* in the presence of carnocyclin A (Gong et al., 2009; Liu et al., 2014), to the best of our knowledge this is the first report of resistance to carnocyclin A in strains of *L. monocytogenes*. In the current study, all of the strains used were able to grow in the presence of carnocyclin A, although the growth rate and lag phase were impacted by carbohydrate.

Mannose, glucose and fructose reduced the growth rate for cells treated with leucocin A, with the exception of strain FSL R2-499. The increased sensitivity to leucocin A, as evidenced by the reduced growth rate, may relate to a requirement for use of the Man-PTS for carbohydrate uptake. This supports the hypothesis that growth of *L.*

monocytogenes in presence of a specific carbohydrate increases the sensitivity of *Listeria* to the bacteriocin if the sugar transport system is also used as a bacteriocin docking molecule. The Man-PTS system is the receptor for leucocin A, as well as other Class IIa bacteriocins (Ramnath et al., 2000; Dalet et al., 2001; Diep et al., 2007) and is also used for uptake of glucose and fructose (Erni, 1989; Kotrba et al., 2001). Cells using the Man-PTS for metabolism and growth may not down regulate the Man-PTS even when class IIa bacteriocins are present (Dalet et al., 2001). In the current study, FSL R2-499, which is the most resistant to leucocin A, became sensitive to leucocin A when grown with mannose as the sole carbohydrate. However, this phenomenon was not observed for all strains. The variation in growth rates among strains can be attributed to strain individuality and carbohydrate preference. *L. monocytogenes* has different carbohydrate transport systems for uptake of sugars under normal conditions (Naghmouchi et al., 2006). Tessema et al. (2011) reported similar results for two spontaneously resistant strains of *L. monocytogenes* to sakacin P grown in mannose. The strains had difference growth rates in mannose despite both being resistant to sakacin P. Interestingly, the strain with resistance to a high bacteriocin concentration repressed *mptA* expression, while the strain with resistance to an intermediate level of bacteriocin overexpressed *mptA* despite have a reduced growth rate in mannose. Under situations where cells need to survive, for example in the presence of a bacteriocin, the preferred sugar transport system may change in response to carbohydrate availability. This may indicate that mechanisms other than carbohydrate transport systems are responsible for the development of resistance.

Glucose is the preferred carbohydrate of *L. monocytogenes* (Premaratne et al., 1991); however, strains used in this study had a faster growth rate in the presence of cellobiose and leucocin A. Cellobiose is transported across the cell membrane by the β-glucoside-PTS (Gravesen et al., 2000) and the lactose-PTS (Dalet et al., 2003). *L. monocytogenes* that have lost enzyme II subunit A/B or subunit C of the Man-PTS are resistant to class IIa bacteriocins (Dalet et al., 2001; Gravesen et al., 2002; Ramnath et al., 2004) and up-regulate the β-glucoside-PTS (Gravesen et al., 2000, 2002). This explains the shorter lag phase and faster growth rate when cultures were grown in cellobiose and treated with bacteriocins. The most resistant strain, FSL R2-499, grew fastest in cellobiose and had

the shortest lag phase, indicating a preference for an alternative carbohydrate transport system.

Cultures treated with carnocyclin A had the slowest growth rates, indicating that carnocyclin A is more effective against *L. monocytogenes* than leucocin A. Carnocyclin A also increased the lag phase of *L. monocytogenes*, except in cultures grown in sucrose. In sucrose, carnocyclin A slowed the growth rate of strains and shortened the lag phase, compared to growth in other carbohydrates. There may be a fitness cost to *L. monocytogenes* associated with the use of metabolic pathways involved in the breakdown of disaccharides. The shorter lag phase indicates that although there may be an additional fitness cost to using sucrose as an energy source, the machinery to transport sucrose is probably not involved in bacteriocin docking.

The ability of *L. monocytogenes* to develop resistance to carnocyclin A was decreased when grown in the presence of glucose, fructose, or mannose as the lag phase was substantially extended; however, resistance developed and growth of cultures occurred within 72 h of incubation. The mode of action of carnocyclin A include formation of voltage-dependent anion selective channels; however, docking molecules that would support initial attachment of carnocyclin A to the cytoplasmic membrane have not been described (Gong et al., 2009). Gabrielsen et al., 2012 identified a maltose ABC transporter as a possible docking molecular for garvicin ML, a cyclic bacteriocin produced by *Lactococcus lactis*. Cells of *Lactococcus lactis* IL1403 that were grown in maltose containing media were 44 times more sensitive to garvicin ML than the same strain grown in glucose. The effect of carbohydrates on the development of resistance in *Listeria* was similar in case of leucocin A and carnocyclin A, which may argue in favour of the existence of a carbohydrate-specific docking molecular for carnocyclin A. However, strains of nisin-resistant *L. monocytogenes*, which are also resistant to Class IIa bacteriocins (Crandall and Montville, 1998), have a less hydrophobic cell membrane compared to sensitive strains (Ming and Daeschel, 1993) and a more positive cell surface due to D-alanylation of teichoic acid and lysinilation of phospholipids (Vadyvaloo et al., 2004a; Jacquet et al., 2012). This change in cell surface properties could also explain the formation of resistance to carnocyclin A. It is possible that poor interactions between

carnocyclin A, which is hydrophobic and cationic, and the cell membrane, decrease the formation of anion selective pores and increase resistance to the bacteriocin.

In conclusion, carbohydrate, bacteriocin type, and strain variation all impact the growth of *L. monocytogenes*. *L. monocytogenes* can develop resistance to leucocin A and carnocyclin A. Resistance of *L. monocytogenes* to carnocyclin A takes longer to form than resistance to leucocin A, but after extended lag phases all isolated subpopulations exhibit a stable-resistant phenotype to repeat exposure to the bacteriocins. Cellobiose and sucrose increased the ability of *L. monocytogenes* to form resistance compared to other carbohydrates, which could pose a food safety risk for industry, depending on the composition of the bacteriocin-containing product. It is important to note that not all strains behaved the same and therefore innate metabolic patterns and resistance may influence how *L. monocytogenes* reacts to the application of bacteriocins. Further research is needed to identify key mechanisms in *L. monocytogenes* that are involved in the development of resistance and if there is a general response to bacteriocins regardless of class or mode of action. The result of this study should cause concern to the food industry with regard to bacteriocin application regardless of bacteriocin class.

3.4 References

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Chapter 4

4.0 Introduction

Listeria monocytogenes is a foodborne pathogen that affects an estimated 1,600 individuals each year in the USA; primarily immuno-compromised, elderly, and pregnant individuals (CDC, 2016). Although, *L. monocytogenes* does not have the highest case frequency rate amongst foodborne pathogens, the risk of infection carries severe implications with a fatality rate of 20–40 % (Drevets and Bronze, 2008; Farber and Peterkin, 1991; Gálvez et al., 2008; Thomas et al., 2013). *L. monocytogenes* is associated with post-processing contamination of ready-to-eat (RTE) products and can grow a wide range of temperatures, including refrigeration temperatures, as such strict guidelines have been developed to control *L. monocytogenes* in RTE products (Flint et al., 2005; Government of Canada, Health Canada, 2011; Zhu et al., 2005). However, there is increased pressure from consumers on the food industry to produce more fresh, minimally-processed (natural), RTE foods without chemical preservatives. Novel methods of bio-preservation, such as bacteriocins, are an alternative to traditional chemical preservatives, that align with consumer demands (Gálvez et al., 2010).

Bacteriocins are ribosomally synthesized antimicrobial peptides that can be used to inhibit the growth of foodborne pathogens and are an attractive alternative to traditional preservatives to improve food safety (Perez et al., 2014). Carnocyclin A, a 60-residue circular bacteriocin, produced by *Carnobacterium maltaromaticum* ATCC PTA-5313, is an antilisterial bacteriocins (Martin-Visscher et al., 2008; van Belkum et al., 2011). Carnocyclin A forms anion selective channels in the lipid membrane. Therefore, carnocyclin A does not require a docking molecule, such as class IIa bacteriocins to form a pore on target cells; however, more research is needed to fully understand if ion selective pores are the only mode of action for carnocyclin A (Drider et al., 2006; Gong et al., 2009). *L. monocytogenes*, can develop resistance to bacteriocins, which is attributed to the down-regulation of genes associated with the mannose phosphotransferase system (Man-PTS), a common docking station for class IIa bacteriocins, and changes in cell membrane properties (Ramnath et al., 2004; Vadyvaloo

et al., 2004a, 2004b, 2002). Our previous study suggested that the carbohydrate source may play a role in the resistance formation of *L. monocytogenes* to carnocyclin A (Balay et al., 2018). This poses as a significant obstacle to implement the use of bacteriocins in food systems to control the growth of *L. monocytogenes*.

To better understand the survival ability and resistance formation of *L. monocytogenes* to circular bacteriocins, the global transcriptome was analyzed with RNA-Seq to identify how *L. monocytogenes* gene expression levels change in regards to carbohydrates and bacteriocin stress. In addition to the analysis of coding RNAs (mRNAs) related to resistance formation, the expression of non-coding RNAs (ncRNAs) under such stressors was also investigated to explore their roles in stress regulation. There is evidence that ncRNA is associated with genetic regulation in response to stress in *L. monocytogenes* (Cerutti et al., 2017; Mandin et al., 2007; Mellin and Cossart, 2012; Mollerup et al., 2016; Mraheil et al., 2011a; Oliver et al., 2009; Toledo-Arana et al., 2009; Vivant et al., 2017). It would be beneficial to understand if any ncRNAs play a role in regulation of the stress response in regards to resistance formation to bacteriocins and if carbohydrate plays a role in how resistance is formed. The specific objectives of this research were to assess the transcriptome profiles and if there was any ncRNA present and/or differentially expressed for *L. monocytogenes,* foodborne outbreak isolate FSL C1-056, when grown in mannose or cellobiose and treated with carnocyclin A.

4.1 Materials & Methods

4.1.1 Bacterial Strains and Culture Conditions

An outbreak strain of *Listeria monocytogenes* FSL C1-056 (lineage II, serotype 1/2a) used for challenge studies was obtained from Cornell University (Fugett et al., 2006). *Carnobacterium maltaromaticum* ATCC-PTA 5313, carnocyclin A producer, was obtained from the University of Alberta Lactic Acid Bacteria culture collection. All strains were stored in 30% glycerol at -80 °C until needed. Prior to use in experiments, cultures were streaked onto All Purpose Tween 1.5% agar (APT; Becton, Dickinson and

Company, New Jersey, USA), and a single colony was inoculated into broth media. Both *L. monocytogenes* and C. *maltaromaticum* ATCC-PTA 5313 was grown in APT broth at 32 °C and 25 °C, respectively.

4.1.2 Production and Purification of Carnocyclin A

Carnocyclin A was purified using the protocol outlined in (Balay et al., 2018) in sufficient quantities for use in experiments. A total of 50.8 μg was used in this experiment.

4.1.3 *L. monocytogenes* FSL C1-056 Genome Sequencing

Genomic DNA was purified using Wizard® Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA). To obtain a high concentration of gDNA 3 ml of *L. monocytogenes* FSL C1-056 was grown overnight at 32 °C to ~10^8 CFU/mL. Subsequent steps followed the protocol for 'Isolating Genomic DNA from Gram Positive and Gram Negative Bacteria', (Promega) with one modification. Nuclease free water (50 μL) was used to rehydrate gDNA instead of 100 μL of DNA Rehydration Solution. Quality and the quantity of gDNA was checked on a NanoDrop® ND 1000 Spectrophotometer to ensure A_{260}/A_{280} >1.75 (Thermo Fisher Scientific, Wilmington, DE, USA) and gDNA was ran on a 1% agarose gel to confirm high molecular weight fragments. The library was constructed using the TruSeq DNA PCR-Free Library Preparation Kit (Illumina®, San Diego, CA, USA). Shotgun, paired-end sequencing (2 × 125 bp) was carried out at McGill University Genome Quebec Innovation Centre on an Illumina HiSeq2500 platform.

4.1.4 *L. monocytogenes* FSL C1-056 Grown in the Presence of Different Carbohydrates With or Without the Presence of Carnocyclin A

Prior to experimentation *L. monocytogenes* FSL C1-056 was grown as outlined above (bacterial strains and culture conditions) and sub-cultured (1% v/v inoculum) into a tube containing 5 ml of basal media with or without the addition of (3.3 mM) carnocyclin A. Basal media was prepared and supplemented with the carbohydrates mannose or cellobiose. For each litre of basal media the following formulation was used: 10 g Proteose Peptone No. 3 (Becton, Dickinson and Company), 10 g Beef Extract (Becton, Dickinson and Company), 5 g Yeast Extract (Becton, Dickinson and Company), 1 g Tween® 80, 2 g disodium phosphate, 1 mL sterile basal media salt solution [10 g magnesium sulfate, 5 g manganese sulfate dissolved in 100 mL dH_2O], and for each litre of basal media, 20 g of either mannose or cellobiose was added. Carnocyclin A was added to each basal media supplemented with a different carbohydrate to achieve a final concentration of 3.3mM when required. All strains and treatments grown at 32 °C and total RNA harvested at OD_{630nm} 0.30 (~10^7 CFU/mL). Experiments were independently replicated three times.

4.1.5 Total RNA Isolation and Library Preparation and Sequencing

L. monocytogenes FSL C1-056 was grown at 32 °C in basal media supplemented with mannose or cellobiose in the presence of 3.3 mM carnocyclin A and collected at OD_{630nm} 0.30. Total RNA was extracted from 3 biological replicates and a total of 12 cDNA libraries (treated and untreated) were constructed and sequenced. Cultures were treated with 2 volumes of RNAprotect® Bacteria Reagent (Qiagen Inc., ON, Canada), to 1 volume of bacterial culture and vortexed on high (speed 8; Vortex Genie 2™, Fisherbrand®, Bohemia, NY, USA) for 5 s before incubation at 22 °C for 5 min. Cells were harvested by centrifugation for 10 min at 8,000 x *g*, supernatant was decanted and cell pellet was stored at -80 °C for later downstream processing. Cell pellets were thawed and resuspended in 1 mL of PBS buffer and transferred to a lysing tube for microorganisms containing 2 mL of 0.1 mm glass beads (Precellys, Bertin Technologies,

Inc., Montigny-le-Bretonneux, France). Tubes were centrifuged at 10,000 x *g* for 5 min at 4 °C, the PBS was removed without disturbing the beads, and the tubes were placed on ice. Total RNA was isolated using the *mir*Vana™ miRNA Isolation Kit with Phenol (Ambion®, Life Technologies, Inc.™, Carlsbad, California, USA). Briefly, Lysis/Binding Buffer (600 µL) was added to the lysing tubes on ice, each tube was vortexed twice on high for 30 s, 60 µL of Homogenate Additive was then added to each tube and vortexed on high for 10 s. Samples were incubated on ice for 10 min, and 600 µL Acid-phenol:Chloroform was added to the tubes. Each sample was vortexed three times on high for 10 s, and centrifuged twice at 10,000 x *g* for 5 min, at 22 °C. The aqueous phase was transferred to a fresh tube and manufacturer's directions were followed for the remainder of the isolation. The quality of RNA was assessed on a spectrophotometer (NanoDrop® ND 1000 Spectrophotometer, Thermo Fisher Scientific, Edmonton, Alberta, Canada), a 2200 TapeStation (Agilent Technologies, Inc., Santa Clara, CA, USA) with Agilent Technologies, Inc. RNA Screen Tape (Agilent Technologies, Inc.), and quantity of RNA was assessed using a fluorometer (Qubit® 3.0 Fluorometer, Life Technologies, Inc.™, Invitrogen™, Eugene, Oregon, USA) with Qubit® RNA BR Assay Kit (Life Technologies, Inc.™). RNA sequencing library was constructed with the TruSeq™ RNA Library Preparation Kit v2 Set A (Illumina®), following the low sample protocol from the manufacturer without 'Purify and Fragment mRNA' step. Libraries were validated with the TapeStation (Agilent Technologies, Inc.) with Agilent Technologies, Inc. D1000 Screen Tape (Agilent Technologies, Inc.) and quantified with a fluorometer (Life Technologies, Inc.™) with a Qubit® dsDNA HS Assay Kit (Life Technologies, Inc.™). The libraries were then sequenced at McGill University and Genome Quebec Innovation Centre (Quebec, Canada). Samples were sequenced on two separate platforms; biological replicate 1 was sequenced on a HiSeq2500 (125 bp, PE) platform and biological replicate 2 & 3 were sequenced on HiSeq4000 platform (100 bp, PE).

4.1.6 Data Analysis

Quality control for each sequenced dataset was conducted using Trimmomatic (version 0.36) (Bolger et al., 2014) to trim adapters and low-quality bases (quality scores < 20), and to remove short reads with the length < 75 bp. Filtered genomic DNA reads were de novo assembled to obtain contigs using SPAdes (version 3.11.1) with the multiple k-mer sizes of 21, 33, 55, 77, 99 (Simpson et al., 2009). RAST (Rapid Annotation using Subsystem Technologies, Inc.) (Aziz et al., 2008) and RNAspace (version v1.2.1) (Mariette et al., 2011) were applied to predict/annotate genes and non-coding RNAs for *L. monocytogenes* FSL C1-056, respectively. Because the rRNA depletion step was omitted during the transcriptome library construction, post-QC RNA reads were first aligned to the 16S, 23S, and 5S rRNA genes of *L. monocytogenes* FSL C1-056 using Bowtie2 (Langmead and Salzberg, 2013) to remove these rRNA reads. To estimate the gene expression profiles of each transcriptome dataset, the remaining non-rRNA reads were mapped allowing a 1 bp mismatch in the seed alignment for every 100bp to the genome and predicted ncRNAs using Bowtie2, and then were counted using BBMap built-in pipeline *pileup.sh* (Bushnell). Genes were considered expressed if the normalized reads were >1 in 2/3 replicates. Differential expressed genes were identified using DESeq2 (Love et al., 2014). Briefly, original count of RNA reads mapped to each gene/ncRNA were normalized using the median of ratios method implemented within DESeq2, and pairwise comparisons between treatments were performed. P-values were adjusted into Benjamani–Hochberg false discovery rate (FDR) (Benjamini and Hochberg, 1995), and an FDR value < 0.05 and < 0.1 was applied as the threshold to define differential expressed genes and non-coding RNAs, respectively, between two treatment groups.

4.1.7 Accession Numbers

The whole genome shotgun project for *L. monocytogenes* FSL C1-056 has been deposited at DDBJ/ENA/GenBank under the accession number SJSW00000000. The version described in this paper is version SJSW01000000 (Balay and McMullen, 2019a). The

sequence data for the BioProject- *Listeria monocytogenes* FSL C1-056 RNA Seq, has been deposited to the sequence read archive (SRA) under the accession number PRJNA541339 (Balay and McMullen, 2019b).

4.2 Results

Cultures used in experiments were grown to an OD_{630nm} of 0.30 regardless of the addition of carnocyclin A.

4.2.1 Genome of *L. monocytogenes* FSL C1-056

The genome of *L. monocytogenes* FSL C1-056 was sequenced to provide a reference genome that mRNA profiles could be mapped back to as well as used for the prediction of potential ncRNAs. The raw sequences resulted in a total of 3,048,786,500 bases and 12,195,146 reads, with an average Phred Quality Score of 35. Complete number of sequences and breakdown for each treatment replicate for total RNA, non-rRNA and ncRNA can be found in Appendix B, Table S4.1. The de novo assembly yielded a draft genome with 12 contigs, and after filtering all contigs < 500 bp, 9 contigs were kept for downstream alignment and annotation (N50 = 1,495,380 bp). The total length of draft genome was 2,874,730 bp. The genome was annotated by Rapid Annotation using Subsystem Technology (RAST) and resulted in 2,848 coding sequences (Appendix B, Table S4.2) and 366 predicted ncRNA coding regions.

4.2.2 Mapping Reads From Transcriptomic Libraries for mRNA and ncRNA

To determine the impact of carbohydrates on gene expression in *L. monocytogenes* FSL C1-056 exposed to carnocyclin A, reads obtained from RNA-Seq were mapped to annotated genes and predicted ncRNA regions. For each individual library, a total of 50.7 to 93.0 million reads were obtained, after quality control a total of 46.2 to 80.9 million reads remained. On average the proportion of rRNA for each individual library was

83.4%, and 51% of the remaining non rRNA reads were mapped to the genome of *L. monocytogenes* FSL C1-056 (Table 4.1). The original and normalized number of reads mapped to the annotated *L. monocytogenes* FSL C1-056 genes for each library can be found in Tables S4.3 and S4.4, respectively. The total number of expressed genes, defined as genes with the counts per million (CPM) > 1 in 2 out of 3 replicates, ranged from 2,797 to 2,827, with all 4 experimental groups sharing 2,779 expressed genes (Fig. 4.1). RNAspace predicted a total of 366 ncRNA coding regions based on *L. monocytogenes* FSL C1-056 sequenced genome (Appendix B, Table S4.5; original and normalized transcript reads matched to predicted ncRNA regions Appendix B, Table S4.6, S4.7). In the current study, only ncRNA with CPM > 1 in 2 out of 3 replicates were considered as expressed. According to this cut-off, a total of 121 to 132 predicted ncRNA regions were expressed for all treatment groups, (Table 4.2), and the experimental groups shared 113 expressed ncRNA (Fig. 4.2).

Table 4.1 The number of sequences obtained from early stationary phase *L. monocytogenes* FSL C1-056 grown in different carbohydrates and treated with or without 3.3 mM CclA for mRNA.

Sample[b]	**Original reads (n)**	**Reads after QC**[c] **(n)**	**rRNA (16S + 23S + 5S; %)**	**non-rRNA (n)**	**non-rRNA mapped to genome (n)**
Mannose -CclA	71,170,667 ±2,497.84	62,801,107 ±2,131.59	80.13 ±7.19	14,928,325 ±259.44	8,457,523 ±270.51
Mannose +CclA	70,612,492 ±1,939.27	58,517,273 ±1,634.64	85.74 ±11.56	10,625,340 ±,285.44	5,010,590 ±1,246.96
Cellobiose -CclA	76,909,034 ±3,220.92	67,886,209 ±2,785.56	82.18 ±8.24	14,318,799 ±343.91	8,027,863 ±339.54
Cellobiose +CclA	81,720,771 ±1,476.93	67,946,615 ±891.21	86.31 ±8.96	13,005,183 ±1,501.72	6,381,040 ±2,267.49

[a]carnocyclin A; [b]average of 3 biological replicates for each treatment reported with SEM, [c]quality check

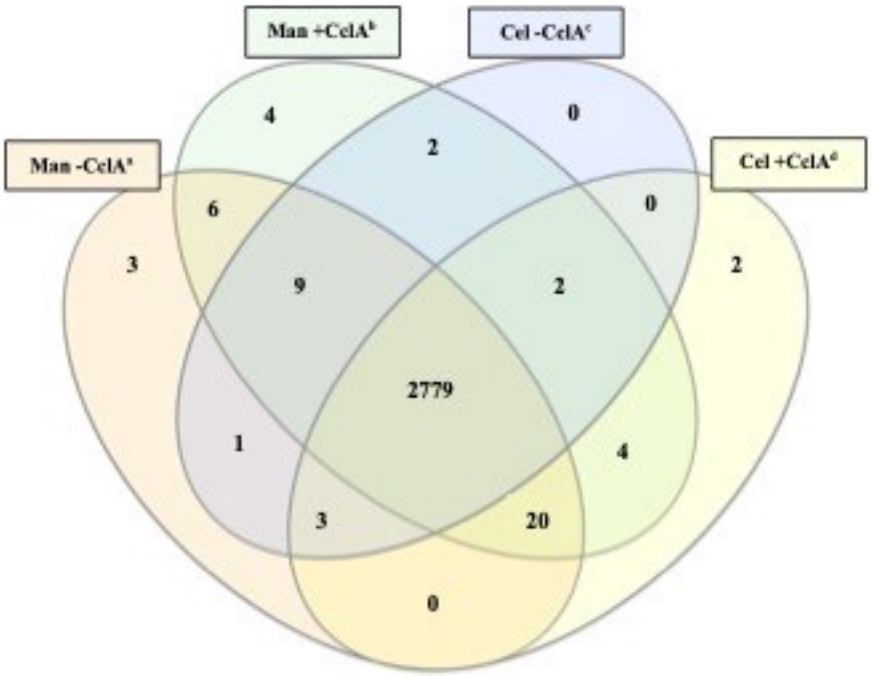

Figure 4.1 Venn diagram of overlapping predicted transcripts from *L. monocytogenes* FSL C1-056 genome grown in [a]mannose without carnocyclin A, [b]mannose with carnocyclin A, [c]cellobiose without carnocyclin A, [d]cellobiose with carnocyclin A at 32°C.

Table 4.2 The number of sequences obtained from early stationary phase *L. monocytogenes* FSL C1-056 grown in different carbohydrates and treated with or without 3.3 mM CclA for ncRNA.

Sample[b]	**Predicted ncRNA transcripts genome (n)[c]**	**Transcripts matched predicted ncRNA (n)[d]**	**Matched ncRNA reads mapped to transcripts (n)**
Mannose -CclA	366	129	743,916 ± 403.92
Mannose +CclA	366	121	670,836 ± 445.44
Cellobiose -CclA	366	127	671,744 ± 409.76
Cellobiose +CclA	366	132	693,867 ± 314.00

[a]carnocyclin A; [b]average of 3 biological replicates for each treatment reported with SEM; [c]predicted ncRNA transcripts from *L. monocytogenes* FSL C1-056 genome; [d]matched normalized transcripts from total sequenced RNA to predicted ncRNA transcripts of *L. monocytogenes* FSL C1-056 genome (CPM > 1 in 2/3)

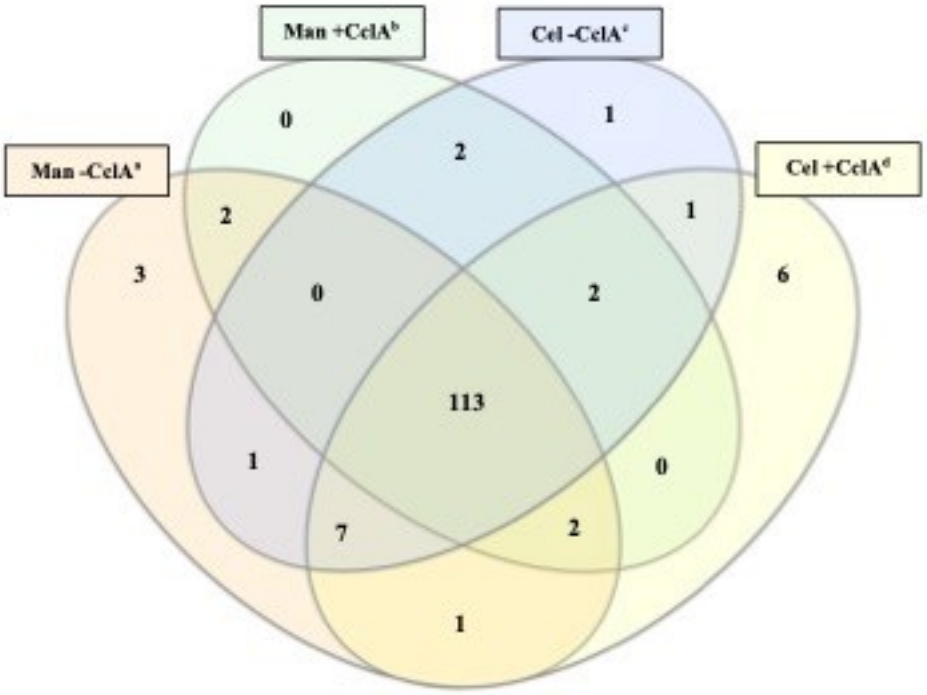

Figure 4.2 Venn diagram of overlapping matched normalized ncRNA transcripts to predicted transcripts from *L. monocytogenes* FSL C1-056 genome grown in [a]mannose without carnocyclin A, [b]mannose with carnocyclin A, [c]cellobiose without carnocyclin A, [d]cellobiose with carnocyclin A at 32 °C.

4.2.3 Differentially expressed genes for mRNA

Expression of genes with a $\log_2$ fold change > 1.5 or < -1.5 (FDR adjusted $P < 0.05$) changes were defined as differentially expressed and they were considered of significance to the cells stress response. *L. monocytogenes* FSL C1-056 grown in mannose without addition of carnocyclin A compared with the addition of carnocyclin A had a total of 29 genes that were differentially expressed (Fig. 4.3A). The functions of the majority of these genes were related to PTS systems (mannose, galactitol), metabolism, transcriptional regulation, cell membrane synthesis, and some virulence related factors. More genes (43) were differentially expressed when grown in cellobiose without carnocyclin A compared to cells with the addition of carnocyclin A (Fig. 4.3B). The functions of these genes were related to PTS systems (mannose, cellobiose, beta-

glucoside, fructose), metabolism, transcriptional regulation, cell membrane synthesis, and some virulence associated factors; however, there were many hypothetical genes.

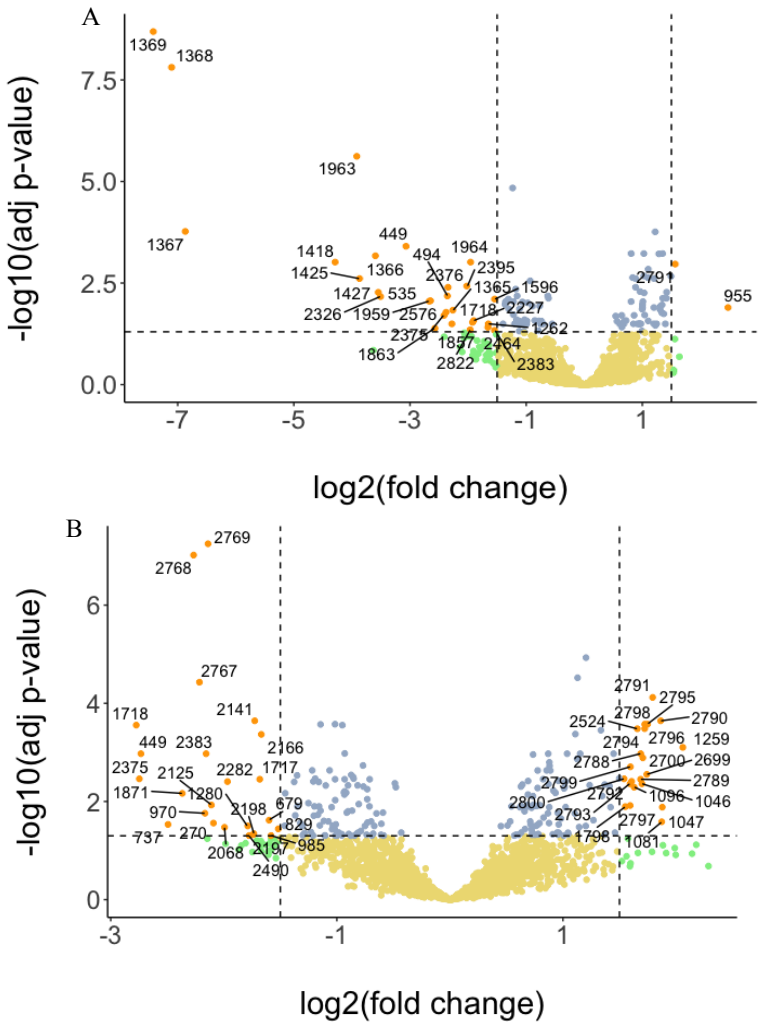

Figure 4.3 Volcano plots for global comparisons of the transcription profiles of *L. monocytogenes* FSL C1-056 grown in mannose with or without the addition of carnocyclin A (A), *L. monocytogenes* FSL C1-056 grown in cellobiose with or without the addition of carnocyclin A (B). Intercepts for x axis indicates threshold values for $\log_2$ fold change < -1.5 or > 1.5. Intercept for y axis indicates a threshold value of FDR < 0.05. Labelled orange spots are down-regulated or up-regulated with a threshold of $\log_2$ fold change < -1.5 or > 1.5 and a FDR < 0.05. Green spots are down-regulated or up-regulated with a threshold of $\log_2$ fold change < -1.5 or > 1.5 and a FDR > 0.05. Blue spots are down-regulated or up-regulated with a threshold of $\log_2$ fold change > -1.5 or < 1.5 and a FDR < 0.05. Yellow spots are down-regulated or up-regulated with a threshold of $\log_2$ fold change > -1.5 or < 1.5 and a FDR > 0.05. Gene ID's correspond to Appendix B, Table S4.2.

When *L. monocytogenes* FSL C1-056 was grown in mannose with carnocyclin A compared to cells without carnocyclin A, expression of genes encoding the enzymes of the Man-PTS (EIIA, EIIB, EIIC) were down-regulated from 6.87 to 7.42- $\log_2$ fold, while that of galactical-specific enzyme IIA was also down-regulated 3.92- $\log_2$ fold (Fig. 4.4A). In cellobiose, expression of genes encoding enzymes relating to the Man-PTS (EIIA, EIIB, EIIC, EIID) were also down-regulated (2.14 to 2.27- $\log_2$ fold) when *L. monocytogenes* was treated with carnocyclin A, while that of genes encoding beta-glucoside PTS enzymes (EIIA/B/C) and cellobiose-specific PTS membrane enzyme EIIC were up-regulated by 1.74 and 1.66- $\log_2$ fold, respectively (Fig. 4.4B). The expression of genes encoding virulence-related proteins N-acetylmuramoyl-L-alanine amidase was down-regulated 2.65- $\log_2$ fold when *L. monocytogenes* was grown in mannose compared to the upregulation of 1.54- $\log_2$ fold of another gene encoding the same protein in *L. monocytogenes* when grown in cellobiose treated with carnocyclin A (Fig. 4.4A, 4.4B). Cultures grown in mannose and carnocyclin A down-regulated the expression of two more virulence related genes encoding a zinc ABC transporter and an internalin-like protein (LPXTG motif) 1.96- $\log_2$ fold and 1.65- $\log_2$ fold, respectively (Fig. 4.4A). In cellobiose, the expression of another virulence gene encoding an ATP-dependent Clp protease was down-regulated 2.49- $\log_2$ fold. Interestingly though the expression for *lmaB* was up-regulated by 1.69- $\log_2$ fold, which encodes for *L. monocytogenes* antigen B (Fig. 4.4B). In addition, the expression of metabolism and quorum sensing genes such as, *cheV*, were up-regulated in *L. monocytogenes* when grown in cellobiose and treated with carnocyclin A.

A total of 27 genes were differentially expressed in cells grown in different carbohydrates (mannose versus cellobiose; Fig. 4.5A). Most of these genes had functions related to PTS systems, specifically for cellobiose and beta-glucosides, metabolism, transcriptional regulation and cell membrane synthesis. A comparison between *L. monocytogenes* FSL C1-056 grown in mannose treated with carnocyclin A to cells grown in cellobiose treated with carnocyclin A (Fig. 4.5B) resulted in 22 differentially expressed genes that were similar to those listed above. When the groups grown in different carbohydrates with or without carnocyclin A were compared, there were 4 differentially expressed genes that overlapped (Fig. 4.6). In contrast, there were 14 differentially expressed genes that

overlapped in the controls without carnocyclin A compared to the controls with carnocyclin A.

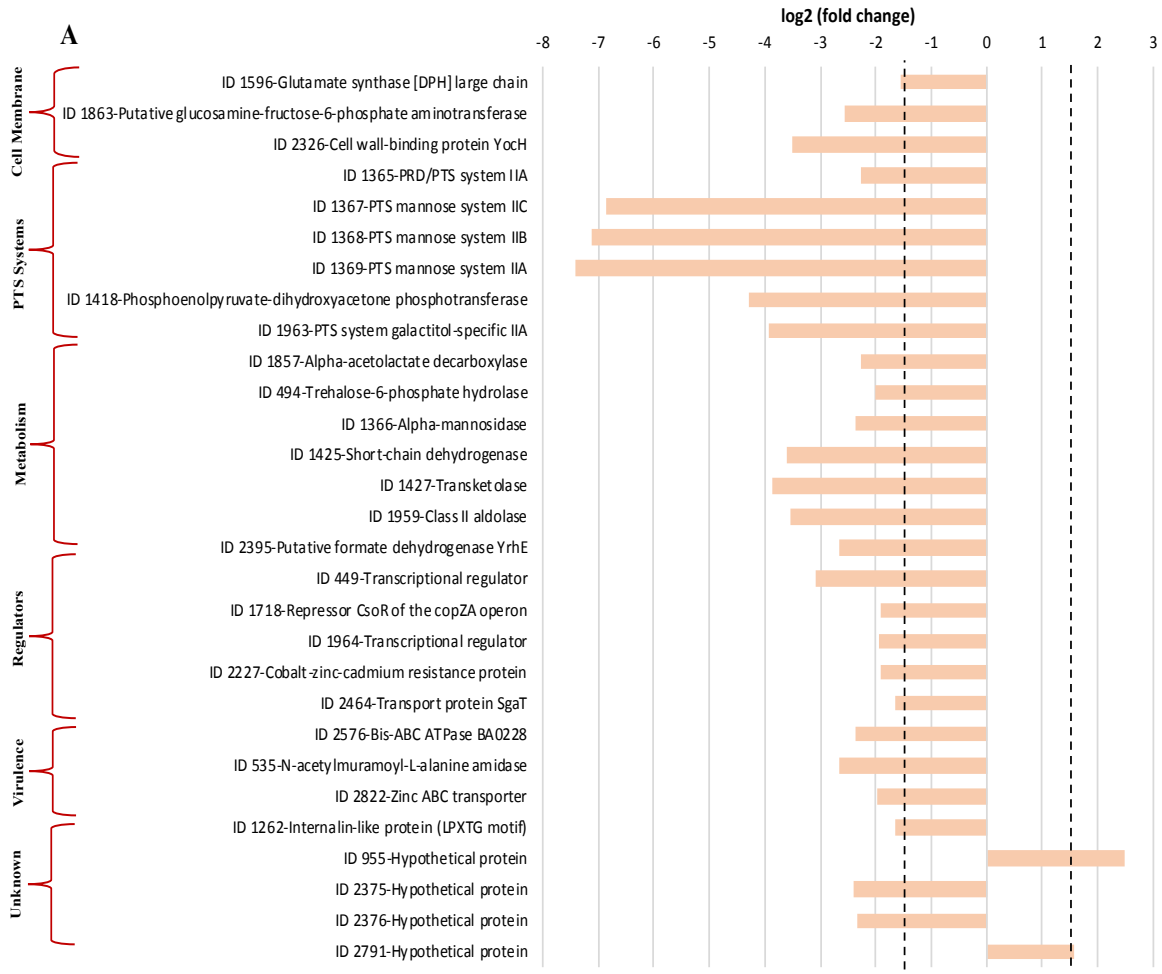

Figure 4.4A Differentially expressed gene transcripts (FDR < 0.05 and $\log_2$ fold change) < 1.5 or > 1.5) for *L. monocytogenes* FSL C1-056 grown in mannose with or without the addition of carnocyclin A (A). Gene ID's correspond to Appendix B, Table S4.2.

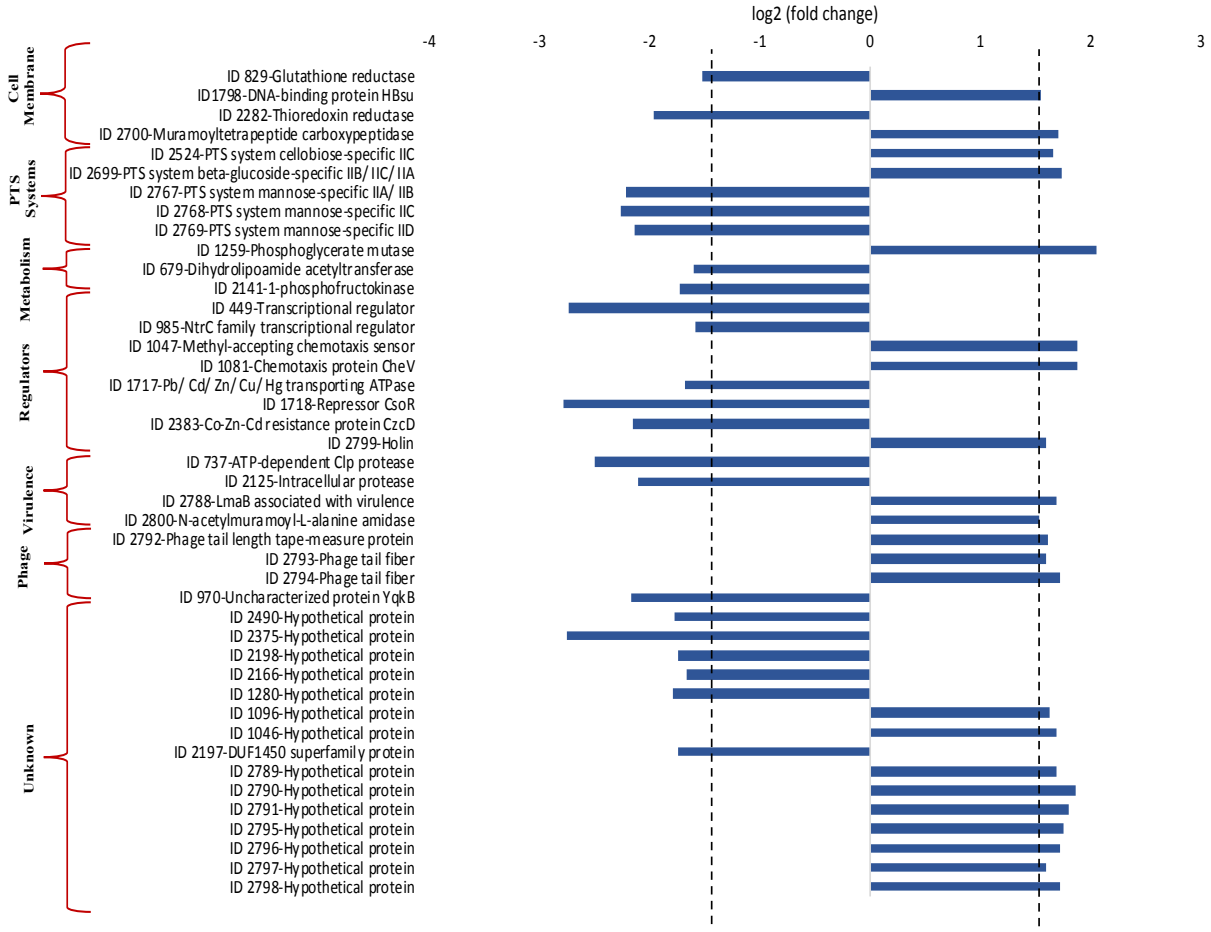

Figure 4.4B Differentially expressed gene transcripts (FDR < 0.05 and $\log_2$ fold change) < 1.5 or > 1.5) for *L. monocytogenes* FSL C1-056 grown in cellobiose with or without the addition of carnocyclin A (B). Gene ID's correspond to Appendix B, Table S4.2.

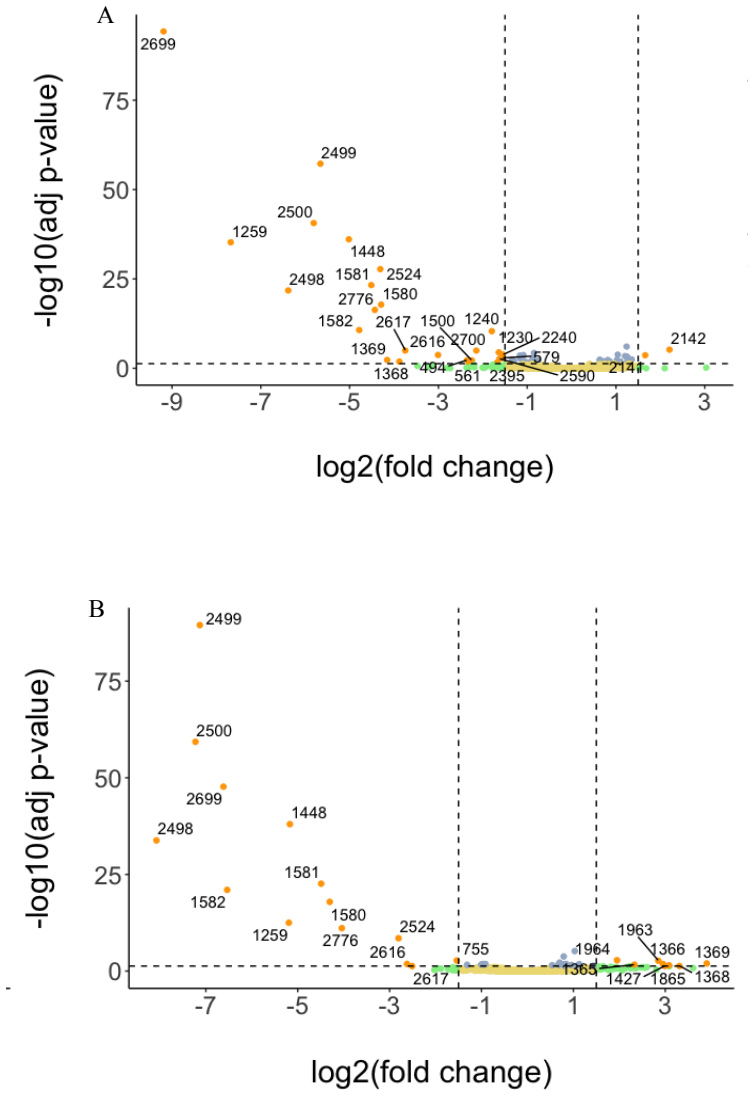

Figure 4.5 Volcano plots for global comparisons of the transcription profiles of *L. monocytogenes* FSL C1-056 grown in mannose or cellobiose without carnocyclin A (A) or *L. monocytogenes* FSL C1-056 grown in mannose or cellobiose with carnocyclin A (B). Intercepts for x axis indicates threshold values for $\log_2$ fold change < -1.5 or > 1.5. Intercept for y axis indicates a threshold value of FDR < 0.05. Labelled orange spots are down-regulated or upregulated with a threshold of $\log_2$ fold change < -1.5 or > 1.5 and a FDR < 0.05. Green spots are down-regulated or up-regulated with a threshold of $\log_2$ fold change < -1.5 or > 1.5 and a FDR > 0.05. Blue spots are down-regulated or up-regulated with a threshold of $\log_2$ fold change > -1.5 or < 1.5 and a FDR < 0.05. Yellow spots are down-regulated or up-regulated with a threshold of $\log_2$ fold change > -1.5 or < 1.5 and a FDR > 0.05. Gene ID's correspond to Appendix B, Table S4.2.

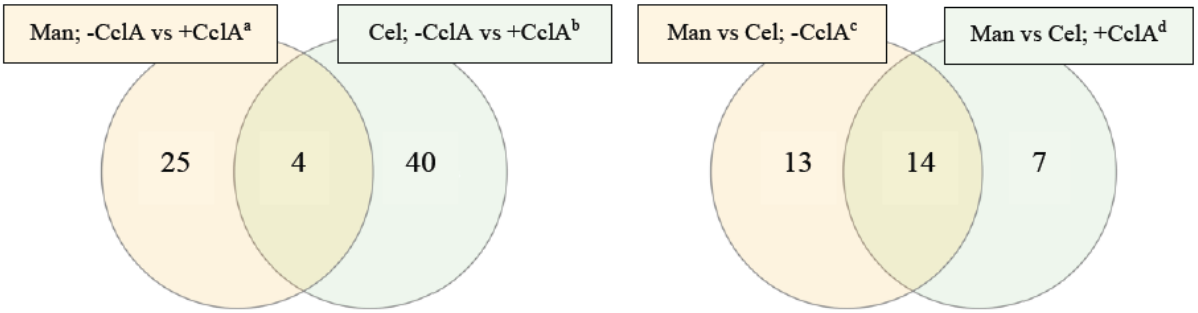

Figure 4.6 The first set displays *L. monocytogenes* FSL C1-056 grown in [a] mannose with or without carnocyclin A compared to being grown in [b] cellobiose with or without carnocyclin A. The second set displays *L. monocytogenes* FSL C1-056 grown in [c] mannose or cellobiose without carnocyclin A compared being grown in [d] mannose or cellobiose with carnocyclin A. Venn diagrams of overlapping differentially expressed gene transcript sets with a FDR < 0.05 and a threshold of log2 fold change < -1.5 or > 1.5.

Differentially expressed genes identified when *L. monocytogenes* FSL C1-056 was grown in either mannose or cellobiose, encoded enzymes involved in phosphoenolpyruvate-dependent sugar PTS systems (beta-glucoside, cellobiose, mannose) (Fig. 4.4C). All PTS systems were down-regulated 3.88 to 9.19-$\log_2$ fold; however the transcriptional repressor of the fructose operon was up-regulated 2.21-$\log_2$ fold. Expression of genes related to enzymes involved in glycolysis were down-regulated. Similarly, differentially expressed genes between *L. monocytogenes* FSL C1-056 grown in mannose or cellobiose and treated with carnocyclin A, had down-regulated expression of genes related to PTS (beta-glucoside, cellobiose) ranged from 2.81 to 8.08-$\log_2$ fold (Fig. 4.4D), while to expression of genes for Man-PTS and galactical-specific enzyme IIA were up-regulated (2.33 to 3.90-$\log_2$ fold) and 2.85-$\log_2$ fold, respectively.

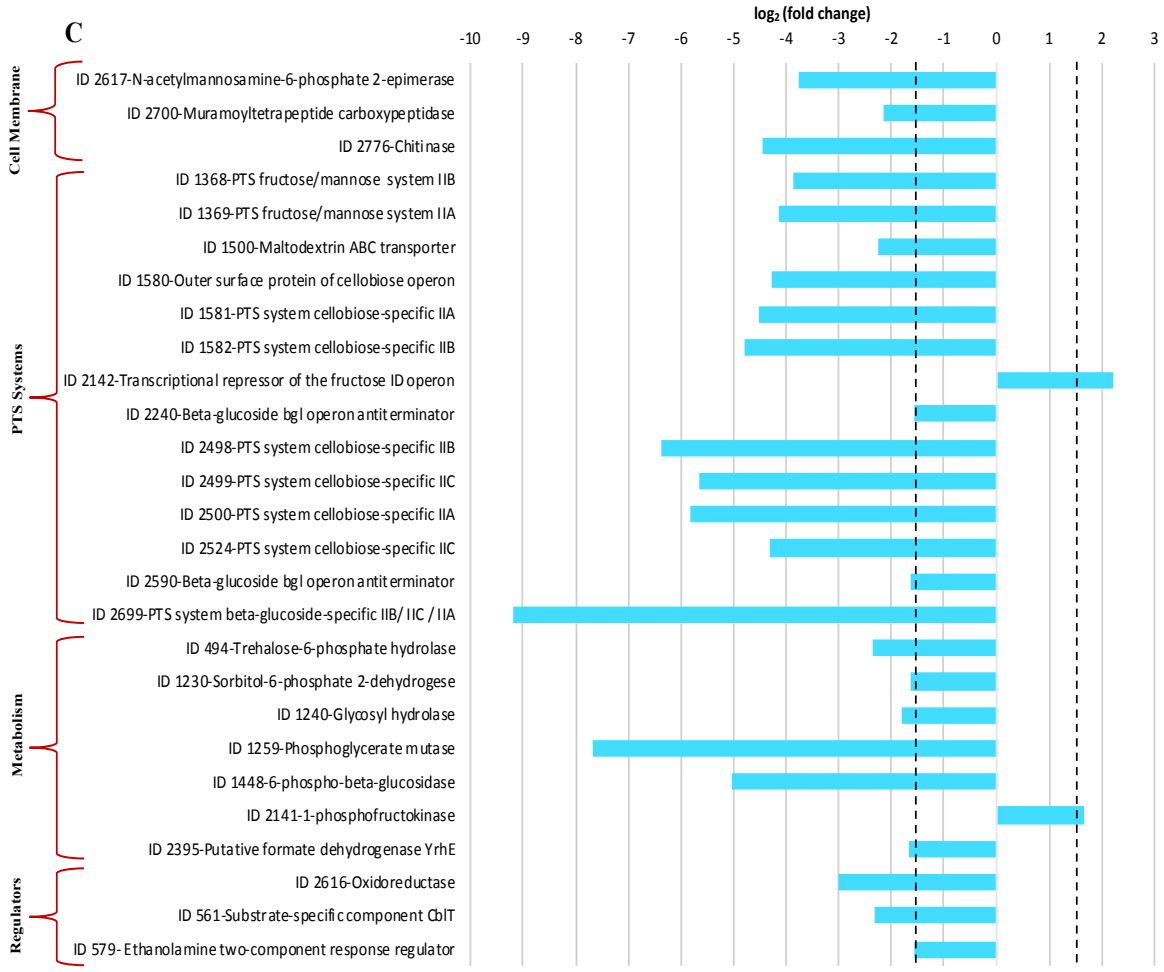

Figure 4.4C Differentially expressed gene transcripts (FDR < 0.05 and $\log_2$ fold change) < 1.5 or > 1.5) for *L. monocytogenes* FSL C1-056 grown in mannose or cellobiose (C). Gene ID's correspond to Appendix B, Table S4.2.

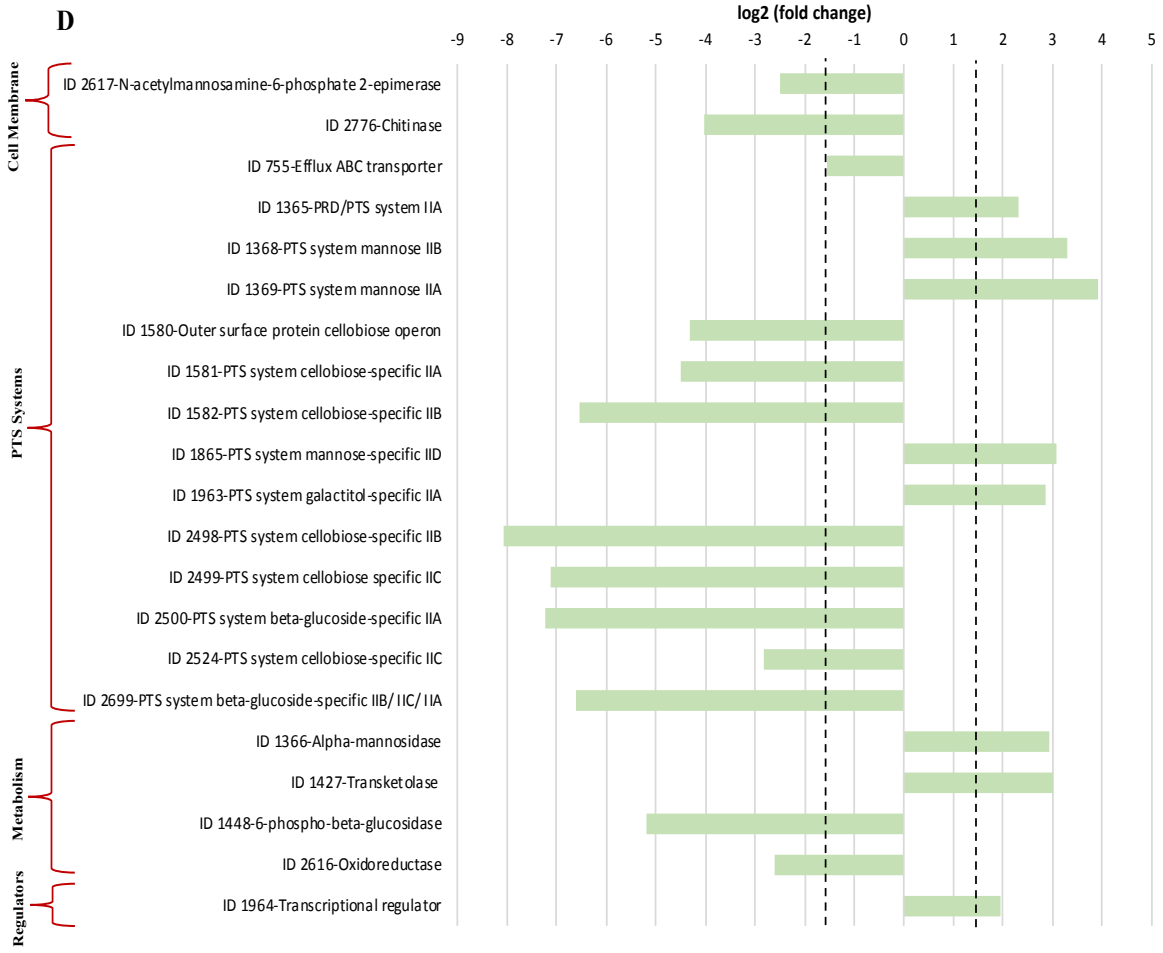

Figure 4.4D Differentially expressed gene transcripts (FDR < 0.05 and $\log_2$ fold change) < 1.5 or > 1.5) for *L. monocytogenes* FSL C1-056 grown in mannose or cellobiose with the addition of carnocyclin A (D). Gene ID's correspond to Appendix B, Table S4.2.

4.2.4 Analysis of ncRNA Transcriptome

The role ncRNAs play in regulating *L. monocytogenes* stress response to carbohydrate availability and bacteriocin exposure was investigated. Differentially expressed ncRNAs were defined as FDR adjusted P < 0.1 and if the $\log_2$ fold change of > 1.5 or < -1.5 the ncRNA was considered to be significant (Appendix B, Table S4.8). There were no

differentially expressed ncRNA when comparing *L. monocytogenes* FSL C1-056 grown in mannose or cellobiose or when grown in either carbohydrate with the addition of carnocyclin A. When comparing the transcriptome of *L. monocytogenes* FSL C1-056 grown in mannose to that grown in mannose with carnocyclin A, there were 7 ncRNA that were differentially expressed. A T-box riboswitch was up-regulated 0.89-$\log_2$ fold while Rli47 was down-regulated 3.54- $\log_2$ fold (Table 4.3). When *L. monocytogenes* FSL C1-056 was grown in cellobiose compared to growth in cellobiose with the addition of carnocyclin A, 5 ncRNA regions had significant differential expression. Three T-box riboswitches and Rli24 did not have $\log_2$ fold change > 1.5; however, Rli53 was down-regulated 2.80- $\log_2$ fold (Table 4.4).

Table 4.3 Matched ncRNA transcripts to *L. monocytogenes* FSL C1-056 genome when grown in mannose compared to mannose with the addition of carnocyclin A.

ncRNA		Strand	Start (bp)	End (bp)	Log2 Fold Change	P-value	FDR
64	unknown	+	1071581	1071680	1.81	0.39	0.98
67	unknown	+	1081344	1081435	2.89	0.22	0.98
103	unknown	+	1465572	1465667	2.33	0.36	0.98
132	unknown	+	1762556	1762627	-1.68	0.70	0.98
140	**unknown**	+	1890814	1890894	-3.32	**0.02**	**0.98**
140	unknown	-	1890814	1890894	-4.76	0.14	0.98
172	unknown	+	2310700	2310769	-1.68	0.70	0.98
174	unknown	+	2335685	2335757	-3.10	0.47	0.98
175	unknown	+	2340181	2340278	1.77	0.25	0.98
183	unknown	+	2471712	2471789	-1.50	0.52	0.98
224	Lysine	+	2183234	2183406	-1.88	0.22	0.98
335	rli49	+	1529442	1529542	2.18	0.19	0.98
489	unknown	-	516497	516629	2.78	0.35	0.98
492	unknown	-	143741	143824	-2.58	0.52	0.98
498	unknown	+	2627275	2627433	-2.70	0.53	0.98
499	unknown	+	1348201	1348347	1.67	0.20	0.98
505	unknown	+	734940	735047	2.06	0.18	0.98
505	unknown	-	734940	735047	1.89	0.07	0.98
506	unknown	+	1175245	1175404	-1.50	0.53	0.98
760	**T-box**	-	2591050	2591272	0.89	**0.00**	**0.00**
783	bantam	+	2243636	2243694	-1.68	0.70	0.98
896	snoM1	+	1738447	1738549	1.74	0.19	0.98
2065	**rli47**	+	2038772	2039279	-3.54	**0.00**	**0.03**
2117	**Cobalamin**	-	596537	596726	-2.16	**0.03**	**0.98**
2159	LhrC	+	59963	60073	2.35	0.08	0.98
2161	LhrC	+	60368	60479	2.24	0.12	0.98

[*] full list for differentially expressed ncRNA transcript (Appendix B, Table S4.8); [a] ncRNA transcript number and name from predicted ncRNA list (Appendix B, Table S4.5); [b] values in bold are statistically significant (P-value < 0.05)

Table 4.4 Matched ncRNA transcripts to *L. monocytogenes* FSL C1-056 genome when grown in cellobiose compared to cellobiose with the addition of carnocyclin A.

ncRNA[a]		Strand	Start (bp)	End (bp)	Log2 Fold Change	P-value	FDR[b]
41	unknown	+	569896	569971	-1.78	0.68	0.99
82	unknown	-	1323026	1323115	-1.78	0.68	0.99
90	unknown	+	1398725	1398832	-2.48	0.54	0.99
140	unknown	+	1890814	1890894	-2.01	0.35	0.99
159	unknown	+	2195022	2195094	-1.78	0.68	0.99
172	unknown	+	2310700	2310769	-2.15	0.62	0.99
173	unknown	+	2321137	2321217	1.53	0.71	0.99
183	**unknown**	+	2471712	2471789	-5.03	**0.04**	**0.80**
224	Lysine	+	2183234	2183406	-1.56	0.33	0.99
287	unknown	-	2081369	2081561	-2.62	0.55	0.99
489	unknown	-	516497	516629	-1.59	0.61	0.99
502	unknown	+	164760	164903	3.80	0.25	0.8
502	**unknown**	-	164760	164903	2.63	**0.04**	**0.99**
506	unknown	+	1175245	1175404	-2.61	0.33	0.99
670	unknown	+	2442404	2442614	1.64	0.32	0.99
724	PyrR	-	2731584	2731680	-2.13	0.63	0.99
759	**T-box**	+	277890	278094	1.04	**0.00**	**0.07**
760	**T-box**	-	2591050	2591272	0.76	**0.00**	**0.01**
762	**T-box**	-	572196	572424	-1.13	**0.00**	**0.07**
2028	**rli24**	-	1545671	1545828	1.18	**0.00**	**0.04**
2065	**rli47**	+	2038772	2039279	-2.40	**0.01**	**0.39**
2078	rli43	-	1672815	1673068	2.18	0.06	0.87
2117	Cobalamin	-	596537	596726	1.53	0.11	0.99
2147	**rli53**	-	834338	834510	-2.80	**0.00**	**0.04**

[*] full list for differentially expressed ncRNA transcript (Appendix B, Table S4.8); [a] ncRNA transcript number and name from predicted ncRNA list (Appendix B, Table S4.5); [b] values in bold are statistically significant (P-value < 0.05)

4.3 Discussion

The growth of *L. monocytogenes* can be controlled with bacteriocins, such as carnocyclin A (Martin-Visscher et al., 2008); however, *L. monocytogenes* can develop resistance to bacteriocins (Balay et al., 2018; Gravesen et al., 2002; Kaur et al., 2011; Ramnath et al., 2000; Vadyvaloo et al., 2004b, 2004a, 2002). *L. monocytogenes* uses different carbohydrate transport systems for uptake of sugars and metabolism under normal conditions (Deutscher et al., 2014, 2006; Galinier and Deutscher, 2017; Naghmouchi et al., 2006). Sugar transport systems are commonly used as bacteriocin docking stations for

pore formation and cell death (Dalet et al., 2001; Garcia De Gonzalo et al., 2015; Kjos et al., 2010, 2009; Ramnath et al., 2000); however, they have been implicated in the formation of resistance to bacteriocins (Gravesen et al., 2002; Kaur et al., 2011; Ramnath et al., 2000). When a single carbohydrate is available for metabolism, cells may change the expression of genes related to specific sugar transport systems to become resistant to a bacteriocin and survive.

When *L. monocytogenes* FSL C1-056 was grown in mannose with or without carnocyclin A, the Man-PTS system was down-regulated. This may indicate that carnocyclin A resistance is associated with the Man-PTS system as this system would be required for survival if the only carbohydrate present was mannose. This aligns with a decreased growth rate and increased lag phase when *L. monocytogenes* FSL C1-056 was grown in mannose with or without the addition of carnocyclin A (Balay et al., 2018). When *L. monocytogenes* FSL C1-056 was grown in cellobiose, PTS systems associated with the transport of cellobiose (β-glucoside-PTS and cellobiose-PTS) (Dalet et al., 2003; Gravesen et al., 2000) were up-regulated, while the Man-PTS system was down-regulated. Again, this is an indication that the Man-PTS is associated with the formation of resistance of *L. monocytogenes* FSL C1-056 to carnocyclin A. In cases where *L. monocytogenes* has lost enzyme II subunit A/B or subunit C of the Man-PTS and are resistant to class IIa bacteriocins, β-glucoside-PTS systems are up-regulated (Dalet et al., 2001; Gravesen et al., 2002, 2000; Ramnath et al., 2004). With the up-regulation of cellobiose transport systems when *L. monocytogenes* is grown in cellobiose, resistance to carnocyclin A can occur quicker than when cells are grown in mannose. When treated with 3.3 mM of carnocyclin A and grown in cellobiose *L. monocytogenes* FSL C1-056 had a significantly shorter lag phase (21.83 h) than when grown in mannose (40.39 h) with carnocyclin A (Balay et al., 2018). The cell can use an alternate PTS system for carbohydrate transport while down-regulating the Man-PTS system to induce resistance to carnocyclin A. In confirmation the maximal growth rate of *L. monocytogenes* grown in cellobiose and exposed to carnocyclin A was significantly faster than *L. monocytogenes* grown in mannose when exposed to carnocyclin A (Balay et al., 2018).

The mode of action of carnocyclin A has previously been described as the formation of voltage-dependent anion selective channels (Gong et al., 2009). Results presented in this study indicate there may be a second mode of action that would involve a bacteriocin docking-molecule, as indicated by the down-regulation of the Man-PTS systems in resistant cells. Garvicin ML, a cyclic bacteriocin produced by *Lactococcus lactis,* may use a maltose ABC transporter as a docking station for the molecule as *Lactococcus lactis* IL1403 had increased sensitivity to garvicin ML when grown in maltose compared to when grown in glucose (Gabrielsen et al., 2014, 2012). Previous reports showed that *L. monocytogenes* grown in mannose and treated with carnocyclin A had increased sensitivity to the bacteriocin compared to growth in other carbohydrates; however, it was able to become resistant to the bacteriocin given sufficient time (Balay et al., 2018).

Genes related to virulence in *L. monocytogenes* are in part regulated by catabolite repression (Milenbachs and Brown, 1997). Regulation of virulence factors are repressed by β-glucosides (cellobiose) (Park and Kroll, 1993). Interestingly, transcriptional analysis revealed up-regulation of genes encoding N-acetylmuramoyl-L-alanine amidase (an autolysin) and LmaB, which are associated with virulence (Chatterjee et al., 2006; Nielsen et al., 2012; Wu et al., 2018) when *L. monocytogenes* FSL C1-056 was grown in cellobiose and treated with carnocyclin A. Carnocyclin A may stimulate a stress response that triggers increased expression of these genes despite the presence of cellobiose, rather than repression of their expression. In contrast, when cells were grown in mannose and treated with carnocyclin A there was down-regulation of an internalin-like protein (LPXTG motif) as well as the N-acetylmuramoyl-L-alanine amidase. This could be due to the down-regulation of Man-PTS enzyme IIA/B as it is suspected of repressing PfrA (Aké et al., 2011).

Carnocyclin A elucidated a stress response in *L. monocytogenes* that resulted in resistance to the bacteriocin. It is possible that changes in gene expression could be due to regulatory ncRNA. When *L. monocytogenes* FSL C1-056 was grown in mannose with the addition of carnocyclin A, expression of ncRNA Rli47 (*sbrE*) (Izar et al., 2011; Mellin and Cossart, 2012; Mujahid et al., 2013; Vivant et al., 2017) was down-regulated significantly. Rli47 is a σ^B dependent (Mujahid et al., 2013), trans-acting ncRNA that

affects the expression of the regulator related to virulence, PfrA, by binding to a untranslated region of its mRNA (Vivant et al., 2017). As σ^B is often related to the general stress response of *L. monocytogenes* (Kazmierczak et al., 2003; Palmer et al., 2009) and the presence of carnocyclin A would be considered stress upon the cell, it is possible that Rli47 is affected by the presence of the bacteriocin and may play a role in the formation of resistance. Another indication that ncRNAs may play a role in development of resistance is that when the experimental strain was grown in cellobiose with or without carnocyclin A, two additional ncRNAs were differentially expressed, Rli53 and Rli24. Rli53 is related to lincomycin resistance and behaves as a terminator/anti-terminator and switches to the open state in the presence of the antibiotic allowing for the downstream transcription of an ABC transporter (Lebreton and Cossart, 2016). This ncRNA could be related to *L. monocytogenes* ability to form resistance to carnocyclin A. A lesser known ncRNA Rli24 was significantly up-regulated in the presence of cellobiose and carnocyclin A and has been shown to be up-regulated in macrophages (Kuenne et al., 2013). Again, this response is linked to stress in the cell and perhaps could play a role in regulating resistance. Moreover, the expression of T-boxes were also significantly up-regulated and down-regulated in both experimental groups. These represent the largest class of riboswitches in *L. monocytogenes* and act as cis-regulators that control gene expression by binding to tRNA (Izar et al., 2011; Mraheil et al., 2011a). These riboswitches may also play a role in adaptation and resulting physiological changes seen when *L. monocytogenes* is exposed to carnocyclin A (Balay et al., 2018). Together as a part of a larger stress response ncRNAs are likely playing a role regulating gene expression related to the *L. monocytogenes* ability to form resistance to carnocyclin A.

In conclusion, when exposed to carnocyclin A, *L. monocytogenes* down-regulated the expression of genes related to sugar transport, metabolism and virulence, among others, when grown in mannose; however, in cellobiose more genes were up-regulated compared to growth in mannose. This indicated the cells focused on slowing down cellular function and regulation in hopes of surviving the stress from carnocyclin A. It suggests that high levels of carnocyclin A may cause resistance in *L. monocytogenes* and may be better suited to be used in conjunction with other interventions to control the growth of *L.*

monocytogenes in food (Allen et al., 2014; Chen and Hoover, 2003; Gálvez et al., 2008; Parada et al., 2007; Perez et al., 2014; Tiwari et al., 2009; Zhu et al., 2005). The carbohydrate present for growth and metabolism played a role in the transcriptional response of *L. monocytogenes* FSL C1-056. The Man-PTS was down-regulated in *L. monocytogenes* grown in mannose or cellobiose. The Man-PTS has previously been linked to bacteriocin resistance and we suspect it to be related to carnocyclin A resistance, and may be a possible docking station for the bacteriocin. In addition to an effect on sugar transport systems, there was also an indication that the presence of carnocyclin A can impact the transcription of virulence genes, which needs further validation. The differentially expressed ncRNAs likely play a role regulating gene expression related to the formation of resistance to carnocyclin A. The analysis of *L. monocytogenes* FSL C1-056 transcriptome allowed for a more global perspective of the stress response and development of resistance to carnocyclin A that can be impacted by carbohydrates.

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Chapter 5

5.0 General Discussion

Listeria monocytogenes, is a foodborne pathogen typically associated with ready-to-eat (RTE) foods due to post-processing contamination and a long storage life where the organism can grow (PHAC, 2012; Zhu et al., 2005). Most RTE foods are consumed without an additional heating step, which increases the risk of eating contaminated food, and developing listeriosis. Consumers are looking for more convenient RTE meals that are fresh and preserved with minimal addition of chemical (traditional) preservatives along with and the globalization of the food market further increases the risk of consuming contaminated RTE foods (Gálvez et al., 2008). It is important to control the risk of illness from consuming foods contaminated with *L. monocytogenes* while aligning with consumer demands for a more natural approach to preservatives.

Biopreservatives are antimicrobial products of living organisms making them a natural alternative to traditional preservatives (Gálvez et al., 2010). Bacteriocins are considered a biopreservatives and are an attractive alternative to traditional preservatives to increase food safety against foodborne pathogens (Perez et al., 2014). Bacteriocins are ribosomally synthesized by lactic acid bacteria and are excreted out of the cell and have a narrow-spectrum of activity (Cotter et al., 2005), which is important for targeted control of *L. monocytogenes* without disrupting the natural microflora of the foods. There are bacteriocins that have been approved for use in foods such as nisin (Gálvez et al., 2008), which indicates bacteriocins are a viable option to be implemented in foods to control the risk of *L. monocytogenes*. However, nisin cannot be used with raw meat as it reacts with glutathione, which inactivates the peptide (Rose et al., 1999) limiting its use in RTE meat products, making it necessary to investigate alternative bacteriocins for use in RTE meat products that can control *L. monocytogenes*. An alternative class of bacteriocins that are known for their potent antilisterial activity, that are not inactivated in raw meat, are class IIa bacteriocins, which are attractive candidates to investigate for use in RTE meat products (Drider et al., 2006; Zhu et al., 2005). A new and emerging class of bacteriocins known as circular bacteriocins (class IIc) also have demonstrated antilisterial activity

with increased stability against proteases, which may be able to degrade bacteriocins (Kumariya et al., 2019; Martin-Visscher et al., 2008). These two bacteriocins are ideal candidates to further study their efficacy and application in RTE meat products to control *L. monocytogenes*. However, *L. monocytogenes* can form resistance to bacteriocins (Kaur et al., 2011). Although not all strains of *L. monocytogenes* display resistance to bacteriocins it would be advantageous for the food industry to understand if this phenomenon associated with class IIa bacteriocins can be linked to the new emerging circular bacteriocins.

The research reported in this thesis investigates and contrasts the efficacy and resistance mechanisms of class IIa (leucocin A) and class IIc circular (carnocyclin A) bacteriocins (Kumariya et al., 2019) against strains of *L. monocytogenes* that were developed for use in challenge studies (Fugett et al., 2006).

Traditional methods of purification are tedious involving many steps, which increases the cost of bacteriocin recovery, and only recover very small amounts of peptide for application in food (Garsa et al., 2014). Although bacteriocins are often active in the nanomolar range (Bastos et al., 2015) their use as a food preservative for mass production would require quantities larger than research scale purification processes would be able to produce efficiently. Investigation into alternate methods of bacteriocin purification with a simplified purification process, that produced sufficient yields of bacteriocin for food application are imperative to bacteriocin success in the food industry. Therefore, to carry out *in vitro* and *in vivo* experiments to determine the efficacy of leucocin A to inhibit the growth of *L. monocytogenes* in the presence of natural spoilage microbiota on vacuum packaged RTE meat, a novel purification method was developed to obtain sufficient quantities of leucocin A in as few steps as possible. The purification procedure used as a basis for the process follows work done by Hastings and Stiles (1991), which only produced ~0.41 mg/1L culture. This was insufficient for the experimental use so, the method was modified to increase yield. Modifications included the removal of Tween® 80, the centrifugation step to separate the cells from the bacteriocin containing supernatant; therefore the complete cell ferment was poured over the affinity chromatography column to capture free bacteriocin in suspension and possible

bacteriocin that was still bound to the cells increasing the overall amount of peptide obtained for downstream purification. As a result, the affinity chromatography protocol was altered to account for changes to the media and cells. Lastly, the ammonium sulfate precipitation was eliminated from the complete purification protocol. MALDI-TOF confirmed the active substance purified was similar in mass to that reported for leucocin A, ~3,903 Da and LCMS/MS confirmed that the amino acid sequence of the purified peptide was that of leucocin A. The new purification procedure resulted in a 5-fold increase in the amount obtained (~2.33 mg/1 L culture). Additionally, a leucocin A analogue was synthesized to determine if changes to the amino acid structure could increase the bacteriocins efficacy and stability against *L. monocytogenes*.

The efficacy of leucocin A to inhibit the growth of *L. monocytogenes* differed in solid and liquid-phase systems. The MIC value could not be determined for *L. monocytogenes* strains in broth due to the lack of inhibition after 18 h incubation; however, on agar an MIC value could be obtained. Further investigation was pursued on the killing kinetics of leucocin A against *L. monocytogenes* strains to determine why it was active on agar and not in broth. The synthesized, leucocin A analogue, LeuN17L, was not an option for use in foods as the concentration needed to inhibit the growth of *L. monocytogenes* was 6 to 8 times the amount of leucocin A needed to inhibit the same strains of *L. monocytogenes*; thus the use of the analogue was not investigated further.

L. monocytogenes growth in broth was assessed over 48 h in the presence of leucocin A and bactericidal and bacteriostatic effects were observed. A subpopulation of each strain was able to overcome the adverse effects of the leucocin A and was able to grow within 1 to 2 log of the original cell density within 24 h. This indicated that a subpopulation of *L. monocytogenes* either was innately or spontaneously resistant to leucocin A. The initial kill of the sensitive population could be observed on agar as a zone of inhibition, while the spontaneously resistant subpopulations were better identified in broth where the strains grew to almost maximum cell numbers. This accounted for the discrepancy in MIC values for solid- and liquid-phase matrices.

Interestingly, when leucocin A was applied to a wiener (solid-phase matrix) in the presence of *L. monocytogenes*, leucocin A was able to inhibit the growth of a cocktail of

5 strains of *L. monocytogenes* during two weeks of refrigerated, vacuum packaged, storage. When *L. monocytogenes* inoculated onto wieners with natural spoilage organisms, *Carnobacterium divergens* and *Brochothrix thermosphacta*, and leucocin A was added to the surface, there was a reduction in the counts of *L. monocytogenes* during the two weeks of storage. The antimicrobial effect of leucocin A was not impacted by the presence of spoilage organisms on wieners. In contrast, when tested in broth containing leucocin A, *B. thermosphacta* caused inactivation of the bacteriocin, resulting in growth of *L. monocytogenes*. This may have been in part due to the production of enzymes by *B. thermosphacta*, such as α-chymotrypsin (Casaburi et al., 2014; Nowak and Piotrowska, 2012), which can degrade leucocin A and cause inactivation (Hastings and Stiles, 1991). These results suggest that leucocin A has potential for application in solid-phase systems, such as the surface of wieners, but further steps must be taken to ensure the formation of resistance and outgrowth of *L. monocytogenes* does not occur with extended storage times or in liquid products. It is important to consider that the cocktail of *L. monocytogenes* used in this study is recommended for use in challenge studies (Fugett et al., 2006), yet all the strains had resistance to leucocin A, which may bias the results of challenge studies that use class IIa bacteriocins or other antimicrobials with similar modes of action.

Resistance of *L. monocytogenes* to class IIa bacteriocins can be attributed to different mechanisms including a more positive cell surface, more fluid membrane, and/or most notably a decrease in the expression of mannose permease operons (sugar phosphotransferase systems; PTS) leading to interference with the efficacy of a bacteriocin (Gravesen et al., 2002; Jacquet et al., 2012; Vadyvaloo et al., 2004b). The mannose phosphotransferase system (Man-PTS) is used by *L. monocytogenes* to uptake sugars (mannose and glucose) into the cell for energy. If the Man-PTS is the main mechanism of resistance to class IIa bacteriocin, an investigation using different carbohydrates may reveal varying levels of innate or spontaneous resistance to leucocin A. If *L. monocytogenes* down regulates the transcription of the Man-PTS enzymes in response to exposure to a bacteriocin, the growth kinetics will be affected due to the residual effects on carbohydrate transport and resulting downstream metabolism. The application of bacteriocins in food to inhibit the growth of *L. monocytogenes* requires a

better understanding of how carbohydrates affect the efficacy of bacteriocins and the development of resistance.

The growth kinetics of the same *L. monocytogenes* strains previously investigated were assessed when the strains were grown in different carbohydrates (mannose, glucose, cellobiose, fructose, sucrose) in the presence of leucocin A and carnocyclin A. The bacteriocins are reported to have two separate modes of action, and belong to different classes, class IIa and class IIc, respectively (Kumariya et al., 2019).

To obtain sufficient carnocyclin A for use in experiments, the original purification protocol (Martin-Visscher et al., 2008) was altered to increase the amount of peptide purified. The new purification procedure was modeled after the adapted purification procedure developed for leucocin A. The main adaptations that increased the yield of peptide obtained was the elimination of the centrifugation step and altered hydrophobic affinity chromatography procedure to accommodate the complete cell ferment to be applied to the column. The new purification protocol produced 4.8 mg/1 L culture of carnocyclin A, which was 2.5 times more purified peptide than the original protocol. The mass of the purified bacteriocin was confirmed by MALDI-TOF to be 5,863.5 Da, which is in agreement with the reported mass of carnocyclin A.

Leucocin A decreased the growth rate of most strains of *L. monocytogenes* when grown in broth supplemented with either sucrose, mannose, fructose, glucose or cellobiose, but only for a few strains the lag phase was extended significantly by the presence of leucocin A. When the growth of the *L. monocytogenes* was delayed due to the presence of leucocin A, development of resistance to the bacteriocin occurs allowing the growth of the culture after an initial reduction to the cell counts as observed by the growth curves of each *L. monocytogenes* strain grown in the presence of leucocin A, regardless of the carbohydrate present. In general mannose, glucose, and fructose reduced the growth rate for cells treated with leucocin A, with the exception of strain FSL R2-499, which was the most resistant to leucocin A. However, this strain became more sensitive to leucocin A when grown with mannose as the sole carbohydrate. The increased sensitivity to leucocin A, as evidenced by the reduced growth rate, may relate to a requirement for use of the Man-PTS for carbohydrate uptake. This supports the hypothesis that growth of *L.*

monocytogenes in the presence of a specific carbohydrate increases the sensitivity of *Listeria* to the bacteriocin if the sugar transport system is also used as a bacteriocin docking molecule.

Glucose was thought to be the preferred carbohydrate of *L. monocytogenes* (Premaratne et al., 1991); however, strains used in this study had the fastest growth rate in the presence of cellobiose. Cellobiose is transported across the cell membrane by cellobiose-PTS, β-glucoside-PTS, and lactose-PTS (Gravesen et al., 2000; Kotrba et al., 2001; Stoll and Goebel, 2010). *L. monocytogenes* that have lost enzyme II subunit A/B or subunit C of the Man-PTS are resistant to class IIa bacteriocins (Dalet et al., 2001; Gravesen et al., 2002; Ramnath et al., 2004) and up-regulate the β-glucoside-PTS (Gravesen et al., 2002, 2000). This explains the shorter lag phase and faster growth rate when cultures were grown in cellobiose and treated with bacteriocins.

Carnocyclin A increased the lag phase and shortened the growth rates for each strain of *L. monocytogenes* compared to leucocin A for the same carbohydrate indicating that carnocyclin A was more effective against *L. monocytogenes* than leucocin A. When *L. monocytogenes* was grown in sucrose and treated with carnocyclin A the growth rate was slowed, but had a shortened lag phase compared to *L. monocytogenes* growth in other carbohydrates. It is plausible that there is an additional fitness cost associated with the breakdown of disaccharides by *L. monocytogenes*. The shorter lag phase indicates that although there may be an additional fitness cost to using sucrose as an energy source, the machinery to transport sucrose into the cell is probably not involved in bacteriocin docking. The ability of *L. monocytogenes* to develop resistance to carnocyclin A was decreased when grown in the presence of glucose, fructose, or mannose as the lag phase was substantially extended; however, resistance still developed after 72 h of incubation. Carnocyclin A was reported to form voltage-dependent anion selective channels in the target cell membrane (Gong et al., 2009), which is different to the mechanism reported for class IIa bacteriocins such as leucocin A. In the current research, similar effects of carbohydrates on the development of resistance in *Listeria* was observed in cells treated with either leucocin A or carnocyclin A, which may argue in favor of the existence of a carbohydrate-specific docking molecule for carnocyclin A. However, strains of *L.*

monocytogenes that are resistant to class IIa bacteriocins (Crandall and Montville, 1998), have a less hydrophobic cell membrane compared to sensitive strains (Ming and Daeschel, 1993) and a more positive cell surface due to D-alanylation of teichoic acid and lysinilation of phospholipids (Jacquet et al., 2012; Vadyvaloo et al., 2004a). This change in cell surface properties could also explain the formation of resistance to carnocyclin A. It is possible that poor interactions between carnocyclin A, which is hydrophobic and cationic, and the cell membrane, decrease the formation of anion selective pores and increase resistance to the bacteriocin.

The development of resistance to leucocin A or carnocyclin A resulted in a stable phenotype. This was confirmed by the observation that bacteriocins increased the lag phase in the original cultures but not in cultures that were adapted to the respective bacteriocins.

L. monocytogenes can develop resistance to leucocin A and carnocyclin A and can be affected by the carbohydrate source for metabolism and innate strain variation. Resistance of *L. monocytogenes* to carnocyclin A takes longer to form than resistance to leucocin A, but after an extended lag phase all isolated subpopulations exhibit a stable-resistant phenotype to repeat exposure to the bacteriocins. Cellobiose and sucrose increased the ability of *L. monocytogenes* to form resistance compared to other carbohydrates. It is important to note that not all strains behaved the same and therefore innate metabolic patterns and resistance may have influenced how *L. monocytogenes* reacted to the application of bacteriocins. Further research is needed to identify if there is a global stress response to bacteriocin exposure or if there is specific pathways that lead to resistance for each specific class of bacteriocin and carbohydrate. The results of this research raise concerns regarding the application of bacteriocins in foods, as growth in different carbohydrates supported development of resistance to bacteriocins, but the resistance was dependent on the strain and the carbon source.

L. monocytogenes FSL C1-056 was the most sensitive strain to both bacteriocins and was used in downstream experimentation to better understand the ability to survive and form resistance to carnocyclin A when grown in mannose or cellobiose. The global transcriptome was analyzed with RNA-Seq to identify how *L. monocytogenes* gene

expression levels changed in response to carbohydrate and bacteriocin stress and to discover if any non-coding RNA (ncRNA) were affected under such stressors to explore their roles in stress regulation.

When *L. monocytogenes* FSL C1-056 was grown in mannose with or without carnocyclin A, the Man-PTS system was down-regulated. This may indicate that resistance to carnocyclin A is associated with the Man-PTS system as this system would be required for survival if the only carbohydrate present was mannose. This aligns with a decreased growth rate and increased lag phase when *L. monocytogenes* FSL C1-056 was grown in mannose with carnocyclin A in the previous study. When *L. monocytogenes* FSL C1-056 was grown in cellobiose, PTS systems associated with the transport of cellobiose (β-glucoside-PTS and cellobiose-PTS) were up-regulated, while the Man-PTS system was down-regulated. Again, this is an indication that the Man-PTS is associated with the formation of resistance of *L. monocytogenes* FSL C1-056 to carnocyclin A. With the up-regulation of cellobiose transport systems when *L. monocytogenes* is grown in cellobiose, resistance to carnocyclin A can occur quicker than when cells are grown in mannose as indicated in the previous study with a significantly shorter lag phase (21.83 h) than when grown in mannose (40.39 h).

Genes related to virulence in *L. monocytogenes* are in part regulated by catabolite repression (Milenbachs and Brown, 1997). Regulation of virulence factors are repressed by β-glucosides (cellobiose) (Park and Kroll, 1993). There was up-regulation of N-acetylmuramoyl-L-alanine amidase (an autolysin) and LmaB, which are associated with virulence (Chatterjee et al., 2006; Nielsen et al., 2012; Wu et al., 2018) when *L. monocytogenes* FSL C1-056 was grown in cellobiose and treated with carnocyclin A. Carnocyclin A may stimulate a stress response that triggers increased expression of these genes in the presence of cellobiose, rather than repression of virulence gene expression. In contrast, when cells were grown in mannose and treated with carnocyclin A there was down-regulation of an internalin-like protein (LPXTG motif) as well as the N-acetylmuramoyl-L-alanine amidase. This could be due to the down-regulation of Man-PTS enzyme IIA/B as it suspected of repressing PfrA (Aké et al., 2011).

There were 366 predicted ncRNA sequences found in the genome of *L. monocytogenes*; however only 6 of these ncRNAs were differentially expressed in the present study. Most of the ncRNA that were affected relate to *L. monocytogenes* ability to deal with environmental stress, including antibiotic resistance. These same ncRNAs may also play a role in *L. monocytogenes* ability to become resistant to bacteriocins. Rli47 (*sbrE*), a σ^B dependent ncRNA (Mujahid et al., 2013) was significantly down-regulated when the strain was grown in mannose and treated with carnocyclin A. *L. monocytogenes* general stress response is often regulated by σ^B (Kazmierczak et al., 2003; Palmer et al., 2009) and the presence of carnocyclin A would be considered stress upon the cell, which makes it possible that Rli47 may play a role in the formation of resistance. Rli53 and Rli24 were differentially expressed when *L. monocytogenes* was grown in cellobiose with carnocyclin A. Rli53 is related to lincomycin resistance (Lebreton and Cossart, 2016) and Rli24 has been shown to be up-regulated in macrophages (Mraheil et al., 2011), both of which could relate to *L. monocytogenes* ability to form resistance to carnocyclin A. There were also T-boxes, which are the largest class of riboswitches in *L. monocytogenes*, that can regulate gene expression by binding to tRNA (Izar et al., 2011; Mandin et al., 2007). Together, as a part of a larger stress response ncRNA are likely to play a role in regulating gene expression related to the ability of *L. monocytogenes* to become resistance to carnocyclin A; however, further research needs to be done to confirm this. Expression of each significant ncRNA transcript would need to be investigated by designing custom probes for each of the predicted ncRNA sequences, followed by RT-qPCR to provide validation that ncRNA is actually present and being transcribed under the prescribed conditions. It would be necessary to knockout each ncRNA of interest from the wildtype strain and then test each mutant under the same conditions to determine if *L. monocytogenes* remains resistant to carnocyclin A or becomes sensitive. It would also be interesting to investigate the presence of the same target ncRNA in other strains of *L. monocytogenes*, such as FSL R2-499, as this strain is more innately resistant to bacteriocins to see if the expression of these ncRNAs is the same in a more sensitive strain, such as FSL C1-056. Additionally, it would be interesting to investigate the expression of these ncRNAs in the presence of other bacteriocins.

When exposed to carnocyclin A, *L. monocytogenes* down-regulates the expression of genes related to sugar transport, metabolism and virulence, among others, when grown in mannose. However, when grown in cellobiose more genes were up-regulated as compared to growth in mannose. This indicated that the cell focused on slowing down cellular function and regulation in hopes of surviving the stress from carnocyclin A. The carbohydrate present for growth and metabolism plays a role in the transcriptional response of *L. monocytogenes* FSL C1-056 and how quickly the cell can form resistance to carnocyclin A. The Man-PTS, which has previously been linked to bacteriocin resistance, was down-regulated in *L. monocytogenes* grown in mannose or cellobiose linking it to carnocyclin A resistance. It may in fact be an alternate docking station for the bacteriocin; however, further research is needed to test this hypothesis. In addition to affected sugar transport systems, there was also an indication that the presence of carnocyclin A impacted the transcription of virulence genes, which needs further validation. Lastly, ncRNA likely play a role regulating gene expression related to the formation of resistance to carnocyclin A due their determined associations with stress response pathways and role in antibiotic resistance. The analysis of *L. monocytogenes* FSL C1-056 transcriptome allowed for a more global understanding of the stress response and development of resistance to carnocyclin A.

5.1 Conclusions

Ultimately, bacteriocins have the ability to enhance food safety by inhibiting the growth of pathogens, such as *L. monocytogenes*. This will help alleviate the detrimental effects this organism has on our healthcare system and the food industry. In addition to enhancing food safety, the use of bacteriocins aligns with consumer trends of increased demand for natural preservatives in fresh RTE products. The research reported in this thesis focused on the efficacy of leucocin A and carnocyclin A to control *L. monocytogenes.* It is advantageous for the industry and researchers to understand that the cocktail strains of *L. monocytogenes* designed for use in challenge studies can become resistant to leucocin A and carnocyclin A. However, *in situ* leucocin A can control the growth of *L. monocytogenes* in the presence of spoilage organisms, but there is concern

that some spoilage organisms may result in less inhibition. The extended storage of RTE meat products could allow subpopulations of *L. monocytogenes* to become resistant to bacteriocin present. The proposed mechanisms for resistance to leucocin A were confirmed to play a role in the formation of resistance for these specific strains of *L. monocytogenes* and in the formation of resistance to carnocyclin A, indicating potential for cross resistance between class IIa and class IIc bacteriocins. Carbohydrates influence the ability of *L. monocytogenes* to develop resistance, although given enough time and nutrients *L. monocytogenes* will be able to outgrow the stress of bacteriocins *in vitro*. Additionally, there are ncRNA differentially expressed in *L. monocytogenes* FSL C1-056 exposed to carnocyclin A, and they may be involved in regulating the ability of cells to develop resistance to bacteriocins. The information gained from the RNAseq analysis also indicated that virulence factors may be affected by the presence of carnocyclin A and there may be a secondary mode of action for the bacteriocin, which uses a docking station.

All together bacteriocins elucidate what may be considered more of a general stress response in the presence of bacteriocins that allow for altered gene expression that results in resistant strains of *L. monocytogenes*. Due to the overlap in stress response pathways between bacteriocins and carbohydrates present for metabolism a singular resistance mechanism is unlikely to be the sole cause for a resistant phenotype. Arguably, the research presented in this thesis could suggest that resistance to bacteriocins may in fact actually be a prospect of *L. monocytogenes* ecological niche. The resistance shaped by the nutrients available, competitive microflora, innate strain specificities, interaction time, and type of bacteriocin intervention. Each aspect triggers a unique response in *L. monocytogenes* to better respond to the stress of a bacteriocin treatment. This may be why *L. monocytogenes* behaves in an overwhelmingly similar manner to bacteriocins of differing classes, as any stress in the environment has already signaled the regulatory actions of the cell to prepare for a stress response, and only when the bacteriocin is introduced *L. monocytogenes* fine tunes its response to survive by becoming resistant to the bacteriocin present. Ecological forces may drive *L. monocytogenes* to adapt to stressors like bacteriocins more completely and efficiently, which would account for the differences seen amongst isolates of *L. monocytogenes* even in the presence of the same

bacteriocin. However, further research is needed to conclude these insights that the development of resistance to bacteriocins in *L. monocytogenes* is an ecological adaptation compared to a regulated mechanism of response triggered by the bacteriocin itself.

These investigations will provide insight for how to better use these bacteriocins in food systems and what other hurdles may need to be in place to create a safe product. All proposed avenues of research provide the food industry with valuable information for the safe and effective application of leucocin A and carnocyclin A to control the growth of *L. monocytogenes* in foods. Although it appears leucocin A and carnocyclin A are active against food isolates of *L. monocytogenes*, they may be better suited for use in conjunction with other interventions as part of a hurdle system to prevent the outgrowth of *L. monocytogenes* to ensure the safety of RTE food during storage (Allen et al., 2014; Chen and Hoover, 2003; Gálvez et al., 2008; Parada et al., 2007; Perez et al., 2014; Tiwari et al., 2009; Zhu et al., 2005). Bacteriocins have the potential to enhance food safety and reduce the economic impact of foodborne illness on society, though special attention must be paid to the composition of the RTE product and how it may affect the formation of resistance, growth kinetics, and virulence potential of *L. monocytogenes*.

5.2 References

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Appendices

Appendix A

Supplemental Figures S3.5

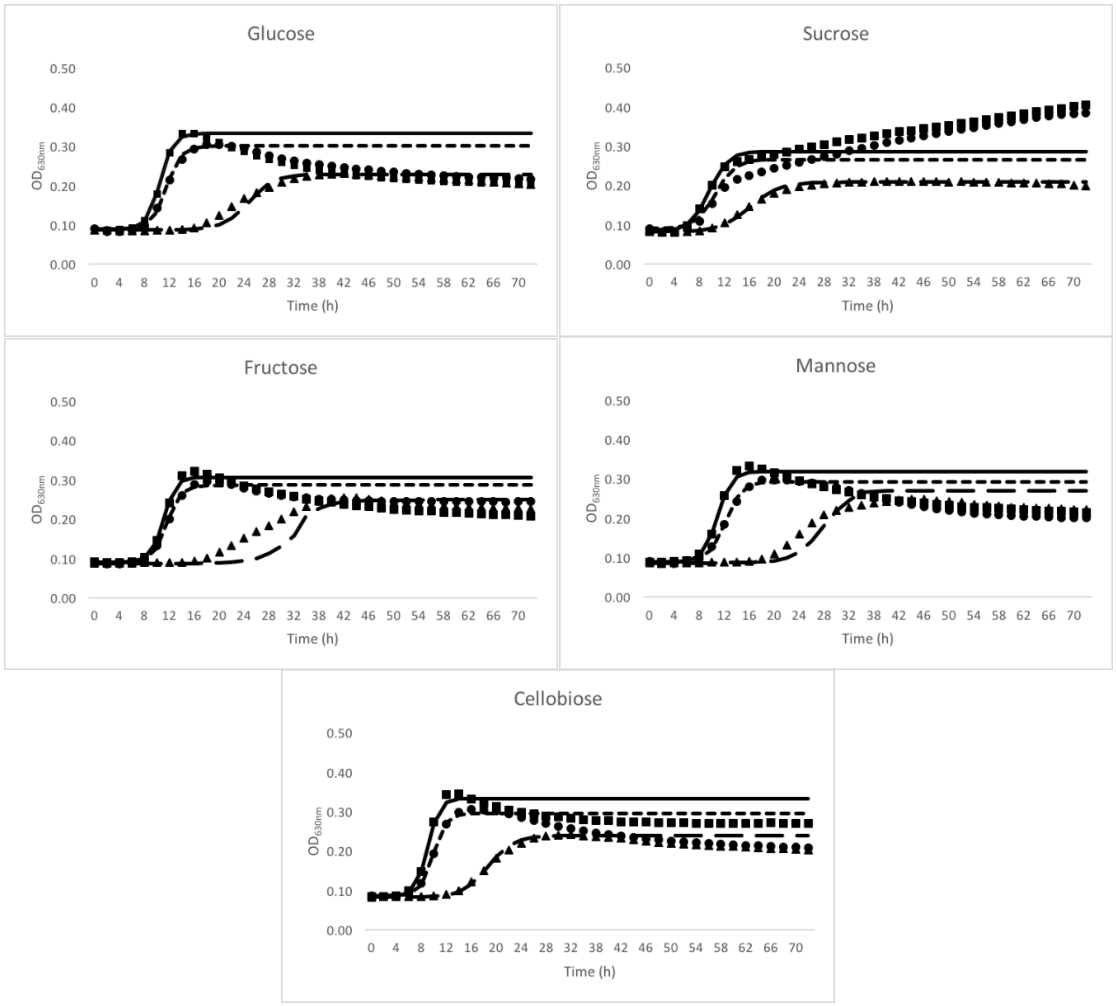

Figure S3.1 Experimental growth curve data and modelled growth curve data for *L. monocytogenes* (FSL N1-227). Experimental growth curve data (control, ■; treated with leucocin A, ●; treated with carnocyclin A, ▲) and modelled growth curve data (control, ——; treated with leucocin A, - - - ; treated with carnocyclin A, — -) grown in different carbohydrates at 25 °C. Optical density (630 nm) values displayed as the average of three independent replicates.

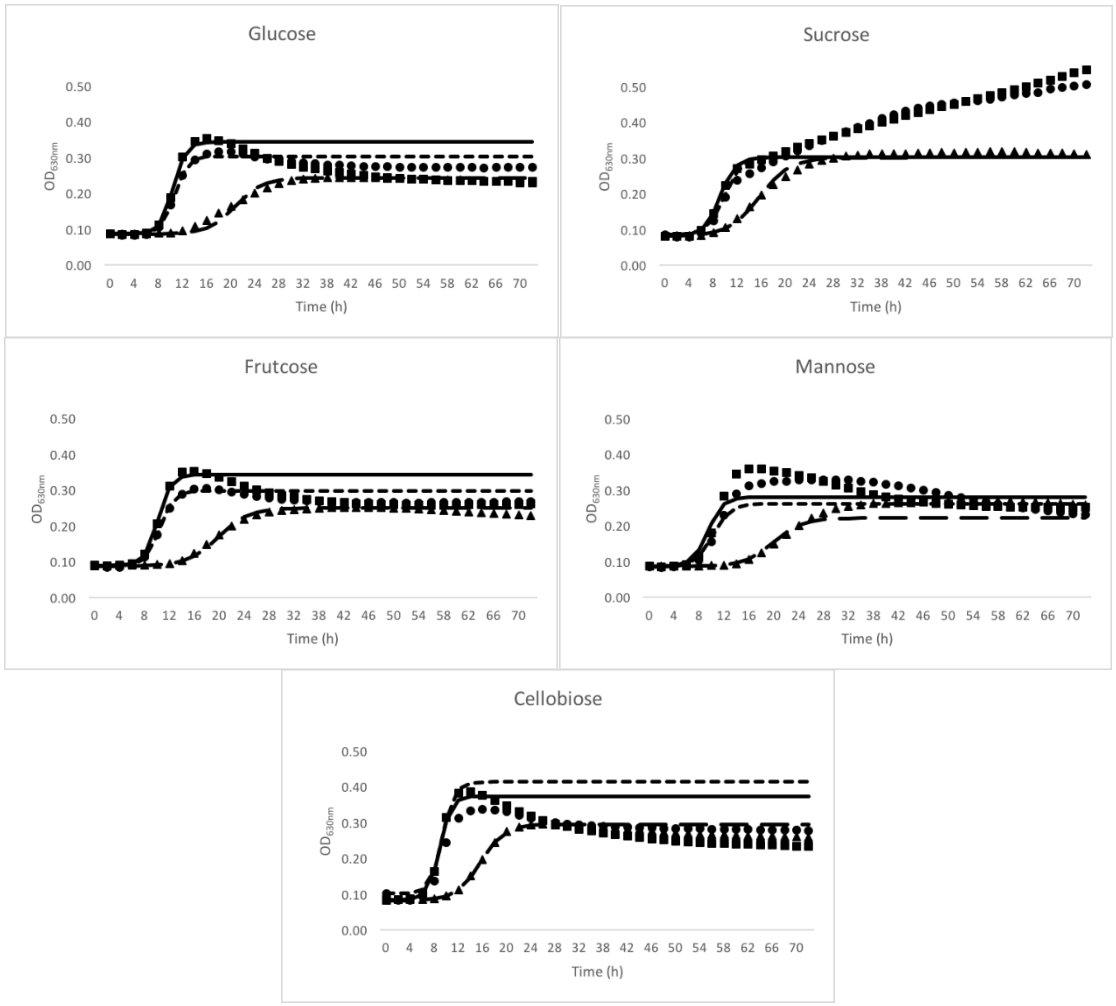

Figure S3.2 Experimental growth curve data and modelled growth curve data for *L. monocytogenes* (FSL R2-499). Experimental growth curve data (control, ■; treated with leucocin A, ●; treated with carnocyclin A, ▲) and modelled growth curve data (control, ——; treated with leucocin A, - - - ; treated with carnocyclin A, — -) grown in different carbohydrates at 25 °C. Optical density (630 nm) values displayed as the average of three independent replicates.

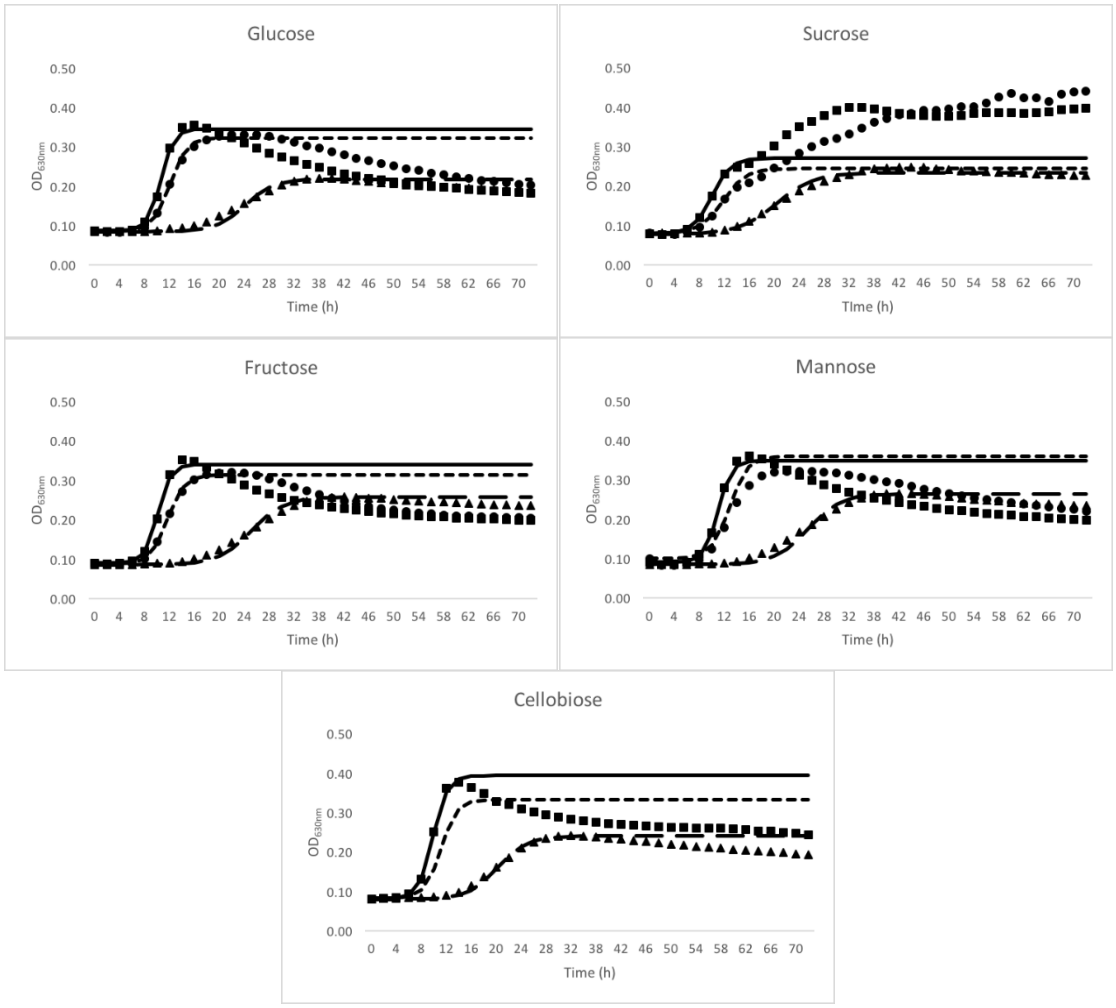

Figure S3.3 Experimental growth curve data and modelled growth curve data for *L. monocytogenes* (FSL N3-013). Experimental growth curve data (control, ■; treated with leucocin A, ●; treated with carnocyclin A, ▲) and modelled growth curve data (control, ——; treated with leucocin A, - - -; treated with carnocyclin A, — - -) grown in different carbohydrates at 25 °C. Optical density (630 nm) values displayed as the average of three independent replicates.

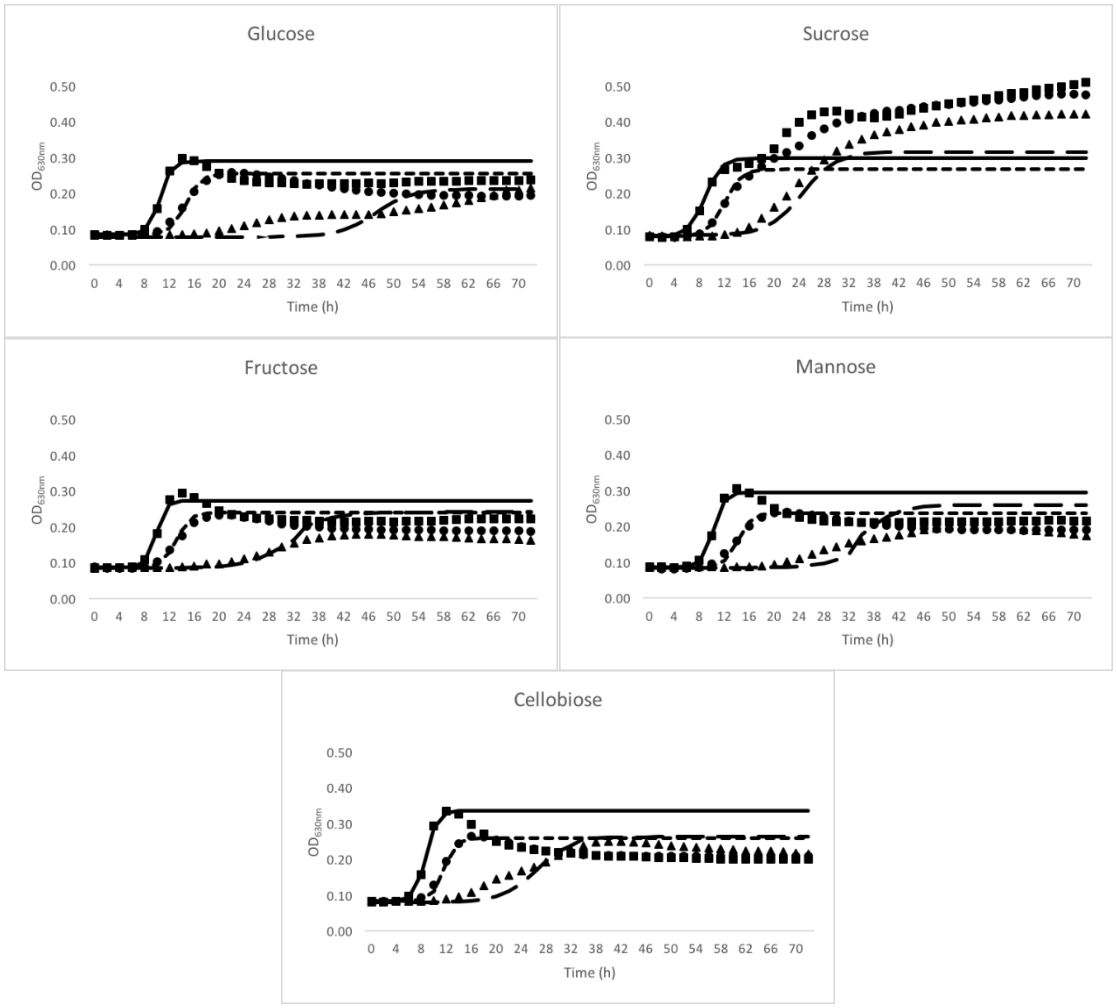

Figure S3.4 Experimental growth curve data and modelled growth curve data for *L. monocytogenes* (FSL J1-177). Experimental growth curve data (control, ■; treated with leucocin A, ●; treated with carnocyclin A, ▲) and modelled growth curve data (control, ——; treated with leucocin A, - - -; treated with carnocyclin A, — - -) grown in different carbohydrates at 25 °C. Optical density (630 nm) values displayed as the average of three independent replicates.

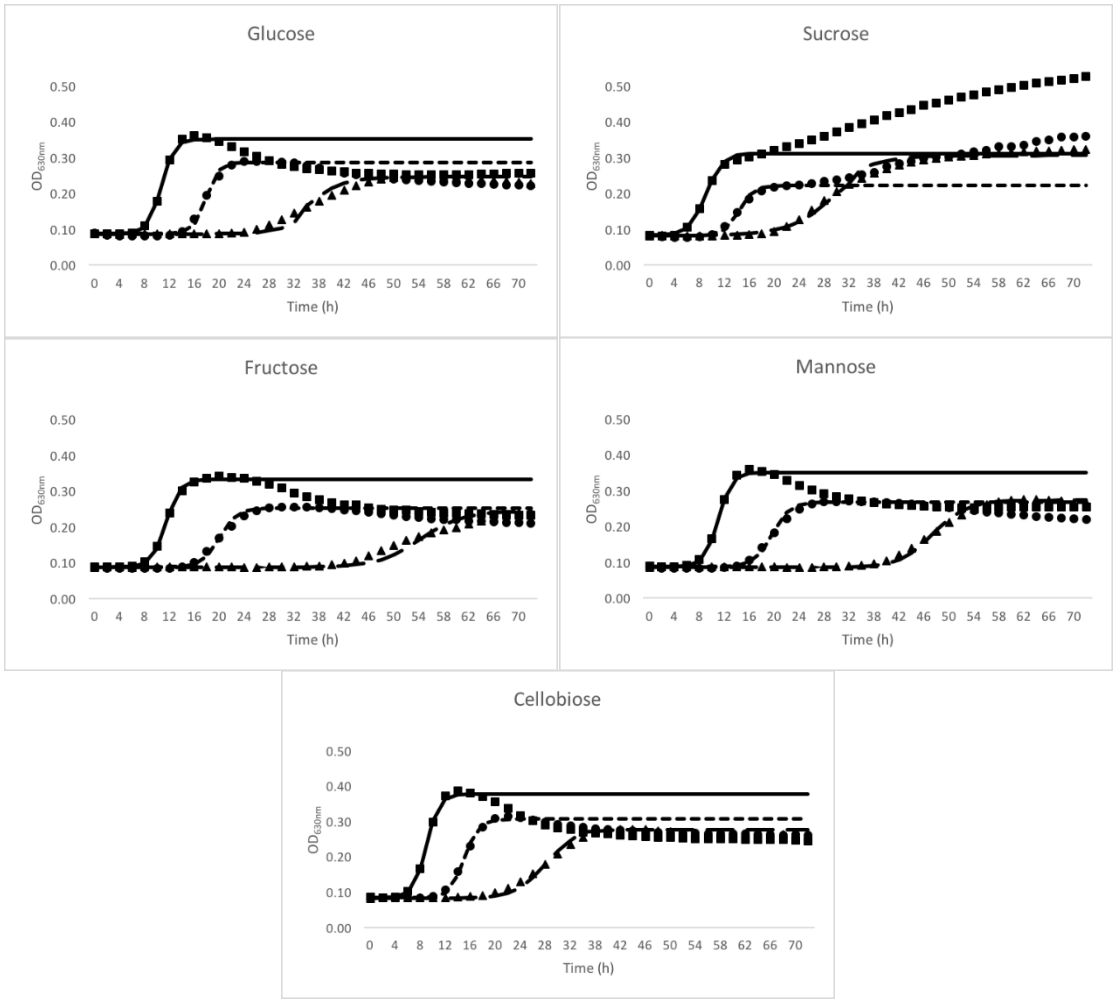

Figure S3.5 Experimental growth curve data and modelled growth curve data for *L. monocytogenes* (FSL C1-056). Experimental growth curve data (control, ■; treated with leucocin A, ●; treated with carnocyclin A, ▲) and modelled growth curve data (control, ——; treated with leucocin A, - - -; treated with carnocyclin A, — - -) grown in different carbohydrates at 25 °C. Optical density (630 nm) values displayed as the average of three independent replicates.

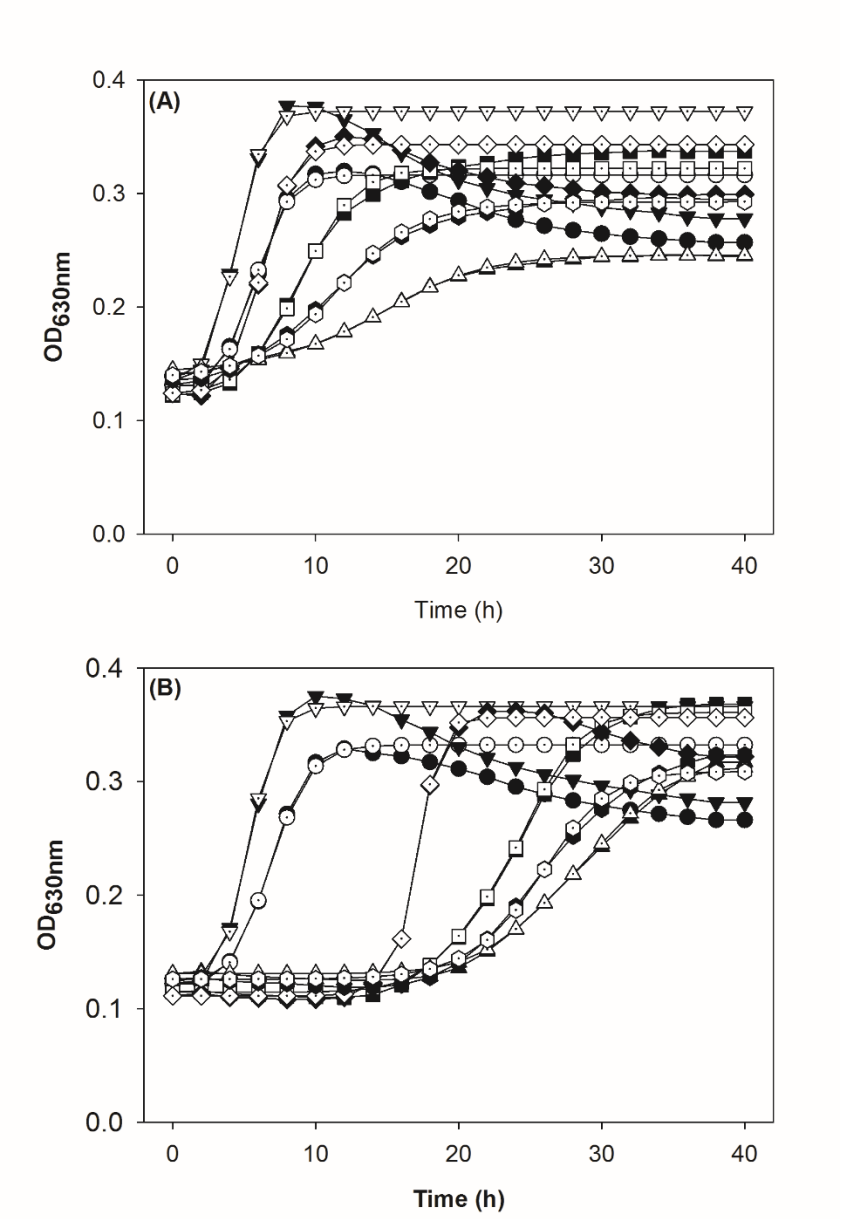

Figure S3.6 Experimental growth curve data (closed symbols) for original cultures of *L. monocytogenes* strains FSL R2-499 (A) and FSL C1-056 (B) compared to the modelled growth curve data (open symbols). *L. monocytogenes* was grown in mannose (●,○); grown in mannose and treated with leucocin A (■,□); grown in mannose and treated with carnocyclin A (▲,△); grown in cellobiose (▼,▽); grown in cellobiose treated with leucocin A (◆,◇); grown in cellobiose treated with carnocyclin A (□,□) at 25 °C for 40 h. Optical density was measured at 630 nm and data is displayed as the average of four independent replicates.

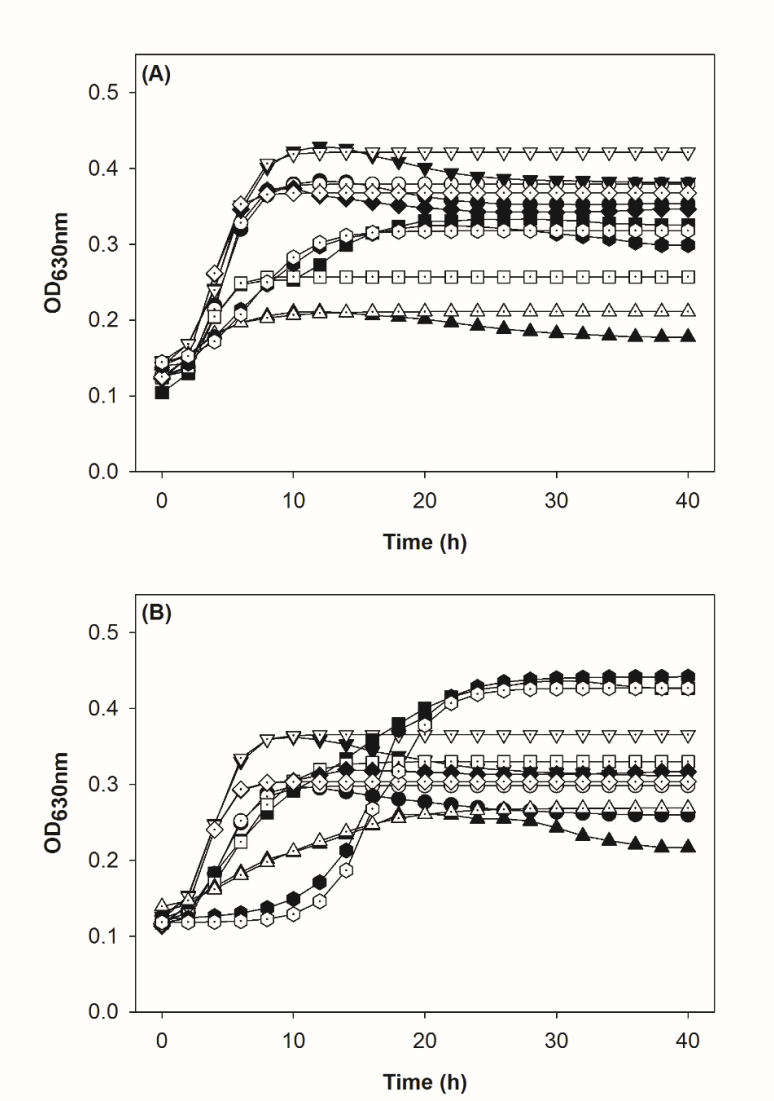

Figure S3.7 Experimental growth curve data (closed symbols) from isolated subpopulations from original cultures of *L. monocytogenes* strains FSL R2-499 (A) and FSL C1-056 (B) compared to the modelled growth curve data (open symbols). *L. monocytogenes* was grown in mannose (●,○); grown in mannose and treated with leucocin A (■,□); grown in mannose and treated with carnocyclin A (▲,△); grown in cellobiose (▼,▽); grown in cellobiose treated with leucocin A (◆,◇); grown in cellobiose treated with carnocyclin A (□,□) at 25 °C for 40 h. Optical density was measured at 630 nm and data is displayed as the average of four independent replicates.

Supplemental Tables S3.6

Table S3.1 Comparisons of maximal growth rate and lag phase of the original cultures and isolated subpopulations of *L. monocytogenes* determined at 25 °C in basal medium supplemented with mannose or cellobiose in the presence of 3.3 mM leucocin A or carnocyclin A (n=3).

			original culture		Subpopulation	
			μ_m*	λ†	μ_m	λ
FSL C1-056	mannose	control	0.22±0.02	3.85±0.55	0.23±0.07	2.04±0.35
		leucocin A	0.17±0.04	18.79±2.99	0.15±0.05	1.69±0.52
		carnocyclin A	0.10±0.02	23.43±4.20	0.07±0.01	1.76±1.62
	cellobiose	control	0.24±0.05	3.25±0.51	0.28±0.06	1.33±0.37
		leucocin A	0.45±0.21	15.00±1.36	0.30±0.11	1.38±0.28
		carnocyclin A	0.11±0.04	20.37±3.32	0.16±0.06	9.78±3.73
FSL R2-499	mannose	control	0.30±0.07	2.96±0.37	0.31±0.04	2.28±0.28
		leucocin A	0.14±0.04	4.20±1.07	0.23±0.13	1.86±1.33
		carnocyclin A	0.04±0.03	4.83±3.38	0.09±0.06	1.41±0.86
	cellobiose	control	0.33±0.04	2.39±0.81	0.25±0.09	1.68±0.99
		leucocin A	0.27±0.04	3.78±0.48	0.44±0.26	1.68±0.97
		carnocyclin A	0.07±0.05	3.98±1.97	0.11±0.02	2.31±0.80

* maximal growth rate (h^{-1}), † lag phase (h)

Appendix B

Supplemental Tables S4.5

Table S4.1 The number of sequences obtained from early stationary phase *L. monocytogenes* FSL C1-056 grown in different carbohydrates and treated with or with-out 3.3 mM carnocyclin A for mRNA and ncRNA.

Treatment Group	Original reads (n)	Reads after QC (n)	rRNA (16S + 23S + 5S)	reads mapped to ncRNA	reads mapped to ncRNA (n)	non-rRNA (n)	non-rRNA mapped to genome	non-rRNA mapped to genome (n)
Mannose Rep 1	50,787,746	46,259,024	73.07%	2.41%	1,114,718	16,081,682	56.47%	9,081,277
Mannose Rep 2	79,064,800	68,845,880	84.26%	2.30%	1,582,475	14,060,490	53.59%	7,535,149
Mannose Rep 3	83,659,454	73,298,418	83.06%	2.68%	1,966,260	14,642,804	59.80%	8,756,144
Mannose +CclA Rep 1	54,703,992	49,973,848	74.26%	2.25%	1,125,005	16,571,492	56.52%	9,365,952
Mannose +CclA Rep 2	82,556,522	71,665,322	90.86%	2.72%	1,949,768	8,582,076	39.77%	3,412,839
Mannose +CclA Rep 3	74,576,962	53,912,648	92.09%	2.11%	1,138,049	6,722,452	33.51%	2,252,979
Cellobiose Rep 1	50,654,478	46,168,656	74.23%	2.07%	955,148	15,532,594	56.73%	8,811,797
Cellobiose Rep 2	87,116,004	76,623,526	84.41%	2.29%	1,753,205	14,567,520	57.53%	8,380,870
Cellobiose Rep 3	92,956,620	80,866,444	87.89%	1.60%	1,295,438	12,856,282	53.60%	6,890,922
Cellobiose +CclA Rep 1	67,694,706	61,704,968	89.66%	1.75%	1,081,653	13,080,920	32.13%	4,203,530
Cellobiose +CclA Rep 2	88,392,444	75,516,128	92.61%	1.75%	1,321,161	7,536,368	42.73%	3,220,515
Cellobiose +CclA Rep 3	89,075,162	66,618,750	76.66%	2.59%	1,727,644	18,398,260	63.70%	11,719,075

[a] carnocyclin A

Table S4.2 *L. monocytogenes* FSL C1-056 annotated genome features.

https://doi.org/10.7939/r3-16qn-hy91

Table S4.3 Original number of reads (n) mapped to predicted genes of *L. monocytogenes* FSL C1-056 for each treatment.

https://doi.org/10.7939/r3-16qn-hy91

Table S4.4 Normalized number of reads (n) mapped to predicted genes of *L. monocytogenes* FSL C1-056 for each treatment.

https://doi.org/10.7939/r3-16qn-hy91

Table S4.5 Predicted ncRNA regions from *L. monocytogenes* FSL C1-056 genome.

https://doi.org/10.7939/r3-16qn-hy91

Table S4.6 Original number of ncRNA reads (n) mapped to predicted genes of *L. monocytogenes* FSL C1-056 for each treatment.

https://doi.org/10.7939/r3-16qn-hy91

Table S4.7 Normalized number of ncRNA reads (n) mapped to predicted genes of *L. monocytogenes* FSL C1-056 for each treatment.

https://doi.org/10.7939/r3-16qn-hy91

Table S4.8 Differential expression for predicted and matched ncRNA regions of *L. monocytogenes* FSL C1-056.

https://doi.org/10.7939/r3-16qn-hy91