Lactococcus lactis subsp. cremoris JFR1 suppresses virulence gene expression of Salmonella in intestinal epithelial cells

by

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ABSTRACT

*LACTOCOCUS LACTIS SUBSP. CREMORIS JFR1 SUPPRESSES VIRULENCE GENE EXPRESSION OF SALMONELLA IN INTESTINAL EPITHELIAL CELLS*

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University of Guelph, 2018

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A major foodborne pathogen, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, causes disease in humans by expressing virulence genes to invade the host epithelial cells. Interactions between food or food components, pathogens, and the host environment could alter the fate of pathogen colonization. Thus, the search for food/food components bearing antivirulence properties could contribute to the reduction of *Salmonella* invasion. This research revolves around utilizing *Lactococcus lactis* subsp. *cremoris* JFR1 (JFR) treatments on intestinal epithelial cells to determine the effects on *Salmonella* invasion and virulence properties. Comparisons of JFR exopolysaccharide producing and nonproducing strains demonstrated that both strains could down regulate the virulence gene expression and reduce attachment of *Salmonella* to HT-29 epithelial cells. However, further analysis utilizing exopolysaccharides extracted from JFR revealed that there was no significant effect on *Salmonella* virulence gene expression or attachment onto HT29 epithelial cells at the concentrations ranging between 0-0.2 mg/mL. In intestinal epithelial cell models comprised of mucus producing cells (HT29-MTX), absorptive type cells (Caco-2) and cocultures, JFR treatments of epithelial cells suppressed and reduced invasion of only *Salmonella* Typhimurium DT104 but not *Salmonella* lacking OppA. This was also demonstrated when milk fermented with JFR strains were applied to epithelial cells. Suppression of *Salmonella* virulence, lower numbers of invasive bacteria as well as reduced epithelial cell production of inflammatory cytokine and higher transepithelial electrical resistance was observed only when
infected by *S. Typhimurium* DT104. Infection of epithelial cells by *S. Typhimurium* mutant ∆oppA was unresponsive to treatments applied and did not show differences in virulence and invasion compared to controls. Furthermore, treatments of epithelial cells by digested of JFR fermented milk treatments lost its ability to suppress virulence genes in both *Salmonella* strains, regardless of a functional OppA. These results indicate the importance of OppA and its role in virulence gene suppression in the presence of JFR treatments. A possible mode of action could be the presence of peptides, of a certain length, in JFR treatments which harbour antivirulence properties perhaps by acting as quorum sensing inhibitors or interacting directly with the enzyme responsible for autoinducer production.

Keywords: *Salmonella enterica* Typhimurium, *Lactococcus lactis* subsp. *cremoris* JFR1, exopolysaccharides, virulence, *in vitro*, oligopeptide transport, fermented milk
DEDICATION

To my friends and family, always and forever.
FOREWORD

All of the work presented in this dissertation was completed at the University of Guelph, Canadian Research Institute for Food Safety, Department of Food Science. Financial support was provided by the Canadian Dairy Commission, Ontario Dairy Council (M. Corredig), and NSERC/DFO Industrial Research Chair in Dairy Microbiology (G. LaPointe).

Chapter 1. A review of literature providing background on necessary information needed to understand the thesis.

Chapter 2. A version of this material has been published as Zhang, J.S., Guri, A., Corredig, M., Morales-Rayas, R., Hassan, A., Griffiths, M., and LaPointe, G. (2016) entitled “Lactococcus lactis subsp. cremoris strain JFR1 attenuates Salmonella adhesion to human intestinal cells in vitro” in Food Research International. Purification of exopolysaccharides was performed in the department of Food Science with the help of Eleana Kristo. Confocal microscopy imaging was completed with the assistance of Sandy Smith. All additional experiments were performed by the author, Justina Zhang.

Chapter 3. This section contains original and unpublished work completed primarily by Justina Zhang with help from summer students. Rocio Morales-Rayas provided assistance in the design the methodologies presented in sections 3.2.4, 3.2.5, and 3.2.6. This manuscript was written by me and edited by Rocio Morales-Rayas and Gisèle LaPointe. It is in preparation for submission to Probiotics and Antimicrobial Proteins.
Chapter 4. This chapter has been submitted to the Journal of Dairy Science. The manuscript was written by Justina Zhang and edited by Rocio Morales-Rayas, Mansel Griffiths, and Gisèle LaPointe. Experiments were completed and analyzed by Justina Zhang. Methodologies in sections 4.2.6-8 were designed by Rocio-Morales-Rayas and I.

Chapter 5. Provides a general discussion and conclusion of the research. The limitations to the work and future directions were also discussed.
ACKNOWLEDGEMENTS

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I would like to send hugs over to Rocio, Sandy and Tricia for all of their help, reassurance, and listening ear during the last few years. Of course, all the lab mates and summer students that I have had the pleasure of working with, especially in our cozy tissue culture lab, thank you all for a great learning experience. I won’t go on to thank each person one by one because, knowing me, I am going to forget someone!

Last but not least, I want to thank my friends, family and Penny for their unconditional love and support. I am extremely grateful to have all of you, through the good times and the bad. Your patience and comforting words (or barking, in Penny’s case) helped me through the rough patches and temper tantrums. To my husband, who I met in CRIFS, thank you for putting up with me and staying by my side. To my parents, you guys are amazing and none of this would have been possible without the two of you, literally! I also could not have made it without McDonalds, Pokemon, and the gym! In conclusion, I love you all!
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<th>Description</th>
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<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin-I converting enzyme</td>
</tr>
<tr>
<td>AHL</td>
<td>Acyl-homoserine lactone</td>
</tr>
<tr>
<td>AI-2</td>
<td>Autoinducer-2</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATR</td>
<td>Acid tolerance response</td>
</tr>
<tr>
<td>C</td>
<td>Carbon</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control</td>
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<tr>
<td>CFU</td>
<td>Colony forming units</td>
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<tr>
<td>CRIFS</td>
<td>Canadian Research Institute for Food Safety</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dpp</td>
<td>Dipeptide permease</td>
</tr>
<tr>
<td>DT104</td>
<td><em>Salmonella enterica</em> Typhimurium DT104</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EPS</td>
<td>Exopolysaccharides</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GDL</td>
<td>Glucono-delta lactone</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
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<tr>
<td>HSL</td>
<td>Acylated homoserine lactone</td>
</tr>
<tr>
<td>IBD</td>
<td>Intestinal bowel disease</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>JFR+</td>
<td><em>Lactococcus lactis</em> subsp. <em>cremoris</em> JFR1 (EPS producing)</td>
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<tr>
<td>JFR-</td>
<td><em>Lactococcus lactis</em> subsp. <em>cremoris</em> JFR1 (EPS nonproducing)</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>LAB</td>
<td>Lactic acid bacteria</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>Lrp</td>
<td>Leucine responsive regulatory protein</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>N</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NEAA</td>
<td>Nonessential amino acids</td>
</tr>
<tr>
<td>Opp</td>
<td>Oligopeptide permease</td>
</tr>
<tr>
<td>OppA</td>
<td>Oligopeptide permease A</td>
</tr>
<tr>
<td>ΔoppA</td>
<td><em>Salmonella enterica</em> Typhimurium ΔoppA</td>
</tr>
<tr>
<td>P</td>
<td>Phosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>QS</td>
<td>Quorum sensing</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RTE</td>
<td>Ready-to-eat</td>
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<tr>
<td>RT-qPCR</td>
<td>Real time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SCV</td>
<td><em>Salmonella</em> containing vacuole</td>
</tr>
<tr>
<td>SHIME</td>
<td>Simulator of the human intestinal microbial ecosystem</td>
</tr>
<tr>
<td>SPI1</td>
<td><em>Salmonella</em> pathogenicity island 1</td>
</tr>
<tr>
<td>SPI2</td>
<td><em>Salmonella</em> pathogenicity island 2</td>
</tr>
<tr>
<td>SRB</td>
<td>Sulforhodamine B</td>
</tr>
<tr>
<td>SSR</td>
<td>Starvation stress response</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TEER</td>
<td>Transepithelial electrical resistance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>TJP</td>
<td>Tight junction proteins</td>
</tr>
<tr>
<td>Tpp</td>
<td>Tripeptide permease</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
</tr>
<tr>
<td>T3SS</td>
<td>Type 3 secretion system</td>
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Introduction

Among all the measures taken for foodborne illness, food hygiene and surveillance are stressed as the most effective ways to prevent foodborne illness. In Canada, for instance, approximately 4 million episodes of foodborne illness occurs each year. Of the estimated total of 11,632 hospitalizations and 238 deaths, over one third of hospitalizations and deaths are caused by norovirus, nontyphoidal *Salmonella* subspecies, *Campylobacter* subspecies, *Escherichia coli*, and *Listeria monocytogenes*. Human salmonellosis is a worldwide public health concern with over 90% of cases associated with the consumption of contaminated foods such as poultry, eggs, pork and fresh produce. The most common form of salmonellosis is enterocolitis, which arises within 12 to 36 hours after consumption of contaminated food. Symptoms of enterocolitis include abdominal pain, nausea, vomiting, diarrhea, and headache.

Pathogenesis of *Salmonella* involves the expression of multiple virulence genes located on *Salmonella* pathogenicity islands. The pathogen invades nonphagocytic host cells, such as epithelial cells, by forming a needle-like complex to deliver effector proteins to the host cell. This process causes host cell cytoskeleton rearrangement and finally uptake of the *Salmonella*. Once inside the host cell, *Salmonella* reside in membrane-bound compartment knows as the *Salmonella*-containing vacuole where it survives and replicates. Subsequently, more effector proteins are released to modify the cellular environment and induce the release of *Salmonella* allowing it to reach deeper tissue.

The human gastrointestinal tract is the largest body surface in contact with the exterior environment, inhabited by over $10^{10}$ colony forming units (CFU) of microorganisms. The intestinal epithelium acts as the first line of defense to prevent pathogen invasion and
colonization leading to increased intestinal permeability, which is implicated with many gastrointestinal disorders. The relationship and balance between the host, microbes as well as food components has been reported to play an important role in gastrointestinal health. The development of antibiotic resistant strains is an extremely challenging issue, especially with the rise of multi-drug resistant strains such as *Salmonella* Typhimurium DT104. Several gastrointestinal disorders including diarrheal diseases such as traveller’s diarrhea, antibiotic-associated diarrhea, as well as inflammatory bowel disease have shown to be prevented or alleviated by probiotic treatments. Foods of interest, which provide benefits beyond nutrition, have also been reported to positively effect several gastrointestinal disorders. For instance, milk fermented by LAB including *Lactobacillus casei*, *L. paracasei*, *L. acidophilus*, and *L. rhamnosus* fermented milk have shown preventative and inhibitory effect of different pathogens such as *Salmonella* and *Staphylococcus aureus* as well as antibiotic-associated diarrhea and gastrointestinal infection. Moreover, research on lactic acid bacteria and probiotics as well as their metabolites have shown the potential to treat gastrointestinal disorders and regulate pathogen virulence. Recent evidence has reported the production of bioactive components during milk fermentation by lactic acid bacteria revealing health promoting effects. For instance, bioactive fractions isolated from cell free spent medium of *Lactobacillus acidophilus* La-5 and *Lactobacillus helveticus* demonstrated immunomodulating effects on the host both *in vitro* and *in vivo* as well as virulence gene suppression of pathogens. The potential demonstrated by lactic acid bacteria and/or their components exerting effects on pathogen virulence leads to the purpose of this study: to investigate the effects of *Lactococcus lactis* subsp. *cremoris* JFR1 on *Salmonella* virulence and invasion of intestinal epithelial cells.
Chapter 1. Literature Review

1.1 *Salmonella enterica*

*Salmonella* are motile, gram-negative, rod shaped facultative intracellular anaerobes capable of causing disease in a wide range of hosts including humans, sheep, chicken and cattle (Blaser & Newman, 1982; Underwood et al., 2015). *Salmonella* are divided into two species, *Salmonella bongori*, which consists of 22 serovars (Grimont & Weill, 2008), and *Salmonella enterica* consisting of six subspecies (I to VI), the most important being subspecies I, *enterica*, containing over 2500 serovars (Percival & Williams, 2014). Most *Salmonella enterica* serovars associated with disease in humans belong to subspecies I, which consists of typhoid (e.g. *Salmonella* Typhi) and nontyphoid (e.g. *Salmonella* Typhimurium) serovars (Percival & Williams, 2014).

1.1.1 *Salmonella as a foodborne pathogen*

*Salmonella* is a foodborne pathogen causing major public health concern. Worldwide, it is estimated that *Salmonella* causes 93.8 million human cases annually (Majowicz et al., 2010). In the United States alone, over one million cases of salmonellosis are reported each year with approximately 600 deaths (Mead et al., 1999). Between 2000 and 2010, a Canadian case study reported that non-typhoidal *Salmonella* was ranked third among the major foodborne pathogens with an approximate number of 88,000 salmonellosis cases recorded in Canada each year (Thomas et al., 2013).

Although there are over 2500 serovars of *Salmonella enterica*, most human cases are confined to a limited number of serotypes (Jones et al., 2008). The Centers for Disease Control and Prevention (CDC) informed the most common serotypes causing foodborne outbreaks in the
U.S. include *S. Enteritidis*, *S. Typhimurium*, *S. Newport*, *S. Heidelberg* and *S. Javiana* (CDC, 2010), but the two most frequently reported causes for human salmonellosis are *Salmonella enterica* serovars Enteritidis (Rodrique, Tauxe, & Rowe, 1990) and Typhimurium (Hendriksen et al., 2011).

Major sources of outbreak arise from animals, in particular food animals, such as poultry and pigs through the contamination of food or water (Helms, Ethelberg, & Mølbak, 2005; Hendriksen et al., 2011). Approximately 95% of *Salmonella* infections are foodborne through the consumption of contaminated food products as well as improper storage or cooking of ready-to-eat foods (RTE) while the remaining cases result from direct exposure to animal carriers (Linam & Gerber, 2007). Contamination and temperature abuse of RTE foods readily leads to a sufficient outbreak dose of $10^1$ to $10^3$ (Todd, Greig, Bartleson, & Michaels, 2008), furthermore, the majority of food handler associated outbreaks of *Salmonella* contamination happen in RTE egg-based mayonnaise salad items (Todd et al., 2007). The infectious dose of salmonellosis in humans ranges from $10^1$ to $10^9$ CFU depending on the strain type and the status of the individual (Blaser & Newman, 1982; Hara-Kudo & Takatori, 2011; Kothary & Babu, 2001; McCullough & Eisele, 1951). More susceptible groups include the elderly, very young children or immune-compromised individuals. Symptoms may include fever, vomiting, diarrhea and in severe cases septicemia and death (Helms et al., 2005).

### 1.1.2 *Salmonella* pathogenesis

The gastric acidity and rapid transit time of the bolus through the stomach (approximately 30 minutes) may discourage the colonization of many bacteria (Dethlefsen, Eckburg, Bik, & Relman, 2006; Schwarz, Kaspar, Seelig, & Kunnecke, 2002). Additionally, it is reported that the infectious dose of *Salmonella* is lower when contaminated foods have quicker transit time
through the stomach (e.g. liquids) or when stomach acidity is raised by accompanying food (Lynch & Tauxe, 2009). Therefore, the interaction between *Salmonella* and the intestinal mucosa to initiate attachment is crucial for its invasion and colonization.

1.1.2.1 Attachment

*Salmonella* are pathogens which induce their own uptake into normally non-phagocytic cells by inducing the rearrangement of the host cell cytoskeleton through secretion of virulence factors at close proximity to the target host cell (Altier, 2005; Darwin & Miller, 1999). Finger-like projections known as fimbriae, present on the bacterial cell surface, play a critical role in attachment to epithelial cell surfaces by attaining close contact (Edwards, Schifferli, & Maloy, 2000). Several fimbrial systems have been identified in *Salmonella* which synergistically contribute as well as compensate one another for pathogenesis. The best characterized systems include type 1 fimbriae, long polar fimbriae, plasmid-encoded fimbriae and thin aggressive fimbriae (Darwin & Miller, 1999). Type 1 fimbriae bind specifically to α-D-mannose receptors present on many eukaryotic cell types. Although it has been reported that type 1 fimbriae aid in attachment of *S. Typhimurium* to HeLa cells, others have reported no apparent role of type 1 fimbriae in HEp-2, T84, Int-407 or MDCK cell lines (Bäumler, Tsolis, & Heffron, 1996a). Long polar fimbriae have been shown to mediate attachment of *S. Typhimurium* to murine Peyer’s patches (Bäumler, Tsolis, & Heffron, 1996b) though a mutation of *lpfC*, which encodes outer membrane fimbrial usher protein, resulted in decreased colonization of Peyer’s patches and adhesion to HEp-2 cells (Bäumler, Tsolis, & Heffron, 1996a; 1996b). Furthermore, there is evidence that long polar fimbriae in *Salmonella* Typhi preferentially target enterocytes, as shown in a Caco-2 model (Gonzales, Wilde, & Roland, 2017). The third fimbriae system, plasmid-encoded fimbriae (*pef*) is encoded on the *Salmonella* virulence plasmid (pSLT). It is considered a necessity for full virulence of *Salmonella* and cause of systemic infection in *in*
vivo studies (Gulig & Curtiss, 1987; Gulig, Caldwell, & Chiodo, 1992). Experiments using an intestinal-organ culture model showed that S. Typhimurium pefC mutant could not adhere to the small intestine as well as the wild type indicating its importance for colonization (Bäumler, Tsolis, Bowe, Kusters, Hoffmann, & Heffron, 1996c). The autoaggregative phenotype exhibited by some strains are dependent on the production of thin aggregative fimbriae or curli, which is believed to improve survival ability of Salmonella in hostile environments such as the acidic stomach and other antimicrobials secreted by the host (Collinson et al., 1993). Thin aggregative fimbriae aid in the binding of S. Enteritidis to fibronectin found in the eukaryotic extracellular matrix (Collinson et al., 1993). Additionally, a mutation eliminating thin aggregative fimbriae production decreased attachment of S. Typhimurium to murine small intestinal cells (Sukupolvi et al., 1997). In addition, Van Der Velden et al., (1998) reported that after mutation of all four fimbriae systems just mentioned, S. Typhimurium was still able to colonize, though in lower numbers, murine models, moreover, electron microscopy imaging presented uncharacterized fimbrial structures (Van Der Velden et al.1998). Collectively, fimbriae systems play a role in bringing the bacteria into closer proximity to the host cell for effective secretion of effectors to carry out the next step of invasion.

1.1.2.2 Salmonella pathogenicity island 1

Once close to the host cell, Salmonella prompts its own uptake into several cell types within the host gastrointestinal tract including non-phagocytic epithelial cells (Tsolis et al., 1999). The SPI1 (Salmonella pathogenicity island 1) encodes the type III secretion system (T3SS) which is necessary for intestinal infection of Salmonella (Altier, 2005; Galán, 1999; Ochman, Soncini, Solomon, & Groisman, 1996). The SPI1 encodes at least 33 proteins including regulatory and effector proteins responsible for components of the T3SS and invasion process (Bajaj, Lucas, Hwang, & Lee, 1996; Collazo, Zierler, & Gatan, 1995; Fu & Galán, 1998; Johnston, Pegues,
Hueck, Lee, & Miller, 1996). It has been demonstrated that a T3SS deficient *Salmonella* Typhimurium was drastically less virulent compared to the wild type exhibiting defects in colonization and invasion (Murray & Lee, 2000). The central regulator HIL (hyperinvasive locus) is essential for the expression of the T3SS apparatus; the activation of structural genes and another regulatory gene *invF*, which triggers expression of bacterial effectors (Gerlach, Jäckel, Geymeier, & Hensel, 2007; Lostroh, Bajaj, & Lee, 2000). The formation of a syringe-like apparatus is used to inject effectors into the host cell that promote cytoskeleton rearrangement and subsequent uptake (Galán, 1999). *Salmonella* invasion proteins (Sip) such as SipB and SipC are secreted through the T3SS to form a translocon pore on the plasma membrane of the host cell allowing for further delivery of effectors into the cytoplasm (Bakowski, Cirulis, Brown, Finlay, & Brumell, 2007; Darwin & Miller, 1999). Rearrangement of the cytoskeleton causing membrane ruffles are a result of effectors such as SopB, SopD, and SipA facilitating engulfment of the *Salmonella* by actin remodeling and micropinosome formation (Bakowski et al., 2007; Hernandez, Hueffer, Wenk, & Galán, 2004; Lostroh et al., 2000). The membrane-bound compartment which now harbors *Salmonella* is known as the *Salmonella* containing vacuole (SCV), where replication is promoted by SPI2 (Méresse, Steele-Mortimer, Finlay, & Gorvel, 1999).

1.1.2.3 *Salmonella* pathogenicity island 2

The SPI2 encoded T3SS is expressed after *Salmonella* internalization and translocates bacterial effectors that advance SCV maturation, intracellular survival, replication leading finally to the systemic infection phase (Bakowski et al., 2007; Gorvel & Méresse, 2001; Waterman & Holden, 2003). SPI2 encoded T3SS produces over 20 effector proteins which are translocated across the SCV membrane to modify the cellular environment allowing *Salmonella* to replicate and cause apoptosis of the host cell for systemic infection in murine models (Hensel et al.,
A mutant of *Salmonella* lacking *sifA* (*Salmonella*-induced filament) function demonstrated inability of SIF biogenesis, the formation of a complex network throughout the host cell promoting interaction with organelles, implicated with intracellular survival and proliferation of *Salmonella* (Knuff & Finlay, 2017). Furthermore, SPI2 mutants showed impaired ability to multiply within murine macrophages and human epithelial cells (Cirillo, Valdivia, Monack, & Falkow, 1998). Within macrophages, *Salmonella* are able to cause apoptosis through the interaction of SipB, which is secreted by SPI1 T3SS, with caspase-1 leading to cell death and bacterial dissemination into the surrounding tissues for further infection and colonization of other organs including the liver and spleen (Fink & Cookson, 2005; 2006). Whereas in epithelial cells, the onset of cell apoptosis is delayed compared to macrophage phagocytosis (Paesold et al. 2002). Paesold et al., (2002) suggested that specific effectors secreted during the beginning of intracellular invasion, which are translocated across the SCV, provoke a pro-apoptotic signal in the host cell causing the execution phase of apoptosis to take place after SPI1 invasion genes are no longer expressed (Paesold et al., 2002). It is presumed that delayed cell death could benefit *Salmonella* by allowing sufficient time for adaptation and gene expression for invasion of deeper mucosal tissue as well as the host cell allowing for cell signalling and communication and inflammatory response in attempt to restore epithelial cell regulation and maintenance of the intestinal barrier (Paesold et al., 2002). The process of *Salmonella* invasion and infection is not a direct process but rather a simultaneous cooperation of many pathways and networks for the numerous bacterial-host interactions striving for survival.

### 1.1.3 Nutritional stress and survival of *Salmonella*

*Salmonella* can enter the host through the consumption of contaminated food or beverages. Once ingested, the microenvironment surrounding the bacteria changes, so in order to survive,
*Salmonella* must adapt and respond to various stress factors. *Salmonella* first encounters a decrease in pH as it reaches the stomach, ranging from 1 to 2, although the presence of other food substances, especially those high in fat or protein content may protect *Salmonella* against the low pH (Waterman & Small, 1998). Upon exposure to low pH, *Salmonella* exhibits well developed systems for acid survival including the acid tolerance response (ATR). The ATR system induces the production of many preshock and acid shock proteins to enhance acid resistance and *Salmonella* survival (Foster, 1991; Foster & Spector, 1995). Under these acid stress conditions, several regulons are responsible for the detection and adaptation in the stomach (Slauch, Taylor, & Maloy, 1997). RpoS functions in controlling the expression of specific genes under stress conditions to improve resistance and survival of *Salmonella* (Rychlik & Barrow, 2005) and is responsible for stress tolerance in both exponential and stationary growth phases. During the exponential growth phase, *rpoS* dependent acid tolerance requires 60 to 120 minutes of adaptation time, during which, more than 50 proteins are induced, specifically *rpoS* dependent (Foster, 1991; Lee, Lin, Hall, Bearson, & Foster, 1995; Lin, Lee, Frey, Slonczewski, & Foster, 1995). Although a slight ATR is still demonstrated in mutants defective in *rpoS*, the level of acid resistance is significantly reduced due the importance of the 7 *rpoS* dependent shock proteins (Lee et al., 1995). Acid shocked *Salmonella* also demonstrate a level of cross protection to osmostic stress (Foster & Spector, 1995), which is encountered as the bacteria moves further down the gastrointestinal tract.

Leaving the stomach, *Salmonella* enters the small intestine where it is exposed to multiple stress factors including oxygen and osmolarity. As oxygen availability decreases through the intestine, *Salmonella* shift from an aerobic metabolism to an anaerobic metabolism. Genes encoding the enzymes for the TCA (tricarboxylic acid) cycle are suppressed under less favorable conditions to save energy sources and to decrease the production of harmful oxygen
radicals (Sevcík, Sebková, Volf, & Rychlík, 2001). Moreover, oxygen is an important factor in expression of invasion genes where SPI-1 virulence genes are maximally expressed under oxygen limiting conditions (i.e. the intestines) (Bajaj, Lucas, Hwang, & Lee, 1996; B. D. Jones & Falkow, 1994). In addition, the osmotic pressure may also act as a signal for invasion by inducing hilA expression through OmpR (Bajaj, Lucas, Hwang, & Lee, 1996; Galán & Curtiss, 1990; Lucas & Lee, 2001). The osmotic pressure is more than 300 mOsm in the small intestine (Fordtran, Rector, Ewton, Soter, & Kinney, 1965) and in response to this increase in osmolarity, Salmonella can increase the uptake of solutes such as potassium to maintain turgor (Sutherland, Cairney, Elmore, Booth, & Higgins, 1986) or to modify the composition of the outer membrane. Modifications to the outer membrane are sensed and controlled by the EnvZ sensor and OmpR regulator (Chatfield, Dorman, Hayward, & Dougan, 1991). Under high salt environments, the OmpF porin is replaced by the OmpC porin forming small pores of smaller diameter to reduce the influx of solutes (Chatfield et al., 1991). Furthermore, through the passage of the small intestine, pH levels rise to near neutral which provides another environmental signal for invasion inducing hilA expression (Bajaj, Lucas, Hwang, & Lee, 1996). Another environmental cue for Salmonella invasion is the level of acetate. In the distal ileum, where the primary site of Salmonella invasion is located, the acetate concentration rises to approximately 15-30 mM, and in conjunction with the neutral pH, expression of SPI-1 is induced through hilA and invF regulators (Durant et al., 1999; Lawhon, Maurer, Suyemoto, & Altier, 2002).

The starvation stress response (SSR) of Salmonella can be induced by starvation for phosphate (P), carbon (C), and/or nitrogen sources (N). Because of their lifestyle, Salmonella occupy a wide range of niches and must be able to tolerate and adapt to periods of nutrient starvation. For instance, during invasion of the host cell, several unique proteins are induced in Salmonella within the host environment (i.e. host cell; SCV) to enhance survival. Several P starvation-
inducible (psi) loci, C starvation-inducible (csi) loci, and multiple nutrient starvation-inducible (sti) loci have been identified, where stiA, stiB, and stiC are indispensable for *Salmonella* survival during simultaneous P, C, and N starvation (Spector & Cubitt, 1992). The regulation of starvation inducible genes is positively and negatively controlled by CRP (cyclic AMP receptor protein) and RpoS. In non-starved *Salmonella*, CRP (cyclic AMP receptor protein) functions to negatively regulate genes including stiA, stiB, and stiC (Spector & Cubitt, 1992). However, during starvation, *rpoS* positively regulates starvation-inducible gene expression of stiA and stiC as well as virulence operon spvABCD expressed in the liver/spleen during infection (Fang et al., 1992; Guiney, Libby, Fang, Krause, & Fierer, 1995). It has been demonstrated in the mouse virulence model that *rpoS* is necessary for full oral virulence of *Salmonella* (Fang et al., 1992; Ibanez-Ruiz, Robbe-Saule, Hermant, Labrude, & Norel, 2000). Furthermore, mutations in *rpoS*, stiA, stiB, and stiC reduce starvation survival by up to 100-fold (O’Neal et al., 1994; Spector & Cubitt, 1992).

### 1.1.4 Quorum sensing in enteric pathogens

Quorum sensing (QS) refers to cell to cell bacterial communication both among and between species via small signaling molecules known as autoinducers (Gellatly & Hancock, 2013). The accumulation of these membrane-diffusible molecules increases as the bacterial cell density increases; and once the threshold level is reached, the bacterial entity can then express genes for certain phenotypes, especially virulence associated genes (Podbielski & Kreikemeyer, 2004). This competitive mechanism allows for bacteria to monitor, adapt and modify gene expression to increase their survival in various environments. For instance, *Pseudomonas aeruginosa* do not express their virulent phenotype until the bacterial population size has become large enough to overpower the defense mechanisms of the host (Gellatly & Hancock, 2013; Kalia, 2013).
QS was first identified in two luminescent marine bacteria *Vibrio harveyi* and *Vibrio fischeri* where light emission was only observed at high bacterial densities corresponding to autoinducer molecule accumulation (Nealson & Hastings, 1979). The signaling molecules AIP (autoinducing peptide) and HSL (acylated homoserine lactone), produced by *V. harveyi* and *V. fischeri* function mainly in bacterial communication within their own species, but more often than not, multiple species will inhabit the same ecosystem such as the oral cavity or human gut (Jayaraman & Wood, 2008; Palmer, Kazmerzak, Hansen, & Kolenbrander, 2001). Originally found in *V. harveyi*, the luxS gene is associated with the synthesis of autoinducer-2 molecules (AI-2), which functions both in intraspecies and interspecies communication (De Keersmaecker, Sonck, & Vanderleyden, 2006; Surette, Miller, & Bassler, 1999). Homologues of luxS have been identified in a number of Gram-positive and Gram-negative bacteria including *Bacillus subtilis, Mycobacterium tuberculosis, Escherichia coli, S. Typhimurium, S. Typhi, Clostridium perfringens and Clostridium difficile*, many of which are enteric pathogens (Anand & Griffiths, 2003; Bassler, 1999; De Keersmaecker et al., 2006; Sperandio, Mellies, Nguyen, Shin, & Kaper, 1999; Surette et al., 1999).

Among the many bacterial species that contain the luxS gene are two of the major foodborne pathogens, *S. Typhimurium* and *E. coli* (Perrett et al., 2009; Sperandio et al., 1999; Surette et al., 1999). The LuxS enzyme is directly involved in the formation of AI-2 signaling molecules in these microorganisms (Perrett et al., 2009; Schauder, Shokat, Surette, & Bassler, 2001; Surette et al., 1999). S-ribosylhomocysteine, which is a product of the S-adenosylmethionine utilization pathway, is cleaved to by the LuxS enzyme to release homocysteine and 4,5-dihydroxy-2,3-pentanedione, the precursor of AI-2 (Schauder et al., 2001; Taga, Semmelhack, & Bassler, 2001; Winzer et al., 2002). Contrary to the differing structures of signaling
molecules produced during intraspecies communication, the AI-2 molecules secreted from different species of bacteria appear to be structurally identical (Taga et al., 2001). For example, the inactivation of luxS in S. Typhimurium LT2 resulted in the inability to produce AI-2 molecules, however, complementation with either the E. coli O157:H7 or V. harveyi BB120 luxS genes restored production (Surette et al., 1999).

Although Salmonella possess a receptor encoded by sdiA which can detect autoinducer AHL (acyl-homoserine lactone) produced by many gram-negative bacteria, they do not produce AHL for communication (Walters & Sperandio, 2006). In S. Typhimurium, luxS is responsible for the regulation of the luxS-regulated operon (lsr) for AI-2 production (Taga et al., 2001). The lsr operon encodes a transporter homologous to the ribose ABC transporter of both S. Typhimurium and E. coli, which functions in the uptake of AI-2 molecules (Perrett et al., 2009; Taga et al., 2001). There are 6 genes encoded on the lsr operon in E. coli responsible for AI-2 uptake and modification: lsrA, lsrC, lsrD, lsrB encode the transport apparatus while lsrF, lsrG are putative AI-2 processing genes for the removal of phosphorylated AI-2 (Hooshangi & Bentley, 2011; L. Wang, Hashimoto, Tsao, Valdes, & Bentley, 2005a; L. Wang, Li, March, Valdes, & Bentley, 2005b). A highly homologous system is used in S. Typhimurium (Taga et al., 2003). The LsrR transcriptional regulator inhibits the lsr operon by binding to the promotor site (Taga et al., 2003). However, upon AI-2 translocation into the cell, phosphorylation of the signaling molecule allows for the interaction with LsrR preventing lsr operon inhibition (Taga et al., 2001; 2003). Thus, a positive feedback loop is created where increased concentration of AI-2 inside the cell results in more uptake of AI-2 (Hooshangi & Bentley, 2011). The lsr complex has been reported to exist predominantly in pathogens associated with endotherms suggesting the importance of QS in the interactions between bacteria and host (Rezzonico & Duffy, 2008).
1.1.5 In vitro models to investigate Salmonella pathogenesis

The use of intestinal epithelial cell models presents a time and cost-efficient model to study the many interactions that may occur in the human gastrointestinal tract, including drug absorption, toxicity, host-pathogen or host-probiotic interactions and even the role of epithelial cells in immune responses (CenciČ & Langerholc, 2010; Eriksen, Vegarud, Langsrud, Almaas, & Lea, 2008; Gopal, Prasad, Smart, & Gill, 2001). The epithelial barrier is an indispensable part of the human gastrointestinal tract providing protection and selectively controlling permeability, thus, different in vitro models have been developed for various scenarios and environments. To investigate the mechanisms of intestinal colonization of enterocolitis-causing Salmonella serovars such as Typhimurium and Enteritidis, several in vitro models have been reported. Most common cell models include HT-29, Caco-2, HT29-MTX, T84 and HeLa cell lines (Bakowski et al., 2007; Gagnon, Zihler Berner, Chervet, Chassard, & Lacroix, 2013; Greene & Klaenhammer, 1994; Mellor, Goulter, Chia, & Dykes, 2009; Nandakumar, Pugazhendhi, & Ramakrishna, 2009; Vizoso Pinto et al., 2009).

Epithelial cells differentiate and proliferate from stem cells located at the base of the crypts located in the small and large intestine. The 4 main types of differentiated epithelial cells include enteroendocrine, Paneth, absorptive enterocytes and goblet cells (Kim & Ho, 2010; Mahida, 2004). Absorptive enterocyte cells and goblet cells are the two most abundant epithelial cell types. Enterocytes form a polarized layer containing an apical brush functioning in nutrient and fluid uptake as well as select transporter proteins (van der Flier & Clevers, 2009; Verburg et al., 2000). Goblet cells are specialized epithelial cells by which at maturation secrete the mucins that offer the protective layer over the intestinal epithelium (Brittan & Wright, 2004). The membrane bound secretory vesicles are located at the apical region of the cell and as maturation is reached, increased number of mucus vesicles are formed (Deplancke &
Thus, enterocytes and goblet cells are widely used either alone in a mono-culture cell model or in combination as a co-culture cell model to more closely mimic the environment of the intestine.

The HT-29 cell line was originally isolated from a human colon carcinoma (Fogh & Trempe, 1975) and forms a multilayer of undifferentiated cells when in culture (Lesuffleur et al., 1990). However, when these cells are cultured under conditions causing metabolic stress, differentiation into specialized cells occur (Lesuffleur et al., 1990). Deprivation of glucose and glutamine to the cells (Huet, Sahuquillo-Merino, Coudrier, & Louvard, 1987; Zweibaum et al., 1985), as well as treatment with sodium butyrate (Augeron & Laboisse, 1984), 5-fluorouracil (Lesuffleur, Kornowski, et al., 1991b) or methotrexate (MTX) (Lesuffleur et al., 1991a) result in the formation of polarized monolayers or absorptive or mucus producing cells (Lesuffleur et al., 1990). There are many subpopulations of HT-29 such as HT29-MTX and HT29-16E, which, depending on the adaptation process, express different mucin genes. HT29-MTX is one of the most commonly used mucus secreting intestinal cell lines due to the broad range of mucin genes expressed: MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, and MUC12 (Gouyer et al., 2001; Lesuffleur et al., 1993; Moal et al., 2002; Pigny et al., 1996).

Most commonly used in combination with HT29-MTX cells in co-culture models are Caco-2 cells, which was also derived from a colon carcinoma. In culture, Caco-2 cells spontaneously differentiate into polarized enterocyte-like cells which represent the most abundant cell type found in the intestinal epithelium (Hilgendorf et al., 2000). Caco-2 cells form a columnar epithelial cell with a well-developed brush border on the apical surface and are characterized by the formation of tight junction complexes which act as a seal near the apical surface of adjacent epithelial cells (Hidalgo, Raub, & Borchardt, 1989). In vitro models of Caco-2 cells have been used extensively to investigate drug and nutrient absorption, however, when cultured
on permeable filter membranes, intestinal barrier permeability can be evaluated (Anderson, Cookson, McNabb, Kelly, & Roy, 2010). Measurement of transepithelial electrical resistance (TEER) is a non-invasive technique to determine barrier permeability in studies of host-microbe interactions and host response to bioactive compounds. The inverse relationship of TEER and barrier permeability is demonstrated by higher TEER measurements corresponding to less permeability across the epithelial membrane indicating tightness of the barrier (McCormick, 2003). Co-culturing Caco-2 and HT29-MTX cells provides an in vitro model consisting of two representative cell types in addition to the mucus layer present on the apical cell surfaces similar to the intestine (Walter, Janich, Roessler, Hilfinger, & Amidon, 1996) allowing for a wide range of study applications. Several studies employing co-culture models of Caco-2 and HT29-MTX cells include drug permeability studies, investigating seeding ratios, host-microbe interactions and barrier permeability (Gagnon et al., 2013; Hilgendorf et al., 2000).

1.2 Lactic acid bacteria and gastrointestinal health

The human gastrointestinal tract (GIT) harbors between 300 to 500 different bacterial species in an adult commonly presenting 6 genera including Bacteroides, Bifidobacterium, Lactobacillus, Eubacterium, Streptococcus, and Clostridium (Hooper, Midtvedt, & Gordon, 2002; Mahida, 2004). Generally, a symbiotic relationship exists between the host and commensal intestinal microbiota playing a key role in maintaining intestinal homeostasis. The proportion of lactobacilli colonization in the gastrointestinal tract is quite high, ranging from $10^3$ to $10^4$ CFU/mL in the oral cavity to $10^5$ to $10^7$ CFU/mL in the ileum and approximately $10^4$ to $10^8$ CFU/mL in the colon (Borriello et al., 2003; Hill, Eschenbach, & Holmes, 1984) as they are capable of utilizing a wide variety of carbohydrates (Borriello et al., 2003). These bacteria
produce lactic acid as the major end product during carbohydrate fermentation by may also yield ethanol, carbon dioxide, acetate, butyrate and formic acid (Lebeer, Vanderleyden, & De Keersmaecker, 2008; Soomro, Masud and Kiran Anwaar, 2002). Cumulative evidence shows the beneficial effects of probiotics, including certain strains of LAB, on health as well as the prevention and treatment of disease.

1.2.1 Benefits of lactic acid bacteria

Dietary supplementation of beneficial microbes as the form of probiotics either in fermented food products or capsules has become increasingly accepted. Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). Health benefits include prevention and treatment of irritable bowel syndrome (IBD), antibiotic associated diarrhea, traveler’s diarrhea, colorectal cancer, atopic eczema, and dental caries (Allaker & Stephen, 2017; Ghasemi, Mazaheri, & Tahmourespour, 2017; J. Kim et al., 2017; Navarro-López et al., 2018; Ritchie & Romanuk, 2012; Surendran Nair, Amalaradjou, & Venkitanarayanan, 2017; Wardill, Van Sebille, Ciorba, & Bowen, 2018). Mode of action generally occurs by lowering the luminal pH through lactic acid production, directly or indirectly competing with pathogens, secreting bioactive molecules, enhancing barrier function, competing for nutrients, inhibiting quorum sensing, as well as exhibiting immunomodulatory activity (Gibson & Roberfroid, 1995; Johnson-Henry, Hagen, Gordonpour, Tompkins, & Sherman, 2007; Madsen et al., 2001; Mattar et al., 2002; Sherman, Ossa, & Johnson-Henry, 2010; Surendran Nair et al., 2017; Ulluwishewa et al., 2011; Vizoso Pinto et al., 2009; Zhang et al., 2016). For instance, pretreatment of MDCK-I and T84 epithelial cells by Lactobacillus rhamnosus GG prior to infection by enterohemorrhagic E. coli O157:H7 not only decreased the number of lesions formed but also attenuated the decline of transepithelial electrical resistance caused by infection (Johnson-Henry et al., 2008). Disruption and
redistribution of the tight junction proteins claudin-1 and zonula occludens normally induced during EHEC infection, was prevented in epithelial cells in the presence of *L. rhamnosus* GG, contributing to preserving barrier integrity (Johnson-Henry et al., 2008). Maintenance of barrier function in the intestinal epithelium is crucial as it acts at the front line of defense between the nonsterile external environment and the sterile internal milieu. Similarly, tight junction proteins zonula occludens and actin were both maintained in human intestinal cell lines HT29/cl. 19A and Caco-2 during exposure to enteroinvasive *E. coli* O29:NM when treated by *Streptococcus thermophilus* and *Lactobacillus acidophilus* (Resta-Lenert & Barrett, 2003). Pretreatment of epithelial cells also resulted in a lower number of adhered and invasive *E. coli*, presumably through the inference and competition between the microorganisms (Resta-Lenert & Barrett, 2003). Comparable observations have also been demonstrated in epithelial cells infected by *S. Typhimurium*. *Lactococcus lactis* subsp. *cremoris* pretreatment of HT-29 cells not only decreased the adhesion of *S. Typhimurium*, but also suppressed virulence gene expression of *hilA* and *ssrB* (Zhang et al., 2016). In addition to the beneficial effects incurred by lactic acid bacteria and potential probiotics, molecules produced by certain strains under various conditions have also demonstrated potential health applications. Cell extracts produced by *L. acidophilus* GP1B administered at 10 µg/mL exerted antivirulence effect of *C. difficile*, which causes antibiotic associated diarrhea and colitis in both animals and humans (Yun, Oh, & Griffiths, 2014). Furthermore, the survival rates of C57BL/6 mice challenged with *C. difficile* was drastically increased when administered *L. acidophilus* cell extracts demonstrated beneficial effects both *in vitro* and *in vivo* (Yun et al., 2014). Under different conditions, many fermented milk products including their metabolites have also been widely reported to be associated with gut health. Milk fermented by *Lactobacillus paracasei* strains produced metabolic products have been associated to strengthening intestinal epithelium function by protecting against inflammatory damage *in vitro* (Zagato et al., 2014; Chen et al., 2016).
1.2.1.1 Fermented dairy products

Fermentation is a common food processing method utilizing bacterial starter cultures to produce foods with increased microbial safety, organoleptic properties, nutrition as well as health benefits (Leroy & De Vuyst, 2004). LAB are most commonly used in a symbiotic combination for optimal conversion of carbohydrates to organic acids such as lactic acid and acetic acid as well as other metabolic products including exopolysaccharides and peptides (AMPs) (Leroy & De Vuyst, 2004). Some examples of food and beverages produced by fermentation include yogurt, cheese, sauerkraut, kimchi, kefir, pickles, kombucha, wine and beer.

During milk fermentation, LAB play key roles in: (1) preservation of the product by the production of acids and possible antimicrobial compounds; (2) production of metabolites contributing to flavor (e.g. acetaldehyde in yogurt) (Chaves et al., 2002) and texture (e.g. exopolysaccharides in cheese) (Duboc & Mollet, 2001) properties; (3) enhancing the nutritional value through release of free amino acids or synthesis of vitamins (Crittenden, Martinez, & Playne, 2003; Leroy & De Vuyst, 2004; Patel, Shah, & Prajapati, 2013); and (4) improving and controlling gastrointestinal health (Parvez, Malik, Kang, & Kim, 2006; Rolfe, 2000). The amount, bioavailability and digestibility of some dietary components are improved during LAB or Bifidobacterium milk fermentation. Increased levels of production of folic acid, niacin, and riboflavin have been demonstrated in yogurt and acidophilus milk (Alm, 1982; Crittenden et al., 2003; Deeth, 1981; Patel et al., 2013). Aside from increased vitamin levels, the bioavailability of protein and fat may be elevated by bacterial enzymatic hydrolysis through the release of free amino acids and short chain fatty acids (Clare & Swaisgood, 2000; Fernandes, Shahani, & Amer, 1987; Rolfe, 2000). Additionally, symptoms of lactose intolerance can be
alleviated through the activity of bacterial lactase enzyme present in the fermented dairy product produced by certain LAB strains (Alm, 1982; Martini, Kukielka, & Savaiano, 1991). Furthermore, lactase activity is enhanced in environments of lower pH generated by lactic acid production (Alm, 1982; Fernandes et al., 1987; Marteau et al., 1990). The low pH of intestinal content also contributes to the prevention of invasive pathogens such as Salmonella and E. coli (Mack, Michail, Wei, McDougall, & Hollingsworth, 1999a; Millette, Luquet, & Lacroix, 2007; Noto Llana et al., 2013; Sharma et al., 2014).

Many studies have reported the effects of bioactive components found in lactic acid bacteria fermented milk which lower the adhesion and invasion of pathogens, as well as exhibit immune modulation (Bayoumi & Griffiths, 2010; Kemgang, Kapila, Shanmugam, Reddi, & Kapila, 2016; Millette et al., 2007; Vizoso Pinto et al., 2009; Zagato et al., 2014). Purified components produced during L. acidophilus La-5 fermentation of milk added into commercial yogurt demonstrated attenuated pathogenicity and virulence of E. coli O157:H7 in vivo (Zeinhom et al., 2012). Similarly, molecules secreted by Bifidobacterium bifidum have demonstrated antivirulence effect on Salmonella as well as decreased colonization in vitro (Bayoumi & Griffiths, 2012). Tellez et al. (2011) determined that a peptidic fraction isolated from Lactobacillus helveticus fermentation of milk could protect mice from Salmonella infection and also demonstrated attenuated virulence capacity (Tellez et al., 2011).

Many genera of LAB including Lactococcus, Lactobacillus, Leuconostoc, and Streptococcus produce and secrete exopolysaccharides (EPS) during milk fermentation (Patel et al., 2010). Some studies have revealed that EPS produced by several strains of LAB offer beneficial effects in vivo; Lactobacillus casei fermentation produced an EPS fraction harboring anti-hypertensive effect in rats (Ai et al., 2008) while S. thermophilus EPS demonstrated anti-
gastritis effect in mice (Rodríguez et al., 2009). The broad range of beneficial effects shown in these studies in addition to the advantages of *in situ* EPS production, illustrates the potential of EPS produced by LAB.

1.2.1.2 *Exopolysaccharides from LAB*

Bacterial EPS are released into the extracellular medium by bacteria. It can be secreted in the form of “ropy” polysaccharides or in forms that are more associated with the cell surface – often referred to as “capsular” (Sutherland, 1972). Bacterial EPS can be broadly classified by the sugar composition of one or different types of monosaccharides into homopolysaccharides (e.g. cellulose and levan) and heteropolysaccharides (e.g. xanthan) (Patel, Michaud, Singhania, Soccol, & Pandey, 2010). Homopolysaccharides and heteropolysaccharides are both employed in food applications; for example, homopolysaccharides are used in bread products to improve structural, textural and moisture properties (Tieking et al., 2005) and heteropolysaccharides in dairy products such as kefir to improve organoleptic properties (Patel et al., 2010).

The amounts of EPS produced by LAB vary from 40 to 800 mg/mL depending on the strain (Patel et al., 2010). *L. lactis* subsp. *cremoris* produces approximately 600 mg/mL of EPS consisting of rhamnose, glucose, and galactose with lactose as the carbon source (Higashimura, Mulder-Bosman, Reich, Iwasaki, & Robijn, 2000) while a change in carbon source results in a different sugar composition (Patel et al., 2010). The type of medium and cultural conditions used for the growth of LAB is a very important factor for EPS production. Generally, a high carbon and low nitrogen substrate ratio is favoured which includes many complex media containing peptone, beef and yeast extracts as well as whey and skim milk based media (Cerning, 1990; Purushothaman, Wang, & Cleary, 2003; Sanni, Onilude, Ogunbanwo, Fadahunsi, & Afolabi, 2002; Vaningelgem et al., 2004). It is reported that some LAB are
capable of secreting more than one type of EPS under identical growth conditions while other LAB secrete different EPS in different growth conditions (Grobben, Smith, Sikkema, & de Bont, 1996; Grobben et al., 1997; Marshall, Cowie, & Moreton, 1995). Under the same experimental conditions different amounts of EPS with varying viscosities were produced by *S. thermophilus* (Cerning, Bouillanne, Desmazeaud, & Landon, 1988). Prolonged incubation times, unstable temperature or spontaneous mutations are all factors affecting EPS production (Bouzar, Cerning, & Desmazeaud, 1996; Gancel & Novel, 1994; Robijn, van den Berg, Haas, Kamerling, & Vliegenthart, 1995).

Several studies have shown the benefits of bacterial EPS including anti-hypertensive (Ai et al. 2008), immune modulating (Bleau et al. 2010; Cui, Goh, Archer, & Singh, 2007; Kitazawa, Itoh, Tomioka, Mizugaki, & Yamaguchi, 1996; Makino et al., 2006), antioxidant (Xu, Shang, & Li, 2011), and anti-gastritis effects (Rodríguez, Medici, Rodríguez, Mozzi, & Font de Valdez, 2009). It has been also reported that EPS produced by *Lactobacillus reuteri* strains could interfere with enterotoxigenic *E. coli in vitro* (Wang, Gänzle, & Schwab, 2010). Moreover, evidence has indicated the presence of different types of EPS producing LAB in the oral cavity and intestinal system suggests that EPS may be significant for survival of these EPS producing strains in the survival of harsh conditions (Patel et al., 2010). This protective action of EPS could be advantageous to probiotics for competitive colonization to exclude pathogenic bacteria inside the human intestinal tract (Gänzle & Schwab, 2009; Ruas-Madiedo, Gueimonde, Margolles, de los Reyes-Gavilán, & Salminen, 2006).

### 1.2.2 Mechanisms of action

The human gut microbiota contains one of the most complex ecosystems comprised of over 100 trillion microorganisms living in harmony (Human Microbiome Project Consortium, 2012).
The inherent microbiota work together with the host to achieve host-microbe homeostasis preserving normal intestinal morphology, function and response to stimuli (Belizário & Napolitano, 2015). One of the most important roles of commensal microbes is utilizing nutrients that the hosts are not capable of (e.g. cellulose) for survival while at the same time promoting host cell performance. The host-microbiota relationship can be modified and disrupted by many environmental factors such as diet, pathogen, and drugs (Carding, Verbeke, Vipond, Corfe, & Owen, 2015). When imbalance or dysbiosis in the gut occurs between the host immune system, resident microbiota and their metabolic products, it could lead to development of several disorders such as Crohn’s disease, ulcerative colitis and irritable bowel syndrome (Carding et al., 2015).

1.2.2.1 Immune modulation

The interaction between the host and certain LAB and potential probiotics may trigger the release of signaling factors by epithelial cells causing host immune modulation (Surendran Nair et al., 2017). Numerous cytokines are secreted upon stimulation by environmental factors such as the appearance of a pathogen and are responsible for initiating the cascade of events leading to the host immune modulation response. Several LAB such as *Lactobacillus plantarum*, *L. rhamnosus* GG and *L. paracasei* can elicit an immunomodulatory response both *in vitro* and *in vivo* (Bleau et al., 2010; Chen, Hsu, Hung, & Chen, 2016; Kim et al., 2008a; Kozuch & Hanauer, 2008; Zagato et al., 2014). Milk fermented by *L. paracasei* CBA L74 and its supernatant demonstrated strong immunomodulatory activity inhibiting the production of pro-inflammatory cytokine IL-12p70 while increasing anti-inflammatory cytokine IL-10 secretion *in vitro* (Zagato et al., 2014). Milk fermented by another strain of *L. paracasei* 01 also demonstrated immune modulating effects inhibiting production of inflammatory cytokines IFN-γ and TNF-α by intestinal epithelial cells (Chen et al., 2016). In an animal study using
BALB/c mice, the production of TNF-α induced by lipopolysaccharide stimulation was significantly suppressed by lipoteichoic acid harvested from *Lactobacillus plantarum* K8 (Kim et al., 2008a).

1.2.2.2 *Improved barrier function*

The intestinal epithelium creates a barrier between the nonsterile external environment and the sterile internal milieu. Exposure to intraluminal microorganisms and their by-products is prevented by this physical barrier, sealed and connected together by tight junctions and further shielded by a layer of mucous secreted by goblet cells. Tight junctions regulate barrier function of the epithelium by controlling the passage of luminal nutrients, water and ions while restraining harmful substance and pathogens (Farquhar & Palade, 1963; Ulluwishew et al., 2011). Thus, disruption of barrier function may lead to disorders such as inflammatory bowel disease, enteric infections and autoimmune diseases (Sakaguchi, Köhler, Gu, McCormick, & Reinecker, 2002; Schmitz et al., 1999; Watts et al., 2005). It has been widely reported that LAB including *L. reuteri*, *L. paracasei*, *L. acidophilus*, and *L. helveticus* have been associated with strengthening barrier function through immune modulation in both *in vitro* and *in vivo* models (Chen et al., 2016; Madsen et al., 2001; Peng, 2014). Barrier function can also be enhanced by stimulation of increased MUC gene expression and mucus secretion as demonstrated by LAB such as *L. plantarum* 299v, *L. rhamnosus* GG, and *L. casei* GG which increased expression of *MUC2* and *MUC3* in intestinal epithelial cells (Mack, Michail, Wei, McDougall, & Hollingsworth, 1999b; Mattar et al., 2002).

1.2.2.3 *Antimicrobial and bioactive compounds*

The ability of many LAB to produce antimicrobial compounds is one of the competitive advantages LAB have over pathogens. The main groups of inhibitory compounds produced by
LAB include organic acids, bacteriocins, and secondary metabolites (Mishra & Lambert, 1996). Depending on the availability of nutrient substrate, the two main organic acids produced by LAB include lactic acid and acetic acid, both containing antimicrobial activity. By lowering pH, whether in a food system or host, it creates an unfavorable environment for the pathogen disrupting the cellular metabolism and delaying growth (Maloney, 1990). In addition to the production of organic acids during LAB growth, other metabolites such as hydrogen peroxide are also produced. By chemical oxidation of cellular components, hydrogen peroxide is capable of inhibiting the growth of many microorganisms including Psuedomonas, Staphylococcus aureus, and Salmonella (Mishra & Lambert, 1996; Pridmore, Pittet, Praplan, & Cavadini, 2008). The majority of LAB produce hydrogen peroxide during growth and it was demonstrated that production by eight different strains of Lactobacillus johnsonii and Lactobacillus gasseri were effective in killing a major food pathogen S. Typhimurium (Pridmore et al., 2008).

Bacteriocins function by permeabilizing the cell membrane of selective bacteria interfering with cell wall biosynthesis and forming pores which eventually lead to cell death (Duquesne, Destounieux-Garzón, Peduzzi, & Rebuffat, 2007). These proteinaceous antimicrobial compounds are produced by a number of Gram-positive bacteria including several members of LAB such as Lactobacillus salivarius UCC118 and L. lactis (Collins et al., 2002; Corr et al., 2007; Delves-Broughton, 1990). Lactobacillus salivarius UCC118 produces bacteriocin ABP-118 which inhibits the growth of Streptococcus, Bacillus, and Enterococcus species in addition to in vivo inhibition of Listeria monocytogenes (Collins et al., 2002; Corr et al., 2007). Another antimicrobial peptide, Nisin, is produced by Lactococcus lactis fermentation of modified milk medium (Delves-Broughton, 1990). Nisin is effective against Gram-positive bacteria and almost all spore forming species of Bacillus and Clostridium and is used a preservative in dairy
and canned foods (Delves-Broughton, 1990; Delves-Broughton, Blackburn, Evans, & Hugenholtz, 1996). Additionally, LAB are also known for production of peptides that are physiologically active. Bioactive peptides are harbored in the sequence of the parent protein molecule and can be released by enzymatic digestion, gastrointestinal digestion, or fermentation (Korhonen & Pihlanto, 2006). Bioactive peptides can be produced from precursor proteins by enzymatic hydrolysis (i.e. digestive enzymes), fermentation or proteolysis by enzymes from microorganism/plant origin (Korhonen & Pihlanto, 2006). In some cases, combinations of these treatments have also been used to produce short bioactive peptides (Korhonen & Pihlanto, 2003). Enzymatic hydrolysis is the most common method used to produce bioactive peptides, employing digestive enzymes pepsin and trypsin. For instance, angiotensin-I converting enzyme (ACE) inhibitory peptides have been widely reported to be produced during digestion with pepsin or trypsin (Gobbetti, Minervini, & Rizzello, 2004; Tauzin, Miclo, & Gaillard, 2002; Vermeirssen, Camp, & Verstraete, 2007). Milk is a major source of bioactive peptides, specifically casein as the major precursor protein. The proteolytic system of certain LAB such as *L. lactis* and *Lactobacillus delbrueckii* subsp. *bulgaricus* have been well characterized, consisting of cell wall bound proteinases and several intracellular peptidases including aminopeptidases, endopeptidases, dipeptidases and tripeptidases (Christensen, Dudley, Pederson, & Steele, 1999). LAB fermentation of milk has derived a variety of bioactive peptides with various functions including antihypertensive, antioxidative, immunomodulatory, ACE inhibitory and antivirulence activity (Ashar, Milchwissenschaft, 2004; Gagnaire, Mollé, Herrouin, & Léonil, 2001; Tellez et al., 2011; Tsai, Chen, Pan, Gong, & Chung, 2008). However, the potential biological and functional effect of a bioactive peptide depends on its ability to remain intact through its gastrointestinal journey. The amino acid composition of the peptide affect digestion where the presence of proline and hydroxyproline aids in resisting hydrolysis by digestive enzymes (Segura-Campos, Chel-Guerrero, Betancur-
Moreover, amino acids located near the C- or N-terminal positions of the peptide plays a large role in the stability and survival during digestion. When the C- or N-terminal groups are blocked, peptides can pass through the intestinal epithelium intact (Gardner, 1984) as shown by thyrotropin-releasing hormone and luteinizing hormone-releasing hormone (Arhewoh, Ahonkhai, & Okhamafe, 2005; Segura-Campos et al., 2011).

1.2.2.4 Limitation of nutrients

In a system as complex as the human gut where microorganisms coexist with each other and the host, many resources need to be shared. The competition for nutrients is a key factor in shaping the gut microbiota to preserve intestinal balance and health. It has been shown that two strains of lactobacilli, *L. reuteri* strain 100-23 and *L. johnsonii* strain 100-33, both of which utilize glucose and maltose substrates for survival, are able to coinhabit in mouse models through partitioning of available carbohydrate resources (Tannock et al., 2011). This is a prevalent phenomenon demonstrated in 102 pairs of commensal bacteria allowing for coinhabitation in the gut (Metagenomics of the Human Intestinal Tract (MetaHIT) Consortium et al., 2016). However, in the presence of pathogens, commensal bacteria and LAB function by limiting nutrient sources. For instance, *L. acidophilus* and *L. delbrueckii* are capable of binding iron (Elli, Zink, Rytz, Reniero, & Morelli, 2000) and therefore limiting the availability to pathogens such as *Salmonella* which produce siderophores to gather iron to enhance its survival in the inflamed gut (Raffatellu & Bäumler, 2010). Additionally, the competition for glucose, sialic acid as well as N-acetyl-glucosamine in an animal study promoted the removal of *C. difficile* from the mouse cecum (Wilson & Perini, 1988).

1.2.2.5 Antivirulence effects
Once pathogen adhesion to the host cell is successful, invasion into the cell is required to infect the host. Many LAB have exhibited antivirulence activity interfering with virulence gene expression of many enteric pathogens. There is sufficient evidence demonstrating the antivirulence activity of molecules secreted by \textit{L. acidophilus} La-5 against \textit{E. coli} as well as \textit{Campylobacter jejuni} (Medellin-Peña, Wang, Johnson, Anand, & Griffiths, 2007; Mundi, Delcenserie, Amiri-Jami, Moorhead, & Griffiths, 2013). In addition, incorporating \textit{L. acidophilus} La-5 molecules into commercial yogurt also resulted in antivirulence effect of \textit{E. coli} (Zeinhom et al., 2012). Furthermore, down regulation of major foodborne pathogen \textit{Salmonella} Typhimurium virulence expression of \textit{hilA} and \textit{ssrB} was demonstrated by cultures of \textit{L. lactis} subsp. cremoris (Zhang et al., 2016) as well as \textit{in vivo} via peptidic fractions purified from \textit{L. helveticus} milk fermentation (Tellez et al., 2011). Another pathogen causing colitis and diarrhea in humans, \textit{C. difficile}, displayed suppressed virulence gene expression when treated by cell extracted collected from \textit{L. acidophilus} GP1B (Yun et al., 2014).

1.2.2.6 Competitive exclusion

One of the most beneficial mechanisms of LAB and potential probiotic microorganisms is the ability to competitively exclude pathogen colonization. Broadly, competitive exclusion of pathogens by beneficial microbes is also in part dependent on the availability of nutrients, mucosal adhesion sites and presence of inhibitory compounds (Collado, Grześkowiak, & Salminen, 2007; Surendran Nair et al., 2017). For instance, competitive exclusion of \textit{E. coli} O157:H7 to the attachment of intestinal epithelial cells was achieved following \textit{MUC2} upregulation stimulated by \textit{L. acidophilus} A4 (Kim, Kim, Whang, Kim, & Oh, 2008b). In addition, exclusion of \textit{Salmonella}, \textit{E. coli}, and \textit{Clostridium} were demonstrated in exposure to swine intestinal mucus by \textit{L. rhamnosus} LGG (Collado et al., 2007). Furthermore, human intestinal cells exposed to \textit{L. acidophilus} strains also displayed properties of exclusion of
several enteropathogens including *S. Typhimurium* and *E. coli* (Bernet, Brassart, Neeser, & Servin, 1994).

1.2.2.7 Inhibition of quorum sensing

Bacterial communication through chemical signaling is known as quorum sensing (QS). QS between pathogens allows gene expression for different phenotypes, especially genes associated with bacterial virulence (Kalia, 2013). With the increased number of multi-drug resistant bacterial strains, QS inhibitors could target QS systems in order to inhibit pathogenicity. It has been demonstrated that *L. acidophilus* La-5 secreted molecules can substantially decrease the level of autoinducer-2 molecules produced by *E. coli* while suppressing virulence gene expression of *LEE1* (Medellin-Peña et al., 2007). Similarly, soluble molecules secreted by *L. paracasei* subsp. *paracasei* were capable of inhibiting *Staphylococcus aureus* QS gene expression (Cotar et al., 2010). Another example demonstrated QS inhibition by *L. reuteri* RC-14 via the production of small signaling molecules that obstructed the staphylococcal QS system *agr*, which is also a key regulator of virulence genes (Li, Wang, Xu, Magarvey, & McCormick, 2011).

There is evidence that lactic acid bacteria and/or their components may impact the behaviour of pathogens including *Salmonella* both *in vitro* and *in vivo* via different modes of actions. The potential of lactic acid bacteria treatments to modify the pathogenicity and virulence activity of foodborne pathogens like *Salmonella* could be a successful alternative to the use of antibiotic treatments. Further investigations into the interactions between *Salmonella*, lactic acid bacteria, as well as the host cell response may provide new knowledge for the overall improvement of gut health through the addition of fermented food products/components.
1.3 Hypotheses and aims

The overall aim of this research was to investigate the interactions between *Lactococcus lactis* subsp. *cremoris* strain JFR1 (both exopolysaccharide producing and nonproducing strains) and its impact on *Salmonella enterica* serovar Typhimurium, using *in vitro* models of human intestinal epithelial cells, to examine their effects on *Salmonella* virulence gene expression as well as the following host cell response. Therefore, the main hypotheses of this research are:

1) Exopolysaccharides (EPS) produced by *L. lactis* subsp. *cremoris* JFR1 may impact the attachment of *Salmonella* in *in vitro* models of human intestinal epithelial cells.

2) Overnight cultures of *L. lactis* subsp. *cremoris* JFR1 could reduce *Salmonella* invasion of human intestinal epithelial cells by suppressing *Salmonella* virulence gene expression. A possible mechanism of action could be due to the transport of peptides present in the overnight culture treatments via the *Salmonella* oligopeptide permease.

3) Based on the second hypothesis, milk fermented with *L. lactis* subsp. *cremoris* JFR1 could also down regulate virulence gene expression of *Salmonella* in HT29-MTX, Caco-2, as well as cocultured cell models. Similarly, lack of a function *Salmonella* oligopeptide transport system will not show change in virulence and invasion activity.

The specific aims of this study are:

1) To explore and compare EPS-producing and nonproducing strains of *L. lactis* subsp. *cremoris* JFR1 on *Salmonella* adhesion to HT-29 intestinal epithelial cells. Purified EPS at different concentrations and its impact on *Salmonella* adhesion to epithelial cells will also be explored.

2) Investigate how *L. lactis* subsp. *cremoris* JFR1 overnight cultures treatments of intestinal epithelial cells influences invasion of *Salmonella*. To also examine the mode
of action by which invasion is affected by the treatments using a *Salmonella* mutant lacking oligopeptide transport system.

3) Assess the effect of milk fermented by *L. lactis* subsp. *cremoris* JFR1 on intestinal epithelial cells and how it impacts *Salmonella* invasion and virulence expression. *Salmonella* mutant lacking oligopeptide transport system will also be used to assess the possible mode of action.
Chapter 2. *Lactococcus lactis* subsp. *cremoris* strain JFR1 attenuates *Salmonella* adhesion to human intestinal cells *in vitro*

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**Abstract**

*Lactococcus lactis* subsp. *cremoris* JFR1 has been studied in reduced fat cheese due to its ability to produce exopolysaccharides (EPS) *in situ*, contributing to improved textural and organoleptic properties of fermented milk products. In this study, the effect of strain JFR1 on virulence gene expression and attachment of *Salmonella* on HT-29 human colon carcinoma cell models was investigated. Overnight cultures of *L. lactis* subsp. *cremoris* JFR1 containing EPS, grown in M17 media with 0.5% glucose supplementation, decreased attachment as well as down regulated virulence gene expression in *Salmonella enterica* subsp. *enterica* when tested on HT-29 cells. However, EPS isolated from milk fermented with *L. lactis* subsp. *cremoris* JFR1 did not affect *Salmonella* virulence gene expression or attachment to HT-29 cells. These results suggest that EPS does not contribute to the attachment of *Salmonella* to HT-29 cells. However, the possibility that the isolation process may have affected the structural features of EPS cannot be ruled out.

**Keywords:** Exopolysaccharides, *Lactococcus lactis* subsp. *cremoris* JFR1, pathogen attachment, invasion, *Salmonella*, HT-29
2.1 Introduction

Over the past few decades, consumers have become more health conscious, demanding foods with additional benefits beyond taste and convenience. Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). Bacterial EPS play a number of important roles in LAB physiology including cell protection, colonization, and cellular regulation (De Vuyst, De Vin, Vaningelgem, & Degeest, 2001; Krinos et al., 2001; Looijesteijn, Trapet, De Vries, Abee, & Hugenholtz, 2001; Roberts, 1996). Colonization may be associated with unique structural components of cells such as fimbriae/pili, lipoteichoic acid (LTA), lipopolysaccharides (LPS), proteins or other cell wall components. For attachment to eukaryotic cells, the presence of a receptor, commonly carbohydrate or peptide residues, and adhesins (bacterial ligand) on the surface of the bacterium is required (Beachey & Courtney, 1989; Nobbs, Lamont, & Jenkinson, 2009).

Many genera of lactic acid bacteria (LAB) are capable of producing exopolysaccharides (EPS), including *Lactococcus*, *Lactobacillus*, *Leuconostoc*, and *Streptococcus* (Badel, Bernardi, & Michaud, 2011; Degeest, Vaningelgem, & De Vuyst, 2001; Patel, Michaud, Singhania, Soccol, & Pandey, 2010). The amounts of EPS produced by LAB vary from 40 mg/ml to over 2000 g/L depending on strain and medium (Bergmaier, Champagne, & Lacroix, 2003; Macedo, Lacroix, Gardner, & Champagne, 2002; Patel et al., 2010). *Lactococcus lactis* subsp. *cremoris* JFR1 can produce approximately 450 to 500 mg/L of EPS (Miao, 2015).

To date, the effect of EPS on probiotic as well as pathogen adhesion in the gastrointestinal (GI) tract has begun to be characterized. The impact of the EPS produced by three different potential probiotics was tested on the adhesion of probiotic and enteropathogenic strains, showing that
purified EPS, up to 5 mg/ml, increased the attachment of *Salmonella* Typhimurium as well as *Clostridium difficile* to human intestinal mucus (Ruas-Madiedo, Gueimonde, Margolles, de los Reyes-Gavilán, & Salminen, 2006). More research is needed in this area to understand how this increased attachment is related to EPS composition and structure.

The pathogenesis of some enteric pathogens such as *Salmonella* requires colonization of a normally non-phagocytic cell (e.g., epithelial cell) by attachment and invasion, followed by multiplication inside the host cell. *Salmonella* invade by stimulating the epithelial cell to engulf and internalize them. The process initiates when the bacterium is in close proximity to the host cell. Type III secretion systems controlled by *Salmonella* pathogenicity island I (SPI1) triggers the release of effector proteins which cause “membrane ruffling”, a process in which the cytoskeleton of the epithelial cell rearranges itself to engulf the *Salmonella* into the cytoplasm (Altier, 2005; Galán & Curtiss, 1989; Kaniga, Bossio, & Galán, 1994). SPI1 contains genes such as *hilA* for the control of type III secretion systems which are needed for invasion while SPI2 contains genes such as *ssrB* needed to carry out systemic infection (Galán & Curtiss, 1989; Ochman, Soncini, Solomon, & Groisman, 1996; Shea, Hensel, Gleeson, & Holden, 1996). By investigating the expression of virulence genes, it is possible to study the behaviour of *Salmonella* during attachment to and invasion of epithelial cells such as those of the HT-29 or Caco-2 cell lines (Kim et al., 2008; Mellor, Goulter, Chia, & Dykes, 2009). Human intestinal cell culture models are widely used for *in vitro* studies and thus are good candidates for investigating interactions between host and enteric pathogens in the presence of EPS and/or EPS producing LAB. The aim of this research is to explore the potential effect of EPS produced by *Lactococcus lactis* subsp. *cremoris* on *Salmonella* attachment and invasion of intestinal cells *in vitro*.
2.2 Materials and methods

2.2.1 Materials
Buffered peptone water, Dulbecco’s Modified Eagle Medium (DMEM) (containing 25 mM glucose), HEPES buffer and peptone water were obtained from Sigma-Aldrich Corporation. Heat-inactivated fetal bovine serum (FBS), nonessential amino acids (NEAA), 0.25 % trypsin 1 mM EDTA 4Na (1×), L-glutamine, penicillin-streptomycin (10,000 U of penicillin and 10,000 µg of streptomycin per ml), and phosphate-buffered saline (PBS) were purchased from Invitrogen (Canada Inc., Burlington, Ontario, Canada). Glass bottom dishes used for microscopy were from MatTek Corporation, Ashland, MA, US while 24 well plate from Corning Glass Works, Mississauga, ON, Canada. M17 broth and agar were purchased from Oxoid Microbiology, Nepean, ON, Canada while Luria-Bertani broth and agar, glucose and lactose were purchased from Difco, Detroit, USA. Milli-Q water from Millipore purification system was used during EPS extraction as well as trichloroacetic acid (TCA) obtained from Sigma-Aldrich Corporation.

2.2.2 Strains and growth conditions
*Lactococcus lactis* subsp. *cremoris* JFR1 EPS producing as well as non-EPS producing strains (donated by Prof. Hassan, South Dakota State University) were grown in M17 broth (Oxoid Microbiology, Nepean, ON, Canada) supplemented with either lactose (Difco, Detroit, USA) or glucose (Difco, Detroit, USA) at 0.5%. *L. lactis* subsp. *cremoris* JFR1 was incubated overnight at 30°C under modified atmospheric conditions (4% O₂, 20% CO₂, 76% N₂). Solid media preparation for all strains were carried out under the same conditions but with the addition of 15 g/L of agar and incubated under the same conditions. *Salmonella enterica* subsp. *enterica* serovars Enteritidis Lux CDABE and Typhimurium *hilA:*lux and *ssrB:*lux constructs
obtained from the Canadian Research Institute for Food Safety (CRIFS) were grown in Luria-Bertani (LB; Difco, Detroit, USA) broth supplemented with 50 µg/ml of ampicillin and grown aerobically overnight on a shaking incubator at 37°C. *Salmonella enterica* subsp. *enterica* serovar Typhimurium expressing green fluorescent protein (GFP) (also obtained from CRIFS) was grown overnight in tryptic soy broth (TSB) aerobically on a shaking incubator at 37°C. For solid media preparation, 15 g of agar was added per litre of broth.

### 2.2.3 Cell culture preparation

Human colon carcinoma cell line HT-29 was grown in DMEM supplemented with 25 mM glucose, 10% FBS, 2 mM of l-glutamine and 1% penicillin-streptomycin solution. Cells were incubated in 75-cm² flasks at 37°C and 5% CO₂ at constant humidity until a confluent monolayer was formed. During growth, the media was changed every two days.

### 2.2.4 EPS production and isolation

*L. lactis* subsp. *cremoris* JFR1 was sub-cultured twice in M17 broth then 200 µL was added to 7 ml of sterile skim milk and incubated overnight at 30°C. Serial dilutions were made using 0.1% peptone water and by adding 2 ml of each diluted culture to 90 ml of sterile skim milk and incubated overnight at 30°C. After incubation, the most diluted tube showing milk coagulation was used as the inoculum for the purpose of maximum acidification activity at the start of the experiment (Kristo, Biliaderis, & Tzanetakis, 2003). The gel-like coagulum (15 ml) was mixed with 485 ml of pasteurized skim milk (purchased from a local supermarket) and incubated until pH reached 4.6. When fermentation was complete, the pH was adjusted to 7 for EPS purification. In brief, 95% ethanol was added at a 3:1 (v/v) (ethanol: fermented milk) ratio, stirred well and left at 4°C overnight for EPS precipitation. The sample was then centrifuged at 8000 × g for 20 min at 4°C (Beckman Instruments J2-MC Centrifuge, Palo Alto, CA, USA).
and the pellet was dissolved in 10 ml of Milli-Q water and left to stir overnight at 4°C. TCA was added to a final concentration of 10% (w/v) to precipitate proteins and was mixed well and centrifuged. The supernatant was kept and EPS precipitated with 3:1 volumes of 95% ethanol at 4°C, then suspended in 10 ml Milli-Q water and dialyzed in 6-8 kDa cut-off dialysis tubing against water for three days at room temperature, followed by freeze-drying. The EPS was dissolved in PBS for 3 h at 90°C at a concentration of 2 mg/ml for the stock solution.

2.2.5 Adhesion/attachment assay

HT-29 cells were harvested from 75-cm² flasks once 80-90% confluency was reached. Trypsin-EDTA (0.25% trypsin, 1 mM EDTA (1x), was used to detach cells and 1 ml of 5×10⁵ cells/ml was seeded onto a 24 well plate. Cells were then incubated at 37°C and 5% CO₂ until confluent again (80-90%). The spent media was removed, and cells were washed four times with PBS followed by the addition of bacteria diluted in incomplete DMEM (lacking antibiotics and fetal bovine serum). Bacterial strains were sub-cultured twice in broth before each attachment experiment. A sample of 100 µL from overnight L. lactis subsp. cremoris JRF1 culture or from the 2 mg/ml stock solution of isolated EPS was added into the well with 100 µL of diluted Salmonella (final concentration of 10⁵ CFU/ml) and incubated for 3 h at 37°C and 5% CO₂. After incubation, the plates were washed again with PBS followed by 100 µL of trypsin-EDTA to detach cells. Samples were measured for bioluminescence (gene expression), serially diluted and plated for bacterial enumeration.

2.2.6 Gene expression of Salmonella enterica serovar Typhimurium

To evaluate the possible impact of purified EPS on virulence gene expression, bioluminescent reporter strains S. Typhimurium hilA::lux and ssrB::lux constructs were used (Bayoumi & Griffiths, 2010). In brief, the luxCDABE operon from Xenorhabdus luminescens was extracted
and cloned with an ampicillin resistance gene into a plasmid (pSB377), which was further integrated into either the hilA or ssrB promoter regions. The hilA and ssrB promoters control the expression of the lux genes so light is emitted when the gene is expressed. S. Typhimurium containing hilA::lux or ssrB::lux constructs were cultured as described above. After 18 h of growth at 37°C, the bacterial suspension was diluted in fresh broth containing 50 µL/ml ampicillin. A total of 200 µL of each sample (controls and treatments) was incubated in opaque, sterile 96 well plates for 3 h at 37°C. Cell density and bioluminescence of Salmonella was measured using the Victor multi-label counter (Wallac, PerkinElmer Life Sciences Canada, Woodbridge, Ontario, Canada). Bioluminescence is expressed as counts per minute.

2.2.7 Confocal microscopy

Cell and bacterial strains were imaged using a confocal laser scanning inverted microscope (Leica TCS SP2, model Leica DM IRE2, Leica Microsystems CMSGmbH, Mannheim, Germany) with an Ar/Kr visible light laser and 63× (oil) objective (Bakowski, Cirulis, Brown, Finlay, & Brumell, 2007). Cell cultures were grown in glass bottom dishes and incubated for 3 h at 37 °C and 5% CO₂ with the addition of Salmonella and specified treatments (either overnight culture or isolated EPS) before imaging.

2.2.8 Statistical analysis

All experiments were carried out twice with triplicates of each sample. Means and standard deviations were analyzed using ANOVA followed by Bonferroni’s multiple comparison post-test with significance at $P < 0.05\%$, after verification that the data followed a normal distribution.

2.3 Results and Discussion
The attachment to HT-29 cells and virulence gene expression of *S. Typhimurium* *hilA::lux* and *ssrB::lux* were studied in the presence of overnight cultures of *L. lactis* subsp. *cremoris* JFR1 or in the presence of purified EPS. The two strains of *L. lactis* subsp. *cremoris* JFR1 used were the EPS producing strain (JFR+) and non-EPS producing strain (JFR-). *Salmonella*, when combined over a 3-hour period in solution with overnight whole culture media from either JFR+ or JFR-, showed a down regulation in both *hilA::lux* and *ssrB::lux* gene expression (Fig. 2.1A, B). When the same treatments were added to HT-29 human intestinal cell cultures (Fig. 2.1C-F) a decrease in both plate count and virulence gene expression was observed. The final pH of the *S. Typhimurium* and *L. lactis* subsp. *cremoris* JFR1 mixtures at the end of the 3-h incubation period remained near neutral at pH 6.8. Previous studies have indicated that neutral pH does not affect the adhesion of *S. Typhimurium* or intestinal cell viability (Greene & Klaenhammer, 1994; Lehto & Salminen, 1997). Decreased attachment of *S. Typhimurium* GFP was also visually confirmed by laser scanning confocal microscopy. More *Salmonella* cells were noted on the surface of control HT-29 cells after washing (Fig. 2.2A) compared with the treatments where the cells were incubated with *Salmonella* in the presence of JFR1+ or JFR1- (Fig. 2.2B - E).

EPS isolated from overnight cultures were used to further investigate effects on *Salmonella* attachment and virulence gene expression. Under the incubation conditions used, *L. lactis* subsp. *cremoris* JFR1 produced approximately 450 to 500 mg/L of EPS with an average molecular weight of 7480 kDa determined by size exclusion chromatography coupled with light scattering detection (Miao, 2015). The EPS produced under these conditions is composed of rhamnose, glucose, galactose, and mannose (Miao, 2015). Purified EPS up to 0.2 mg/ml did not have any effect on *S. Enteritidis* attachment to HT-29 cell cultures after 3 h simultaneous
incubation (Fig. 2.3). Comparable outcomes were seen in a study conducted with EPS purified from three probiotic strains where concentrations up to 1 mg/ml of EPS added did not change the adhesion of *C. difficile* ATCC 9689 (Ruas-Madiedo et al., 2006). However, adhesion of *E. coli* NCTC 8603 did increase after 1 mg/ml treatments with two of the three probiotic EPS (Ruas-Madiedo et al., 2006).

![Figure 2.1 Bioluminescence of *S. Typhimurium* hilA::lux (A) and ssrB::lux (B) measured after 3 h of incubation in solution (Control M17 G: M17 medium supplemented with glucose; Control M17 L: M17 media supplemented with lactose; JFR+: *L. lactis* subsp. cremoris JFR1 producing EPS; JFR-: *L. lactis* subsp. cremoris JFR1 non-EPS producing. HT-29 attachment assay of *S. Typhimurium* hilA::lux construct using 10% overnight culture media (C) and ssrB::lux (D). *S. Typhimurium* enumeration after 3 h incubation in E (hilA::lux) and F (ssrB::lux). Experiments were performed in triplicate and statistical analysis was carried out using ANOVA followed by Bonferroni’s post-test (*P* < 0.05). Letters indicate significant differences (*P* < 0.05) within each subsection of the figure.

The results from the current study suggest that the *L. lactis* subsp. cremoris JFR1 EPS, at the
concentrations used (0.2 mg/ml), did not aid in pathogen attachment. This is in contrast with some other reports (Ruas-Madiedo et al., 2006). When EPS concentrations were increased to 5 mg/ml, heightened attachment was seen to human intestinal mucus for S. Typhimurium ATCC 29631, Enterobacter sakazakii ATCC 29544, E. coli NCTC 8603 and C. difficile ATCC 9689 (Ruas-Madiedo et al., 2006). However, higher concentrations could have been possible due to the higher solubility of their EPS extracts. Solubilisation of L. lactis subsp. cremoris JFR1 EPS was a challenge at higher concentration. A reason for the discrepancy may be due to a loss of functionality/bioactivity of EPS as a result of the modification of structural features (such for example, dephosphorylation) during the extraction and purification process. Furthermore, the EPS was rehydrated, and the dehydration and rehydration may have disrupted the native structure of EPS and its association with other molecules when present in solution. More information is needed on the differences in physical characteristics among these different EPS structures and their impact on viscosity. EPS viscosity may impact cell attachment due to steric hindrance and shielding of the cell surface adhesins that are involved in cell attachment (Orgad, Oren, Walker, & Herzberg, 2011; Ruas-Madiedo et al., 2006). Fimbrial adhesins are capable of aiding the in vitro attachment of Salmonella Typhimurium to various mammalian epithelial cells (Humphries et al., 2001; Sirsat et al., 2011).

**Figure 2.2** Representative laser scanning confocal microscopy image of HT-29 cell line with attached Salmonella Typhimurium GFP on its surface (green). A: Control, only Salmonella Typhimurium GFP, B: Salmonella Typhimurium GFP combined with L. lactis subsp. cremoris JFR1, C: Salmonella Typhimurium GFP combined with L. lactis subsp. cremoris non-EPS.
producing variant of JFR1.

**Figure 2.3** HT-29 attachment assay of purified EPS produced from *L. lactis* subsp. *cremoris* JFR1 in 3 concentrations showed no significant difference in attachment of *Salmonella* Enteritidis Lux CDABE after 3 h of incubation. Experiments were performed in triplicate and statistical analysis was carried out using ANOVA followed by Bonferroni’s post-test (*P* < 0.05).

Despite using two different *Salmonella* serovars (Fig. 2.1 and 2.4), similar trends were observed for attachment and gene expression. There was no significant effect of isolated EPS with *S. Typhimurium* in LB broth (Fig. 2.4A, B) or with treatments (Fig. 2.4C - F) on HT-29 cells. Cell cultures were used to show that purified EPS not only has no effect on virulence gene expression but also no effect on the physical attachment to cell monolayers of HT-29 (Fig. 2.5). A medium control was also added to confirm no changes in bioluminescence resulted from its addition. Again, these results would suggest no impact of EPS on *S. Typhimurium* virulence gene expression. However, the EPS extraction process or concentration may have affected its ability to influence the virulence of *Salmonella*. To visualize the attachment of *Salmonella*, a strain expressing the green fluorescence protein (GFP) was exposed to HT-29 cells and imaged with laser scanning confocal microscopy. After washing, *Salmonella* (green dots) were present on the surface of the HT-29 cells (dark grey, circular objects) (Fig. 2.5). The
attachment of *S. Typhimurium* GFP showed no significant difference between the concentrations of EPS applied (0 to 0.2 mg/ml). These images confirm the data from virulence gene expression and attachment studies. Further experiments (data not shown) with overnight culture medium containing an increased amount of EPS (20% overnight culture) or with lower *Salmonella* (approximately $10^4$ CFU/ml) to cell ratio showed the same trends, with decreased bioluminescence and attachment in the presence of *L. lactis* subsp. cremoris JFR1.

Figure 2.4 Bioluminescence of *S. Typhimurium* containing either *hilA::lux* (A) or *ssrB::lux* (B) constructs in the presence of purified EPS in solution. Bioluminescence of *S. Typhimurium* *hilA::lux* (C) or *ssrB::lux* (D) with EPS treatments on HT-29 cell cultures. Plate counts (colony forming units/ml) of *S. Typhimurium* after 3 h of incubation with EPS treatments on HT-29 cell cultures of *hilA::lux* (E) and *ssrB::lux* (F). Experiments were performed twice, with
triplicate samples and statistical analysis was carried out using ANOVA followed by Bonferroni’s post-test \( P < 0.05 \).

The two virulence gene constructs represent two different phases of the invasion process. A down regulation in the \textit{hilA::lux} construct may indicate that the LAB strains affect the SPI1 type III secretion system, the genes responsible for initial attachment and invasion. On the other hand, a down regulation of the \textit{ssrB} construct could indicate a change in SPI2 type III secretion system, the system responsible for carrying out systemic infection. These results also suggest that the down regulation and decreased attachment of \textit{Salmonella} may not be due to the presence of EPS but instead to the LAB presence and their culture medium. Although the interacting mechanisms between potential probiotics and pathogens require further investigation, studies have suggested that probiotics may interrupt the early interactions of pathogens and host cells (Gopal, Prasad, Smart, & Gill, 2001; Resta-Lenert & Barrett, 2003; Tuomola, Ouwehand, & Salminen, 1999). Coaggregation of probiotics and pathogens is strain specific and subject to incubation conditions (Collado, Meriluoto, & Salminen, 2007). Some coaggregation properties have been observed with \textit{Salmonella} subspecies and different lactic acid bacteria including Lactobacilli and \textit{L. lactis} 3, 8, and KN, indicating strain specificity (Li, Liu, Zhou, & Wang, 2015). This property should be considered when selecting potential probiotics (Collado et al., 2007; Li et al., 2015).
Some probiotics have been shown to inhibit food pathogens such as *Salmonella* and this may be due partly to down regulating virulence genes. Bayoumi & Griffiths (2010) showed that cell free spent medium containing molecules secreted by *Bifidobacterium bifidum* had the ability to down regulate the expression of both *hilA::lux* and *ssrB::lux* gene constructs (Bayoumi & Griffiths, 2010). Although down regulation was confirmed, the mode of action was not identified. Searle et al. (2009) found that the presence of approximately 2 mM of Bimuno® (a commercial prebiotic galacto-oligosaccharide) produced from the galactosyltransferase activity of *B. bifidum* NCIMB 41171 significantly reduced infection of *S. Typhimurium* SL1344nal' on HT-29-16E cells (Searle et al., 2009). Oral administration of 2500 mg Bimuno1/kg in BALB/c mice resulted in a significant decrease of *S. Typhimurium* SL1344nal' colonization in five organs (Searle et al., 2009). Despite the *in vivo* and *in vitro* findings by Searle et al. (2009), mechanisms were unclear on how the oligosaccharide interfered with
invasion.

In our study, an effect was seen only with overnight cultures of *L. lactis* subsp. *cremoris* JFR1 (EPS producing and non-producing) in both down regulation of virulence gene expression (*hilA* and *ssrB*) and decreased attachment. Further experiments with isolated EPS showed no changes. Some possible explanations include: 1) demonstration of survival behaviour competitiveness between *Salmonella* and probiotic strains (Etienne-Mesmin et al., 2011) since both EPS+ and EPS- strains had significant effects on attachment and virulence gene expression, 2) disruption of native EPS structure during the isolation process (characteristics such as charge or the presence of phosphate groups), and 3) the range of EPS concentrations applied to the cells, which was 0.05-0.2 mg/ml compared to 1-5 mg/ml tested by Ruas-Madiedo et al. (2006).

2.4 Conclusions

In the present study, overnight cultures of EPS-producing *L. lactis* subsp. *cremoris* JFR1 and non-EPS producing *L. lactis* subsp. *cremoris* JFR1 both contributed to lowering the adhesion of *Salmonella* to HT-29 colon cancer cells as well as down regulating virulence genes *hilA* and *ssrB*. However, when EPS isolated from milk fermentation was employed up to a concentration of 0.2 mg/ml, no changes were seen in either attachment or virulence gene expression of *Salmonella*. More studies are needed to further confirm the role of EPS in pathogen attachment, notably the impact of the physical characteristics on viscosity, such as structure and charge of the EPS. From our study, we may conclude that EPS may not be the only factor that can influence bacterial attachment, and not all EPS can facilitate pathogen adhesion. The isolated EPS used in our study may not be able to exert beneficial effects either, such as interfering with pathogen attachment.
2.5 Acknowledgements

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Chapter 3. *Lactococcus lactis* subsp. *cremoris* JFR1 lowers *Salmonella* virulence in intestinal epithelial models

Abstract

Invasion and infection of *Salmonella* into host intestinal epithelial cells requires the expression of several virulence genes. In this study, cell culture models of human intestinal cells (mucous producing HT29-MTX cells and absorptive Caco-2 cells) were used to determine the effects of *Lactococcus lactis* subsp. *cremoris* treatments on virulence gene expression of *Salmonella* Typhimurium and its mutant lacking the OppA permease (*ΔoppA*). During the course of infection by *Salmonella* Typhimurium DT104, virulence gene expression was down regulated, accompanied with lower numbers of invasive bacteria into epithelial cells in the presence of *L. lactis* subsp. *cremoris* treatment. However, *Salmonella* lacking the OppA permease function remained virulent. Additionally, HT29-MTX cells treated with *L. lactis* subsp. *cremoris* produced significantly less pro-inflammatory cytokine IL-8. Furthermore, improved barrier function was reflected with increased transepithelial electrical resistance in epithelial cells treated with *L. lactis* subsp. *cremoris*. Contrarily, these responses were not observed during infection with *Salmonella* Typhimurium *ΔoppA*. Our results show that an intact peptide transporter is essential for the suppression of *Salmonella* virulence genes which leads to the protection of the barrier function in the cell culture models studied.

Keywords: human epithelial cells, *Salmonella* virulence, *Lactococcus lactis* subsp. *cremoris*, IL-8, oligopeptide permease
3.1 Introduction

The human intestinal epithelium provides the first barrier of defense shielding from potentially harmful antigens, molecules and bacteria. The mucus layer, normally 50 to 800 µm thick in healthy individuals, covers the entire length of the intestinal tract (Swidsinski et al., 2007). During infection, this layer must be penetrated by pathogens to reach the epithelial cells and subsequently deeper tissue. The intestinal epithelium is composed of a monolayer of epithelial cells dividing the intestinal lumen from the lamina propria. Neighboring epithelial cells are sealed by tight junctions which are crucial for regulating the intestinal barrier permeability. Tight junctions are multi-complex structures composed of transmembrane proteins which can interact with the actin cytoskeleton of epithelial cells. Chronic inflammation is often correlated with barrier dysfunction where passage of microbial antigens enters underlying tissue (Ohland & MacNaughton, 2010). Many disorders such as Crohn’s disease, ulcerative colitis and celiac disease are associated with increased epithelial permeability (Kamada, Seo, Chen, & Núñez, 2013; Ng, Hart, Kamm, Stagg, & Knight, 2009; Suenaert et al., 2002; Vogelsang, Schwarzenhofer, & Oberhuber, 1998). It has been reported that IBD patients are often associated with high pro-inflammatory cytokines (e.g. TNF-α, IFN-γ, IL-13, IL-1β) production levels in their gastrointestinal epithelium which contribute to increasing epithelial permeability and inflammation of surrounding tissue (Al-Sadi et al., 2014; Amasheh et al., 2009; Wang et al., 2005; Ye, Ma, & Ma, 2006).

Many factors and stimuli are believed to increase intestinal permeability by inducing junctional disassembly. During infection, pathogens have developed multiple strategies to increase the host epithelial permeability to gain access into the underlying tissue, including but not limited to secreting a number of intestinal barrier disrupting molecules such as pore-forming toxins and cytoskeleton modifying proteins (Ivanov, Parkos, & Nusrat, 2010). Rearrangement of the
host cell cytoskeleton is one of the key steps of pathogen invasion. Pathogenic *Escherichia coli* strains, *Citrobacter rodentium*, *Salmonella* Typhimurium and *Vibrio cholerae* operate by disrupting the host cell cytoskeleton through induction of actin contraction and polymerization causing increased epithelial permeability (Boyle, Brown, & Finlay, 2006; Fasano et al., 1995; Flynn & Buret, 2008; Gerhard, Schmidt, Hofmann, & Aktories, 1998; Matsuzawa, Kuwae, Yoshida, Sasakawa, & Abe, 2004). *S. Typhimurium* is a unique pathogen as it can induce its own uptake into the epithelial cell through the type III secretion system (T3SS) (Galán, 2001). *Salmonella* pathogenicity island 1 (SPI1), a chromosomal region encoding most essential genes for the T3SS, is ubiquitous in *Salmonella* and essential for invasion (Altier, 2005). During *Salmonella* invasion, promoted by hilA, a “syringe-like” apparatus is formed functioning in the delivery of effector proteins (i.e. the SOP family) causing host cytoskeleton rearrangement leading to the engulfment of the microorganism (Altier, 2005; Shea, Hensel, Gleeson, & Holden, 1996; Zhou & Galán, 2001).

Increased epithelial permeability is directly reflected in the measurement of transepithelial electrical resistance (TEER). TEER is a quantitative technique used to analyze the integrity of cell tight junctions in cell culture models without causing cell damage (Srinivasan et al., 2015). Numerous studies have reported that probiotic bacteria can prevent the decrease in TEER caused by the disruption of tight junctions during infection, inflammation and stress (Ait-Belgnaoui et al., 2006; Czerucka, Dahan, Mograbi, Rossi, & Rampal, 2000; Dahan et al., 2003; Ewaschuk et al., 2008; Madsen et al., 2001; Moal et al., 2002; Mumy, Chen, Kelly, & McCormick, 2008; Resta-Lenert & Barrett, 2006; Sherman et al., 2005). The ability of some LAB (lactic acid bacteria) to induce reduction of inflammatory marker production of intestinal epithelial cells through interactions with the host (e.g. cell tight junction proteins) contributes to the decrease in barrier permeability and preventing increased movement of harmful
substances (Madsen et al., 2001). HT-29 and Caco-2 epithelial cells exposed to *Streptococcus thermophilus* and *Lactobacillus acidophilus* demonstrated a protective effect against enteroinvasive *E. coli* causing lowered adhesion, invasion, as well as an increase of TEER (Resta-Lenert & Barrett, 2003). *Lactobacillus rhamnosus* also demonstrated a protective effect of epithelial cells by inhibiting enterohemorrhagic *E. coli* and increasing TEER resulting in decreased epithelial permeability (Johnson-Henry, Donato, Shen-Tu, Gordanpour, & Sherman, 2008). It has been also suggested that lactic acid bacterial secreted factors may directly or indirectly affect epithelial cells. Surface active proteins released by certain strains of LAB functioning in the inhibition of pathogen adhesion may act as signals to intestinal epithelial cells to decrease the paracellular permeability to preserve barrier function (Heinemann, 2000). This concept was also demonstrated when enterohemorrhagic *E. coli* pathogenesis was hindered by surface layer proteins purified from *Lactobacillus helveticus* and resulted in no increase of epithelial cell permeability (Johnson-Henry, Hagen, Gordonpour, Tompkins, & Sherman, 2007). In addition, molecules produced by *Bifidobacterium bifidum* interfered with attachment and invasion of both *E. coli* and *Salmonella* to HeLa cells and macrophages (Bayoumi & Griffiths, 2012). Treatment with cell free fractions also caused down regulation of virulence gene expression in both pathogens (Bayoumi & Griffiths, 2012). The presence of bioactive molecules produced by LAB have shown suppressive effects on the virulence of some foodborne pathogens, however, the mode of action still remains unexplained. Thus, the objective of this study was to investigate whether *Lactococcus lactis* subsp. *cremoris* JFR1 could reduce virulence gene expression of *Salmonella* Typhimurium DT104 utilizing cell culture models. *S. Typhimurium ΔoppA*, defective in peptide transport, was used in order to determine whether peptide transport was involved in the mechanism of antivirulence action.

### 3.2 Materials and Methods
3.2.1 Preparation of cell culture models

Human colon carcinoma cell line HT29-MTX and human adenocarcinoma cell line Caco-2 were obtained from the CRIFS (Canadian Research Institute for Food Safety, Guelph, ON) Culture Collection. Cells were cultured in T-75 cm² flasks with growth media consisting of Dulbecco’s modified eagle medium (DMEM) (Sigma-Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum (Invitrogen Canada Inc., Burlington, Ontario, Canada), 1% of penicillin-streptomycin solution (Invitrogen Canada Inc., Burlington, Ontario, Canada), and 2 mM L-glutamine (Invitrogen Canada Inc., Burlington, Ontario, Canada) for HT29-MTX and an addition of 25 mM HEPES buffer (Sigma-Aldrich, St. Louis, USA) and 1% of nonessential amino acids (Sigma-Aldrich, St. Louis, USA) for Caco-2 cells. Cell cultures were maintained at 37°C and 5% CO₂ at constant humidity. Cell culture media was replaced every two days until 80-90% confluent. Cocultures of epithelial cells were made combining 75% Caco-2 cells and 25% HT29-MTX cells (Mahler et al., 2009). Epithelial cells were grown separately until confluency and were mixed before seeding into polycarbonate membrane inserts (BD Biosciences, Mississauga, ON, Canada) as described in the Salmonella invasion assay section.

3.2.2 Bacterial strains

Two Lactococcus lactis subsp. cremoris JFR1 strains were used in this study (donated by Prof. Hassan, South Dakota State University). The exopolysaccharide producing strain (JFR+) was grown in M17 broth (BD Biosciences, Mississauga, ON, Canada) supplemented with 0.5% lactose while the exopolysaccharide nonproducing strain JFR- was grown in M17 supplemented with 0.5% glucose. Strains were incubated overnight at 30°C under modified atmospheric conditions (4% O₂, 20% CO₂, 76% N₂. Salmonella enterica subsp. enterica serovar Typhimurium DT104 (obtained from CRIFS Culture Collection) and Salmonella enterica subsp. enterica serovar Typhimurium ΔoppA (donated by Prof. Brown, McMaster
University) were grown aerobically in Luria-Bertani broth (Difco, Detroit, USA) at 37°C in a
shaking incubator at 200 rpm for 18 hours. The S. Typhimurium ΔoppA mutant strain was
supplemented with 50 µg/mL of kanamycin.

3.2.3 SRB (sulforhodamine B) method for cell proliferation
The SRB method was performed for each HT29-MTX, Caco-2 as well as cocultures of Caco-2:HT29-MTX cells. Confluent cells were detached and harvested with trypsin-EDTA then
diluted in fresh DMEM. Caco-2 and HT29-MTX cells were each diluted and seeded directly
at a concentration of 2×10³ cells/mL. For coculture, cells were diluted to 2×10³ cells/mL
separately before combining at 75:25 ratio. 100 µL of diluted cells were seeded into a 96 well
tissue culture treated flat bottom plate (Corning 96 well TC treated microplates) and allowed
to grow for 24 hours at 37°C and 5% CO2. After 24 hours, old media was removed, and cells
were stimulated with treatments (prepared in serum free DMEM) for another 24 hours. The
treatments were 50%, 25%, 10% and 5% of JFR+ overnight culture or JFR- overnight culture
performed in at least triplicates. Following the treatments and aspiration of old DMEM, 200
µL of serum free DMEM with 50 µL of 50% cold TCA (Trichloroacetic acid, Sigma-Aldrich,
St. Louis, USA) was added to each well to help reduce background for 1 hour at 4°C. TCA was
then removed and wells were washed 5 times with 300 µL of sterile water. Plates were air dried
until all traces of water were removed followed by the addition of 50 µL of SRB dye solution
(0.4% w/v in 1% acetic acid; Sigma-Aldrich, St. Louis, USA). Plates were kept at room
temperature on a low speed orbital shaker. After 30 minutes, wells were washed with 300 µL
of 1% acetic acid 4 times and air dried again. Before reading plates at 570 nm (Synergy™ HT
Multi Detection Microplate Reader; Bio-Tek Instruments, Winooski, Vermont, USA), the dye
was solubilized with 100 µL of 10 mM Tris buffer (Sigma-Aldrich, St. Louis, USA).
3.2.4  Effect of JFR on Salmonella virulence gene expression in the absence of epithelial cells

The Salmonella strains were grown in presence of JFR +/- until mid-exponential phase in broth to determine the effect of JFR +/- on the S. Typhimurium (DT104 and ΔoppA) gene expression. For this, Salmonella strains were grown as described in the bacterial strains section, Salmonella was then centrifuged and washed twice with phosphate buffered saline (PBS; Invitrogen Canada Inc., Burlington, Ontario, Canada) after 18 hours. Following centrifugation, the pellet was reconstituted with fresh LB broth to the same volume. JFR+ and JFR- overnight cultures were centrifuged to remove spent M17 broth. The pellet was reconstituted with fresh M17 broth and vortexed thoroughly before use. 50 mL sterile tubes were filled with 4.5 mL of fresh LB broth followed by 10% of pretreatment (JFR+, JFR-, M17 broth as negative control for JFR strains, or LB broth as negative control for the Salmonella cultures) and 1% of washed S. Typhimurium (DT104 or ΔoppA). All tubes were incubated for 3 hours at 37°C in a shaking incubator at 200 rpm. Bacterial pellets were then collected and prepared for RNA extraction. Each treatment was done in triplicate with three independent studies.

3.2.5  Salmonella invasion assay

All confluent epithelial cells were harvested from 75-cm² flasks and detached with Trypsin-EDTA (0.25% trypsin, 1 mM EDTA (1x) (Invitrogen Canada Inc., Burlington, Ontario, Canada). Epithelial cells diluted to a concentration of 1×10⁵ cells/mL were seeded into transwell plates (Corning Glass Works, Mississauga, ON, Canada) and incubated at 37°C and 5% CO₂ for 21 days until morphological and functional differentiation was complete (Lesuffleur et al., 1991). On day 21, cell culture media was removed, and the cells were washed
with PBS two times followed by the addition of 10% pretreatment prepared in DMEM with no additives. Cell monolayers were pretreated for 1 h at 37°C and 5% CO2 prior to *Salmonella* infection with each strain, DT104 or ΔoppA. The pretreatments consisted of 10% JFR+, JFR-, LB broth or M17 broth. Following 1 hour pretreatment, 10% of diluted *Salmonella* (centrifuged and washed twice with PBS) was added into each well and incubated for another 2 hours at 37°C and 5% CO2. The integrity of the cell monolayers was monitored at 0, 1, and 3 hours by TEER (transepithelial electrical resistance) measurement using an EVOM2 epithelial voltmeter (WORLD Precision Instruments, Sarasota, FL, USA). After the 2 h invasion, the basal layer media was retrieved and stored at -20°C for cytokine analysis via commercial ELISA kit. The epithelial cells were then washed with PBS again followed by two periods of gentamicin incubation for removal of non-invaded *Salmonella* (350 μg/mL for 1 hour and 200 μg/mL for 30 minutes). After another washing step, epithelial cells were lysed with 1 mL of 1% Triton X-100 for 10 min at 37°C to release invaded *Salmonella*. Lastly, the samples were collected for RNA extraction as well as bacterial enumeration. An aliquot of 100 μL for each invasion experiment (JFR+, JFR-, with/without *S. Typhimurium* DT104 or ΔoppA) was serially diluted in sterile peptone water prior plating in duplicate on LB agar. Colony counts were done after incubation at 37°C during 18 h.

### 3.2.6 Determination of *Salmonella* virulence gene expression using RT-qPCR

Following the invasion assay or the interaction in solution, the entire contents inside the transwells/tubes were collected and centrifuged for 5 minutes at 10,000 × g (Spectrafuge 16M, Labnet International, Edison, USA) to remove the supernatant. The remaining pellet was suspended in 100 μL of PBS and 600 μL of RNAProtect Bacteria Reagent (Qiagen, Toronto, ON) and incubated for 5 minutes at room temperature following manufacturer’s instructions. Qiagen’s RNeasy Plus Mini Kit and MinElute Cleanup Kit were used for RNA extraction and
purification. In brief, pellets were dissolved in 200 μL of Tris-EDTA buffer (BioUltra for molecular biology, pH 8.0, Sigma-Aldrich Co., St. Louis, USA), 20 μL of proteinase K (Qiagen, Toronto, ON) and 60 μL of freshly prepared lysozyme (20 mg/mL) (Sigma- Aldrich Co., St. Louis, USA) and incubated for 1 hour at 450 rpm (Thermomixer, Eppendorf, Hamburg, Germany). The remaining steps were completed using kit materials and instructions. The final product was analyzed using the NanoDrop 1000 spectrophotometer (Thermoscientific, Wilmington, DE) as well as gel electrophoresis (1% agarose gel) to confirm the quality of the RNA. Later, the extracted RNA was converted into its complementary deoxyribonucleic acid (cDNA) using the the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Burlington, Ontario, Canada). The RT reaction mixture was composed of 1000 ng/mL of extracted RNA, 2 μL of 10× RT buffer, 2 μL of 10× random hexamer primers, 1 μL of Multiscribe reverse transcriptase and 0.8 μL of 25× deoxyribonucleoside triphosphate adjusted with nuclease-free H2O to a final volume of 20 μL. The Mastercycler Gradient Thermocycler (Eppendorf, Mississauga, Ontario, Canada) was used under the following conditions for cDNA synthesis: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and a final step holding the temperature at 4°C. After cDNA synthesis gene expression of *Salmonella* was determined using the ViiATM 7 Real-Time PCR System (Applied Biosystems, Burlington, Ontario, Canada). A list of housekeeping and target genes is presented in Table 3.1. The qPCR reaction volume was 20 μL, which contained 5 μL cDNA, 10 μL PowerUp SYBR Green Master Mix, 1.6 μL each forward and reverse primers, and 1.8 μL RNase free water. All samples were analyzed at least in triplicate in 96 well plates (MicroAmp® Fast Optical 96 Well Reaction Plate, Life Technologies, Canada) under the following conditions: UDG activation at 50°C for 2 min, 40 cycles of denaturation (95°C for 15 s), annealing (54°C for 30 s) and amplification (72°C for 45 s) followed by the dissociation curve. Transcript levels were quantified by
normalizing target gene expression to expression of the housekeeping gene to calculate relative fold change using the ΔΔCt method (Livak & Schmittgen, 2001; Pfaffl, 2001).

### 3.2.7 Statistical analysis

All experiments were performed three times with triplicates of each sample. Means and standard deviations of RT-qPCR data were analyzed using the t-test analysis with significance at \( P < 0.05 \). Statistical analysis of remaining experiments was carried out using analysis of variance (ANOVA) followed by Tukey’s post-test with significance at \( P < 0.05 \).

**Table 3.1** List of housekeeping and target genes and primers used for *Salmonella* virulence gene expression experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoD</td>
<td>Housekeeping gene</td>
<td>F: GTGAAATGGGCACCTGTTGAACCTG</td>
<td>(Velayudhan et al., 2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TTCCAGCAGATAGGTAATGGGTTTC</td>
<td></td>
</tr>
<tr>
<td>hilA</td>
<td>Transcriptional regulator of SPI-1</td>
<td>F: TGTCGGAAGATAAGAGCAT</td>
<td>(Sharma, 2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AAGGAAGTATCGCCAAATGTA</td>
<td></td>
</tr>
<tr>
<td>invA</td>
<td>Required for invasion of cells</td>
<td>F: GAAATTATCGCCACGTTCGCCGA</td>
<td>(Rahn et al., 1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TCATCGCACCCTCAAAGGAACC</td>
<td></td>
</tr>
<tr>
<td>sopD</td>
<td>Secreted effector protein</td>
<td>F: ATTAATGCGCGTAACTTTGA</td>
<td>(Sharma, 2014)</td>
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<td></td>
<td></td>
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### 3.3 Results

#### 3.3.1 Effect of *L. lactis* overnight cultures on cell proliferation

The effect of 50%, 25%, 10%, and 5% overnight culture treatment concentrations on the viability of epithelial cells was determined (Fig. 3.1). A treatment of M17 broth was added to confirm no effect on cell viability. At 50% concentration of JFR+ or JFR- overnight cultures, cell viability was significantly lower (50-60% viability) than the controls for HT29-MTX, Caco-2, and coculture cells. When treatment concentration was decreased to 25%, viability in all cell models used increased to approximately 70-75%. HT29-MTX, Caco-2, and coculture cells were unaffected at 10% and 5% thus suitable for further use.
Figure 3.1 SRB cell proliferation assay of HT29-MTX cells (A), Caco-2 cells (B) and cocultures of Caco-2:HT29-MTX cells (C) treated with 50% (checkered pattern), 25% (horizontal lines), 10% (vertical lines), or 5% (diagonal lines) of overnight culture. Data were analyzed by ANOVA and Tukey’s multiple comparison post-test with significance at $P < 0.05$.

3.3.2 Internalization of Salmonella into cell culture models
The M17 control was added to verify no effect of M17 broth during *Salmonella* invasion; showing no significant difference to the *Salmonella* control. Internalized DT104 was reduced approximately by 1 log when epithelial cells (HT29-MTX, Caco-2, and cocultures) were pretreated with JFR+/JFR- compared to the cells without JFR addition (Fig. 3.2-A, C, E). The number of *S. Typhimurium ΔoppA* internalized into epithelial cells remained unaffected compared to the cells that were not treated with JFR strains (Fig. 3.2-B, D, F).
**Figure 3.2** Number of internalized *S. Typhimurium* DT104 (A, C, E) or *S. Typhimurium* Δ*oppA* (B, D, F) during invasion of HT29-MTX (A, B), Caco-2 (C, D), or coculture cells (E, F) by *S. Typhimurium* DT104 (A, C, E) or *S. Typhimurium* Δ*oppA* (B, D, F). Statistical significance was calculated using analysis of variance (ANOVA) followed by Tukey’s post-test. Letters represent significant difference (*P* < 0.05).

### 3.3.3 TEER of epithelial cells

The TEER of HT29-MTX cells showed an increase regardless of the presence of pretreatment from 0 to 1 h (Fig. 3.3-A, B). After the addition of *S. Typhimurium* DT104 (1 to 3 h), the TEER was maintained in HT29-MTX cells pretreated with JFR+ or JFR- at over 150% (Fig. 3.3-A). On the contrary, this effect was not seen when cells were infected with *S. Typhimurium* Δ*oppA*, as TEER dropped (Fig. 3.3-B). Caco-2 cells showed a similar trend where the increased TEER from time 0 to 1 h was only maintained (at approximately 150%) by cells pretreated with JFR+ or JFR- during DT104 infection (Fig. 3.3-C). Similar to HT29-MTX, the addition of *S. Typhimurium* Δ*oppA* to Caco-2 cells resulted in a drop in TEER for both controls and pretreatments (Fig. 3.3-D). Cocultures comprised of both cell types displayed similar behavior where TEER was only maintained during DT104 infection on cells pretreated by either JFR +/- (Fig. 3.3-E, F).
Figure 3.3 Transepithelial electrical resistance of HT29-MTX (A, B), Caco-2 (C, D), and coculture cells (E, F) measured at times 0, 1 (time of Salmonella addition), and 3 hours of invasion assay. Treatment groups include: S. Typhimurium DT104 (A, C, E) or S. Typhimurium ΔoppA (B, D, F) (black bars), M17 control (horizontal lines), L. lactis subsp. cremoris JFR+ overnight culture (dotted pattern), and L. lactis subsp. cremoris JFR- overnight culture (diagonal lines). Statistical significance was calculated using analysis of variance (ANOVA) followed by Tukey’s post-test. Letters represent significant difference (P< 0.05).

3.3.4 Production of IL-8

HT29-MTX cells showed the highest levels of IL-8 produced at the end of the invasion assay (Fig. 3.4). Invasion of HT29-MTX by S. Typhimurium DT104 in the presence of overnight
cultures demonstrated lower production (approximately 205 ± 33 pg/mL) of IL-8 cytokine compared to both the DT104 (287 ± 12 pg/mL) and M17 control (266 ± 20 pg/mL) (Fig. 3.4-A). Production of IL-8 in cocultures of HT29-MTX and Caco-2 cells displayed a similar trend with pretreated cells producing approximately 205 ± 14 pg/mL compared to the control (260 ± 238 pg/mL) (Fig. 3.4-E). The amount of IL-8 produced by Caco-2 cells was relatively low compared to HT29-MTX and cocultures but remained stable with and without pretreatment, ranging from 42 to 57 pg/mL (Fig. 3.4-C). When epithelial cells were invaded by S. Typhimurium ΔoppA, no decrease in IL-8 production was observed among all treatments (Fig. 3.4-B, D, F).
Figure 3.4 Pro-inflammatory cytokine IL-8 production by epithelial cell lines HT29-MTX (A, B), Caco-2 (C, D), and cocultures of Caco-2:HT29-MTX (E, F) during S. Typhimurium DT104 (A, C, E) or S. Typhimurium ΔoppA (B, D, F) infection. Statistical significance was calculated using analysis of variance (ANOVA) followed by Tukey’s post-test. Letters represent significant difference ($P < 0.05$).

3.3.5 Expression of Salmonella virulence genes in presence and absence of epithelial cells

Both Lactococcus lactis subsp. cremoris strains, JFR+ and JFR-, significantly suppressed the virulence gene expression of hilA (>5-fold), invA (>6-fold), and sopD (>3-fold) in S. Typhimurium DT104 when the interaction was in solution (Table 3.2). However, gene
expression in *S. Typhimurium* ∆*oppA* was not significantly different, showing < 2-fold changes for *hilA* and *invA* with JFR+ and in *sopD* with JFR-. The expression of the same virulence genes was slightly different when invading the epithelial cells than in solution. DT104 *hilA* expression was down regulated by approximately 8-fold in the presence of both JFR+ and JFR- during invasion of HT29-MTX, Caco-2, and coculture cells (Table 3.2). The expression of DT104 *invA* was down regulated by approximately 6-fold only in pretreated HT29-MTX and coculture cells; whereas *sopD* expression was suppressed only in Caco-2 and cocultures. This resulted in suppression of all tested virulence genes of DT104 in pretreated coculture cells. Although significant suppression was observed in DT104 virulence gene expression, the *S. Typhimurium* ∆*oppA* mutant did not exhibit the same behavior (Table 3.2). No down regulation was displayed by *S. Typhimurium* ∆*oppA* (<2-fold change) during invasion of any of the epithelial cell culture models.
**Table 3.2** Virulence gene expression of *Salmonella* Typhimurium treated with 10% overnight cultures of JFR+ or JFR-. Statistical significance was calculated using t-test analysis; each letter represents significant difference $P<0.05$.

<table>
<thead>
<tr>
<th>Absence of epithelial cells</th>
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<th>S. Typhimurium ΔoppA</th>
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<td>Treatment</td>
<td><strong>hilA</strong></td>
<td><strong>invA</strong></td>
</tr>
<tr>
<td>10% JFR+</td>
<td>-5.3±0.2$^{a}$</td>
<td>-7.8±0.5$^{a}$</td>
</tr>
<tr>
<td>10% JFR-</td>
<td>-5.6±0.3$^{a}$</td>
<td>-6.7±0.5$^{b}$</td>
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<tbody>
<tr>
<td>Treatment</td>
<td><strong>hilA</strong></td>
<td><strong>invA</strong></td>
</tr>
<tr>
<td>10% JFR+</td>
<td>-8.7±1.0$^{a}$</td>
<td>-5.9±0.6$^{a}$</td>
</tr>
<tr>
<td>10% JFR-</td>
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<tr>
<td>Treatment</td>
<td><strong>hilA</strong></td>
<td><strong>invA</strong></td>
</tr>
<tr>
<td>10% JFR+</td>
<td>-8.2±0.6$^{a}$</td>
<td>-0.6±0.4$^{a}$</td>
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<td>10% JFR-</td>
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<tr>
<td>Treatment</td>
<td><strong>hilA</strong></td>
<td><strong>invA</strong></td>
</tr>
<tr>
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<td>-8.1±0.7$^{a}$</td>
<td>-6.7±0.9$^{a}$</td>
</tr>
<tr>
<td>10% JFR-</td>
<td>-7.4±0.3$^{a}$</td>
<td>-4.9±0.7$^{b}$</td>
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3.4 Discussion

*Salmonella* are unique pathogens which can internalize into nonphagocytic cells such as the epithelial cells used in this study. The inclusion of a coculture model allows for an improved representation of an *in vivo* system, consisting of two of the most abundant cell types found in the human intestinal tract (Brittan & Wright, 2004; Hilgendorf et al., 2000; Walter, Janich, Roessler, Hilfinger, & Amidon, 1996). In such models, many external factors such as temperature and pH must be carefully controlled to maintain healthy cell development. The pH of the human intestinal tract ranges from approximately pH 6 to pH 7 (Evans et al., 1988; Fallingborg, 1999), accordingly, intestinal cell models should also be cultivated at near neutral pH values (Greene & Klaenhammer, 1994). The acidic pH of the JFR+ and JFR- overnight cultures affected the viability of the intestinal epithelial cells at high concentrations since the buffering effect of DMEM was not enough. The chosen concentration of 10% overnight culture prepared in DMEM, was buffered to approximately pH 6.7, which is suitable for cell culture work while eliminating any pH effect.

For successful uptake, *Salmonella* must be in close proximity to the host cell for apparatus formation and internalization. Expression of SPI-1 genes, regulated by HilA, are responsible for the type III secretion system encoding the secretion apparatus for subsequent delivery of effector proteins into the host cell (Altier, 2005). In this study, epithelial cells pretreated with *L. lactis* subsp. *cremoris* strains were less infected by *S. Typhimurium* DT104, however, *S. Typhimurium ΔoppA* remained virulent regardless of epithelial cell pretreatment indicating the critical role of peptide transport either directly or indirectly affecting *Salmonella* virulence. The structural organization of the *Salmonella Typhimurium* oligopeptide transport system is very similar to *Bacillus subtilis, Bacillus thuringiensis, Listeria monocytogenes,* and *Streptococcus suis* consisting of *oppA, B, C, D,* and *F* (Gominet, Slamti, Gilois, Rose, &
Lereclus, 2001; Zheng et al., 2018). The OppA transport protein is one of the most abundant permeases found in both gram-negative and gram-positive microorganisms. It functions in the uptake of oligopeptides two to five amino acids in size (Hiles, Gallagher, Jamieson, & Higgins, 1987), thus a lack of this permease potentially indicates a shortage of oligopeptides being taken up into the *Salmonella*. A mutation in the gene encoding OppA resulted in impaired uptake of peptides in *Salmonella* and *E. coli* whereas Dpp and Tpp mutations did not affect peptide transport into the bacteria (Goodell & Higgins, 1987). In *L. monocytogenes*, it was demonstrated that OppA plays a role in the intracellular survival of the pathogen both *in vitro* and *in vivo* (Borezee, Pellegrini, & Berche, 2000). Deletion of oppA in *L. monocytogenes* displayed delayed growth, however, the pathogen still remained fully virulent (Borezee et al., 2000). Comparison of the wild type and oppA mutant during infection of macrophages showed that the number of mutant *L. monocytogenes* able to escape phagosomes was approximately half of the wild type (Borezee et al., 2000). This demonstrated the significance of OppA in growth and multiplication of the pathogen inside the phagosomes and finally release into the cytoplasm possibly by means of signaling pathways modulated by peptide uptake (Borezee et al., 2000). Delayed growth was thought to be attributed to the lack of nutrients due to a nonfunctional OppA permease. Not only is Opp implicated in nutrition, but it has been reported that several gram-positive pathogens utilize the Opp system for transportation of pheromones needed to activate virulence genes in pathogens such as *B. thuringiensis* and *Streptococcus agalactiae* (Gominet et al., 2001; Samen, Gottschalk, Eikmanns, & Reinscheid, 2004).

The oligopeptides present in the JFR cultures are derived from hydrolysis of proteins supplied in the nutrient media, including digests of casein and soy proteins. The extracellular proteinase of *L. lactis* strains cleave caseins to form smaller peptides ranging from 4 to 30 amino acid residues (Juillard et al., 1995) which can be subsequently imported into the bacterial cell by
dipeptide, tripeptide, and oligopeptide transport systems which both *L. lactis* and *S. Typhimurium* carry. During invasion, *Salmonella* enters a niche where limited nutrient resources (i.e. peptides produced by JFR) are available for growth and survival. In addition, the high-level and constitutive expression of *opp* indicates that oligopeptide uptake plays an important role in *Salmonella* metabolism and survival compared to Tpp and Dpp transport systems (Hiles & Higgins, 1986; Jamieson & Higgins, 1984). The presence of antimicrobial peptides affecting *S. Typhimurium* virulence has been previously reported (Bader et al., 2003), however, the culture medium produced by both JFR strains showed no direct inhibition of the growth of *S. Typhimurium* DT104 or *S. Typhimurium ΔoppA* (data not shown). It was reported that proteinaceous molecules produced by *B. bifidum* have demonstrated interference in attachment and virulence of *Salmonella* and *E. coli* on enterocytes (Bayoumi & Griffiths, 2012). Furthermore, in an in vivo study, peptidic fractions produced during *Lactobacillus helveticus* milk fermentation demonstrated an antivirulence effect in mice models (Tellez, Corredig, Turner, Morales, & Griffiths, 2011). In addition, in an animal model, commercial yogurt combined with purified bioactive molecules produced by *L. acidophilus* La-5 resulted in decreased attachment and colonization of *E. coli* O157:H7 (Zeinhom et al., 2012). Comparison of the gene expression levels of *hilA*, *invA*, and *sopD* of *S. Typhimurium* DT104 and *S. Typhimurium ΔoppA* also indicate that the oligopeptide transport mutant is insensitive to the treatment with both JFR strains. In pretreated HT29-MTX cells, suppression of *hilA* and *invA* in *S. Typhimurium* DT104 suggest interference with the formation of the T3SS apparatus (Darwin & Miller, 1999). The T3SS apparatus is involved in the translocation of effector proteins crucial for formation of the translocon pore and further cytoskeleton remodeling of the host cell membrane required for *Salmonella* internalization (Bakowski, Cirulis, Brown, Finlay, & Brumell, 2007; Darwin & Miller, 1999). Lowered expression of genes controlling the T3SS apparatus formation interfere with the ability of *Salmonella* to enter the host cell thus
resulting in lower numbers of internalized DT104. Moreover, pretreated Caco-2 cells showed down regulation of DT104 hilA and sopD, which are effector proteins promoting invasion by altering host cell membrane dynamics (Raffatellu et al., 2005), and accordingly, during infection of the coculture model, S. Typhimurium DT104 displayed suppressed expression of hilA, invA, and sopD associated with intervention of the T3SS apparatus and following effectors. However, the fact that Salmonella lacking oligopeptide transport did not show the same response to JFR treatments suggests that a link exists between Salmonella virulence and peptide transport. It is possible that oligopeptides found in JFR treatments could be responsible for interference with specific steps of initial invasion, as observed with comparisons between S. Typhimurium DT104 S. Typhimurium ΔoppA.

In addition, it has been reported that the Leucine-responsive regulatory protein (Lrp) plays a role in regulating Salmonella virulence (Baek, Wang, Roland, & Curtiss, 2009). Lrp is capable of direct interactions with the promotor regions of hilA, invF and ssrA resulting in virulence suppression; as demonstrated by a constitutively expressed lrp Salmonella mutant (Baek, Wang, Roland, & Curtiss, 2009). In addition, it was shown that the presence of leucine did not affect the expression of hilA or ssrA. However, invF expression was reduced in the presence of leucine via stronger interactions and increased binding of Lrp to the promotor of invF with no changes in lrp expression. Lrp is a global transcriptional regulator important in amino acid metabolism and transport, regulation of branched-chain amino acid synthesis, and pilus assembly (Cowles, Cowles, Richards, Martens, & Goodrich-Blair, 2007; Friedberg, Platko, Tyler, & Calvo, 1995; Tani, Khodursky, Blumenthal, Brown, & Matthews, 2002). It affects more than 10% of E. coli genes (Tani et al., 2002) and is thought to act similarly in Salmonella since there is 99% homology in amino acid sequence identity (Friedberg et al., 1995). The general nutritional status of the bacteria is monitored by Lrp by sensing L-leucine.
concentrations in the bacterial cell (Landgraf, Wu, & Calvo, 1996). In *E. coli*, *lrp* expression is inversely related to growth rate; an increase in guanosine 3',5'-bispyrophosphate, as a result of amino acid limitation, stimulates the expression of *lrp* (Landgraf et al., 1996). Similarly, Baek et al. (2009) reported that under conditions with less available nutrients (minimal media compared to LB), *lrp* expression was increased in wild type *Salmonella* which resulted in reduced invasion *in vitro*. This antivirulence mechanism of Lrp functions to enhance survivability of *Salmonella* by achieving efficient transmission and survival in host environments. In this research, the removal of *oppA* in *Salmonella* is likely to decrease the amino acid pool resulting in a possible limitation of amino acids which could lead to modified Lrp activity and ultimately affect virulence. The relationship between oligopeptide transport systems and Lrp has not been established, however, there is indication that the control of virulence traits is interrelated to the nutritional status (i.e. function of OppA permease) of the pathogen from this study.

It could be anticipated that oligopeptides present in the overnight cultures could be transported and accumulated in the *Salmonella* before causing an interference with virulence (i.e. quorum sensing inhibiting molecules). Tellez et al. (2011) purified peptides produced by *L. helveticus* milk fermentation and reported that only peptide concentrations over 25 µg/mL exhibited down regulation of *Salmonella* virulence gene *ssrB*, suggesting perhaps 10% overnight culture treatment contained enough oligopeptide of interest to cause a disruption in virulence gene expression of *hilA*, *invA*, and *sopD*. On the other hand, these oligopeptides could play a role in quorum quenching of *S. Typhimurium*. *Salmonella* and *E. coli* both harbor the LuxS quorum sensing system for bacterial cell communication through the production of autoinducer molecules (Surette, Miller, & Bassler, 1999). In *E. coli* O157:H7, this system is involved with intestinal colonization where virulence genes encoded on LEE pathogenicity islands are
activated by quorum sensing (DeLisa, Wu, Wang, Valdes, & Bentley, 2001; Sperandio, Mellies, Nguyen, Shin, & Kaper, 1999). It was reported that cell free spent medium produced by *L. acidophilus* La-5 was able to inhibit *E. coli* O157:H7 production of autoinducer-2 (AI-2) signaling molecules interfering with virulence (Medellín-Peña, Wang, Johnson, Anand, & Peng, 2007). Furthermore, *L. lactis* oligopeptides could also interfere with quorum sensing by interacting directly with LuxS, the enzyme needed for AI-2 production, as shown in another Gram-negative pathogen *Edwardsiella tarda* (Zhang, Jiao, Hu, & Sun, 2009). Virulence of *E. tarda* was mitigated by the presence of small peptides which were generated with sequence similaries to LuxS via hydrophobic interactions with LuxS or occupation of the LuxS metal binding site which is necessary for its catalytic activity (Zhang, Jiao, Hu, & Sun, 2009). Although the peptides present in JFR cultures were not studied, it is possible that perhaps *L. lactis* oligopeptides may function in a similar manner.

Other studies have also reported LAB and probiotic species such as *B. bifidum, L. acidophilus* and *L. helveticus* that are capable of attenuating virulence of pathogens including *Salmonella, E. coli* and *Clostridium difficile* through the secreted peptides as well as metabolites present in the cell extract (Bayoumi & Griffiths, 2010; Tellez et al., 2011; Yun, Oh, & Griffiths, 2014). Although the JFR+ strain used in this study produces exopolysaccharides (EPS); a previous *in vitro* investigation comparing the EPS production/no production, showed that both strain treatments effectively suppressed virulence gene expression in *S. Typhimurium* ruling out the effect of the EPS (Zhang et al., 2016). Moreover, the use of cell free *L. acidophilus* treatments resulted in inhibited virulence of *Campylobacter jejuni* indicating molecules produced exert antipathogenic effects (Mundi, Delcenserie, Amiri-Jami, Moorhead, & Griffiths, 2013). It has also been reported that peptides secreted by *Bifidobacterium infantis* enhanced barrier function in T84 human epithelial cells as well as immune modulation in *in vivo* colitis models.
In epithelial cells, intercellular junctional complexes are comprised of several constituents including tight junctions, adherent junctions, gap junctions and desmosomes (Ohland & MacNaughton, 2010). Not only did the JFR treatments exert an effect on Salmonella, but also provided benefits to the intestinal epithelial cells. Several reports have shown the positive effect brought by LAB and components produced/found in the cell free spent medium on host cells (Anderson, Cookson, McNabb, Kelly, & Roy, 2010; Chen, Hsiao, Hong, Dai, & Chen, 2012; Madsen et al., 2001; Peng, 2014; Sherman et al., 2005). For instance, epithelium barrier function was strengthened by treatment with Lactobacillus kefiranofaciens M1 isolated from milk kefir grains shown by increased TEER (Chen et al., 2012). In vivo evidence demonstrated decreased production levels of inflammatory cytokines and higher levels of anti-inflammatory cytokine which have been suggested to be contributing factors in maintaining epithelium barrier function (Chen et al., 2012; Madsen et al., 2001). The decrease in intestinal barrier permeability, reflected in higher TEER values, was demonstrated in epithelial cells treated with overnight cultures of JFR+ and JFR- during infection with S. Typhimurium DT104 but not with S. Typhimurium ΔoppA. However, information describing the link between oligopeptide permease in Salmonella and bioactive peptides is limited, thus, no comparison can be made to corroborate the potential role of oligopeptide transport in these specific situations.

Many cell types including epithelial cells have been observed to produce cytokines. IL-8 has been observed in vitro in many cell types including neutrophils, endothelial cells, hepatocytes and epithelial cells (Strieter et al., 1989; Thornton, Strieter, Lindley, Baggiolini, & Kunkel, 1990). Several studies have reported IL-8 production from epithelial cells including HT-29 and Caco-2 cells from pathogen or TNF-α stimulation (Bahrami, Macfarlane, & Macfarlane, 2011; Haller et al., 2000; Imaoka et al., 2008; Kolios et al., 1996). In addition, similar to this study,
production of IL-8 from Caco-2 cells compared to HT-29 were significantly less when stimulated with TNF-α, LPS, IL-1β and IFN-γ (Eckmann et al., 1993). Co-incubation of HT-29 epithelial cells with Lactobacillus rhamnosus GG and Salmonella or V. cholerae markedly reduced the production of IL-8 (Wagar, Champagne, Buckley, Raymond, & Green-Johnson, 2009), in agreement with observations in this study. JFR treated epithelial cells infected by S. Typhimurium DT104 resulted in lowered IL-8 secretion compared to S. Typhimurium ΔoppA. This corresponds to the previous concept discussed where immune modulation could contribute to preserving barrier function. In a diseased state, intestinal epithelial cell shedding is increased, largely due to the release of inflammatory cytokines such as TNF (Williams et al., 2014). During cell shedding, tight junction proteins assist the remaining epithelial cells to cover the newly exposed basement membrane and restore epithelium continuity (Williams et al., 2014). However, during inflammation, redistribution of the tight junction proteins may not be able to keep up with pathological epithelial cell shedding at multiple sites resulting in increased permeability (Jackson, Dai, & Sewell, 2000; Williams et al., 2014).

In a less complex system without the interactions of epithelial cells, the effects of Lactococcus strains on Salmonella virulence was also shown. The main findings further demonstrate the importance and effect of oligopeptide transport on Salmonella virulence. The continuous presence of oxygen during the gene expression experiment may have influenced the observed differences in the gene expression when compared to the cell culture models. SPI1 genes are optimally expressed at 37°C, which was the temperature used in both experiments, but oxygen-limiting conditions (Ernst, Dombroski, & Merrick, 1990; Lee & Falkow, 1990) were used only during cell culture invasion. The pH of both cell culture and in medium virulence studies ranged between pH 6.5 to pH 7 (data not shown) which support the maximum expression of SPI1 genes (Bajaj, Lucas, Hwang, & Lee, 1996). Even without the interactions with intestinal
cells in culture, an antivirulence effect was observed in broth culture and S. Typhimurium DT104 co-incubation, but not in the presence of S. Typhimurium ΔoppA. The potential role of oligopeptide transport in Salmonella virulence in the presence of JFR treatments was illustrated, presenting a potential contributing factor to the mode of action.

3.5 Conclusion

The findings of this study provide evidence that the oligopeptide transport system of Salmonella plays a crucial role in response to L. lactis subsp. cremoris JFR+ and JFR- treatments of epithelial cells. JFR treatments offer protection and maintenance of epithelial cells against S. Typhimurium DT104 but not S. Typhimurium ΔoppA invasion. The mechanism may involve quorum quenching of S. Typhimurium DT104, thus down regulating virulence expression resulting in lowered numbers of invasive Salmonella. This contributed to maintaining the transepithelial resistance of epithelial cells while also offering immune modulating effects of IL-8. The study represents one step towards establishing the connection between Salmonella oligopeptide transport and its influence on treatments of epithelial cells.

3.6 Acknowledgements

This research was supported by M. Corredig on behalf of the Ontario Dairy Council as well as the NSERC/DFO Industrial Research Chair on Dairy Microbiology held by G. LaPointe.
Chapter 4. Effect of fermented milk from *Lactococcus lactis* subsp. *cremoris* strain JFR1 on *Salmonella* invasion of intestinal epithelial cells

**Abstract**

The process of fermentation contributes to the organoleptic properties, preservation and nutritional benefits of food. Fermented food may interfere with pathogen infections through a variety of mechanisms, including competitive exclusion or improving the intestinal barrier integrity. In this study, the effect of milk fermented with *Lactococcus lactis* subsp. *cremoris* JFR1 on *Salmonella* invasion of intestinal epithelial cell cultures HT29-MTX, Caco-2 and cocultures of the two was investigated. Epithelial cells were treated for 1 h with *Lactococcus lactis* subsp. *cremoris* JFR1 fermented milk before infection with *Salmonella enterica* subsp. *enterica* Typhimurium. Treatment with fermented milk resulted in increased cell membrane integrity shown by monitoring the transepithelial electrical resistance (TEER) of epithelial cell models. The TEER values of treated epithelial cells increased during fermented milk treatment and remained constant for the duration of infection (up to 3 h), illustrating a protective effect. After gentamicin treatment to remove adhered bacterial cells, enumeration revealed a reduction in numbers of intracellular *Salmonella*. RT-qPCR data indicated a down regulation of *Salmonella* virulence genes *hilA*, *invA*, and *sopD* after treatment with fermented milk. Fermented milk treatment of epithelial cells also exhibited an immunomodulatory effect reducing the production of pro-inflammatory IL-8. In contrast to this, chemically acidified milk (glucono delta-lactone) failed to show the same effect on monolayer integrity, *S.* Typhimurium invasion and gene expression as well as immune modulation. Furthermore, treated epithelial cells infected with an *oppA* knockout *S.* Typhimurium did not show suppressed virulence gene expression. Collectively, these results suggest that milk fermented with *Lactococcus lactis* subsp. *cremoris* JFR1 is effective *in vitro* in the reduction of *Salmonella* invasion into intestinal
epithelial cell models. A functional OppA permease in *Salmonella* is required to obtain the antivirulence effect of fermented milk.

Keywords: *Lactococcus lactis* subsp. *cremoris* JFR1, *Salmonella* Typhimurium DT104, virulence gene expression, intestinal epithelial cells
4.1 Introduction

The human gastrointestinal tract is inhabited by $10^{10}$ to $10^{14}$ colony forming units (CFU) of microorganisms (Ley, Peterson, & Gordon, 2006). Commensal and beneficial strains as well as dietary components have shown to benefit the intestinal barrier function aiding in the prevention of “leaky gut”. Increased intestinal permeability is implicated in many inflammatory, autoimmune and atopic disorders such as inflammatory bowel disease and celiac disease (Suenaert et al., 2002; Ulluwishewa et al., 2011; Vogelsang, Schwarzenhofer, & Oberhuber, 1998; Wells, Rossi, Meijerink, & van Baarlen, 2010). Modes of action include boosting immune function through immunomodulatory stimulation (Mattar et al., 2002), lowering the luminal pH through lactic acid production (Gibson & Roberfroid, 1995; Sherman, Ossa, & Johnson-Henry, 2010), and by competitive exclusion of pathogens (Bernet, Brassart, Neeser, & Servin, 1994; Johnson-Henry, Hagen, Gordonpour, Tompkins, & Sherman, 2007).

It has been shown in mice that orally administered fermented milk containing *Lactobacillus casei* have demonstrated immunomodulatory capacity as well as the ability to conserve intestinal homeostasis through the elevated secretion of major effector molecules in the mucosal system (Noto Llana et al., 2013; Perdigon, Nader de Macias, Alvarez, Oliver, & Pesce de Ruiz Holgado, 1990). Furthermore, enteropathogenic *Escherichia coli* demonstrated lowered colonization and infection of several organs in mice treated with fermented milk (Sharma et al., 2014). In addition, when active fractions, isolated from *Lactobacillus acidophilus* La-5 cell free spent medium, were combined into commercial yogurt and fed to mice, attenuated attachment and colonization of *E. coli* was demonstrated (Zeinhom et al., 2012). Protective effects were also demonstrated by *Lactobacillus paracasei* CBA L74 fermented milk against *in vitro* invasion of *Salmonella* Typhimurium (Zagato et al., 2014). Furthermore, germ-free mice challenged with *S. Typhimurium*, through oral consumption of milk fermented by two strains isolated from artisanal cheese, *Lactobacillus rhamnosus* D1 and
Lactobacillus plantarum B7 also exhibited protection against Salmonella infection as well as immunomodulating effects (Acurcio et al., 2017).

The human intestinal epithelium is one of the front-line defense barriers to pathogen invasion and colonization. It actively commits to maintaining a balance between the host and microbes as well as host antimicrobial defense. Epithelial barrier selectively functions in controlling substance passage from one side of the epithelium to the other through transcellular and/or paracellular pathways (Balda et al., 1996). The intestinal epithelium is comprised of two major cell phenotypes, absorptive enterocytes and mucous producing goblet cells, which are most commonly represented in in vitro studies by Caco-2 cells and HT29-MTX cells, respectively (CenciČ & Langerholc, 2010; Noah, Donahue, & Shroyer, 2011). In order to maintain a healthy barrier between the luminal environment and internal environment of the body, it is necessary to maintain the connections, known as tight junctions, between individual epithelial cells. Tight junction proteins are located near the apical surface of epithelial cells and act as a seal between adjacent cells securing the paracellular space between them (Farquhar & Palade, 1963; Ulluwishewa et al., 2011). The barrier integrity can be quantified through the measurement of transepithelial electrical resistance (TEER) evaluating the “tightness” of neighboring epithelial cells. When this barrier is disrupted by harmful materials such as bacterial antigens and pathogens, a decrease in TEER will result.

Salmonella is a foodborne pathogen causing major public health concerns. Worldwide, it is estimated that Salmonella causes 93.8 million human cases annually (Majowicz et al., 2010). There are over 2,500 serovars of Salmonella enterica but most human cases are caused by Salmonella enterica Enteritidis and Typhimurium (Hendriksen et al., 2011; Rodrigue, Tauxe, & Rowe, 1990). Salmonella invasion into host epithelial cells involves the expression of
bacterial virulence genes regulated by transcriptional and post transcriptional regulators located on *Salmonella* pathogenicity islands (SPI) (Helms, Ethelberg, & Mølbak, 2005; Hendriksen et al., 2011; Jones et al., 2008). Invasion genes such as *hilA* and *invA*, which are part of the type III secretion system-1 in addition to effector genes such as *sopD*, are necessary for bacterial colonization (Helms et al., 2005; Jones et al., 2008; Martin et al., 2004). *Salmonella* enter the epithelial cell by microbial directed endocytosis (Kaniga, Bossio, & Galán, 1994), which requires a needle-like apparatus, encoded by SPI1, that stretches the external and internal cell membrane (Altier, 2005; Kubori, 1998). When the bacteria are in close proximity to the epithelial cell, this needle complex delivers secreted effector proteins into the host cell cytoplasm (Altier, 2005). This will cause the epithelial cell to rearrange its cytoskeleton and form membrane ruffles (Kaniga et al., 1994) leading to the engulfment of the bacteria inside vacuoles (Galán, 2001). The formation of *Salmonella*-containing vacuoles allows it to survive and multiply inside the host until dissemination when *Salmonella* can reach deeper tissue and induce inflammatory responses in the host. The down regulation of the *Salmonella* invasion genes could, therefore, lead to lower colonization of the intestinal epithelium prior to multiplication and inflammation. For instance, Bayoumi and Griffiths (2010) reported that cell-free spent medium produced from *Bifidobacterium infantis* and some lactic acid bacteria such as *Lactobacillus plantarum* and *L. acidophilus* were able to reduce the expression of *S. 5* Typhimurium invasion genes *hilA* and *ssrB* (Bayoumi & Griffiths, 2010). *L. acidophilus* GP1B cell extracts and live cell treatments demonstrated an antivirulence effect on *Clostridium difficile* *in vitro* as well as lowering mortality of CDAD mice (Yun, Oh, & Griffiths, 2014). Ding et al., (2005) observed lower expression of *flaA* (polar flagellum virulence determinant) in *Campylobacter jejuni* after treatment with cell-free extracts of milk fermented with strains of *Bifidobacterium* and lactic acid bacteria (Ding, Wang, & Griffiths, 2005). Thus, it has been reported that some lactic acid bacteria as potential probiotics can exert anti-virulence activity
(Bayoumi & Griffiths, 2012; Surendran Nair, Amalaradou, & Venkitanarayanan, 2017; Upadhyay, Upadhyaya, Mooyottu, & Venkitanarayanan, 2016). In a previous report, the down regulation of Salmonella virulence genes by spent medium produced by Lactococcus lactis subsp. cremoris strain JFR1 was demonstrated (Zhang et al., 2016). Therefore, in an effort to determine the potential effect of milk fermented with Lactococcus lactis subsp. cremoris JFR1 (JFR) on Salmonella invasion, intestinal epithelial cell models were used as an in vitro model to explore the potential mode of action.

4.2 Materials and methods

4.2.1 Bacterial strains and growth conditions

Lactococcus lactis subsp. cremoris JFR1 exopolysaccharide (EPS) producing strain (JFR+) and the non-EPS producing strain (JFR-) (donated by Prof. Hassan, South Dakota State University) were grown in M17 broth (BD Biosciences, Mississauga, ON, Canada) supplemented with either lactose (Difco, Detroit, USA) or glucose (Difco, Detroit, USA) at 0.5% (w/v). M17 agar (BD Biosciences, Mississauga, ON, Canada) was used when solidified medium was required. L. lactis subsp. cremoris JFR1 strains were incubated overnight at 30°C under modified conditions using a GasPak system (BD Biosciences, Mississauga, Canada). Salmonella enterica subsp. enterica serovar Typhimurium DT104 was obtained from the Culture Collection of the Canadian Research Institute for Food Safety (CRIFS) and grown in Luria-Bertani (LB; Difco, Detroit, USA) aerobically overnight on a shaking incubator at 37°C and 200 rpm. LB agar (Difco, Detroit, USA) was used for solid media preparation for Salmonella. Salmonella enterica subsp. enterica serovar Typhimurium ΔoppA, kindly donated by Dr. Eric Brown (McMaster University, Hamilton, Ontario), is a mutant lacking the oligopeptide permease gene oppA. The oppA mutant was grown in Luria-Bertani supplemented with kanamycin (50 µg/mL) aerobically overnight on a shaking incubator at 37°C and 200 rpm.
LB-kanamycin agar was used for solid media preparation for the mutant strain. *Salmonella enterica* subsp. *enterica* serovar Typhimurium GFP (also obtained from CRIFS Culture Collection) was grown overnight in tryptic soy broth (TSB, Difco, Detroit, USA) aerobically on a shaking incubator at 37°C and 200 rpm. For solid media preparation, 15 g of agar were added per litre of broth.

### 4.2.2 Cell culture preparation: HT29-MTX, Caco-2, and cocultures

The human colon carcinoma cell line HT29-MTX was differentiated from HT-29 cells (CRIFS Culture Collection) according to Lesuffleur et al. (1990). Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, USA) containing 10% heat inactivated fetal bovine serum (Invitrogen Canada Inc., Burlington, Ontario, Canada), 2 mM of l-glutamine and 1% penicillin-streptomycin solution (both from Invitrogen Canada Inc., Burlington, Ontario, Canada). Cells were grown in T-75-cm² flasks at 37°C and 5% CO₂ at constant humidity until a confluent monolayer was formed. Similarly, human colon adenocarcinoma Caco-2 cells were also obtained from the CRIFS Culture Collection. Cells were grown in DMEM supplemented with 10% FBS, 25 mM HEPES buffer (Sigma-Aldrich, St. Louis, USA), and 1% of each NEAA (Invitrogen Canada Inc., Burlington, Ontario, Canada), penicillin–streptomycin solution, and 2 mM L-glutamine. Caco-2 cells were cultured in T-75 cm² flasks and maintained at 37°C and 5% CO₂ at constant humidity. Cell culture media was changed every two days until 80-90% confluency was reached. Epithelial cells were then harvested from 75-cm² flasks by first detaching with Trypsin-EDTA (0.25% trypsin, 1 mM EDTA (1×) (Invitrogen Canada Inc., Burlington, Ontario, Canada). Cells were diluted and seeded at a final concentration of $1 \times 10^4$ cells/mL into 12-well plate transwells (Corning Glass Works, Mississauga, ON, Canada). Cocultures of epithelial cells consisted of Caco-2 cells combined with HT29-MTX mucous producing cells at a ratio of 75:25 (Caco-2: MTX). Each
cell culture was grown, detached, counted and diluted separately before combining. A final concentration of $1 \times 10^4$ cells/mL was seeded into 12-well plate transwells. Combined cells were maintained in DMEM supplemented with 10% FBS, 25 mM HEPES buffer, and 1% of each NEAA, penicillin–streptomycin solution, and 2 mM L-glutamine. All cell cultures were grown at 37°C and 5% CO$_2$ for 21 days (medium changed every 2 days) until completion of morphological and functional differentiation (Hilgendorf et al., 2000; Lesuffleur et al., 1991; Pontier, Pachot, Botham, Lenfant, & Arnaud, 2001).

4.2.3 Preparation of milk: fermentation, acidification and digestion

The milk used in these experiments was subjected to three different treatments: i) JFR+ fermentation or JFR- fermentation, ii) chemical acidification, and iii) digestion. The milk used for all conditions was skim milk powder (Difco, Detroit, USA) prepared according to the manufacturers’ instructions, combining 100 g of skim milk powder with 1 L of distilled water followed by sterilization. The prepared 10% milk was then stored at 4°C no more than 72 h until use. The fermentation with either JFR+/− was as follows: JFR strains were grown separately overnight and then centrifuged at $5000 \times g$ for 5 minutes (Beckman Instruments J2-MC Centrifuge, Palo Alto, CA, USA). Each pellet was then washed twice with sterile PBS (phosphate buffered saline; Invitrogen Canada Inc., Burlington, Ontario, Canada) and reconstituted in fresh M17 broth to its original volume. These reconstituted pellets were used to inoculate skim milk at 1% (v/v), separately. After this, fermentation was carried out by incubating aliquots at 30°C until a pH of 4.6 was reached. Subsequently, tubes were fast cooled by immersion into ice water and stored at -20°C (Miao, 2015). Acidified milk was prepared by adding 1% glucono delta-lactone (GDL) to skim milk. After acidification, GDL milk was also stored at -20°C. Three tubes of each milk preparation were used for the third condition, enzymatic digestion. These milk aliquots were batch digested following the protocol described
by (Majumder & Wu, 2009). In brief, the pH was adjusted to 2 by the addition of sterile 1 N HCl and then digested by pepsin (4%, w/w of protein) (Sigma-Aldrich Co., St. Louis, USA) for 3 h. The pH of the slurries was then increased to pH 7.5 by addition of sterile 1 N NaOH and further digested by pancreatin (2%, w/w of protein) (Sigma-Aldrich Co., St. Louis, USA) for another 3 h. The hydrolysis was then terminated by increasing the temperature to 95 °C and maintaining it for 10 min. All digestates were then stored at -20 °C until further use. A digestate control was also prepared treating sterile water according to the same protocol. The milk subjected to each condition is further referred to as: JFR+, milk fermented with EPS producing Lactococcus lactis subsp. cremoris JFR1; JFR-, milk fermented with EPS nonproducing L. lactis subsp. cremoris JFR1; GDL, chemically acidified milk; JFR+ dig, digested JFR+; JFR- dig, digested JFR-; GDL dig, digested GDL; and dig. juice, digested water control.

4.2.4 SRB (sulforhodamine B) method for cell proliferation

The SRB method was performed for HT29-MTX, Caco-2 cells as well as cocultures of Caco-2:HT29-MTX cells. Confluent epithelial cells were detached with trypsin-EDTA and then diluted in fresh DMEM. HT29-MTX and Caco cells were diluted and seeded directly at a concentration of 2×10^3 cells/mL. For coculture models, cells were diluted to 2×10^3 cells/mL separately before combining at a 75:25 ratio. Following, 100 μL of diluted cells were seeded into a 96 well tissue culture treated flat bottom plate (Corning 96 well TC treated microplates) and grown for 24 hours at 37°C and 5% CO₂. After 24 hours, the old media was removed, diluted treatments, prepared in serum free DMEM, added to epithelial cells, which were incubated for another 24 h. Treatments included 100% (undiluted), 50%, 25%, 10%, 5%, and 2.5% of GDL, JFR+ or JFR-, as well as 50%, 25%, 10% and 5% of digested GDL, JFR+ or JFR-, all performed in triplicate. Following the treatments and removal of DMEM, 200 μL of serum free DMEM with 50 μL of 50% cold TCA (Trichloroacetic acid, Sigma-Aldrich, St.
Louis, USA) was added to each well for 1 hour at 4°C. TCA was then removed and wells were washed 5 times with 300 μL of sterile water. Plates were air dried to remove all traces of water followed by the addition of SRB dye solution (50 μL; prepared 0.4% w/v in 1% acetic acid; Sigma-Aldrich, St. Louis, USA). Plates were kept at room temperature on a low speed orbital shaker for 30 minutes. After incubation, wells were washed with 300 μL of 1% acetic acid 4 times and air dried again. Before reading plates at 570 nm (Synergy™ HT Multi Detection Microplate Reader; Bio-Tek Instruments, Winooski, Vermont, USA), the SRB dye was solubilized with 100 μL of 10 mM Tris buffer (Sigma-Aldrich, St. Louis, USA).

4.2.5 Salmonella virulence gene expression

After 18 h of overnight growth, both S. Typhimurium DT104 and ΔoppA were centrifuged at 5,000 × g for 5 min (Beckman Instruments J2-MC Centrifuge, Palo Alto, CA, USA) and then washed twice with sterile PBS. Each pellet was then reconstituted in the same volume of fresh LB broth. Both strains were then mixed separately with each prepared milk mentioned in the previous section (fermented, acidified, digested) at four different concentrations (v/v) 5%, 10%, 20%, or 40%. Each prepared milk/concentration was considered a treatment and each treatment was prepared by adding milk into fresh LB broth inoculated with 1% of washed Salmonella cells. For example, for the 10% treatment with JFR+, 500 μL of JFR+ were mixed with 4.5 mL of fresh LB broth containing 50 μL of S. Typhimurium DT104. The same amounts were used for the 10% treatment with S. Typhimurium ΔoppA. A positive control of only Salmonella and LB was also used. After Salmonella-milk mixes were prepared, all treatments were incubated for 3 h at 37°C in a shaking incubator at 200 rpm. At the end of the incubation, aliquots were removed for RNA extraction, as described in the RNA extraction section.

4.2.6 Salmonella invasion assay
Intestinal epithelial cells were cultured in tissue culture flasks before detachment, dilution and seeding to investigate *Salmonella* internalization in the presence of the milk treatments. On day 21, spent medium was removed and cells were washed twice with sterile PBS followed by the addition of prepared milk treatments. Each prepared milk (JFR+, JFR-, GDL, JFR+ dig, JFR-dig, GDL dig) as well as the digestive juice control prepared in serum-free DMEM. The positive control, consisting of *S. Typhimurium DT104* or Δ*oppA*, DMEM and cells, was also included. Cells were then incubated for 1 h for each treatment. After 1 h, 100 µL (final concentration of $10^6$ CFU; MOI of 10:1) of either *S. Typhimurium DT104* or Δ*oppA* (washed twice with PBS) were added into the milk-treated cells and incubated for another 2 h at 37°C and 5% CO$_2$ to determine invasion. The integrity of the monolayers was checked at 0, 1, and 3 h by monitoring the TEER (transepithelial electrical resistance) using an EVOM2 epithelial voltmeter (WORLD Precision Instruments, Sarasota, FL, USA). At the end of the *Salmonella* invasion, epithelial cells were washed with PBS followed by incubation with gentamicin (350 µg/mL for 1 h and 200 µg/mL for 30 min). After removal of gentamicin, epithelial cells were washed again with PBS followed by the addition of 1% Triton X-100 into each transwell and incubated for 10 min at 37°C. Samples were then harvested for RNA extraction and serially diluted for bacterial enumeration. The basal layer liquid of each treated insert was retrieved once invasion was completed and kept frozen for cytokine analysis.

### 4.2.7 *Salmonella* RNA isolation and purification

Samples of lysed epithelial cells containing internalized *Salmonella* that were harvested from the transwells were centrifuged for 5 min at 10,000 × g (Spectrafuge 16M, Labnet International, Edison, USA) to remove the supernatant. The bacterial cell pellet was then suspended in 100 µL PBS and 600 µL of RNAprotect Bacteria Reagent (Qiagen, Toronto, ON) and incubated for 5 min at room temperature. The supernatant was again removed by centrifuging for 5 min at
10,000 \times g\) and the pellet was kept for RNA extraction by the RNeasy Plus Mini Kit (Qiagen, Toronto ON). In summary, the pellets were dissolved in 20 \mu L of proteinase K (Qiagen, Toronto, ON), 200 \mu l of Tris-EDTA buffer (BioUltra for molecular biology, pH 8.0, Sigma-Aldrich Co., St. Louis, USA) and 60 \mu L of 20 mg/mL lysozyme for molecular biology (Sigma-Aldrich Co., St. Louis, USA). The reconstituted pellets were incubated at 37°C for 1 h, shaking at 450 rpm (Thermomixer, Eppendorf, Hamburg, Germany). The remaining extraction steps including the removal of DNA from the samples were handled using kit materials and following the manufacturer’s instructions. The RNeasy MinElute Cleanup Kit (Qiagen, Toronto, ON) was used to purify extracted RNA also following the manufacturer’s instructions. The concentration of the clean RNA was determined using the NanoDrop 1000 spectrophotometer (Thermoscientific, Wilmington, DE) and also subjected to gel electrophoresis (1% agarose gel) to confirm quality of RNA.

4.2.8 Gene expression of Salmonella

The effect of epithelial cell treatments on gene expression of \textit{Salmonella} was determined with a 2-step qPCR. First, RNA was converted into complementary deoxyribonucleic acid (cDNA) using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Burlington, Ontario, Canada). A final concentration of 1,000 ng/mL of RNA was used in the reaction mixture consisting of 2 \mu L of 10\times random hexamer primers, 0.8 \mu L of 25\times deoxyribonucleoside triphosphate (100 mM), 2 \mu L of 10\times RT buffer, and 1 \mu L of Multiscribe reverse transcriptase (50 U /mL), adjusted to a final volume of 20 \mu L. The Mastercycler Gradient Thermocycler (Eppendorf, Mississauga, Ontario, Canada) was used to perform cDNA synthesis with the following conditions: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and a final holding step at 4°C. Second, the cDNA was used as qPCR templates in a ViiA™ 7 Real-Time PCR System (Applied Biosystems, Burlington, Ontario, Canada) to determine gene
expression of *Salmonella*. The housekeeping and target genes used along with their PCR primers are listed in Table 4.1. A total reaction volume of 20 µL was used, containing 10 µL of PowerUp SYBR Green Master Mix (Fisher Scientific, Nepean, Ontario, Canada), 1.6 µL of each forward and reverse primers, 1.8 µL of RNAse free water, and 5 µL of cDNA (diluted 2×). Samples were measured in triplicate in 96-well plates (MicroAmp® Fast Optical 96 Well Reaction Plate, Life Technologies, Canada) under the following conditions: UDG activation (50°C for 2 min), 40 cycles of denaturation (95°C for 15 s), annealing (54°C for 30 s) and amplification (72°C for 45 s) followed by the default dissociation curve. Quantification of transcript levels were determined by normalizing to the expression of housekeeping gene *rpoD*, which exhibited expression stability across all conditions and samples. All qPCR reactions were optimized based on the efficiency of each gene’s standard curve. Relative fold changes were calculated according to the ∆∆Ct method (Livak and Schmittgen, 2001; Pfaffl, 2001).

### Table 4.1 List of target gene sequences used for performing RT-qPCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Sequence (5'-3')</th>
<th>Reference</th>
</tr>
</thead>
</table>
| *rpoD* | Housekeeping gene | F: GTGAAATGGGCACTGTTGAACTG  
R: TTCCAGCAGATAGGTAATGGCTTC | (Velayudhan et al., 2014) |
| *hilA* | Transcriptional regulator of SPI-1 | F: TGTCGGAAGATAAAGAGCAT  
R: AAGGAAGTATCGCCAATGTGA | (Sharma, 2014) |
| *invA* | Required for invasion of cells | F: GAAATTATCGCCACGTTCGGGCAA  
R: TCATCGCACCGTCAAAGGAACC | (Rahn et al., 1992) |
| *sopD* | Secreted effector protein | F: ATTAATGGCGTGTTTTTTGA  
R: CTCTGAAACCGGTGAATAGC | (Sharma, 2014) |

**4.2.9 Confocal laser scanning microscopy**

The attachment of *S. Typhimurium* DT104 to HT-29-MTX cells was imaged using a confocal laser scanning inverted microscope (Leica TCS SP2, model Leica DM IRE2, Leica Microsystems CMSGmbH, Mannheim, Germany) with an Ar/Kr visible light laser and 63× (oil) objective as described by (Gagnon, Zihler Berner, Chervet, Chassard, & Lacroix, 2013). In brief, cell cultures were grown for 21 days in glass bottom dishes (MatTek Corporation,
Maine, USA) and incubated for 2 h at 37 °C and 5% CO₂ with or without S. Typhimurium DT104 before imaging. Staining was carried out as follows: after Salmonella invasion, cells were fixed with chilled formaldehyde (3.7% in PBS) for 15 minutes; permeabilized with 0.5% Triton X-100; blocked with 3% BSA (bovine serum albumin) for 1 h; and incubated with tetramethyl-rhodamine B isothiocyanate-phalloidin (TRITC-phalloidin) for 20 min.

4.2.10 Determination of pro-inflammatory cytokine IL-8

The secretion of pro-inflammatory IL-8 into the basal layer liquids of treated epithelial cells was determined by ELISA (enzyme linked immunosorbent assay) following the manufacturer’s instructions (Becton Dickinson and Company, Mississauga, ON, Canada). In brief, a 96-well plate was coated with 100 µL of IL-8 capture antibody and kept overnight at 4°C. The next morning, wells were washed 3× with wash buffer then blocked with 200 µL of assay diluent for 1 h at room temperature. Wells were washed again (3×) followed by the addition of standards (provided in the kit) and basal layer liquid samples (100 µL) then incubated at room temperature for 2 h. After another washing step (5×), 100 µL of working detector were added and incubated for 1 h at room temperature. Wells were then washed again (7×) followed by the final incubation with 100 µL of substrate solution for 30 min in the dark. The reaction was terminated with 50 µL of stop solution and absorbance was read at 405 nm using the Victor multi-label counter (Wallac, PerkinElmer Life Sciences, Canada, Woodbridge, Canada). Concentrations were calculated based on the linear equation produced from the standard curve.

4.2.11 Statistical analysis
All experiments were performed in at least two independent experiments in triplicate. Means and standard deviations were analyzed by analysis of variance (ANOVA) followed by Tukey’s post-test with significance at $P<0.05$.

4.3 Results

4.3.1 Effect of prepared milk treatments on cell proliferation

The optimum concentration of treatment preserving epithelial cell viability was determined following the SRB method. All treatments were compared to the epithelial cell control which consisted of only epithelial cells (HT29-MTX, Caco-2 or cocultures) in DMEM. Treatment of epithelial cells with 100% fermented/acidified milk resulted in low cell proliferation, approximately 25%-50% (Fig. 4.1-A, B, C). At fermented/acidified milk treatment concentrations of 50%, epithelial cell proliferation still remained significantly lower than the cell controls. However, beginning at 25%, including 10% and 5%, HT29-MTX cells showed no significant difference in cell viability compared with the control (Fig. 4.1-A). In contrast, at 25% treatment concentration, GDL still caused a significant depression in cell proliferation (Fig. 4.1-B, C). At 10% and 5% concentrations of fermented/acidified milk, cell viability showed no significant difference compared to the controls. Similar to fermented/acidified milk, 50% digested fermented/acidified milk treatments on HT29-MTX, Caco-2 and cocultures showed drastically lower cell proliferation compared to the cell control as well as the digestive juice control (Fig. 4.2). At 25%, 10%, and 5% digested treatments, HT29-MTX cells displayed no significant difference compared to the control cells alone or treated with digestive juice (Fig. 4.2-A). However, in Caco-2 and coculture models, treatment concentrations of 10% and 5% caused no effect on epithelial cell viability (Fig. 4.2-B, C).
Figure 4.1 SRB cell proliferation assay of HT29-MTX (A), Caco-2 (B) and cocultured cells (C) treated with 100%, 50%, 25%, 10%, 5%, or 2.5% of fermented or acidified milk. Letters represent significant differences within each concentration group. Data were analyzed by ANOVA and Tukey’s multiple comparison post-test ($P < 0.05$).
Figure 4.2 SRB cell proliferation assay of HT29-MTX (A), Caco-2 (B) and cocultured cells (C) treated with 50%, 25%, 10%, or 5% of digested fermented or acidified milk. Letters represent significant differences within each concentration group. Data were analyzed by ANOVA and Tukey’s multiple comparison post-test ($P < 0.05$).

4.3.2 Virulence gene expression of Salmonella in the absence of epithelial cells

Co-incubation of either 5% of *L. lactis* subsp. *cremoris* JFR+ fermented milk or JFR- fermented milk both showed a significant down regulation (> 2-fold changes) of *S. Typhimurium DT104*. 
virulence gene expression compared to GDL chemically acidified milk treatment (Table 4.2). When *S. Typhimurium* DT104 was exposed to 10% fermented milk, *hilA*, *invA*, and *sopD* were down regulated more than 6-fold by JFR+ treatment but not to the same extent by the JFR- treatment when compared to GDL acidified milk. When the fermented milk treatment was increased to 20%, there was similar down regulation of *S. Typhimurium* DT104 observed in the presence of both JFR+ and JFR- fermented milks for all virulence genes tested (approximately 5-fold down regulation). In contrast, when the *S. Typhimurium* Δ*oppA* mutant was co-incubated with JFR+ or JFR- treatments, the down regulation of virulence genes was no longer observed (< 2-fold changes) at any of the concentrations (10, 20, and 40%) of JFR+, JFR- or GDL. However, co-incubation of digested fermented/acidified milk with either *S. Typhimurium* DT104 or *S. Typhimurium* Δ*oppA* resulted in no significant suppression of virulence genes (< 2-fold changes) (Table 4.3). The digestive juice control also showed no significant effect on virulence gene expression.
Table 4.2 Effect of fermented and acidified milk treatments on the expression of three virulence genes from *Salmonella* Typhimurium DT104 and Δ*oppA* in the absence of epithelial cells. Relative fold change was determined using a two-step RT-qPCR method followed by ∆∆Ct computations described previously.

<table>
<thead>
<tr>
<th></th>
<th>Gene target</th>
<th>S. Typhimurium DT104</th>
<th>S. Typhimurium Δ<em>oppA</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Absence of epithelial cells</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Treatment (v/v)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% GDL</td>
<td></td>
<td></td>
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<tr>
<td>hilA</td>
<td>-2.1±0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-1.4±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-1.3±0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>invA</td>
<td>-1.4±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.0±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>sopD</td>
<td>-1.3±0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-2.5±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>5% JFR+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hilA</td>
<td>-3.2±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-1.6±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.5±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>invA</td>
<td>-1.6±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>sopD</td>
<td>-2.0±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-2.5±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
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</tr>
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</tr>
<tr>
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</tr>
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</tr>
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<td>-0.6±0.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>sopD</td>
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<td>-1.7±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-1.5±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>10% GDL</td>
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</tr>
<tr>
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<td>-0.6±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
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</tr>
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</tr>
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<tr>
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</tr>
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</tr>
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<td>sopD</td>
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<td>-5.3±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-1.1±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>abc</sup> Statistical significance (P<0.05) corresponding to each gene (within columns).
Table 4.3 Effect of digested fermented and acidified milk treatments on the expression of three virulence genes from *Salmonella* Typhimurium DT104 and Δ*oppA* in the absence of epithelial cells. Relative fold change was determined using a two-step RT-qPCR method followed by ∆∆Ct computations described previously.

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Treatment (v/v)</th>
<th>hilA</th>
<th>invA</th>
<th>sopD</th>
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<td>-1.3±0.2^a</td>
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<td>-0.7±0.2^a</td>
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<td>-1.5±0.2^a</td>
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<td>10% JFR-</td>
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<td>-1.8±0.2^a</td>
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<td>-1.4±0.3^a</td>
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<td>20% GDL</td>
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<td>-1.5±0.7^a</td>
<td>-1.2±0.8^a</td>
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<td>-1.1±0.8^a</td>
<td>-1.1±0.7^a</td>
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<td>-0.5±0.3^a</td>
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<table>
<thead>
<tr>
<th>Gene target</th>
<th>Treatment (v/v)</th>
<th>hilA</th>
<th>invA</th>
<th>sopD</th>
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<td>20% JFR+</td>
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<td>-1.9±0.5^a</td>
<td>-0.7±0.5^a</td>
<td>-0.6±0.4^a</td>
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<td>20% JFR-</td>
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<td>-0.5±0.5^a</td>
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<td></td>
<td>40% GDL</td>
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<td>-0.8±0.3^a</td>
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<tr>
<td>40% JFR+</td>
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<td>-0.5±0.4^a</td>
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<tr>
<td>40% JFR-</td>
<td></td>
<td>-1.9±0.5^a</td>
<td>-0.5±0.4^a</td>
<td>-0.8±0.5^a</td>
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</tbody>
</table>

^abc^ Statistical significance (*P*<0.05) corresponding to each gene (within columns).

4.3.3 *Salmonella* invasion of intestinal epithelial cells

After the extracellular *Salmonella* were eliminated by gentamicin treatment, the number of invasive *Salmonella* were enumerated (Fig. 4.3). The number of *S.* Typhimurium DT104 internalized by mucosal HT29-MTX cells treated with GDL acidified milk showed no significant difference compared to the *S.* Typhimurium DT104 control (5.0 ± 0.1 log_{10} CFU/mL and 5.5 log_{10} CFU/mL ± 0.4 respectively); while both fermented milk treatments lowered the number of invasive *S.* Typhimurium DT104 (JFR+ fermented milk 3.8 ± 0.2 log_{10} CFU/mL; JFR- fermented milk 4.3 ± 0.2 log_{10} CFU/mL) (Fig. 4.3-A). In contrast, numbers of invasive *S.*
Typhimurium $\Delta oppA$ were not significantly different compared to both the S. Typhimurium $\Delta oppA$ control and GDL acidified milk (approximately $5\text{-}5.5$ log$_{10}$ CFU/mL) (Fig. 4.3-B). Additionally, the number of invasive S. Typhimurium DT104 determined in Caco-2 cells after treatment with fermented milk was significantly less than the S. Typhimurium DT104 control (JFR+ $4.5$ log$_{10}$ CFU/mL ± $0.6$; JFR- $4.4$ log$_{10}$ CFU/mL ± $0.2$; Salmonella $5.4$ log$_{10}$ CFU/mL ± $0.7$) (Fig. 4.3-C). Similar to HT29-MTX cells, invasive S. Typhimurium $\Delta oppA$ was not affected by any treatments (approximately $5$ log$_{10}$ CFU/mL) (Fig. 4.3-D). In the coculture cell model, the same trend was observed; JFR fermented milk treatment of epithelial cells reduced the number of invasive S. Typhimurium DT104 by approximately $1$ log$_{10}$ CFU/mL compared to both GDL and the control (Fig. 4.3-E) whereas numbers of internalized S. Typhimurium $\Delta oppA$ were unaffected by treatments used ($5.2$ to $5.4$ log$_{10}$ CFU/mL).
Figure 4.3 Enumeration of intracellular *Salmonella* Typhimurium DT104 (A, C, E) or *Salmonella* Typhimurium ΔoppA (B, D, F) into HT29-MTX (A, B), Caco-2 (C, D), or cocultures of Caco-2:HT29-MTX (E, F) epithelial cells after invasion. Experiments were performed with 3 independent replicates in triplicate for each sample. Statistical analyses were performed using ANOVA followed by Tukey’s post test (letters represent significant differences between treatments $P<0.05$).

The TEER of epithelial cells was monitored before the addition of acidified/fermented milk, after 1 h of treatment, and again after 2 h of *Salmonella* invasion, represented as time (h) 0, 1,
and 3, and expressed as a percentage of each corresponding cell control (Fig. 4.4). The TEER values of the *Salmonella* positive control, GDL acidified milk, JFR+ and JFR- fermented milks were not significantly different at time 0. However, a significant increase in the TEER of HT29-MTX cells was displayed at 1 h for all treatments (Fig. 4.4-A, B). Two hours after *S. Typhimurium* DT104 addition, only JFR+ and JFR- fermented milks maintained the elevated TEER values (173% ± 47 and 211% ± 16, respectively). Addition of *S. Typhimurium* ΔoppA caused a drop in TEER after 2 hours of infection where TEER at time 3 was not significantly different across all treatments, including nontreated cells containing *Salmonella* (Fig. 4.4-B). TEER measurements of Caco-2 and coculture cells infected with *S. Typhimurium* DT104 displayed a gradual increase in TEER from time 0 to time 3 after JFR+/- treatments, but not cells treated with GDL (Fig. 4.4-C, E). At time 3, TEER of both JFR+ and JFR- treated Caco-2 cells increased to approximately 150% of the Caco-2 cell control during *S. Typhimurium* DT104 invasion. However, when Caco-2 cells were infected with *S. Typhimurium* ΔoppA, after the initial increase of TEER from the first hour, TEER declined to near its initial value at time 3 (Fig. 4.4-D), showing no significant difference compared to GDL or *S. Typhimurium* ΔoppA control. Similarly, the coculture model demonstrated comparable behaviour to Caco-2 cells during infection by *S. Typhimurium* ΔoppA (Fig. 4.4-F).

Microscopy shows the difference between a healthy network and disrupted tight junction network of HT29-MTX monolayers (Fig. 4.5). The disruption of tight junctions (black portions disrupting the red network) is clearly visible 2 h after *S. Typhimurium* DT104 invasion.
Figure 4.4 Transepithelial electrical resistance (TEER) of HT29-MTX epithelial cells (A, B), Caco-2 epithelial cells (C, D), or cocultures of Caco-2:HT29-MTX cells (E, F) measured before and after invasion with *Salmonella* Typhimurium DT104 (A, C, E) or *Salmonella* Typhimurium ΔoppA (B, D, F), added at time 1 h. Grouped bars represent cell treatments: ■ *S. Typhimurium* control, □ GDL acidified milk, ☀ *L. lactis* subsp. cremoris JFR+ fermented milk, and ☀ ☀ *L. lactis* subsp. cremoris JFR- fermented milk. Experiments were performed with 3 independent replicates in triplicate for each sample. Statistical analyses were performed using ANOVA followed by Tukey’s post test (letters represent a significant difference within each sample group *P*<0.05).
Figure 4.5 Confocal laser scanning micrograph of HT29-MTX epithelial cells stained with TRITC-phalloidin before (A) and after (B) Salmonella Typhimurium DT104 invasion. Tight junctions (red network) are disrupted in the image on the right by Salmonella GFP (green dots) after invasion.

4.3.4 Virulence gene expression of internalized Salmonella in intestinal epithelial cells

HT29-MTX epithelial cells pretreated with 10% GDL acidified milk did not cause down regulation of *S.* Typhimurium DT104 virulence genes whereas JFR+ and JFR- fermented milks significantly suppressed the expression of *hilA*, *invA*, and *sopD* (Table 4.4). For instance, the gene expression of *hilA* was significantly reduced in the presence of JFR+ or JFR- fermented milk treatment (-4.9-fold and -5.3-fold, respectively) compared to GDL acidified milk (-1.3-fold). The addition of 20% fermented milk treatments on HT29-MTX cells also displayed similar behavior compared to 20% GDL acidified milk, which did not suppress virulence gene expression of *S.* Typhimurium DT104. *S.* Typhimurium DT104 infection of Caco-2 and coculture cells treated with 10% JFR+/− also showed suppression of virulence *hilA*, *invA* and *sopD*. Caco-2 cells treated with GDL, JFR+ and JFR- all lead to down regulation of *hilA* whereas *invA* and *sopD* were only suppressed by JFR+ and JFR- fermented milk. However, the extent of *hilA* suppression induced by JFR+/− fermented milk was significantly more than GDL (15.1- and 19.8-fold compared to 4.8-fold). In the coculture cell model, treatment with
10% GDL did not suppress expression of *hilA* as much as JFR+ and JFR- treatment (2.4-fold compared to 4.4- and 8.4-fold, respectively). While both JFR+ and JFR- significantly suppressed *hilA* expression, *invA* expression was suppressed only by JFR+ treatment of cocultures (-9.3-fold) while *sopD* was only suppressed by JFR- treatment (-2.9-fold). When infection of epithelial cells was carried out using *S. Typhimurium* Δ*oppA*, no significant down regulation (< 2-fold change) of virulence genes was seen following any of the treatment types. The same was observed with *S. Typhimurium* Δ*oppA* infection of epithelial cells after treatment by digested acidified/fermented milk (Table 5). Compared to *S. Typhimurium* DT104 infection of epithelial cells treated with nondigested fermented milk (Table 4.4), enzymatic digestion of the acidified or fermented milk greatly reduced the suppressive action on virulence genes (Table 4.5). For example, nondigested JFR- fermented milk treatment of HT29-MTX cells reduced *hilA* expression by 5.3-fold, whereas after digestion, only 1.7-fold reduction was observed. However, in some cases, digested fermented milk was still able to significantly suppress virulence gene expression when compared to digested GDL or the digestive juice control. For instance, the down regulation observed in Caco-2 cells treated by JFR+/fermented milk decreased from 15.1-fold/19.8-fold to 3.1-fold/3.8-fold after digestion. The action of digestive juices used during batch digestion did not cause any consequent responses in *Salmonella* virulence gene expression.
Table 4.4 Effect of fermented and acidified milk treatments on the expression of three virulence genes from *Salmonella* Typhimurium DT104 and *oppA* during invasion of epithelial cells. Relative fold change was determined using a two-step RT-qPCR method followed by ∆∆Ct computations described previously.

<table>
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<tr>
<th>Gene target</th>
<th>Treatment (v/v)</th>
<th>HilA</th>
<th>InvA</th>
<th>SopD</th>
</tr>
</thead>
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<td>-1.4±0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-0.9±0.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10% JFR+</td>
<td>-4.9±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.9±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
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</tr>
<tr>
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<td>10% JFR-</td>
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</tr>
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</tr>
<tr>
<td>Coculture cells</td>
<td>10% GDL</td>
<td>-2.4±1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-0.5±0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-1.9±1.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10% JFR+</td>
<td>-4.4±1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-9.3±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-1.8±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10% JFR-</td>
<td>-8.4±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-1.1±0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-2.9±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

abc Statistical significance (*P<0.05*) corresponding to each gene (within columns).
Table 4.5 Effect of digested fermented and acidified milk treatments on the expression of select virulence genes from *Salmonella* Typhimurium DT104 and ΔoppA during invasion of epithelial cells. Relative fold change was determined using a two step RT-PCR method followed by ΔΔCt computations described previously.

<table>
<thead>
<tr>
<th>Treatment (v/v)</th>
<th>Gene target</th>
<th>Treatment (v/v)</th>
<th>Gene target</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% dig. juice</td>
<td>hila</td>
<td>10% GDL</td>
<td>hila</td>
</tr>
<tr>
<td></td>
<td>invA</td>
<td></td>
<td>invA</td>
</tr>
<tr>
<td></td>
<td>sopD</td>
<td></td>
<td>sopD</td>
</tr>
<tr>
<td>1.6 ±0.7</td>
<td>-1.5 ± 0.6</td>
<td>1.9 ± 0.8</td>
<td>-0.85 ± 0.7</td>
</tr>
<tr>
<td>1.3 ± 0.7</td>
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<td>-2.8 ± 0.7</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>1.7 ± 0.6</td>
<td>3.3 ± 0.9</td>
<td>-2.1 ± 0.8</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>1.3 ± 0.2</td>
<td>1.2 ± 0.6</td>
<td>-</td>
</tr>
</tbody>
</table>

abcd Statistical significance (*P*<0.05) corresponding to each gene (column).

4.3.5 Production of inflammatory cytokine IL-8 by infected epithelial cells
Untreated HT29-MTX cells released $7.04 \pm 1.2$ pg/mL of IL-8 during the 3 h incubation. Comparison of IL-8 levels among GDL acidified milk, JFR+ and JFR- fermented milk demonstrated an overall modulating effect of milk ($162.6 \pm 11.1$ pg/mL, $127.9 \pm 8.8$ pg/mL, and $115.4 \pm 26.7$ pg/mL respectively) compared to the S. Typhimurium DT104 positive control ($193.8 \pm 5.1$ pg/mL) (Fig. 4.6-A). However, JFR+ and JFR- fermented milk exhibited a more significant modulating effect compared to GDL acidified milk. In contrast to HT29-MTX cells, Caco-2 cells produced significantly less IL-8 ($< 40$ pg/mL) over the duration of the invasion assay regardless of treatment type (Fig. 4.6-B). In the coculture cell model, production of IL-8 was slightly lower than that of HT29-MTX monocultures. JFR+/- fermented milk treatments resulted in significantly less IL-8 secretion ($103.3 \pm 7.1$ pg/mL and $112.2 \pm 13$ pg/mL respectively) compared to both GDL and S. Typhimurium DT104 ($144.6 \pm 13$ pg/mL and $158.1 \pm 11$ pg/mL respectively) (Fig. 4.6-C).
Figure 4.6 Levels of pro-inflammatory IL-8 cytokine produced by of HT29-MTX (A), Caco-2 (B), or cocultures (C) after 3 h. Experiments were performed with 3 independent replicates in triplicate for each sample. Statistical analyses were performed using ANOVA followed by Tukey’s post test (letters represent significant differences between treatments $P<0.05$).
4.4 Discussion

Intestinal epithelial cell lines Caco-2 and HT29-MTX are representative of enterocytes and goblet cells respectively, which are two of the major cell types found in the intestine. The pH of the human gastrointestinal tract gradually increases from the acidic stomach to pH 6-7.4 in the small intestine and slightly acidic to neutral in the large intestine (pH 5.7 to 7) (Evans et al., 1988; Fallingborg, 1999). Thus, experiments using intestinal epithelial cells should be carried out at neutral or near neutral pH values (Greene & Klaenhammer, 1994). Prepared milk treatments were all of acidic pH, thus an optimum concentration preserving epithelial cell viability was determined through the SRB protocol. The chosen concentration of 10% of the prepared milk treatments was adequately buffered through the DMEM used. Under the optimal concentration, any changes seen in TEER of epithelial cells were not caused by low pH.

Tight junctions are maintained via the actin cytoskeleton of epithelial cells which are fused together by transmembrane proteins such as zonula occludens, claudins and junctional adhesion molecules (Hartsock & Nelson, 2008). Tight junctions help maintain the barrier function of the epithelium by controlling passage of luminal nutrients while preventing access of bacteria (Farquhar & Palade, 1963; Ulluwishewa et al., 2011). Internalization of Salmonella into epithelial cells causes rearrangement of the host cell cytoskeleton leading to disruption of the tight junctions (Ebnet, 2004), which can be measured through TEER. Measurement of TEER is a quantitative technique used to evaluate the cellular barrier function of epithelial cells in culture models such as the ones used in this study. Serving as an indispensable part of the intestinal barrier, tight junction proteins (TJPs) help regulate the selectively permeable membrane, thus controlling transport processes to maintain gut homeostasis. Therefore, a higher TEER value corresponds to enhanced cell membrane integrity and decreased epithelium permeability. Observations seen during invasion showed that only the JFR+ and JFR-
fermented milk treatments were able to elevate and maintain TEER in epithelial cells during *Salmonella* infection, contributing to improved barrier function. Gagnon et al. (2013) observed that *S. Typhimurium* significantly reduced the TEER of HT29-MTX cells at 90 min of invasion, agreeing with the results obtained in this study. Moreover, it was demonstrated that cell free spent medium from *L. acidophilus* La-5 and *Lactobacillus helveticus* LH-2 could attenuate the drop of TEER induced by *Salmonella* invasion of HT-29 epithelial cells (Peng, 2014).

*Salmonella* interfere with the cytoskeleton of epithelial cells, weakening tight junctions, by depolymerization of the actin filaments causing localized membrane ruffling to engulf the bacteria (Galán, 2001). This action contributes to a decrease in TEER, which can be seen in this study. The disrupted network shown in the micrograph reflects the change induced by *Salmonella* effector proteins in the HT29-MTX cell membrane. Kemgang et al. (2016) described a modified method of phenol red diffusion to study barrier integrity and demonstrated the ability of *Salmonella* to interfere with monolayer integrity while exposure to *L. rhamnosus* S1K3 aided in maintaining barrier function in the presence of *Salmonella*. The authors also investigated the expression of *Claudin-1*, a tight junction gene, observing down regulation in *Claudin-1* when exposed to *Salmonella* and up regulation when incubated with *L. rhamnosus*. A similar study employed Caco-2 intestinal epithelial cells to compare TEER values with and without incubation with *Lactobacillus gasseri* LF221 (Fajdiga, Koninkx, Tooten, Marinšek-Logar, 2006). It was observed that *L. gasseri* pre-treatment prevented the TEER value from decreasing compared to that of the *S. Enteritidis* positive control. Similarly, the JFR fermented milk treatments used in this study not only prevented the decrease in TEER, but also helped maintain heightened TEER indicating the beneficial effect of the treatments on host cell tight junctions. Although host cell genes were not analyzed in this study, it is presumed that increased TEER would reflect an increased expression of TJPs of the HT29-MTX cells.
In the present study, gentamicin treatment was used to remove surface attached *Salmonella* leaving only internalized bacteria. Previously, *S. Typhimurium* DT104 had been verified for sensitivity to gentamicin (Peng, 2014). The number of invasive *Salmonella* into intestinal epithelial cells after 2 h was reduced by both JFR+ and JFR- treatments suggesting the presence of bioactive compounds. During fermentation, molecules produced may have a significant effect on hindering *Salmonella* invasion, as similarly described with other LAB (Tellez, Corredig, Turner, Morales, & Griffiths, 2011). The virulence gene expression of *hilA*, *invA*, and *sopD* in *Salmonella* Typhimurium DT104 co-incubated with prepared milk treatments without the presence of epithelial cells showed similar behavior further confirming the presence of bioactive molecules. Many studies have demonstrated that cell free spent medium prepared from numerous lactic acid bacteria and *Bifidobacteria* have the capability to down regulate virulence gene expression in pathogens such as *E. coli* O157:H7, *Salmonella* and *C. jejuni*, suggesting the possibility of bioactive molecules produced by LAB (Medellin-Peña, Wang, Johnson, Anand, & Peng, 2007; Mundi, Delcenserie, Amiri-Jami, Moorhead, & Griffiths, 2013; Sharma, 2014; Tellez et al., 2011). Tellez et al., (2011) observed changes in *Salmonella* Enteritidis infection in mice when treated by peptide fractions obtained through *L. helveticus* milk fermentation. However, when *S. Typhimurium* DT104 was treated with 10% and 20% digested fermented milk, no fold changes over 2 were observed, which suggest no significant down regulation (Phongsisay, Perera, & Fry, 2007) by digested treatments. During the digestion process, through the addition of enzymes, acids and bases the fermented/digested fermented milk became diluted, thus to account for this dilution, a less concentrated (5%) fermented milk treatment was also tested. However, significant down regulation (> 2-fold changes) by 5% diluted JFR+ and JFR- fermented milks were still observed. Additionally, a digestive juice control was also added to confirm that down regulation was a result of the JFR milks and not the enzymes used. Furthermore, comparison of *S. Typhimurium* DT104
expression in cells treated with fermented milk and cells treated with digested fermented milk imply that compounds present in the fermented milk are responsible for the down regulation of virulence genes. It has been reported previously that several LAB such as *L. helveticus* LH-2 and *L. acidophilus* La-5 produce small bioactive molecules isolated from their cell free spent medium exhibiting antivirulence activity (Bayoumi & Griffiths, 2012; Medellin-Peña et al., 2007), similar to what was observed in this study. Moreover, the amino acid composition of peptides, which was not determined in this study, contributes in part to the susceptibility of enzymatic digestion (Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011). Peptides containing proline residues are more resistant to the action of digestive enzymes, although many *in vitro* digestion studies have reported both increases and decreases in bioactivities of peptides after enzymatic digestion (Maeno, Yamamoto, & Takano, 1996; Segura-Campos et al., 2011). For instance, digestion of a heptapeptide from β-casein increase angiotensin-I converting enzyme inhibitory activity while the same digestion treatment decreased the bioactivity of a hexapeptide derived from αs1-casein (Maeno et al., 1996).

The knockout oligopeptide permease *oppA* mutant (Δ*oppA*) of *S. Typhimurium* used in this study provides insight into a possible connection between peptide uptake, virulence gene expression and invasion. For the uptake of most peptides in *Salmonella* as well as other microorganisms such as *E. coli* and *Listeria monocytogenes*, transport proteins are needed. The OppA protein is one of the most abundant periplasmic transport proteins and transits peptides from two to five amino acid residues (Hiles, Gallagher, Jamieson, & Higgins, 1987). The Opp operon has been previously associated with virulence activity of several Gram-positive pathogens such as *L. monocytogenes* and *Bacillus thuringiensis* (Borezee, Pellegrini, & Berche, 2000; Moraes et al., 2014; Perego, Higgins, Pearce, Gallagher, & Hoch, 1991; Zheng et al.,
2018). It has been reported that the lack of a functional Opp system in *Salmonella* impaired peptide transport ability while the knockout of dipeptide and tripeptide transport systems in *Salmonella* showed no impairment of peptide transport (Goodell & Higgins, 1987). Additionally, the roles of other oligopeptide and tripeptide transport systems in *Salmonella* have been previously reported to contribute to antimicrobial resistance via transportation of antimicrobial peptides away from their membrane targets and instead into the bacterial cytoplasm for activation of antimicrobial resistance determinants and degradation (Eswarappa, Panguluri, Hensel, & Chakravortty, 2008; Gibson, Price, & Higgins, 1984; Parra-Lopez, Baer, & Groisman, 1993). Although the role of the Opp operon in *Salmonella* virulence still remains unclear, it has been reported to influence the virulence of other pathogens (e.g. *Streptococcus suis* and *L. monocytogenes*) and functions in growth and survival inside host cells and macrophages as well as macrophage apoptosis (Borezee et al., 2000; Dasgupta et al., 2010; Moraes et al., 2014; Vorwerk et al., 2014; Zheng et al., 2018). Lactococci possess extensive proteolytic systems for growth in milk to release amino acids and peptides from casein proteins (Børsting et al., 2015). Cell envelope proteinases are located on the outside of the bacterial cell and initiates casein cleavage into small peptides and oligopeptides. When epithelial cells were treated with JFR+ and JFR- fermented milk, *S. Typhimurium* ΔoppA showed no significant down regulation (< 2-fold changes) of virulence genes compared to *S. Typhimurium* DT104. This may be due to the lack of OppA function impacting either directly or indirectly to potential quorum sensing signaling, as discussed below. The suppressive effect was eliminated when the JFR+ and JFR- fermented milks were enzymatically digested. One possibility could be that the small peptides (oligopeptides) present in the fermented milk were digested by the enzymes in the pancreatin, eliminating the antivirulence activity on *Salmonella*. The first step in the batch digestion was the addition of pepsin, which digests proteins and larger peptides at acidic pH, preferably targeting peptide bonds connected to aromatic residues (Fruton, 1971; 2002).
However, the second step of pancreatin addition contains many enzymes including ribonuclease, lipase, amylase, trypsin, and protease (Sigma-Aldrich Co.) in which the latter two are capable of hydrolyzing smaller peptides into tripeptides, dipeptides, and amino acids (Olsen, Ong, & Mann, 2004). Trypsin targets peptides with lysine or arginine residues next to the C-terminal cleaving the peptide bond (Olsen et al., 2004). The OppA permease prefers positively charged oligopeptides containing a lysine residue (Klepsch et al., 2011). Therefore, the loss of virulence suppression demonstrated by both S. Typhimurium DT104 and S. Typhimurium ΔoppA (to some extent) in the presence of digested fermented milk treatment might be due to the cleavage of positively charged residues produced by L. lactis fermentation.

A possible mode of action for the antivirulence activity of JFR+/- fermented milk could be the quorum quenching action of the oligopeptides. Quorum sensing in Salmonella requires the LuxS system for cell communication by the production of autoinducer molecules (Surette, Miller, & Bassler, 1999). It has been previously reported that interference with quorum sensing of E. coli O157:H7 resulting in decreased production of autoinducer signaling molecules is linked to lower bacterial virulence (Medellin-Peña et al., 2007). Furthermore, recent evidence suggests that small peptides of approximately 6 amino acids in length produced by lactic acid bacteria and containing sequence similarities to each other, exhibit antivirulence activity by disrupting autoinducer-2 quorum sensing (M.W. Griffiths, personal communication). In addition, it has been reported that small peptides synthesized to possess sequence similarities with LuxS were capable of inhibiting autoinducer-2 activity of Edwardsiella tarda (Zhang, Jiao, Hu, & Sun, 2009). Moreover, the transport and accumulation of oligopeptides via an intact Opp transport system into the bacterial cell may also act as quorum sensing inhibitors at which a certain concentration allows for antivirulence effect. Purified peptide fractions generated from L. helveticus fermentation of milk is required at concentrations of over 25 µg/mL before
suppression of *Salmonella* virulence genes was observed (Tellez et al., 2011). Although no peptide analysis was carried out in this study, it could be hypothesized that JFR oligopeptides may function in a similar manner.

Pathogens would be quickly eliminated from the gut without the ability to form close associations with the intestinal mucosa through the expression of certain virulence genes. In this study, treatment with milk fermented by JFR+ as well as JFR- caused significant down regulation of three *Salmonella* virulence genes compared to the GDL acidified milk treatment. Previous work demonstrated that overnight cultures of JFR+ and JFR- grown in medium induced a down regulation of virulence genes *hilA* and *ssrB* with no significant difference between the two strains (Zhang et al., 2016). The JFR+ strain used in this study is known to produce exopolysaccharides (EPS) although it has been shown that JFR+ EPS did not affect the attachment of *Salmonella* to HT-29 epithelial cells (Zhang et al., 2016). Furthermore, the bioactive components found in JFR+ and JFR- fermented milk do not exhibit antimicrobial activity (data not shown), similar to the bioactive peptides produced during *L. helveticus* milk fermentation (Tellez et al., 2011). In this study, acidified/fermented milk was digested to gain an insight as to how digestion could affect down regulation of *Salmonella* virulence genes. Notably, down regulation of *hilA* by digested fermented milk was not as significant compared to whole fermented milk. However, the overall down regulating effect on *hilA*, *invA*, and *sopD* was still observed. The protein/peptide profile of fermented milk compared to GDL acidified milk may play a key role in the effects on virulence gene expression of *Salmonella*. This may encourage the release of potentially bioactive peptides from fermented milk that would not be present in digested acidified milk, differentiating the effects of GDL acidified milk compared to the JFR+/- fermented milks.
Interleukin-8 (IL-8) is a pro-inflammatory cytokine produced by a variety of cell types including intestinal epithelial cells (Eckmann et al., 1993; Imaoka et al., 2008). Many reports have shown IL-8 production from epithelial cells including HT-29 and Caco-2 cells as a result of pathogen or TNF-α stimulation (Bahrami, Macfarlane, & Macfarlane, 2011; Haller et al., 2000; Imaoka et al., 2008; Kolios et al., 1996; Zhang, Li, Caicedo, & Neu, 2005). Several studies have also reported considerably less production of IL-8 by Caco-2 cells compared to other cell types including HT-29 and its subsets (Eckmann et al., 1993; Zhang et al., 2005). There is considerable evidence reporting lactic acid bacteria and their fermented milks displaying an overall modulating effect in in vitro studies, specifically in the presence of pathogens, which stimulate inflammatory responses. HT29-MTX cells treated with milk fermented by JFR+/− strains showed lower production of IL-8 compared to both the Salmonella positive control and GDL acidified milk control, suggesting that only bacterial fermented milk contained an immunomodulatory effect. Since the effect may be due to the presence of short peptides (oligopeptides) produced during fermentation with JFR, it may be possible that the use of the purified peptide would decrease the inflammatory response during Salmonella infection. In an induced IBD mice model, oral ingestion of soy milk fermented with L. lactis subsp. lactis S-SU2 prevented several characteristic changes normally observed in IBD, which is commonly associated with increased expression of IL-8 (Grimm, Elsbury, Pavli, & Doe, 1996), such as shortening of colon length and enlargement of the spleen (Kawahara et al., 2015). In another study, a milk blend fermented by Streptococcus thermophilus, Bifidobacterium longum, and L. helveticus was used to pre-treat intestinal epithelial cells for various lengths of time followed by addition of TNF-α to induce production of IL-8 (Wagar, Champagne, Buckley, Raymond, & Green-Johnson, 2009). The authors found that the acidified milk blend demonstrated only a minor decrease in IL-8 production while milk containing B. longum and S. thermophilus induced a significant decrease. In a similar manner, when Lactobacillus
*[^rhamnosus* GG was co-incubated with either *Salmonella* or *Vibrio cholerae* on HT-29 epithelial cells, IL-8 production was markedly reduced (Nandakumar, Pugazhendhi, & Ramakrishna, 2009). Pre-treatment of HT-29 cells with *L. rhamnosus* GG also resulted in decreased production of IL-8 but not to the same extent as co-incubation. These results suggest JFR+ and JFR- fermented milk may contribute to slowing the onset of intestinal inflammation caused by pathogen invasion, although testing of more inflammatory markers is necessary.

4.5 Conclusion

This study provides evidence that milk fermented by bacterial strains JFR+ and JFR- was more efficient than GDL acidified milk in reducing *S. Typhimurium* DT104 infection of intestinal epithelial cells. It has also been demonstrated here that the oligopeptide transport system of *Salmonella* plays an essential part in the response to fermented milk treatments of intestinal epithelial cells. The reduced numbers of internalized *S. Typhimurium* DT104 cells was most likely a result of suppressed *hilA*, *invA*, and *sopD* expression, while simultaneously maintaining TEER during infection indicating preservation of epithelial cell membrane integrity. The decreased secretion of inflammatory IL-8 displayed in the HT29-MTX and coculture cell models contributes to immune modulation and has previously been associated with lower risks of inflammatory onset. However, the loss of beneficial effects after digestion suggests that the potentially bioactive oligopeptides (further studies needed to confirm bioactive component) may have been hydrolyzed thus losing their effect. In addition, the mode of digestion should be further investigated using a different models or in vivo studies to clarify whether antivirulence activity is eliminated due to hydrolysis. Lack of response during infection with an OppA permease mutant *Salmonella* strain confirmed that a functioning oligotransport system is essential for the antivirulence effects of JFR fermented milks.
4.6 Acknowledgements

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Chapter 5. General discussion and future work

5.1 General discussion

*Salmonella enterica* subsp. *enterica* are one of the most prevalent foodborne pathogens causing thousands of hospitalizations and deaths year worldwide (Hendriksen et al., 2011; Thomas et al., 2013). *Salmonella enterica* serovars Typhimurium and Enteritidis are the most common serovars causing salmonellosis from food and beverage sources (Hendriksen et al., 2011). In has been estimated from clinical studies that an infection dose of $10^6$ bacteria can cause illness, however, in many cases, $10^1$ to $10^3$ CFU of *Salmonella* could be sufficient to cause disease and infection in the host (Todd, Greig, Bartleson, & Michaels, 2008). Ingestion of *Salmonella* with certain foods, such as those high in fat or liquid foods, may lower the infection dose by protecting the pathogen from the harsh stomach conditions (Rychlik & Barrow, 2005). Due to the rise of multi-drug resistant strains including *Salmonella* Typhimurium DT104, alternatives to antibiotic treatments should be found. The interactions between host, bacteria and food is constantly occurring in the human intestinal environment thus manipulating food or food components that reach the intestine could alter the behaviour of the host and/or pathogen. The main focus of this dissertation was to investigate the effects of *L. lactis* subsp. *cremoris* JFR1 on *Salmonella* invasion of host cells utilizing intestinal epithelial cells as an *in vitro* model. The research was divided into three major sections: i) explore the effects of exopolysaccharides (EPS) produced by *L. lactis* subsp. *cremoris* JFR1 on the attachment of *Salmonella* to intestinal epithelial cells; ii) further examine the response of intestinal epithelial cells to overnight cultures of *L. lactis* subsp. *cremoris* JFR1 as well as invasion and virulence of *Salmonella*; and iii) to incorporate *L. lactis* subsp. *cremoris* JFR1 into a food matrix, fermented milk, to investigate behaviour of epithelial cell models and *Salmonella* virulence response.
In the first phase, intestinal epithelial cell line HT-29 was used to examine *Salmonella* attachment in the presence of either overnight cultures of *L. lactis* subsp. *cremoris* JFR1 exopolysaccharide producing (JFR+) or nonproducing (JFR-) strains as well as extracted EPS. Both JFR+ and JFR- reduced the virulence expression as well as the number of *Salmonella* attached to HT-29 cells. However, concentrations of purified EPS ranging from 0 mg/mL to 0.2 mg/mL displayed no reduction on attachment or virulence behaviour of *Salmonella* thus the effect of EPS was ruled out. Contrary to these results, previous studies have shown increased attachment of pathogens to intestinal mucus in the presence of isolated exopolysaccharides (Ruas-Madiedo, Gueimonde, Margolles, de los Reyes-Gavilán, & Salminen, 2006) although the source and concentration of EPS as well as *in vitro* model differed between the two studies. The authors utilized concentrations of EPS (up to 5mg/mL), whereas the highest EPS concentration used from JFR+ was 0.2 mg/mL due to difficulty to dissolve. In this research, intestinal epithelial cells HT-29, which do not produce mucus, were used as an *in vitro* model to test adhesion of *Salmonella* in the presence of EPS compared to Ruas-Madiedo’s study, where human intestinal mucus isolated from colon cancer patients was used. The effect of mucus as well as EPS concentrations may have contributed to the differences observed between the studies. Although EPS has been reported to exert several beneficial effects including antihypertensive (Ai et al., 2008), anti-gastritis (Rodríguez, Medici, Rodríguez, Mozzi, & Font de Valdez, 2009), and immunomodulation (Bleau et al., 2010; Kitazawa, Itoh, Tomioka, Mizugaki, & Yamaguchi, 1996; Makino et al., 2006), a conclusive effect of EPS on pathogen adhesion has still not been clearly established. The ecological significance of EPS has been described and plays a role in biofilm formation and stress resistance in LAB (Patel, Michaud, Singhania, Soccol, & Pandey, 2010). Furthermore, the existence of EPS producing LAB strains in the oral cavity and lower gastrointestinal tract indicate the importance of EPS for survival in unfavorable conditions.
Subsequently, JFR+ and JFR- overnight cultures on the invasion and response of host cells was further investigated through the use of Caco-2, HT29-MTX and coculture models in transwells. Epithelial cells grown on permeable membranes allowed for the determination of transepithelial electrical resistance (TEER) providing an indication of cell membrane integrity and permeability. In addition, the use of coculture cell models simulate the composition and function of the human small and large intestine providing a more representative in vitro model compared to a monoculture model. Cocultured cells of Caco-2 and HT29-MTX have been previously reported to display properties similar to those found in ex vivo and in vivo studies (Fernandez Miyakawa, Pistone Creydt, Uzal, McClane, & Ibarra, 2005; Matsuo, Ota, Akamatsu, Sugiyama, & Katsuyama, 1997). It was shown that JFR+ and JFR- treatments lowered the virulence gene expression and the numbers of invasive Salmonella into both Caco-2 and HT29-MTX monoculture models as well as the coculture model. Although several studies have reported the protective effect of lactic acid bacteria on cell membrane integrity against pathogen stimulation (Anderson, Cookson, McNabb, Kelly, & Roy, 2010; Peng, 2014), this is the first report that JFR+/- treatments could provide a protective effect to the epithelial cells by preventing the decline in TEER during Salmonella infection. It was determined that small peptides/oligopeptides present in the JFR treatments were most likely responsible for the antivirulence effects against Salmonella through the use of an OppA knockout Salmonella strain. This was further confirmed by testing Salmonella virulence gene expression in the absence of cell cultures; co-incubating Salmonella with JFR+/- treatments also demonstrated down regulation of hilA, invA, and sopD. Although the bioactive component found in JFR overnight cultures was not identified to be an oligopeptide, it was confirmed that components exhibiting antivirulence effect produced from L. helveticus and L. acidophilus were indeed peptides (Mundi, Delcenserie, Amiri-Jami, Moorhead, & Griffiths, 2013; Sharma, 2014; Tellez,
Corredig, Turner, Morales, & Griffiths, 2011; Yun, Oh, & Griffiths, 2014; Zeinhom et al., 2012). Elimination of the antivirulence effect when an OppA knockout Salmonella was used provided indirect evidence indicating the activity of a bioactive peptide. How this mode of action lead to down regulation of Salmonella virulence gene expression was not characterized in this research. However, it has been shown in E. coli that the presence of quorum sensing inhibitors (i.e. components found in L. acidophilus La-5 cell-free spent medium) could reduce the production of autoinducer signaling molecules (Medellín-Peña, Wang, Johnson, Anand, & Peng, 2007). It has also been demonstrated in Edwardsiella tarda that small peptides with sequence identities resembling the C-terminal of LuxS could interfere with the pathogens’ quorum sensing system and suppressed virulence gene expression (Zhang, Jiao, Hu, & Sun, 2009). Perhaps the bioactive components found in JFR overnight culture function in a similar manner, disrupting the quorum sensing mechanism of Salmonella by either direct or indirect interactions with LuxS. In addition, a global transcriptional regulator found in Salmonella, lrp (leucine-responsive regulator protein) has been associated with virulence repressing activity under certain circumstances (i.e. nutrient limiting conditions) (Baek, Wang, Roland, & Curtiss, 2009). It was demonstrated that constitutive expression of lrp resulted in strong interactions between Lrp and the promotor regions of Salmonella virulence genes which suppressed expression and lowered invasion of epithelial cells (Baek et al., 2009). In this research, the lack of OppA permease in the Salmonella mutant would suggest a decrease in the amino acid pool leading to increased expression of lrp, which in turn represses virulence gene expression.

Lastly, the antivirulence activity of the treatments was persistent even when applied in a food matrix, JFR+/- fermented milk. Again, through confirmation by the OppA mutant strain, this action is likely due to the production of bioactive peptides produced through proteolysis of milk proteins by the JFR strains. Several studies investigating fermented milk have also
demonstrated positive effects both in vitro and in vivo (Chen, Hsu, Hung, & Chen, 2016; Kemgang, Kapila, Shanmugam, Reddi, & Kapila, 2016; Millette, Luquet, & Lacroix, 2007; Zagato et al., 2014; Zeinhom et al., 2012). For instance, bioactive fractions from L. acidophilus La-5 were incorporated into yogurt and orally administered to mice resulting in lowered E. coli infection and virulence (Zeinhom et al., 2012). The authors also suggested that the potential mechanism of action could be linked to quorum sensing inhibition of E. coli (Zeinhom et al., 2012). As mentioned, peptides interfering with quorum sensing of bacteria has been reported to be associated with down regulation of pathogen virulence (Medellin-Peña et al., 2007; Zhang et al., 2009) suggesting a possible mechanism for JFR oligopeptides. It is important to note that the positive effects demonstrated in this study were not observed in the presence of GDL acidified milk, indicating that JFR milk fermentation is essential to the antivirulence effects in Salmonella. It is believed that the active components from both JFR overnight cultures and JFR fermented milk should be identical as the common substrate found in skim milk used for fermentation and M17 media used in preparation of overnight cultures was casein proteins. In the situation of chemical acidification of milk, casein proteins would not be degraded and therefore exhibit no antipathogenic effect against Salmonella. Evaluation of inflammatory marker IL-8 production by intestinal epithelial cells in the presence of acidified milk compared to bacterially fermented milk (B. longum and S. thermophilus) clearly demonstrated significantly less production of IL-8 in the presence of bacterially fermented milk treatment (Wagar, Champagne, Buckley, Raymond, & Green-Johnson, 2009). The presence of JFR treatments also reduced the production of inflammatory cytokine IL-8 which could contribute to maintaining intestinal homeostasis in vivo. Many studies have correlated the reduction of inflammatory markers to the status of host cells (i.e. intestinal epithelial cells and barrier integrity) (Bleau et al., 2010; Madsen et al., 2001; Peng, 2014). Moreover, many gastrointestinal diseases such as IBD, have been associated with increased levels of
inflammatory cytokines and increased intestinal permeability (Grimm, Elsbury, Pavli, & Doe, 1996). *In vivo* studies utilizing induced-IBD mice demonstrated oral ingestion of fermented milk could successfully attenuate the onset of IBD symptoms such as shortening of the colon length (Grimm et al., 1996; Kawahara et al., 2015).

5.2 Limitations and future work

Although this research has provided further understanding into the antivirulence activity of *Salmonella* induced by JFR+/- cultures and fermented milk, limitations and drawbacks do exist. The use of coculture cell models presents advantages over monocultures by providing a more complete representation of *in vivo* and *ex vivo* situations. However, the cell lines used originate from tumours and may exhibit altered regulatory mechanisms compared to normal cells. Levels of mucin expression in cancer cells have been reported to affect cell properties including proliferation, adhesion, motility, and invasion (Kim & Ho, 2010). Expression of MUC2 is predominant in healthy human large intestines whereas in a diseased state, MUC2 levels are significantly lower and levels of MUC5AC increase (Kim & Ho, 2010). Moreover, Caco-2 epithelial cells, although isolated from colon carcinomas, display properties similar to the small intestine enterocytes (Pinto, 1983). Alternatives may include the use of primary cultures derived directly from excised tissue which retain many *in vivo* properties or the use of animal models. Administration of treatments followed by challenge with *Salmonella* in animal models (e.g. mice models) could allow specific analysis of colonization in organs as well as any alterations in the microbial community via 16S rRNA sequencing. In addition, the inclusion of qPCR analysis of host cell tight junction proteins before and after JFR+/- treatments and infection with *Salmonella* could provide indication for improving barrier integrity and preventing intestinal permeability.
In this research, digestion of fermented milk was performed via a simple batch procedure. However, the use of gastrointestinal (GI) simulators such as the simulator of the human intestinal microbial ecosystem (SHIME) would represent a much more realistic process on the influence of the gastrointestinal tract and digestion on food components. The SHIME system mimics the entire GI tract including the stomach, small intestine, and different regions of the large intestine. This system allows for sampling after every step of the process (i.e. every “organ”/chamber) followed by further analysis by cell culture experiments, in vivo trials, as well as peptide analysis. Additionally, peptide profiles of the digested fermented milk can be compared with profiles of fermented milk to determine active fractions as well as sequence similarities. Purified active fractions could be used to ascertain minimal dose to achieve highest level of antivirulence activity. Moreover, further analysis to explore the possible mechanisms of antivirulence action could include determining and comparing autoinducer production during Salmonella invasion in the presence of different milk treatments (i.e. Vibrio harveyi luminescence assay).

5.3 Conclusion

Fermented dairy products are not only a source of nutrition but also provide many functional benefits to the host. Interference with pathogen infections through mechanisms such as competitive exclusion, antimicrobial activity, immune modulation and improving epithelial membrane function contribute to treatment towards several gastrointestinal disorders. In this research, treatment of intestinal epithelial cells HT29-MTX, Caco-2, and cocultures by L. lactis subsp. cremoris JFR1 overnight cultures and fermented milk demonstrated an overall protective effect against invasion of Salmonella through suppressing virulence gene expression as well as preserving cell membrane integrity. Treatments also provided modest immune modulating capacity by reducing the production of inflammatory cytokine IL-8. However,
infection with *Salmonella* lacking OppA permease failed to demonstrate the same effects. Oligopeptides produced through the proteolytic action of *L. lactis* subsp. *cremoris* JFR1 are likely responsible for the antivirulence activity of *Salmonella*, considering GDL acidified milk did not display the same phenomenon. Given that digested fermented milk also did not exert these effects, it is indicated that digestive enzymes may disrupt some of the bioactivity and further characterization is needed. The outcomes of this research advance the understanding of how bioactive peptides produced during milk fermentation contribute to down regulation of *Salmonella* virulence activity while simultaneously offering a protective effect to the host by maintaining TEER and regulating the production of inflammatory markers. Furthermore, the importance of an intact OppA permease was elucidated as it is crucial for the bioactive peptides to exert their effects.
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