Bioactive Components from *Lactobacillus acidophilus* and *Lactobacillus helveticus* Fermented Milk Enhance Epithelial Membrane Integrity against *Salmonella enterica* serovars Typhimurium Infection

by

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ABSTRACT

BIOACTIVE COMPONENTS FROM LACTOBACILLUS ACIDOPHILUS AND LACTOBACILLUS HELVETICUS FERMENTED MILK ENHANCE EPITHELIAL MEMBRANE INTEGRITY AGAINST SALMONELLA ENTERICA SEROVARS TYPHIMURIUM INFECTION

Jingya Peng
University of Guelph, 2014

Advisor: Professor Mansel W. Griffiths

Previous in vitro and in vivo studies showed that bioactive components produced by Lactobacillus acidophilus (La-5) and Lactobacillus helveticus (LH-2) exerted protective effects against a variety of enteric pathogens infection, including Salmonella. The purpose of this study is to determine the mechanism of protective effect from La-5 and LH-2 against Salmonella Typhimurium infection on epithelial cells.

Cell free spent medium (CFSM) were obtained after 48 h fermentation in whey protein-based medium or milk for La-5 and LH-2, respectively. Human colonic carcinoma HT-29 cells were grown in Transwell inserts for 40 days until they were polarized (TEER ~120 ohm×cm²). Cells were pre-incubated with CFSMs 24 h prior to Salmonella infection and co-incubated during infection. Lactate dehydrogenase (LDH) activity and TUNEL apoptotic assay were tested. Finally, an invasion assay was carried out using chicken hepatoma LMH cells as an in vitro model for Salmonella presence in poultry.

Results shown that Salmonella induced TEER loss was attenuated when pre-incubated and co-incubated with non-toxic dose of CFSMs at 8th h post-infection (P <0.05). LDH was reduced up to
49.1% (La-5) and 46.8% (LH-2) \( (P < 0.01) \). In terms of apoptosis, 75.8% (La-5) and 62.5% (LH-2) less apoptotic cells were observed \( (P < 0.01) \). The effectiveness of *Salmonella* invasion on LMH cells was strain dependent. Less than one \( \log_{10} \) cycle reduction was obtained when La-5 or LH-2 CFSM was applied.

These data suggest that LH-2 and La-5 have antagonized function against *Salmonella* infection in epithelial cells by enhancing epithelial membrane integrity and would bring further beneficial evidence of consuming bioactives as functional supplements.
ACKNOWLEDGEMENT

First and foremost, I would like to thank my supervisor, Prof. Mansel W. Griffiths, for his insightful guidance and financial support. I thank him for giving me this chance and becoming someone whom I can always rely on whenever I needed help throughout the course of my research. My sincerest gratitude goes to my charming and intelligent committee members, Prof. Milena Corredig and Dr. Angela Tellez, whose encouragements and suggestions always contributed to improving my work and inspiring me in not only my research but also my life.

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Above all, my greatest gratitude goes to my parents. I thank them for giving birth to me and being my role models. I deeply appreciate their encouragement on challenging myself and chasing my dreams. I thank my aunt, uncle and cousin in Toronto for their care throughout my study in Canada. Finally, I thank my boyfriend for his unconditional love and support. I would not finish the Master degree on time without the support from all of them.

Lastly, I thank the University of Guelph for endowing me with this precious journey. I will cherish everything that I have learnt in Guelph. This moment is not a happy ending, but the beginning to a new adventure, as my motto says, “live and learn”.
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ANOVA</td>
<td>Analysis-of-Variance</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>CDC</td>
<td>Center for Disease Control</td>
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<tr>
<td>CFSM</td>
<td>Cell Free Spent Medium</td>
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<td>CFU</td>
<td>Colony-Forming Unit</td>
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<td>CSLM</td>
<td>Confocal Scanning Laser Microscopy</td>
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<tr>
<td>EHEC</td>
<td>Enterohemorrhagic Escherichia coli</td>
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<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
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<td>FAO</td>
<td>Food and Agriculture Organization</td>
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<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<td>GI Tract</td>
<td>Gastrointestinal Tract</td>
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<td>IEC</td>
<td>Intestinal Epithelial Cell</td>
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<td>IFN-γ</td>
<td>Interferon Gamma</td>
</tr>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>IL-8</td>
<td>Interleukin 8</td>
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<tr>
<td>La-5</td>
<td><em>Lactobacillus acidophilus</em> La-5</td>
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<td>LAB</td>
<td>Lactic Acid Bacteria</td>
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<td>LDH</td>
<td>Lactate Dehydrogenase</td>
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<td>LH-2</td>
<td><em>Lactobacillus helveticus</em> LH-2</td>
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<tr>
<td>NLR</td>
<td>Nod-like Receptor</td>
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<tr>
<td>MDCK</td>
<td>Madin-Darby Canine Kidney</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<td>PE</td>
<td>Phycoerythrin</td>
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<td>PI</td>
<td>Propidium Iodide</td>
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<td>S-IgA</td>
<td>Secretory IgA</td>
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<td>SPI</td>
<td><em>Salmonella</em> Pathogenicity Island</td>
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<td>SRB</td>
<td>Sulforhodamine B</td>
</tr>
<tr>
<td>T3SS</td>
<td>Type Three Secretion System</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic Acid</td>
</tr>
<tr>
<td>TEER</td>
<td>Trans-epithelial Electrical Resistance</td>
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<td>TJ</td>
<td>Tight Junction</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptors</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis Factor Alpha</td>
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<tr>
<td>TSA</td>
<td>Trypticase Soy Agar</td>
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<tr>
<td>TSB</td>
<td>Trypticase Soy Broth</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
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<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER 1

Introduction

The public health effects and financial costs incurred following contamination of food with *Salmonella* can be severe. In addition, the increase in antibiotic resistance of *Salmonella* has stressed the need to find therapeutic alternatives. One such alternative may rest with bioactive peptides, especially those produced following fermentation of milk by lactic acid bacteria (LAB). A variety of studies have revealed the health promoting benefits of these peptides, including their ability to interfere with virulence mechanisms of pathogens. Based on previous *in vitro* and *in vivo* observations made in our laboratory, bioactive fractions from cell-free spent medium (CFSM) of *L. acidophilus* and *L. helveticus* showed potent immunomodulating effects on the host and suppression of infectivity of pathogens (Chin, 2002; Ding, Wang, & Griffiths, 2005; M. J. Medellin-Peña, 2007; Ng, 2000; Tellez Garay, 2009). However, the mechanisms whereby CFSMs exert these protective functions on epithelial cells are still not fully understood. We hypothesize that CFSMs enhance resistance of epithelial cells against *Salmonella* infections by maintaining cell membrane integrity and constrain *Salmonella* induced apoptosis. Herein, we propose to have a better comprehension of the mechanisms behind these effects, which could support the use of bioactive components from LAB fermented milk as supplements in functional food.
Literature Review

1. *Salmonella* spp.

*Salmonella* are one of the main causes of human food-borne illnesses. Digestion of *Salmonella* contaminated food leads to two major disease patterns in humans: a systemic disease (typhoid fever) and a self-limiting gastrointestinal illness, salmonellosis (Caron et al., 2006). Non-typhoidal *Salmonella* are the strains mainly associated with gastrointestinal infections. Approximately 1.2 million illnesses, 23,000 hospitalizations, and 450 deaths result from non-typhoidal *Salmonella* infections every year in the United States, resulting in an estimated $365 million dollars spent in direct medical costs annually (CDC, 2013). No significant decline of *Salmonella* infection has been found in more than a decade (CDC, 2011), whereas the increasing resistance of *Salmonella* to clinical-used antibiotics continues to be noticed since 1996. In 2013, CDC showed that around 5% non-typhoidal *Salmonella* were resistant to five or more types of antibiotic (CDC, 2013).

The taxonomy and nomenclature of the genus *Salmonella* used to be a prevalent topic (Tindall, Grimont, Garrity, & Euzeby, 2005). Over 2500 serotypes are found in the *Salmonella* genus, and possibly all of them are pathogenic (Burkholder & Bhunia, 2009). In 2007, the World Health Organization (WHO) defined that *Salmonella* consisted of only two species according to molecular detection, *S. enterica* and *S. bongori*. There are 6 subspecies in *S. enterica*, namely *S. enterica* subsp. *enterica*; *S. enterica* subsp. *salamae*; *S. enterica* subsp. *arizonae*; *S. enterica* subsp. *diarizonae*; *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica*. Specifically, *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) is one of the most common species involved in human food-borne illnesses, and often contaminates poultry, pork, beef and dairy products (Burkholder & Bhunia, 2009; Gaggia, Mattarelli, & Biavati, 2010). As a highly antibiotic-resistant strain, *S. Typhimurium* DT104
has attracted a great deal of public health attention (Burkholder & Bhunia, 2009; Wu, Carlson, & Meyerholz, 2002). In addition to its clinical value, *Salmonella* is an interesting bacterial model to examine for host-pathogen interaction since it can manipulate the functions of the host cells in order to prolong its own survival. Its virulence functions are connected to *Salmonella* pathogenicity islands (SPI1 and SPI2) and it possesses a needle-like Type Three Secretion System (T3SS) (Bayoumi & Griffiths, 2010). Therefore, *S*. Typhimurium DT104 strains were selected in this study to test for *in vitro* interference of epithelial cell functions.

2. Probiotics and Their Fermented Products

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) defined probiotics as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO & WHO, 2001; Khani, Hosseini, Taheri, Nourani, & Fooladi, 2012). Probiotics include bacteria, molds, and yeasts, among which lactic acid bacteria (LAB) are one of the most intriguing groups due to their presence in yogurt, fermented milks and other fermented foods. After Grigoroff isolated the first LAB in 1905 (Grigoroff, 1905), numerous studies of LAB have been done to ensure LAB fermented foods have health benefits along with a GRAS status (generally recognized as safe) (Divya, Varsha, Nampoothiri, Ismail, & Pandey, 2012). It is reported in many reviews that benefits of consuming LAB and their bioactive components include: (1) aiding in digestion and nutrient assimilation; (2) stimulating the immune system; (3) competing with unfavorable pathogens and producing antimicrobial bioactive molecules; (4) preventing the risk of certain cancers; (5) reducing the prevalence of allergy in susceptible individuals; (6) alleviating symptoms of lactose intolerance and diarrhea; (7) lowering serum cholesterol concentrations and (h) reducing blood pressure in hypertensive individuals (Divya et al., 2012; Khani et al., 2012; Masood,
Qadir, Shirazi, & Khan, 2011; Parvez, Malik, Kang, & Kim, 2006; Schrezenmeir & De Vrese, 2001).

Although it is well known that consuming LAB fermented foods exert positive effects on human and animal health, possible mechanisms behind these effects still remain largely unknown. The ingestion of LAB is considered as a promising alternative for antibiotic in order to deal with pathogens’ increased antibiotic resistance. Many in vivo and in vitro studies have focused on using specific LAB strains, with or without their culture medium, which exert antagonistic effects against various types of pathogens. Tables 1.1 and 1.2 summarize information on protection afforded to the host against specific pathogens by probiotics for both in vivo and in vitro studies, respectively.
Table 1.1 *In vitro* studies focus on pathogenic bacteria inhibitory function of probiotics.

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<th>Targeted pathogen</th>
<th>Findings</th>
<th>Reference</th>
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<td><em>Lactobacillus plantarum</em> 299v &amp;</td>
<td>Live microorganisms</td>
<td><em>Escherichia coli</em></td>
<td>Inhibited enteropathogenic <em>E. coli</em> adherence <em>in vitro</em> by inducing intestinal mucin gene expression.</td>
<td>(Mack, Michail, Wei, McDougall, &amp; Hollingsworth, 1999)</td>
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<td><em>Lactobacillus rhamnosus</em> GG</td>
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<td><em>Lactobacillus</em> spp.</td>
<td>Live microorganisms</td>
<td><em>Yersinia enterocolitica</em> DSM4780</td>
<td>Affected the likelihood of <em>Y. enterocolitica</em> survival by compromising urease functionality and cell viability.</td>
<td>(Lavermicocca, Valerio, Lonigro, Di Leo, &amp; Visconti, 2008)</td>
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<td><em>Lactobacillus casei</em> strain Shirot</td>
<td>Live microorganism &amp; cell-free culture supernatant</td>
<td><em>Helicobacter pylori</em> SS1</td>
<td>Inhibited <em>H. pylori</em> growth on solid agar as well as in liquid medium with the presence of living <em>L. casei</em> strain <em>Shirot</em>.</td>
<td>(Sgouras et al., 2004)</td>
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<td><em>Lactobacillus casei Rhamnosus</em></td>
<td>Live microorganism</td>
<td><em>Escherichia coli</em> C25</td>
<td>Inhibited bacterial translocation of <em>E. coli</em> C25 in a dose-dependent manner.</td>
<td>(Mattar, Drongowski, Coran, &amp; Harmon, 2001)</td>
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<td><em>Lactobacillus casei</em> &amp; <em>Lactobacillus acidophilus</em></td>
<td>Live microorganism</td>
<td><em>Shigella sonnei</em></td>
<td><em>Lactobacillus</em> affected <em>shigella</em> growth rate.</td>
<td>(Apella, Gonzalez, Demacias, Romero, &amp; Oliver, 1992)</td>
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<td>cell-free spent medium</td>
<td><em>Escherichia coli</em> O157:H7</td>
<td><em>L. acidophilus</em> La-5 secreted a molecule(s) that could block or interfere with EHEC’s virulence genes involved in colonization.</td>
<td>(M. Medellin-Peña, Wang, Johnson, Anand, &amp; Griffiths, 2007; M. Medellin-Peña &amp; Griffiths, 2009)</td>
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<td><em>Bifidobacterium</em> bifidum</td>
<td>Cell-free spent medium</td>
<td><em>Salmonella</em> Typhimurium</td>
<td>Down-regulated <em>S. Typhimurium</em> reporter gene expression driven by both <em>hilA</em> and <em>ssrB</em> at a dose dependent manner.</td>
<td>(Bayoumi &amp; Griffiths, 2010)</td>
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<td><em>Bifidobacterium</em> bifidum fraction from Cell-free culture medium</td>
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<td><em>Salmonella</em> Typhimurium &amp; <em>E. coli</em> reporter gene expression driven by <em>hilA</em> and <em>ssrB</em>,</td>
<td>(Bayoumi &amp; Griffiths, 2012)</td>
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</table>
Escherichia coli

Lee1, respectively; reduced pathogens colonization on eukaryotic cells; diminished survival and multiply capacities of Salmonella within macrophages.

Cell-free extracts from ten probiotic bacteria inhibited expression of the C. jejuni flaA s28 promoter, which was independent of pH and lactic acid concentration. Two non-probiotic lactic acid bacterial strains, Lactococcus lactis and Stococcus thermophilus, were less inhibitory. (Ding et al., 2005)

S. Typhimurium stimulated secretion of IL-8 was inhibited basolaterally in the presence of B. licheniformis. (Skjolaas, Burkey, Dritz, & Minton, 2007)

Table 1.2 *In vivo* studies focus on pathogenic bacteria inhibitory function of probiotics.

<table>
<thead>
<tr>
<th>Probiotic</th>
<th>Sample format</th>
<th>Targeted pathogen</th>
<th>Findings</th>
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<tr>
<td>Lactobacillus casei strain Shirota</td>
<td>Live microorganism &amp; cell-free culture supernatant</td>
<td>Helicobacter pylori SSI</td>
<td>Administration of L. casei strain Shirota reduced colonizing H. pylori viable counts and the associated inflammation of the gastric mucosa in the H. pylori SSI murine infection model. Produced bacteriocin Abp118 that could significantly protect mice against infection with the invasive foodborne pathogen L. monocytogenes.</td>
<td>(Sgouras et al., 2004)</td>
</tr>
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<td>Lactobacillus salivarius UCC118</td>
<td>Live microorganism</td>
<td>Listeria monocytogenes</td>
<td></td>
<td>(Corr et al., 2007)</td>
</tr>
<tr>
<td><strong>Lactobacillus casei</strong></td>
<td><strong>Gg</strong></td>
<td>Live microorganism</td>
<td><strong>Escherichia coli K1</strong></td>
<td>Decreased the frequency of <em>E. coli</em> K1A translocation in a neonatal rabbit model. (Lee, Drongowski, Coran, &amp; Harmon, 2000)</td>
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<td><strong>Lactobacillus casei</strong> &amp; <strong>Lactobacillus acidophilus</strong></td>
<td>Fermented milk</td>
<td><strong>Listeria monocytogenes</strong> &amp; <strong>enteroinvasive Escherichia coli</strong></td>
<td>Higher survival rate, higher levels of anti-pathogen sera &amp; intestinal, less pathogen colonization of liver &amp; spleen were observed among LAB treated mice when challenged with pathogens. (Demacias, Romero, Apella, Gonzalez, &amp; Oliver, 1993)</td>
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<tr>
<td><strong>Lactobacillus casei</strong></td>
<td>Live microorganism</td>
<td><strong>Salmonella Typhimurium</strong></td>
<td>Oral administration of LAB increased the mucosal intestinal immunity. (Perdigon, Alvarez, Demacias, Roux, &amp; Holgado, 1990)</td>
<td></td>
</tr>
<tr>
<td><strong>Lactobacillus casei</strong> &amp; <strong>Lactobacillus acidophilus</strong></td>
<td>Fermented milk</td>
<td><strong>Salmonella Typhimurium</strong></td>
<td>Anti-salmonella protective immunity mainly mediated by the mucosal tissue using <em>L. casei</em> + <em>L. acidophilus</em> mixture fermented milk. (Perdigon, Demacias, Alvarez, Oliver, &amp; Holgado, 1990)</td>
<td></td>
</tr>
<tr>
<td><strong>Lactobacillus helveticus</strong></td>
<td>A peptidic fraction from fermented milk</td>
<td><strong>Salmonella Typhimurium</strong></td>
<td>A peptide fraction composed of α-lactalbumin and β-casein derived peptides played a dose-dependent role in protecting mice against <em>Salmonella</em> translocation; mechanism involved cell-mediated immune response and inference with virulence gene expression. (Tellez, Corredig, Turner, Morales, &amp; Griffiths, 2011)</td>
<td></td>
</tr>
<tr>
<td><strong>Lactobacillus helveticus</strong></td>
<td>cell-free fractions from fermented milk</td>
<td><strong>Bioluminescent Salmonella Enteritidis</strong> (<em>lux</em> CDABE)</td>
<td>Bioluminescence emitted by mice and the physical condition of the mice indicated that animals fed with fermented milk or fermented milk components prior to infection were less susceptible to bacterial colonization and, subsequently, bacteremia. (Brovko et al., 2003)</td>
<td></td>
</tr>
<tr>
<td><strong>Lactobacillus acidophilus La5</strong></td>
<td>cell-free spent medium</td>
<td>enterohemorrhagic <em>Escherichia coli</em> (EHEC).</td>
<td>Cell-free spent medium fractions were able to down-regulate several virulence genes of EHEC, including <em>stxB2, qseA, luxS, tir, ler, eaeA</em>, and <em>hlyB</em>. (Zeinhom et al., 2012)</td>
<td></td>
</tr>
</tbody>
</table>
3. Bioactive Peptides

Definition and Characteristics

Bioactive peptides are defined as specific protein fragments that have a positive impact on body functions and conditions, ultimately benefiting health (Kitts & Weiler, 2003). Most bioactive peptides are obtained from proteins of animal origin, such as milk, egg, gelatin, fish, as well as plant proteins such as wheat gluten and soy (Elawadli, 2012). During digestion in the gastrointestinal tract, bioactive peptides can be generated from inactive parental protein sequences through three ways of proteolysis: (1) enzymatic hydrolysis by digestive enzymes, (2) fermentation with proteolytic starter cultures and (3) proteolysis by enzyme derived from microorganisms or plants (Korhonen, 2009; Muro Urista, Alvarez Fernandez, Riera Rodriguez, Arana Cuenca, & Tellez Jurado, 2011). A combination of the above methods could also be used in the effective generation of short functional peptides (Korhonen, 2009). The size of the active peptides ranges from two to twenty amino acid residues. Their bioactivities rely on the parental protein source and the composition of amino acid sequences they have inherited. Many peptides are known to exhibit multi-functional properties (Meisel & FitzGerald, 2003).

Bioactive Peptides Derived from Milk

Bioactive peptides derived from milk protein have received increasing attention due to their positive health-promoting effects on digestive, endocrine, cardiovascular, immune and nervous systems (Korhonen, 2009). Research done on peptide sequences have expanded our horizons allowing us to have a better understanding of antimicrobial, antioxidative, antithrombotic, antihypertensive, immunomodulatory and opioid activities that bioactive peptides possess (Muro Urista et al., 2011;
Silva & Malcata, 2005). Figure 1.1 exhibits the potential health benefits of various milk protein derived bioactive peptides. Moreover, it is revealed that LAB have an ability to degrade milk protein in order to fulfill their nutritional requirements for essential amino acids (Benkerroum, 2010). Because of the many different sources of proteinases and their modes of action, bioactive peptides from milk hydrolysed by lactic acid bacteria or their proteases represent a fundamental difference from those generated by digestive proteinases (Benkerroum, 2010). As a result, there has been growing scientific and commercial interest on the evaluation of milk fermentation with microbial proteolysis on human health; specifically on reducing the risk of chronic diseases or enhancing natural immune protection (Hartmann & Meisel, 2007).

Figure 1.1 Milk protein-derived bioactive peptides and their health promoting targets, adapted from Korhonen, 2009.
The wide range of nutritional, functional and biological benefits of milk proteins place them as the most valuable source of bioactive peptides at present (Korhonen, 2009). The protein concentration of bovine milk is about 32 g/L, 80% of which are caseins and 20% of which are whey proteins. LAB prefer the substrate casein due to its porous structure and poor solubility but whey protein also undergoes limited degradation (Griffiths & Tellez, 2013). Caseins can be divided into α-, β- and κ-caseins (Muro Urista et al., 2011). The whey fraction contains α-lactalbumin, β-lactoglobulin and other proteins, e.g., immunoglobulins, lactoferrin and serum albumin (Ebringer, Ferencik, & Krajcovic, 2008; Haug, Hostmark, & Harstad, 2007).

Since the functionality of peptides is closely related to parental protein in milk, a great number of peptide sequences with specific functionality in caseins and whey milk proteins have been identified (Muro Urista et al., 2011). Previous studies on peptide isolation and purification have been conducted in our laboratory. Tellez Garay (2009) found five putative bioactive peptides from a cell-free supernatant of *L. helveticus* LH-2 fermented milk. The fraction, which included these five peptides, showed an immunomodulatory effect and antagonistic effect against *Salmonella* infection in both *in vitro* and *in vivo* studies (Tellez Garay, 2009). Table 1.3 shows the five putative bioactive peptide sequences within this fraction. Other related research carried out by Medellin-Peña (2007) found three bioactive peptides in cell-free spent modified MRS medium (CFSM) fermented with *L. acidophilus* La-5. The bioactive molecule(s) from the CFSM not only down-regulated different virulence genes in enterohemorrhagic *E. coli* (EHEC) O157:H7 but also interfered with *E. coli* quorum sensing. Both *in vitro* and *in vivo* studies indicated increased resistance against infection and colonization with EHEC O157:H7 when biologically active La-5 CFSM fractions were applied (M. J. Medellin-Peña, 2007). Table 1.4 shows the three bioactive peptide sequences from biological active La-5 CFSM fractions.
Furthermore, databases (BIOPEPE etc.) and programs (BLAST etc.) are available to simulate and demonstrate proteolysis processes targeted at obtaining expected bioactive peptides from precursor protein (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008).

### Table 1.3 Bioactive peptides identification within the fraction of cell-free supernatant from *L. helveticus* LH-2 fermented milk using mass spectrometry and NCBI database, adapted from Tellez Garay, 2009.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Protein</th>
<th>Sequence assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>HQPHQPLPPTVMFPPQ</td>
<td>β –Casein</td>
<td>145–160</td>
</tr>
<tr>
<td>HQPHQPLPPT</td>
<td>β –Casein</td>
<td>145–154</td>
</tr>
<tr>
<td>WMHQPHQPLPPT</td>
<td>β –Casein</td>
<td>143–154</td>
</tr>
<tr>
<td>LYQEPVLGPVR</td>
<td>β –Casein</td>
<td>192–202</td>
</tr>
<tr>
<td>LDQWLCEK</td>
<td>α-Lactalbumin</td>
<td>115–122</td>
</tr>
</tbody>
</table>

### Table 1.4 Bioactive peptides identification within the fraction of cell-free spent modified MRS medium fermented with *L. acidophilus* La-5 using mass spectrometry and BLASTp, adapted from M. J. Medellin-Peña, 2007.

<table>
<thead>
<tr>
<th>Peak sequence/sequence aligned</th>
<th>BLASTp protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPVEPF/YPVEPF</td>
<td>YP 194702 ncopullulanase</td>
</tr>
<tr>
<td></td>
<td><em>L. acidophilus</em> NCFM</td>
</tr>
<tr>
<td>YPPGGP/YPPG</td>
<td>YP 193877 ornithine decarboxylase chain A</td>
</tr>
<tr>
<td></td>
<td><em>L. acidophilus</em> NCFM</td>
</tr>
<tr>
<td>NQPY/NQPY</td>
<td>YP 193484 glutamine ABC transporter</td>
</tr>
<tr>
<td></td>
<td><em>L. acidophilus</em> NCFM</td>
</tr>
</tbody>
</table>
4. Intestinal Epithelial Cells

Enterocytes in the gastrointestinal (GI) tract consist of a single monolayer of intestinal epithelial cells (IECs) and this monolayer is the frontline in host defense. The IEC is the most important physical barrier that separates the vast number of microbes in the intestinal lumen from the host tissue. IEC also plays a role in innate immune defense since the GI tract has the greatest number of lymphoid organs in the human body, which can interface with a myriad of internal and external stimuli (Ouwehand, Salminen, & Isolauri, 2002). IEC helps monitor the luminal bacterial signals in the GI tract, interpreting and transmitting this information to mucosal innate and adaptive immune cells in the lamina propria, and collaborates with lymphoid tissue to initiate an immune response to certain stimuli (Goto & Ivaylo, 2013; Pinto et al., 2009).

Epithelial Cells Polarization

*In vitro* models using isolated epithelia are invaluable for observing intracellular activities in response to external stimuli. The most common epithelial cell lines used in studies are small intestine isolated carcinoma Caco-2 cells and large intestine isolated carcinoma HT-29 cells for their ability to better mimic the natural intestinal mucosal barrier when they are polarized (Backert, Boehm, Wessler, & Tegtmeier, 2013). Numerous *in vitro* studies revealed that epithelial cell polarization improves relevance of real human intestinal epithelia. Human IEC, together with *in vitro* polarized epithelial cells are both endowed with physiological, and biochemical characteristics as well as structural markers. One of the most distinct structural features is the tight junction (TJ). TJs form between adjacent cells leading to apical and basolateral cell membrane formation. Additionally, a brush border composed of microvilli can be observed on the apical cell surface. Each microvillus possesses a bundle of actin filaments, which are cross-linked by numerous actin-bundling proteins like villin,
fimbrin and espin to form the microvilli structural core (Chalghoumi et al., 2009; Cohen, Ophir, & Ben Shaul, 1999; Le Bivic, Hirn, & Reggio, 1988; Rodriguez-Boulan & Nelson, 1989). Transwell permeable inserts with membrane filters in cell culture well plates have long been adopted for cross-epithelial transport assessment (Hubatsch, Ragnarsson, & Artursson, 2007; Zemans et al., 2011). The permeable insert is reported to be beneficial for epithelial cell polarization and differentiation. Due to the similar procedure for polarization of HT-29 cells in the permeable inserts to that observed during intestine embryonic development, HT-29 cells are utilized in epithelial differentiation studies (Le Bivic et al., 1988). When cultured in glucose for a certain period of time, HT-29 cells form very tight junctions and microvillus brush border at the apical surface, indicating cell polarization (Fitzgerald, Omary, & Triadafilopoulos, 1997; Höner zu Bentrup et al., 2006). Consequently, their resemblance to native IEC makes this model more representative than cells grown in mono-culture. Even though mono-cultures of epithelial cells are still useful and continue to be used, their restrictions, such as the inability to transport agents (from small molecules to immune cells) across the epithelial monolayer, are concerned (Höner zu Bentrup et al., 2006).

Trans-epithelial Electrical Resistance

The increase in Trans-epithelial Electrical Resistance (TEER) indicates the polarized status of epithelial cells grown in Transwell permeable inserts. The measurement of TEER is a well-developed approach to quantitatively monitor tight junction integrity and qualitatively observe monolayer health (Grajek & Olejnik, 2004). The Millicell ERS (EMD Millipore, Billerica, MA, USA) is a device designed to facilitate measurements of TEER to determine cultured epithelial monolayer integrity directly in tissue culture wells. The Millicell ERS-2 uses alternating currents to eliminate adverse effects on the cell membrane. It contains a silver electrode with a fixed pair of probes. These “chopstick”
like probes can measure the voltage deflection at 37°C in tissue culture medium (Balda et al., 1996). TEER evaluates tight junction formation under a wide variety of experimental conditions (Ferrell et al., 2010; Höner zu Bentrup et al., 2006). This model has provided invaluable insights into the intracellular events that occur in response to pathogenic bacterial stimuli. Solano et al. (2001) used TEER measurement as one of the methods to compare the virulence of different Salmonella Enteritidis strains, since the more virulent strains produce a greater disruption of the epithelial cell monolayer (Solano et al., 2001). In addition, probiotic bacteria have been reported to strengthen the intestinal barrier function as well as limit pathogen induced TEER loss. L. plantarum MB452 (Anderson et al., 2010), St. thermophilus, L. acidophilus (Resta-Lenert & Barrett, 2003) and L. amylophilus D14 (Yu, Wang, & Yang, 2012) inhibited pathogen-induced cell junction loss and mucosal barrier damage.

Epithelial Cells Immune Response to Salmonella Invasion

The intestine contains a great deal of immunoglobulin producing cells, e.g., IgA, comprising the largest immunological organ in the body (Tellez Garay, 2009). The epithelial cells located in the gastrointestinal tract collaborate with intraepithelial lymphocytes by possessing Toll-like receptors (TLRs) and Nod-like receptors (NLRs) on their surface to sense the existence of pathogens or pathogen-associated molecular patterns (Magrone & Jirillo, 2013; Tlaskalova-Hogenova et al., 2002). Furthermore, cytokines secretion is also involved in the immune responses to pathogen invasion. Cytokines are secreted soluble polypeptide or glycoprotein which play diverse biological roles in normal and pathological events, such as cell growth, differentiation, and immune response activation (Steinke & Borish, 2006; Zanabria Eyzaguirre, 2013). They may affect other cytokines production and action in order to achieve regulated immune response by balancing between provoking and suppressive influences (Zanabria Eyzaguirre, 2013).
Cytokines play essential roles in apoptosis and inflammation on epithelial cells. Accordingly, \textit{Salmonella} infection in all organs stimulates IL-1 and TNF-\textalpha expression (Eckmann & Kagnoff, 2001). In response to bacterial endotoxin, the first released cytokine is tumor necrosis factor TNF (Zanabria Eyzaguirre, 2013). Moreover, TNF often triggers the initiation and development of apoptotic and inflammatory processes (Benderska et al., 2012). Even though the detail of the cytokine’ function still remain to be elucidated, Eckmann & Kagnoff (2001) assumed that they may have indirect functions, like overall endothelial adhesion molecules up-regulation in order to initiate macrophage functions. IFN-\gamma is another most studied cytokine, which plays an important role in the host defense against \textit{Salmonella}, since production of IFN-\gamma is readily detectable in \textit{Salmonella} infected mice (Eckmann & Kagnoff, 2001). Kagaya et al., (1989) hypothesized that the most likely mechanism of operation of IFN-\gamma is the ability to activate macrophages to kill \textit{Salmonella} (Kagaya, Watanabe, & Fukazawa, 1989).

5. Apoptosis

Characteristics

Programmed cell death is essential in the hosts’ normal development. Not only does it help with organs differentiation, but it also eliminates useless and dangerous cells. It serves as a balance with cell division to maintain the normal cell number over time (Raff, 1992). \textit{Salmonella} manipulates the cell death pathway of mammalian cells by inducing their apoptosis (Kim et al., 1998). It is hypothesized that their ability to trigger apoptosis is a crucial step in the pathogenesis of \textit{Salmonella} (Ashida et al., 2011; Gobbato, Galdeano, & Perdigon, 2008; Knodler, Finlay, & Steele-Mortimer, 2005). By inducing cell death, previously replicated \textit{Salmonella} inside the host cells are released in the late apoptosis phase by evading the immune response, hence more effectively re-infecting other cells, resulting in systemic
infection (Ashida et al., 2011; De Moreno De Leblanc et al., 2010; Knodler & Finlay, 2001). Yet this mechanism is not fully proven whether apoptosis is a direct outcome of pathogen infection or is the result of inflammatory mediators released by the host (Torchinsky, Garaude, & Blander, 2010). Indeed, Kim et al. (1998) proposed that human colon epithelial cells generated inflammatory mediators in response to bacterial invasion. Some of these mediators also could induce apoptosis (Kim et al., 1998).

The term apoptosis was first introduced in 1972 by Kerr et al., who differentiated a naturally programmed cell death from severe tissue injury necrosis (Kerr, Wyllie, & Currie, 1972). There are three types of cell death involved in mammalian cells categorized by morphological criteria, namely apoptosis, autophagy, and necrosis. Apoptotic cells exhibit alterations to their nuclear morphology, including DNA fragmentation, chromatin condensation, membrane blebbing, overall cell shrinkage, and formation of apoptotic bodies that contain nuclear or cytoplasmic material (Elmore, 2007; Hans-Jurgen, 2008). Autophagy is characterized by an extensive accumulation of autophagosomes (double-membrane vacuoles) followed by fusion with lysosomes, leading to the degeneration of contents of autophagosomes. Cytoplasmic swelling is the characteristic of Necrosis, and the swelling occurs until the intracellular contents are released through the ruptured plasma membrane resulting in pro-inflammatory leakage (Hans-Jurgen, 2008). Figure 1.2 shows the different morphological alterations in the three forms of cell death. Among the three types, apoptosis has been studied extensively since it is a major form of removing unwanted and harmful cells with little inflammatory response and little disturbance of tissue homeostasis in the host (Ashida et al., 2011; de LeBlanc, Castillo, & Perdigon, 2010; Knodler & Finlay, 2001).
Figure 1.2 Schematic representations of three forms of cell death. See context for detail description, adapted from Hans-Jurgen, 2008.
Molecular Basics of Apoptosis

The molecular mechanism of apoptosis is dependent on a family of cysteine proteases called caspases. They mediate apoptosis with irreversible activation of proteins involved in DNA repair, DNA replication, and RNA splicing (Santini, Rainaldi, & Indovina, 2000). Even though all members of the caspase family have some overlapping amino acid sequences and possess similar structures, individual caspases play different physiological roles in apoptosis or inflammatory responses (Fink & Cookson, 2005). There are two major signaling routes to initiate caspase activation: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway (Ashida et al., 2011; Elmore, 2007; Hans-Jurgen, 2008). Extracellular signals such as toxins (Popov et al., 2002), hormones, growth factors, nitric oxide (Brune, 2003) and cytokines (Benderska et al., 2012), either penetrate through the plasma membrane or transduce through membrane receptors to activate the executioners caspase-3 and -7 (Ashida et al., 2011). These signals may trigger (positively affect) or inhibit (negatively affect) apoptotic activity in order to maintain tissue homeostasis, especially in the immune system (Ashida et al., 2011; Hans-Jurgen, 2008; Levi et al., 2014). Intrinsic signaling pathways are mitochondrial initiated events involved with a diverse array of non-receptor mediated stimuli, which can activate caspase-9 production and directly aim at targets within the cells (Ashida et al., 2011). The “cross-talk” between the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway leads to the final execution phase of apoptosis (Elmore, 2007). In this phase, morphological and biochemical alterations such as DNA fragmentation and membrane blebbing appear (Slee, Adrain, & Martin, 2001).

Assays for Apoptosis

Due to the distinct features of apoptotic cells, there are a variety of assays to determine apoptotic
activity activators, effectors and regulators and count the functional consequences of their actions. These assays can be classified into five categories: (1) morphological alterations detected by microscopy (Elmore, 2007; Fink & Cookson, 2005); (2) DNA fragmentation detected by DNA laddering (Compton, 1992) and TUNEL (Fink & Cookson, 2005; Hans-Jurgen, 2008); (3) membrane alterations detected by Annexin-V binding (van Engeland, Nieland, Ramaekers, Schutte, & Reutelingsperger, 1998), LDH and impermeable DNA dyes (Elmore, 2007; Fink & Cookson, 2005; Hans-Jurgen, 2008); (4) caspase activation detected by western blotting, PCR microarray (Elmore, 2007), colorimetric or fluorometric assays (Fink & Cookson, 2005; Hans-Jurgen, 2008); (5) mitochondrial damage detected by ATP production and mitochondrial dyes (Elmore, 2007; Hans-Jurgen, 2008)

Flow Cytometry

Amongst the assays previously described, the detection of DNA fragmentation is currently the most frequently used method when studying apoptosis (Fink & Cookson, 2005) together with flow cytometry, which can facilitate apoptosis detection. Flow cytometry, also referred to as fluorescence-activated cell sorting (FACS), is laser-based biophysical method, which allows multi-parameter measurements. It requires samples prepared in single cell format, to be measured in a fluid stream, which will be spectrophotometrically measured by the laser light. The properties of the designated single cell are acquired, including cell size (shown on forward-scattered light graph), conformation of inner structure (shown on side-scattered light graph), and relative fluorescence intensity (fluorescent intensity graph).

Fluidics, optics and electronics are the three main systems that make up the flow cytometer. The fluidics
system transports cells in single file through a narrow nozzle with arranged sheath fluid. A fluorescent photon resonance vibrating mechanism helps separate the stream of cells into individual droplets. The optics system consists of lasers at different wavelengths to illuminate cells in the same stream and optical filters to channel designated light to the appropriate detection system. Last, but not least, the electronics system converts detected light signals into a measurement that can be analyzed by computer software. As a result, electronic signals can be interpreted after adjusting the sorting settings of the software in order to obtain valuable information. Dong et al. (2008) concluded that flow cytometry was a versatile method of detecting the apoptotic process, including morphological alteration and chromatin shrinkage, activation of caspases, DNA condensation, etc. (Dong, Kleinberg, Davidson, & Risberg, 2008). Figure 1.3 provides a schematic representation of a flow cytometer (Jahan-Tigh, Ryan, Obermoser, & Schwarzenberger, 2012).

Apoptotic cells share noticeable characteristics, which can be captured by flow cytometry with appropriate fluorescent labels or dyes. From the data, it is able to differentiate the different phases of cell death (Vermes, Haanen, & Reutelingsperger, 2000). In addition, flow cytometry allows several measurements to be obtained on each cell simultaneously by applying different stains such as fluorescein isothiocyanate (FITC), propidium iodide (PI), or phycoerythrin (PE) (Corver, Cornelisse, & Fleuren, 1994). The stains’ fluorescent intensity can be quantified using CellQuest™ software (BD Bioscience, Mississauga, ON, Canada). In this study, the Apo-Direct™ Kit (BD Bioscience, Mississauga, ON, Canada) was employed to assess DNA strand breakage using FITC/PI dual stains.

**Probiotics Protection against Pathogen-induced Apoptosis**

Although the detailed mechanisms of pathogen-induced apoptosis are still under evaluation, the
hypothesis that probiotic strains or bioactive components from LAB culture medium may interfere with pathogen-induced apoptosis was investigated. Myllyluoma et al. (2008), performed a 1 h pretreatment of H. pylori infected polarized Caco-2 cells in the presence of L. rhamnosus GG, L. rhamnosus Lc705 and B. breve Bb99 cells and found that they reduced caspase-3 production significantly at 24 h post-infection (Myllyluoma, Ahonen, Korpela, Vapaatalo, & Kankuri, 2008). An in vitro study undertaken by Valdez et al. (2001) showed that the probiotic strains L. delbrueckii subsp. bulgaricus and St. thermophilus down-regulated Salmonella induced apoptosis in macrophages. In this case, the inhibitory effect did not seem to be related to an increase in the uptake of LAB, but more to secretory IgA (S-IgA) production. The latter is an immunoglobulin known to be associated with inhibition of pathogen internalization and toxin neutralization (Mantis & Forbes, 2010). In addition to pro-inflammatory cytokine production, such as TNF-α, oxidant radicals (superoxide, hydrogen peroxide and nitric oxide) released from macrophages stimulated by LAB may also partially explain the protective capacity of LAB (Valdez, Rachid, Gobbato, & Perdigon, 2001). Further, an in vivo study by Gobbato et al. (2008) found administration of L. delbrueckii subsp. bulgaricus and St. thermophilus strains improved microbiocidal activity and down-regulated apoptosis produced by S. Typhimurium infection, possibly through increasing cytokine IFN-γ, immunoglobulin IgA, and apoptosis related protein Bcl-2 release (Gobbato et al., 2008).

There are a limited number of publications describing the putative mechanisms of the anti-apoptotic effect on epithelial cells induced by cell free preparations of probiotics against pathogens. Li et al. (2001) showed that Lactobacillus acidophilus S-layer protein inhibited caspase-3 production by Caco-2 cells following Salmonella infection. In addition, the extracellular signal-regulated kinase 1 and 2 (ERK 1/2) signaling pathway plays a fundamental role in epithelial recovery after pathogen infection,
and the *L. acidophilus* S-layer protein activated the signaling pathway, reducing further apoptotic cell damage (Li, Yin, Yu, & Yang, 2011). Yan et al. (2007), found *Lactobacillus rhamnosus GG* (LGG) soluble factors p75 and p40 could regulate signaling pathways in order to prevent cytokine-induced apoptosis in human and mouse intestinal epithelial cells. Soluble factors p75 and p40 not only suppressed cytokine-induced apoptosis, but also promoted cell survival by stimulating Akt related proapoptotic pathways (Yan et al., 2007).

**Figure 1.3** Schematic representation of a flow cytometry (adapted from Jahan-Tigh et al., 2012). Samples are prepared in single cell format and are labeled by fluorochrome-linked antibodies or stained with fluorescent nuclear, cytoplasmic, or membrane dyes. The labeled/stained cells go through a beam of laser light with known speed and position. A set of optical system is used to detect forward-scatter, side-scatter and fluorescence. Lastly, an electronic system converts these signals into electronic signals on computer-based software.
Objectives

Bioactive peptides from LAB fermentations have shown potential as a therapeutic alternative of clinic-dependent antibiotic, therefore, attracted attention both from academic research and related industries. Based on previous *in vitro* and *in vivo* studies in our laboratory, cell-free spent medium (CFSM) of *L. acidophilus* and *L. helveticus* showed potent immunomodulatory function on the host and interference of pathogens virulence (Chin, 2002; Ding et al., 2005; M. J. Medellin-Peña, 2007; Ng, 2000; Tellez Garay, 2009). Our hypothesis that CFSMs reinforce epithelial cell membrane integrity when invaded by *Salmonella* is tested in four individual steps.

1. Using Trypan Blue Exclusion, LDH Assay and SRB Assay to determine the non-toxic dose of bioactive compounds from *L. acidophilus* or *L. helveticus* fermented milk (La-5 or LH-2) when applied on epithelial cells;

2. Examine the protective effect on epithelial cell membrane integrity present in the bioactive components against *Salmonella* infection through TEER Measurement and LDH Assay;

3. Employing TUNEL Assay to investigate down-regulation of *Salmonella*-induced apoptosis in epithelial cells when La-5 and LH-2 are applied;

4. Evaluate *Salmonella* internalization interference of La-5 and LH-2 in an *in vitro* chicken model by counting amount of invaded *Salmonella*. 
CHAPTER 2: Methodology

La-5 Cell Free Spent Medium (CFSM) Preparation

*Lactobacillus acidophilus* La-5 strain was obtained from the culture collection of the Canadian Research Institute for Food Safety (University of Guelph, Guelph, Canada), streaked on *Lactobacillus* MRS Agar (BD Difco™, Mississauga, ON, Canada) and incubated under anaerobic conditions with a BBL GasPak system (BD Bioscience, Mississauga, ON, Canada) at 37 °C for 48 h. Chemically defined medium (CDM) was composed of 28 g whey protein isolate (Ergogenics Nutrition, Vancouver, BC, Canada), and 10 ml of 0.25 g/ml sterile sucrose solution (Sigma, Markham, ON, Canada) in 500 ml sterile distilled water. The CDM medium (500 ml) was inoculated with *L. acidophilus* La-5 (50 colonies removed from an MRS agar plate cultured as described above) and incubated anaerobically with a BBL GasPak system (BD Bioscience, Mississauga, ON, Canada) at 37°C for 48 h. Following growth, bacterial cells were removed by centrifugation at 12,000 × g for 30 min at 4 °C (Avanti J-20 XPI, Beckman Coulter, Canada). The supernatant was collected and sequentially filtered through a 0.7 μm pore-size filter (EMD Millipore, Billerica, MA, USA), and then through a 0.45 μm pore-size filter (Fisher Scientific, Mississauga, ON, Canada) in order to remove any bacterial cells present. The cell-free preparation was frozen at -80°C (Thermo Fisher Scientific, Mississauga, ON, Canada), followed by freeze drying (Unitop 600 SL, VirTis Co., Inc. Gardiner, NY, USA). The freeze dried samples were stored at -80 °C (Thermo Fisher Scientific, Canada). The freeze-dried supernatant was reconstituted with cell culture grade water (Water for Injection, WFI, Life technologies, Burlington, ON, Canada) to 1/10 of its original volume before use. Portions of the reconstituted samples were also kept at -20°C until analyzed. The protein concentration was measured spectrophotometrically using the
Protein A280 program in Nanodrop according to manufacturer’s instruction (ND-1000, Thermo Fisher Scientific, Mississauga, ON, Canada). The protein concentration of La-5 CFSM was 99.5 ± 0.4 mg/ml.

LH-2 Cell Free Spent Medium (CFSM) Preparation

*Lactobacillus helveticus* LH-2 strain was obtained from the culture collection of the Canadian Research Institute for Food Safety (University of Guelph, Guelph, Canada), streaked on *Lactobacillus* MRS Agar (BD Difco™, Mississauga, ON, Canada) and incubated under anaerobic conditions with a BBL GasPak system (BD Bioscience, Mississauga, ON, Canada) at 37 °C for 48 h. Skim milk powder (Smucker's, Markham, ON, Canada) was reconstituted 10% (w/w) with distilled water. Reconstituted skim milk was heat treated at 95°C for 30 min and then cooled in the biosafety cabinet until it reached room temperature. Isolated colonies of the *L. helveticus* strain were used to inoculate four replicates of 5 ml sterilized reconstituted milk, which were then incubated anaerobically with a BBL GasPak system (BD Bioscience, Mississauga, ON, Canada) at 37°C for 24 h, followed by aerobic incubation at the same temperature for 26 h. The fermented milk samples were centrifuged at 16,000 × g for 10 min at 15 °C (Avanti J-20 XPI, Beckman Coulter, Canada). The supernatant was filtered through a 0.2 μm pore-size filter (EMD Millipore, Billerica, MA, USA) in order to remove any bacterial cells present. From 800 ml of fermented milk, 500 ml of supernatant were obtained. The cell-free supernatant (10 ml) was dispensed in 50 ml tubes and was frozen at -80°C (Thermo Fisher Scientific, Mississauga, ON, Canada), followed by freeze drying (Unitop 600 SL, VirTis Co., Inc. Gardiner, NY, USA). The freeze dried samples were stored at -80 °C (Thermo Fisher Scientific, Canada). The freeze-dried supernatant was reconstituted with cell culture grade water (Water for Injection, WFI, Life technologies, Burlington, ON, Canada) to 1/10 of its original volume before use. Portions of the reconstituted samples were also kept
at -20°C until analyzed. The protein concentration was measured spectrophotometrically using Nanodrop (ND-1000, Thermo Fisher Scientific, Mississauga, ON, Canada) as described above. The protein concentration of LH-2 CFSM was 37.1 ± 0.1 mg/ml.

**Salmonella** Strain and Growth Conditions

A human isolate (SA1997-0934) and two chicken isolates (SA2000-0406, SA2001-4368) of *Salmonella Typhimurium* DT104 along with a GFP expressing mutant of *Salmonella Typhimurium* were obtained from the culture collection of the Canadian Research Institute of Food Safety (University of Guelph, Guelph, Canada). All strains were stored in 25% glycerol (v/v) (Fisher Scientific, Canada) at -80°C, and transferred once to Trypticase Soy Agar (TSA, BD Bioscience, Mississauga, ON, Canada). Following overnight growth at 37°C, a colony was removed aseptically from the plate and transferred into 5 ml Trypticase Soy Broth (TSB, BD Bioscience, Mississauga, ON, Canada), which was then incubated at 37°C with shaking (200 rpm; orbitary shaker incubator, New Brunswick Scientific, USA) for 18–19 h. An aliquot (50 μl) of this culture was added into 5 ml of fresh TSB, and incubated at 37°C with shaking for 4 h. By the end of 4 h incubation period, all *S. Typhimurium* strains were in the late exponential phase and inoculum absorbance at *OD*<sub>600</sub> was adjusted to 1.00. The bacteria count was approximately 1 × 10<sup>9</sup> CFU (colony forming unit)/ml (≈ 9 log<sub>10</sub> CFU/ml).

All the strains used in these experiments are listed in Table 2.1.
Table 2.1 Bacterial strains used in these studies

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain No</th>
<th>Source</th>
<th>Gram stain</th>
<th>Growth conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella Typhimurium</em></td>
<td>SA 1997-0934</td>
<td>CRIFS*</td>
<td>Negative</td>
<td>TSB/TSA, 37°C</td>
</tr>
<tr>
<td></td>
<td>SA 2000-0406</td>
<td>CRIFS*</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SA 2001-4368</td>
<td>CRIFS*</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GFP</td>
<td>CRIFS*</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td>La-5</td>
<td>CRIFS*</td>
<td>Positive</td>
<td>MRS, 37°C</td>
</tr>
<tr>
<td><em>Lactobacillus helveticus</em></td>
<td>LH-2</td>
<td>CRIFS*</td>
<td>Positive</td>
<td>MRS, 37°C</td>
</tr>
</tbody>
</table>

* CRIFS: Canadian Research Institute for Food Safety, University of Guelph, ON, Canada

Cell Culture and Maintenance

The human colonic carcinoma cell line HT-29 was obtained from the culture collection of the Canadian Research Institute of Food Safety (University of Guelph, Guelph, Canada). Cells were cultured in Dulbecco’s modified Eagle medium (DMEM, high glucose, Life technologies, Burlington, ON, Canada) supplemented with 1% (v/v) 10,000 U/mL penicillin–streptomycin (Life technologies, Burlington, ON, Canada) and 10% heat-inactivated fetal bovine serum (HI FBS, Life technologies, Burlington, ON, Canada). The HT-29 cells (20–40 passages) were kept in a humid atmosphere of 5% CO₂ at 37°C in an incubator (Forma™ Series II 3110 Water-Jacketed CO2 Incubators, Thermo Fisher Scientific, Mississauga, ON, Canada), and grown as a monolayer in 25 or 75 cm² flasks (Corning, NY, USA). The medium was changed every two days and passed (80-90% confluence) using 0.25% trypsin-EDTA reagent (Life technologies, Burlington, ON, Canada).

The chicken hepatoma cell line, LMH (ATCC CRL-2117) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Waymouth’s MB 752/1 medium (Life technologies, Burlington, ON, Canada) supplemented with 1% (v/v) 10,000 U/mL penicillin–streptomycin (Life technologies, Burlington, ON, Canada) and 10% fetal bovine serum (FBS, Life technologies, Burlington, ON, Canada). LMH cells (6-23 passages) were kept in a humid atmosphere of
5% CO2 at 37°C, and grown as a monolayer in 0.1% gelatin (PCS-999-027, Cedar Lane Laboratories, Burlington, Canada) coated 25 or 75 cm² flasks (Corning, NY, USA). The medium was changed every two days and was passed (80-90% confluence) using 0.25% trypsin-EDTA reagent (Life technologies, Burlington, ON, Canada).

Trypan Blue Exclusion Assay

This assay based on the principle that viable cells have intact cell membranes which can resist trypan blue dye penetration while dead cells do not. Hence live cells can be easily differentiated and counted with a light microscope using trypan blue (Strober, 2001). Viable cells are clear, unstained, small, and round under light microscopy, while dead cells are blue, stained, and swollen (Louis & Siegel, 2011).

For this test, approximately 1 × 10⁶ HT-29 cells/well were seeded in a 24-well plate (Fisher Scientific, Canada) and left for twenty-four h at 37°C and 5% CO₂. Cells were then stimulated with different concentrations (v/v) of La-5 (0~12.5%) and LH-2 (0~10%) CFSM adjusted in cell culture medium and left for twenty-four h at 37°C, in a CO₂ (5%) incubator. The cells were trypsinized using 250 µl of 0.25% trypsin-EDTA reagent (Life technologies, Burlington, ON, Canada), and agitated pipetting with 750 µl cell culture medium to make total volume of 1 ml cell suspension. The viable cells were counted by adding 50 µl of the cell suspension in 450 µl of trypan blue dye (0.4% v/v, Life Technologies, Burlington, ON, Canada). 50 µl of above cell suspension in trypan blue dye was loaded on haemocytometer. The exclusion of trypan blue from cells were observed under 10× magnification light microscopy (AE2000, Opti-Tech Scientific Inc., Scarborough, ON, Canada).

Confocal Scanning Laser Microscopy (CSLM)

The green fluorescent protein (GFP) from the jellyfish Aequorea victori can be used as an endogenous
fluorescent tag, which allows visualization of bacterial cells even when present intracellularly in host cells (Ling, Wang, Xie, Lim, & Leung, 2000). The most outstanding advantage of using CSLM to study *Salmonella* invasion is that bacterial internalization on epithelial cells can be observed in three dimensions without physically disturbing the specimens (Takeuchi & Frank, 2001). The GFP protein encoded on the plasmid inserted into *Salmonella* shares a common excitation and emission profile with the commercially available GFP, but is optimized for an excitation wavelength of 488 nm (Auty et al., 2005; Burnett, Chen, & Beuchat, 2000). For Leica CSLM, emitted light was collected through a 480 nm dichroic mirror, a 520 nm long-pass filter, and a 680 nm short-pass filter (Takeuchi & Frank, 2001).

Leica confocal scanning laser microscopy (DMIRB Inverted Fluorescence Microscope, Leica Microsystems, Concord, ON, Canada) was used to observe the intracellular populations of transformed *S. Typhimurium* into the epithelial cell monolayer. In this study, 24 h prior to *Salmonella* infection, $5 \times 10^6$ HT-29 cells were seeded in 35mm glass bottom dishes (MatTek Corporation, Ashland, MA, USA). 2 ml of a non-toxic concentration of CFSMs (1.5% of La-5 or 1% of LH-2) adjusted with antibiotics free cell culture medium was added to designated dishes. The GFP labeled *S. Typhimurium* inoculum containing $5 \times 10^7$ CFU/ml (bacteria: cell MOI of 10) was added to dishes and incubated for 2 h at 37°C and 5% CO$_2$. A negative control was included, comprising HT-29 cells without exposure to bacteria and CFSM. The positive control consisted of non-stimulated cells (not exposed to CFSM) infected with *Salmonella* for 2 h. Test groups consisted of HT-29 cells stimulated with either La-5 or LH-2 CFSM and exposed to *Salmonella* with the presence of CFSM for 2 h. After 2 h of infection, extracellular bacteria were removed by extensive washing with PBS together with 1 ml of 350 μg/ml gentamicin sulfate (Sigma, Markham, ON, Canada) treatment for 1 h at 37°C, in a CO$_2$ (5 %) incubator. Medium was subsequently changed to 1 ml of 200 μg/ml gentamicin for an additional 30 min. Following 2 washing steps with PBS,
1 ml of antibiotic-free cell culture medium was added in each dish. Then all the dishes were visualized under CSLM (DMIRB Inverted Fluorescence Microscope, Leica Microsystem, Concord, ON, Canada) according to manufacturer’s instruction. Random fields were examined for bacterial invasion.

Trans-epithelial Electrical Resistance (TEER)

Millicell-ERS is designed to facilitate measurements of TEER of cultured epithelial monolayer integrity directly in tissue culture wells. Millicell ERS-2 (EMD Millipore, Billerica, MA, USA) is a device which uses alternating current to eliminate adverse effects on the cell membrane. It contains a silver electrode with a fixed pair of probes, which can measure the voltage deflection at 37°C in tissue culture medium (Balda et al., 1996). The change in TEER value is an indication of cell monolayer confluence quantitatively and cell monolayer health qualitatively.

Transwell inserts (BD Falcon™, Mississauga, ON, Canada) are permeable supports which create an apical and a basolateral chamber in each well to allow epithelial and other cell types to be grown and studied in a polarized state.

For TEER measurements, HT-29 cells at a concentration of $1 \times 10^5$ cells/ml were pipetted on top of Transwell inserts, resulting in a seeding density of approximately $5 \times 10^4$ cells/insert. The medium was changed every two days in both apical (500 μl) and basolateral (700 μl) chambers in each well. After 40 days, cells formed a fully polarized monolayer and reached the plateau TEER value of $\sim 120$ ohm $\times$ cm$^2$.

TEER was measured with a Millicell ERS-2 apparatus (EMD Millipore, Billerica, MA, USA) by immersing the shorter tip of electrode in the insert and the longer tip in the outer well at a 90° angle to the plate insert. The shorter tip should not contact cells growing on the insert membrane and the longer tip should just touch the bottom of the outer well. TEER values were obtained from blank inserts.
(without cells) and test inserts (with cells) and was calculated to ohm × cm². The final values were obtained from triplicate inserts by subtracting average value of blank inserts from all samples and multiplying by the area of the monolayer (Hasegawa et al., 1999).

Epithelial monolayer Integrity Challenge Study

In this study, we used polarized HT-29 cells which had been grown in Transwell inserts for 40 days with ~120 ohm × cm² TEER value to investigate whether La-5 or LH-2 CFSM could prevent S. Typhimurium from interfering with the HT-29 monolayer integrity. In each experiment, the monolayer in all inserts was from the same passage number and stage of maturation. 24 h before Salmonella infection, medium in both apical and basolateral chambers created by the Transwell insert in each well was replaced with antibiotic-free cell culture medium or stimulated with 1.5% of La-5 or 1% of LH-2 CFSM adjusted in antibiotic-free cell culture medium. Subsequently, 1 × 10⁷ CFU/ml S. Typhimurium DT104 (SA1997-0934) inoculum (Multiplicity of Infection bacteria: cell = 10) was added to the apical chamber of the inserts and left at 37°C, in a 5% CO₂ incubator for 2 h. Gentamicin sulfate (Sigma, Markham, ON, Canada) was used to eradicate extracellular Salmonella as described above. After calibrating the Millicell ERS-2 using culture medium, the sterile probes were vertically immersed into the apical or basolateral chamber at a 90° angle to the plate insert. The first measurement after the addition of gentamicin was regarded as time t₀. TEER readings were recorded at various time intervals and expressed as the ratio of TEER at time t to the initial value at t₀ for each series. This approach has been utilized in the study of evaluating probiotic activity (Klingberg, Pedersen, Cencic, & Budde, 2005).
**Lactate Dehydrogenase (LDH) Cytotoxicity Assay**

LDH is a stable and soluble enzyme present in the cytoplasm of all mammalian cells. LDH is impermeable in normal cells, but if the cell’s plasma membrane is damaged then LDH is rapidly released into the surrounding medium. In this research, LDH release caused by pathogenic bacteria damage to the plasma membrane can be quantified as an accurate index of cell death or cytotoxicity.

The LDH levels in cell culture medium were determined using the LDH-Cytotoxicity Assay Kit II (Abcam, Toronto, ON, Canada) according to the manufacturer’s instructions. The assay utilizes an enzymatic coupling reaction: LDH oxidized lactate to generate NADH, which then reacts with cell-impermeable Tetrazolium Salt WST to generate yellow color (see Figure 2.1). The yellow color formed can be detected spectrophotometrically at 450 nm and the intensity of the generated color is proportional to the degree of cell lysis.

![Catalytic function of the Lactate Dehydrogenase (LDH) enzyme.](image)

Figure 2.1 Catalytic function of the Lactate Dehydrogenase (LDH) enzyme.

Preliminary studies showed that the optimal cell density for LDH quantification was $1 \times 10^5$ cells/ml/well in 96-well plate. 24 h before *Salmonella* infection, 100 μl of HT-29 cells at a concentration of $1 \times 10^5$ cells/ml were seeded into a 96-well plate, resulting in a seeding density of approximately $1 \times 10^4$ cells/well. Simultaneously, 100 μl of non-toxic concentration of La-5 (1.5%) or LH-2 (1%) CFSM (v/v) were adjusted using antibiotic-free cell culture medium and added in designated wells. Two wells with cell culture medium only were included as background controls. The background value has to be
subtracted from all other values. The negative control consisted of supernatant from cells without exposure to bacteria or CFSM. The positive control consisted of HT-29 cells infected with *S. Typhimurium* DT104 (SA1997-0934; MOI of 10) in the presence of the appropriate CFSM for 2 h. All supernatants were centrifuged (Allegra™ 21R Centrifuge, Beckman Coulter™, Mississauga, ON, Canada) at 600 × g for 10 min to remove bacterial and eukaryotic cells. A 10 μl aliquot from each well was dispensed into a new 96-well plate and reacted with 100 μl of WST substrate mix (within the kit). The absorbance was measured at 450 nm using a Multilabel Counter (Wallac 1420 Victor™ 3V, Perkin-Elmer Life Sciences, Woodbridge, ON, Canada). LDH cytotoxicity % was then determined as shown.

\[
\text{Cytotoxicity (\%)} = \frac{(\text{Test sample} - \text{Low Control})}{(\text{High Control} - \text{Low Control})} \times 100
\]

Low Control: untreated cells

High Control: cells treated with 10 μl Cell Lysis Solution (within the kit)

Values of test groups were expressed as % cytotoxicity relative to the positive controls.

**Apoptosis TUNEL Assay**

Briefly, polarized HT-29 cells were seeded at 1 × 10⁶ cells/well of a 24-well plate 2 days prior to *S. Typhimurium* infection. 24 h later, designated wells were pre-incubated with 1.5% La-5 or 1% LH-2 CFSM in antibiotic-free cell culture medium. 1 × 10⁷ CFU/ml *S. Typhimurium* DT104 (SA1997-0934; MOI of 10) was added in designated well with or without the presence of appropriate concentration of La-5 or LH-2. After 1 h, extracellular bacteria were removed by extensive washing with PBS and addition of gentamicin incubation as described above. The cells were trypsinized using 250 μl of 0.25% trypsin-EDTA reagent (Life technologies, Burlington, ON, Canada), and agitated pipetting with 750 μl
cell culture medium to make total volume of 1 ml cell suspension. After washing with PBS and centrifuged at 500g, 5 min (Allegra™ 21R Centrifuge, Beckman Coulter™, Mississauga, ON, Canada) several times, cells were fixed in 1 ml 1% paraformaldehyde (Sigma, Markham, ON, Canada) for 1 h on ice, followed by PBS washing and suspending in cold 70% (v/v) ethanol and left at 4°C for at least 15 h for cell membrane permeabilization. The Apo-Direct ™ Kit (BD Biosciences, Mississauga, ON, Canada) was used in this research according to the manufacturer’s instruction. Positive and negative controls in this kit were run in parallel to the samples in order to define M1/M2 markers. 1 ml of BD positive and negative control cells and all of 15 h frozen cells were both washed with 1 ml of BD wash buffer (within the kit) and centrifuged at 500 g, 5 min (Allegra™ 21R Centrifuge, Beckman Coulter™, Mississauga, ON, Canada) two times. Subsequently, cells were incubated with 50 μl DNA labeling solution for 2 h. Then cells were washed with 1 ml of BD Rinse Buffer (within the kit) and centrifuged at 500g, 5 min (Allegra™ 21R Centrifuge, Beckman Coulter™, Mississauga, ON, Canada) two times, followed by adding 0.3 ml PI/RNase staining buffer. Based on DNA doublet discrimination and exclusion of cell debris, cells were counted adjusting the sorting threshold. Afterwards, cells were analyzed by flow cytometry (FACScan, BD, Mississauga, ON, Canada) with excitation at 488 nm. Fluorescence emission by FITC was measured using a 530/30 band-pass filter while that of PI was measured using a 585/42 band-pass filter. A total number of 5,000 cells were counted for each sample and the percentage of FITC positive (apoptotic) cells was determined.

Sulforhodamine B (SRB) Assay

SRB is a bright-pink aminoxanthene dye. Under mild acidic conditions, it electrostatically binds to basic amino-acid residues, while it dissociates under weakly basic conditions (Skehan et al., 1990). The
cell mass is correlated with the amount of dye extracted from cells. The SRB colorimetric assay is an accurate and reproducible assay based on the sensitive linearity of quantitative staining of cellular proteins with optical density (OD) between 560 and 580 nm wavelengths. This assay has been widely used as an efficient and highly cost-effective method for screening drug toxicity on different types of cell lines (Vichai & Kirtikara, 2006).

The SRB assay was tested on LMH cells in order to determine non-toxic doses of La-5 and LH-2 CFSM. After seeding $3 \times 10^3$ cells/well in a 96-well plate and incubating for 24 h at 37°C in a 5% CO$_2$ incubator, cells were stimulated with different concentrations of La-5 (0–3%) or LH-2 (0–3%) CFSM, followed by cells fixation with 50 μl of 50% (w/v) Trichloroacetic Acid (TCA, Sigma, Markham, ON, Canada). After removing the TCA, cells were stained with 0.4% (w/v) SRB (Sigma, Markham, ON, Canada) in 1% acetic acid (Glacial Acetic Acid, Fisher Scientific, Canada) for 30 min. Unbound dye was removed with 1% acetic acid, and the protein-bound dye was dissolved when 10 mM Tris-base (Tris-hydroxymethyl-aminomethane, Sigma, Markham, ON, Canada) was added. The developed color was measured spectrophotometrically at 570 nm using a microplate reader (Synergy H5, BioTek, Winooski, VT, USA) and results expressed as percentage with respect to control wells (set as 100%) grown under regular conditions.

Invasion Assay

The invasion assay was carried out on LMH cells in order to determine the protective ability of La-5 and LH-2 CFSM to limit the invasion of S. Typhimurium in in vitro chicken model.

Invasion assay was assessed as described elsewhere (Bishop et al., 2008) with specific minor modifications as follows. LMH cells were seeded at $2 \times 10^6$ cells/well in 0.1% gelatin pre-coated
12-well plate for 24 h at 37°C in a 5% CO₂ incubator. Then 2 ml of a non-toxic concentration 1 % of La-5 or 1% of LH-2 adjusted with antibiotics free cell culture medium was added to designated dishes 24 h prior to infection. Two chicken isolates of S. Typhimurium (SA2000-0406 or SA2001-4368) containing $2 \times 10^7$ CFU/ml (bacteria: cell MOI of 10) were added to appropriate wells and incubated for designated time in 37°C and 5% CO₂ incubator. Negative controls were included, comprising LMH cells without exposure to bacteria or CFSM. The positive control consisted of non-stimulated cells (not exposed to CFSM) infected with Salmonella. Test groups consisted of LMH cells stimulated with either La-5 or LH-2 CFSM and exposed to Salmonella with the presence of CFSM. At each time point, extracellular bacteria were removed by extensive washing with PBS, followed by exposure to 350 μg/ml gentamicin sulfate (Sigma, Markham, ON, Canada) for 1 h, and subsequently 200 μg/ml gentamicin for 30 min. Following 2 times of PBS washing, cells were lysed with 500 μl of 1% Triton X-100 solution (Sigma, Markham, ON, Canada) in high-pure water (WFI water, Life Technologies, Burlington, ON, Canada) for 2 min and suspended in 500 μl of PBS (Life Technologies, Burlington, ON, Canada). Eckmann et al. (1993) stated that within 1 h after cell lysis, Triton would not affect bacterial viability (Eckmann, Kagnoff, & Fierer, 1993). The number of released viable bacteria was determined by serial dilution in PBS (Life technologies, Burlington, ON, Canada) and plating on TSA agar (BD Bioscience, Mississauga, ON, Canada). After overnight incubation at 37°C, the numbers of bacterial colony were enumerated. CFU calculation was shown in followed formula. Values of test groups were expressed as % invasion relative to the positive controls.

$$\text{CFU/ml} = \frac{\text{Number of Colonies}}{\text{Dilution Factor} \times \text{Volume (0.1 ml)}}$$
Statistical Analysis

Statistical analyses were performed using the Microsoft Excel 2010 analysis tool package. Unless otherwise stated, all results are the average ± SD of two independent experiments with at least replicates. Data from each experiment were analyzed using analysis of variance (ANOVA). Differences at $P < 0.05$ were considered to be statistically significant. Differences at $P < 0.01$ were considered to be statistically very significant.
CHAPTER 3: Result and Discussion

Trypan Blue Exclusion Assay

In order to use tissue culture to determine the effect of CFSM on the infectivity of Salmonella, a tolerable dose of CFSM for eukaryotic cells used in these studies needs to be established, since negative effects (e.g. cell death) may be triggered when applying doses beyond the cells’ threshold. Based on previous in vivo studies using mice fed with a partially purified LH-2 CFSM fraction (F5), a high dose of this fraction (0.08 μg/day) caused intolerance and the mice exhibited a lower survival rate after Salmonella infection than the non-stimulated mice. However, a low dose of F5 (0.02 μg/day) protected mice against Salmonella infection. One possible reason for negative effects in mice exposed to the high dose of F5 might be perturbation of the immune system (Tellez Garay, 2009).

The trypan blue exclusion assay is based on the principle that viable cells have intact cell membranes which can resist trypan blue dye penetration while dead cells do not. Hence live cells can be easily differentiated and counted with a light microscope (Strober, 2001). This assay may show a relation between the CFSM concentration and HT-29 cell proliferation. In the case of La-5, the CFSM showed no significant effect ($P \geq 0.05$) on cells at all concentrations (see Fig.3.1.A). Interestingly, HT-29 cells showed highest viability in the presence of 1.6% La-5 CFSM. In the case of LH-2, only 1% showed no effect ($P \geq 0.05$) on cell viability (see Fig.3.1.B). Thus, 1.5% La-5 (protein concentration 1.5 mg/ml) or 1% LH-2 (protein concentration 370 μg/ml) were chosen as a non-toxic dose on HT-29 cells and used in subsequent experiments.
Figure 3.1 La-5 and LH-2 toxic dose test on HT-29 cells, estimated by trypan blue exclusive assay. A: La-5 treatment; B: LH-2 treatment. HT-29 cells were seeded and after 24 h the medium was replaced with new cell culture medium containing different concentrations of La-5 CFSM or LH-2 CFSM and incubated for 24 h. Results represent the means ± SD of two independent experiments performed in triplicate. Bars identified with ** are significantly different from untreated cells (P < 0.01). NS means there is no significant difference compared to untreated cells (P ≥ 0.05).
Confocal Scanning Laser Microscopy

In our preliminary studies, the effectiveness of gentamicin treatment in removing extracellular *Salmonella* was assessed using confocal scanning laser microscopy (CSLM) and optical density monitoring; we also evaluated effectiveness of *Salmonella* internalization through different infection time using CSLM.

It is important to know whether the gentamicin treatment effectively removes all extracellular *Salmonella* since *S. Typhimurium*, phage-type DT104 used in our experiments were reported to be resistant to multiple antibiotics. In addition, some strains possess an enhanced virulence phenotype (Wu et al., 2002). The inhibitory effect of different concentrations of gentamicin (from 50 μg/ml to 350 μg/ml) was tested on the growth of *Salmonella Typhimurium* DT104 (SA 1997-0934, SA 2000-0406 & SA 2001-4368) by monitoring optical density (OD) at 600 nm in TSB for up to 40 h. The results showed that the lowest tested concentration of gentamicin (50 μg/ml) inhibited the growth of all *Salmonella* strains (data not shown). It was concluded that 1 h incubation in the presence of 350 μg/ml gentamicin followed by 30 min incubation with 200 μg/ml gentamicin could effectively remove extracellular bacteria. To confirm this, confocal scanning laser microscopy (CSLM) was used to visualize the internalized and externalized *Salmonella* GFP strain.

By using CSLM it was possible to visualize *Salmonella* invasion status in three dimensions with minimum disruption of the specimen. In Figure 3.2, since *Salmonella* cells are visible, and the cell morphology is close to what has been reported in the literature (Collins & Kennedy, 1999). The cells appeared rod-shaped and 2.0-5.0 μm in size. Furthermore, the use of CSLM allowed the detection of minimum infection time for *S. Typhimurium* to invade HT-29 cells. Different *Salmonella* infection time
courses (1 h, 2 h and 3 h) with MOI of 10 were investigated in our preliminary study. All tested infection times showed internalized *Salmonella*. Figure 3.3 exhibits confocal images for 2 h infection with or without La-5 or LH-2 CFSM treatment. In addition, by adjusting the Z-stacks of the scan field and performing a 3D reconstruction using the Leica confocal software, intracellularly located bacterial cells were distinguished from those remaining outside of the host cells. Rarely motile *Salmonella* cells were observed in the medium. However, a very small portion of bacteria were attached to the external surface of the host cell membrane. These attached but not internalized bacteria to some extent affect the accuracy of internalized *Salmonella* count. However, 1 h incubation with 350 μg/ml gentamicin and 30 min incubation with 200 μg/ml gentamicin removed the majority of extracellular bacteria. Therefore, this gentamicin treatment was used in the further studies.

![Image](image_url)

**Figure 3.2** Confocal image of a single *S. Typhimurium* GFP cell. Light channel + argon channel (set at GFP specific parameters 488 nm) merged confocal image of invaded *S. Typhimurium* GFP in HT-29 cells after 2 h of infection (MOI 10). Image was taken after remove extracellular *Salmonella* using gentamicin. A white arrow indicates one single *S. Typhimurium* GFP cell. Scale bar: 2 μm.
Figure 3.3 Merged confocal images of *S*. Typhimurium GFP invasion of HT-29 cells. HT-29 cells were seeded and incubated for 24 h. Then the medium was replaced with new medium only (A) or new medium containing 1.5% of La-5 CFSM (B) or 1% LH-2 CFSM (C) and incubated for 24 h. *S*. Typhimurium GFP (MOI 10) was added without any CFSM (A) or with the presence of 1.5% La-5 (B) or with the presence of 1% LH-2 CFSM (C) and incubated for 2 h. Images were taken after using gentamicin to remove extracellular *Salmonella*. Scale bar: 20 µm.
Trans-epithelial Electrical Resistance (TEER)

The permeable insert used in this study is reported to benefit epithelial cell polarization and differentiation (Ferruzza, Rossi, Scarino, & Sambuy, 2012). Polarized cells improve physiological relevance of cell culture studies as they possess tight junctions and microvilli composed brush border, which are similar to those of human intestinal epithelial cells of the gastrointestinal tract (Chalghoumi et al., 2009; Cohen et al., 1999; Le Bivic et al., 1988; Rodriguez-Boulan & Nelson, 1989). Therefore, cell culture using permeable inserts provides a useful model that can mimic specific properties of the human intestinal epithelium. When cultured in glucose for a certain period of time, HT-29 cells form very tight junctions and microvillus brush border at the apical surface, indicating cell polarization (Fitzgerald et al., 1997; Höner zu Bentrup et al., 2006). Taking all these benefits into consideration, the following experiments were performed using polarized HT-29 cells.

Initial tests were carried out to determine the optimal HT-29 cell density necessary to seed in Transwell permeable inserts. A 500 μl of four different cell concentrations (1×10^4 cells/ml, 6×10^4 cells/ml, 1×10^5 cells/ml and 2×10^5 cells/ml) was seeded in apical chamber of Transwell inserts. Medium in both apical and basolateral chambers in each well was changed every two days. TEER values for each well were measured by Millicell ERS-2 apparatus as described in Chapter 2. Consequently, recorded TEER value was calculated to ohm × cm^2. The final values were obtained from triplicate inserts of each cell density by subtracting average value of blank inserts (without cell) and multiplying by the area of the monolayer (0.33 cm^2) (Hasegawa et al., 1999). As shown in Figure 3.4, it was found that 1×10^5 cells/ml gave a more linear and stable response in TEER values with less fluctuation. Hence, a 1×10^5 cells/ml level was used as optimal cell density for inoculation of Transwell inserts in subsequent experiments.
In order to know how long it took for cells to become polarized, the growth of HT-29 cells in the Transwell permable inserts was determined. Similar with previous TEER measurement, we grew 1×10^5 cells/ml HT-29 cells and recorded the growth in TEER values. From the Figure 3.5, it can be seen that before the 12th day, the TEER value was lower than 20 ohm × cm^2. Cells showed steady increase in TEER values between the 12th day to the 40th day. After the 40th day, the TEER value was stable in the range of 120~140 ohm × cm^2. Since the increase in TEER value was an index of cell confluence, tight junctions formation, and cell polarization establishment (Ghadimi, de Vrese, Heller, & Schrezenmeir, 2010), 120 ohm/cm² TEER value on day 40 was considered as a mature status of cell polarization. Cells with TEER value ≥ 120 ohm/cm² were used in the following experiments.
**Figure 3.4** Determination of optimal HT-29 cell density in Transwell permeable inserts. A 500 μl of four different cell concentrations (1×10^4 cells/ml, 6×10^4 cells/ml, 1×10^5 cells/ml and 2×10^5 cells/ml) was seeded in apical chamber of Transwell inserts. Medium in both apical and basolateral chambers in each well was changed every two days. TEER values for each well were measured by Millicell ERS-2. Consequently, recorded TEER value was calculated to ohm × cm^2. Results represent the means ± SD of two independent experiments performed in triplicate.
Figure 3.5 Growth of HT-29 cells in Transwell permeable inserts. $1 \times 10^5$ cells/ml HT-29 cells was seeded in the apical chambers of the inserts. Medium in both apical and basolateral chambers in each well was changed every two days. TEER values for each well were measured by Millicell ERS-2. Consequently, recorded TEER value was calculated to ohm $\times$ cm$^2$. Results represent the means $\pm$ SD of two independent experiments performed in triplicate.
Polarization of HT-29 cells occurs after a prolonged period of post-confluence culture. Instead of waiting for 40 days to obtain polarized cells, other researchers have investigated using substances to induce cell differentiation, including drugs, chemicals or by changing the energy source from glucose to galactose in standard culture medium (Mitchell & Ball, 2004). Cohen et al. (1999) determined that 20 h of exposure to the drug forskolin could induce up to 80% cell polarization among 21-day old HT-29 cells. Mocodazole and taxol, functioning as microtubule disrupters, were also discovered to affect brush border formation in cells grown for 21 days. While another microtubule disrupting agent, colchicine, could induce apical polarization of most HT-29 cells in only 7 days (Cohen et al., 1999). Fitzgerald et al. (1997) revealed that HT-29 cells became polarized in 21 days when the extracellular environment was adjusted to pH 5 (Fitzgerald et al., 1997). In addition, molecular engineering of HT-29 accelerates the cell polarization period, as well as expands choices in cell line selection. Cloned HT-29 cell lines exhibiting various cell forms in order to meet different research needs have been produced (Huet, Sahuquillomerino, Coudrier, & Louvard, 1987; Mitchell & Ball, 2004). After treating cells with sodium butyrate, the HT-29cl.19A cell line became differentiated and polarized, whereas HT-29cl.16E was goblet-like and secretory (Mitchell & Ball, 2004). After replacing glucose as an energy source with galactose, H29-18-C1 became absorptive, while HT29-18-N2 became mucus secreting (Huet et al., 1987). In conclusion, the prolonged cell culture times used in this study could be replaced by other methods to create polarized cells, which will shorten the experimental time.

Epithelial Barrier Integrity Study

Many investigators have demonstrated that pathogenic microorganisms have the ability to disrupt epithelial cell integrity, resulting in abnormal effects on the host, e.g., diarrhea (Canil et al., 1993;
Guttman & Finlay, 2008; Solano et al., 2001). A polarized monolayer of epithelial cells form tight
junctions, while S. Typhimurium infection cause a rapid loss in monolayer integrity which can be
detected by TEER (Chalghoumi et al., 2009). TEER loss is correlated with Salmonella invasiveness,
since non-invasive Salmonella does not affect TEER values (Finlay & Falkow, 1990). In addition to
TEER loss, Salmonella can cause apical and basolateral cell depolarization, which is presumable the
reason for tight junction disruption (Finlay et al., 1988; Finlay & Falkow, 1990). When Salmonella
attach to the host cell surface, they use a Type Three Secretion System (T3SS) to inject signals which
assist in their internalization. The host cell membrane becomes ruffled to allow uptake of associated
bacteria (Guttman & Finlay, 2009; Solano et al., 2001).

Probiotics are reported to enhance epithelial barrier integrity and modify the host cell cytoskeleton
Klingberg et al. (2005) concluded that measurement of TEER, where intestinal epithelial cells
monolayer grown in permeable inserts, could be used to evaluate probiotic activity. Hence, in this
epithelial barrier integrity study, the protection afforded by La-5 or LH-2 CFSM on cell integrity
following S. Typhimurium infection in polarized HT-29 cells was evaluated.

A preliminary study found that 24 h incubation with 1.5% La-5 CFSM or 1% LH-2 CFSM produced a
small but insignificant increase in TEER value of polarized HT-29 in the inserts (data not shown). In
accordance with previous findings, HT-29 inserts were used when TEER values were above 120 ohm ×
cm². The initial TEER value in this study was 132 ± 10 ohm × cm². The first measurement at time t₀
was conducted right after the gentamicin step used to reduce extracellular populations of Salmonella.
Following Salmonella infection, the TEER value at t₀ fell broadly (60.4 ± 0.8%) compared to the
initial value. However, cells to which no Salmonella were added also showed a reduction in TEER of
43 ± 3%. Figure 3.6 shows the ratio of TEER values obtained at the time of analysis to that obtained initially for all experimental groups. La-5 infected or LH-2 infected groups were pre-incubated with 1.5% La-5 CFSM or 1% LH-2 CFSM, respectively, and $10^7$ CFU/ml S. Typhimurium (bacteria: cell MOI of 10) were added. The positive control group comprised HT-29 cells, which were exposed to $10^7$ CFU/ml S. Typhimurium (MOI of 10) only. The negative control group consisted of HT-29 cells to which neither CFSM, nor Salmonella were added. There was an increase in TEER values for all groups at $t_1$ and $t_2$ (the first 2 h post-infection). In Fig. 3.6, the negative control group maintained stable TEER values after time $t_2$. On the contrary, there was a gradual decline in TEER value for all cells to which Salmonella was added. However, the reduction in TEER value was significantly alleviated at time $t_8$ (8 h post-infection) when La-5 ($P < 0.05$) or LH-2 ($P < 0.01$) CFSM was present. At $t_{22}$ there was attenuation but insignificantly in TEER loss among La-5 or LH-2 infected groups than positive control.
Figure 3.6 Ratio of TEER of polarized HT-29 cell monolayers after exposure to *Salmonella* Typhimurium to initial values. HT-29 cells were exposed to $10^7$ CFU/ml *S*. Typhimurium (MOI of 10) for 2 h. After 1, 2, 3, 4, 8 and 22 h of recovery in antibiotic-free cell culture medium, the changes in the TEER were measured using a Millicell ERS-2 apparatus. Result was expressed as the ratio of TEER at time $t$ in relation to the time zero $t_0$ (the first TEER measurement after gentamicin step) for each series. Results represent the means ± SD of two independent experiments performed in triplicate. The dot identified with ** is significantly different ($P < 0.01$) compared to positive control group, and of which identified with * is significant difference ($P < 0.05$).
The most vulnerable point to pathogen penetration is the paracellular space between cells. The normal network of tight junction associated proteins is able to seal this pathway for microbial translocation while allowing the transportation of electrolytes and other nutrients (Moorthy, Murali, & Devaraj, 2009). Probiotics have an advantageous impact on tight junction related proteins by increasing expression of protein zonula occludens ZO-1 and preventing adverse alteration of protein occluding, both of which benefit epithelial monolayer integrity (Yu et al., 2012). Klingberg et al. (2005) found a time and dose dependent enhancement of monolayer integrity for polarized Caco-2 cells when probiotic *L. plantarum* MF1298 and *L. salivarius* DC5 were presented. They concluded that the increase in TEER value was correlated to the increase in the expression of ZO-1. Furthermore, they demonstrated that *L. monocytogenes*-induced epithelial barrier malfunction was attenuated when cells were pre-incubated with *L. plantarum* MF129 (Klingberg et al., 2005). Our finding that *Lactobacillus* CFSM improved epithelial barrier integrity 8 h post-infection is in contrast with the Klingberg et al. work, whereby they proposed that only live probiotics could exert the beneficial effects within 8 h since heat-inactivated *L. plantarum* cells and *L. plantarum* cell-free supernatants from culture medium did not increase TEER values (Klingberg et al., 2005). The reason for this difference could be the virulence of the pathogen (Solano et al., 2001) or the probiotic strains applied. Yu et al. (2012) found that *S. Typhimurium* induced more severe damage to cell integrity than *E. coli* (Yu et al., 2012). Interestingly, even though Resta-Lenert & Barrett (2003) used *Salmonella* in their study, they still concluded that only live probiotics improved epithelial barrier integrity since antibiotic killed, heat inactivated and spent medium of *Streptococcus thermophilus* and *Lactobacillus acidophilus* failed to enhance TEER values. This may be due to the amount of spent medium applied since no information was provided on the concentrations (Resta-Lenert & Barrett, 2003). In another probiotic study, Yu et al. (2012) tested
expression of tight junction proteins, including ZO-1, claudin-1, and E-cadherin using a fluorescent dye. The probiotic strain *L. amylophilus* D14 did not change the distribution of tight junction proteins, but it was able to improve the expression of tight junction proteins and moderate their abnormal distribution triggered by the presence of pathogenic *E. coli* or *S. Typhimurium* (Yu et al., 2012).

Probiotics are speculated to modulate *Salmonella*-induced cytokine-mediated alteration in paracellular permeability, including interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) (Capaldo & Nusrat, 2009). Epithelial cells respond to IFN-γ by restructuring actin, reducing tight junction protein expression or displacing scaffolding protein ZO-1 (Blum et al., 1997; Youakim & Ahdieh, 1999). Even though conflicting reports exist, TNF-α has been shown in several studies to directly impair tight junction function associated with NF-κB pathway interruption (Ma et al., 2004; Ye, Ma, & Ma, 2006). Furthermore, IFN-γ and TNF-α co-treatment exerts a synergistic effect, which increases barrier sensitization and malfunction (Fish, Proujansky, & Reenstra, 1999; Wang et al., 2005). The CFSMs used in this study were expected to perform cytokine adjustment, since Tellez Garay (2009) revealed a dose dependent effect for IFN-γ and TNF-α modulation by a peptidic fraction of LH-2 CFSM (F5) *in vivo*. She found that when the fraction was applied at a rate of 0.02 μg/mouse/day, the IFN-γ level was significantly lower than in mice not given the fraction, but the fraction also induced TNF-α production (Tellez Garay, 2009). Hence, the modulation of cytokines when epithelial cells are stimulated with CFSMs may contribute to the epithelial barrier integrity.

*Salmonella* entry into the host cell induces membrane ruffling and rearrangement of actin filaments, resulted in a reduction in TEER values. On the contrary, increase of TEER values were observed at the first two h after *Salmonella* infection (see $t_1$ & $t_2$ in Fig. 3.6). Similar TEER increase trend was also observed by Klingberg *et al.* (2005) when $10^7$ CFU/cm² of *Listeria monocytogenes* was added in
polarized Caco-2 monolayer cells. However, the authors failed to give an explanation for this phenomenon. The reason for the increase in TEER values at time $t_1$ and $t_2$ could be due to temperature and homogeneity of culture medium. Matter and Balda (2003) discovered that the TEER value was dependent on temperature using polarized Madin-Darby Canine Kidney (MDCK) cells. The first measurement of TEER was carried out at 37°C. After 15 min, a second measurement was performed at 24°C and a final measurement after a further 15 min was carried out at 4°C. They found that the higher temperature the lower TEER value was. In order to get stable and representative values, TEER measurements require a highly controlled ambient temperature (Matter & Balda, 2003). Figure 3.7 shows the effect of temperature on TEER value. In our experiment, the TEER value at $t_0$ was obtained after several rinses with PBS at room-temperature (24°C); whereas later TEER values were obtained at 37°C. Different temperature causes TEER value fluctuation. The other factor that may influence TEER value is whether the cell culture medium is in homeostasis. Stable TEER result would be achieved if cell culture medium are regulated and homogenized (Matter & Balda, 2003).

In conclusion, La-5 or LH-2 CFSM enhances the epithelial barrier integrity by protecting cells’ tight junctions. The measurement of TEER showed that CFSMs started to alleviate the TEER loss caused by $S$. Typhimurium late after its infection (8 h post-infection).
**Figure 3.7** Temperature dependent of TEER on polarized MDCK (Madin-Darby Canine Kidney) cells grown on permeable inserts for 5 days. Data were obtained from four separate culture with 15 min time interval under 37°C, 24 °C and 4 °C conditions, adapted from Matter & Balda, 2003.

LDH Cytotoxicity Assay

The extracellular stimuli on plasma membrane damage can be quantified by measuring LDH production. It is an accurate index of cell death or cytotoxicity, as well as a convenient method for the evaluation of protective effect of CFSM on cell membrane integrity. First of all, we confirmed that 1.5% La-5 or 1% LH-2 CFSM were not toxic for cells since the LDH production of cell incubated with CFSM for 24 h was not significant different compared to untreated cells (data not shown). The optimal HT-29 cell seeding concentration was $1 \times 10^5$ cells/ml due to its rapid and reproducible detection of LDH. *Salmonella* concentrations were evaluated to provide a Multiplicity of Infection (MOI, number of bacteria: number of cells) of 10, 20, or 50 with 1, 2, or 3 h infection periods. The LDH percentage cytotoxicity was calculated for each group, as shown in Figure 3.8. 1 h infection time was not adequate for all MOI (data not shown). However, after 2 h of infection and with MOI of 10 the LDH cytotoxicity % (22.3 ± 8.7%) was twice that observed for all the other test conditions. After 3 h exposure to *Salmonella* (MOI of 50), destruction of HT-29 cells was nearly completed (98.6 ± 3.4%). Taking previous confocal microscopy results into consideration that 2 h infection period allowed *S.
Typhimurium to enter cells, the *Salmonella* Typhimurium infection condition with MOI of 10 for 2 h was chosen for the LDH study.

![Figure 3.8](image)

**Figure 3.8** Cytotoxicity (%) of HT-29 cells produced by *S. Typhimurium* at different times of exposure and different MOI. $1 \times 10^5$ cells/ml HT-29 cells were seeded in a 96-well plate. LDH production was measured after adding different MOI of *S. Typhimurium* and incubated for 2 or 3 h. Results represent the means ± SD of two independent experiments performed in triplicate.

In addition, the effect of CFSM on *Salmonella* infection of HT-29 cells was investigated. Pre-exposure to probiotic strains exerted an inhibitory effect on pathogen activities like adhesion, cytotoxicity and invasion (Burkholder & Bhunia, 2009; Jankowska, Laubitz, Antushevich, Zabielski, & Grzesiuk, 2008; Myllyluoma et al., 2008). Therefore, we compared the release of LDH from infected HT-29 cells when they were pre-incubated with CFSM for 24 h prior to *Salmonella* infection or co-incubated with CFSM during 2 h of *Salmonella* infection. From Figure 3.9, it is shown that co-incubation with CFSM had almost triple LDH release than with which were co-incubated. The pre-incubation with La-5 CFSM significantly reduced LDH production caused by *Salmonella* invasion than positive control. Therefore, pre-incubation with CFSM for 24 h improved cell monolayer integrity. To strengthen the protective
effect of CFSM, in addition to pre-incubation, we also co-incubated non-toxic doses of CFSM with HT-29 cells during exposure to *Salmonella*. Both pre-incubation and co-incubation with *Salmonella* were applied in further studies.

**Figure 3.9** Evaluation of CFSMs incubation conditions. 1 × 10⁵ cells/ml polarized HT-29 cells were seeded in a 96-well plate. LDH production was measured after adding *S. Typhimurium* (SA1997-0934, MOI of 10) and incubated for 2 h. Positive control represents *S. Typhimurium* infected cells. Negative control represents untreated cells. Test groups were either pre-incubated with CFSM or co-incubated with CFSM. All the data were normalized to positive control (positive control = 100% cytotoxicity). Results represent the means ± SD of two independent experiments performed in triplicate. Bars identified with ** are very significantly different (*P < 0.01*) compared to positive control group, and of which identified with * is significant different (*P < 0.05*). NS means no significant difference compared to positive control (*P ≥ 0.05*).

As for the preliminary study, 100 μl of 1 × 10⁵ HT-29 cells/ml cells was seeded in a 96-well plate. The cells were pre-incubated with La-5 or LH-2 CFSM for 24 h and co-incubated with the CFSM when exposed to *Salmonella* (MOI 10) for 2 h. **Figure 3.10** shows the effect of CFSM on *Salmonella* infection of epithelial cells. Compared with the positive control, very significant differences (*P < 0.01*) were found for both La-5 treated and LH-2 treated groups on normal HT-29 cells and polarized HT-29 cells.
In the normal HT-29 cell line, cytotoxicity % (as measured by LDH release) in the La-5 treated group and LH-2 treated group declined by 57 ± 1% and 58 ± 2%, respectively; whereas for the polarized HT-29 cell line cytotoxicity % in the La-5 treated group and LH-2 treated group was reduced by 46 ± 3% and 51 ± 2%, respectively. Interestingly, we found on both normal and polarized HT-29 cells, La-5 treated and LH-2 treated groups exhibited very significant reduction (P < 0.01) in cytotoxicity compared to the negative control. Besides protecting cells against *S. Typhmurium* infection, it is speculated that CFSM could also benefit epithelial cells under normal growth conditions by reducing the amount of necrotic cells, which would result in improvement of epithelial membrane integrity.

**Figure 3.10** LDH cytotoxicity of epithelial cells induced by *Salmonella* Typhimurium in the presence or absence of CFSM treatment. Positive control represents *S. Typhmurium* infected cells. Negative control represents untreated cells. La-5 represents La-5 pre-incubation and co-incubation during *S. Typhmurium* infection. LH-2 represents LH-2 pre-incubation and co-incubation during *S. Typhmurium* infection. All the data were normalized to positive control (positive control = 100% cytotoxicity). Results represent the means ± SD of two independent experiments performed in four replications. Bars identified with ** are very significantly different (P < 0.01) compared to positive control group.

LDH cytotoxicity evoked by pathogen invasion was alleviated by bioactive components from probiotic strains. This result was in agreement with previous studies where the cytoprotective effect of probiotics against a variety of pathogens or physiological stress have been reported. LDH liberation is
an indicator of cell membrane damage and cell viability. Hence, a reduction in the amount of LDH released could demonstrate an advantageous impact of probiotics on the host. Burkholder & Bhunia (2009) found that 1 h pre-incubation with \textit{L. rhamnosus} GG (LGG) strain significantly attenuated \textit{S. Typhimurium}-induced cytotoxicity for both normal and thermally stressed (41°C, 1 h) Caco-2 cells. Therefore, LGG effectively improved epithelial cell health and mucosal integrity during infection or exposure to toxins (Burkholder & Bhunia, 2009). \textit{H. pylori}-evoked cell membrane damage was very significantly alleviated ($P < 0.001$) by three probiotic strains, namely \textit{L. rhamnosus} GG, \textit{L. rhamnosus} Lc705, \textit{P. freudenreichii} subsp. shermanii Js (Myllyluoma et al., 2008). In conclusion, La-5 and LH-2 CFSM have potent ability to mitigate pathogen-induced cell damage.

Apoptosis Assay Using Flow Cytometry

To evaluate whether the beneficial effects of CFSM indicated by the LDH assay were reflected in a reduction of \textit{Salmonella}-induced apoptosis of HT-29 cells, flow cytometric TUNEL analysis of apoptotic cells with FITC/PI dual stains was counted out.

The optimum \textit{Salmonella} exposure time to cause cell apoptosis was determined. HT-29 cells were pre-incubated with non-toxic doses of CFSM for 24 h, and this was followed by co-incubation in the presence of CFSM and \textit{Salmonella} (MOI 10). The selection of \textit{Salmonella} infection period was based on the results obtained using the LDH assay, as LDH release indicates the phase of late apoptosis/early necrosis (Milovic et al., 2001). Infection periods of 1 h, 2 h or 3 h were then investigated for the apoptosis assay. 1 h infection period caused up to 95% apoptosis on polarized HT-29 cells (data now shown). Therefore, 1h infection time was chosen for the apoptosis assay.

DNA fragmentation is one of the most distinguishing features of apoptotic cells. When a DNA strand
breaks, the FITC stain labels the 3’-hydroxyl (OH) end of double- and single-stranded DNA catalyzed by the enzyme, terminal transferase (TdT; a template-independent addition). Apo-Direct™ Kit (BD Bioscience, Mississauga, ON, Canada) was used to assess DNA strand breakage using FITC/PI dual stains. Cell viability: vital cells, apoptosis and necrosis were discriminated by different staining. Cells with the staining patterns FITC(-) and PI(-) were designated as vital cells, FITC(+) and PI(-) as apoptotic cells, and FITC(+) and PI(+) as late apoptotic or necrotic cells (Punj et al., 2004). Figure 3.11 shows the effect of CFSM on *Salmonella* Typhimurium induced apoptosis of polarized HT-29 cells.

Cell diameter is reflected by forward-angle light scatter (FSC) and the conformation of inner cellular structure is reflected by side-angle light scatter (SSC) (Vermes et al., 2000). Comparing light scattered figures with different exogenous stimuli (A-D in figure 3.11), positive control (B), La-5 treatment group (C) and LH-2 treatment group (D) resulted in a slight decrease of both FSC and SSC compared to the negative control (A). Vermes *et al.* (2000) stated that during the initial phases of apoptosis, FSC decreased while SSC increased or remained unchanged. After several h both FSC and SSC diminished; indicating that cells underwent apoptosis (Vermes et al., 2000). Therefore, it was an evident that 1h *Salmonella* infection (MOI 10) induced apoptosis of polarized HT-29 cells. To check the effects of CFSM, FITC signals (Fig 3.11 E-H) and cell viability figures (Fig. 3.11 I-L) were examined.

Figure 3.11 E-H shows the intensity of FITC stain. The M1 and M2 regions in FL1-H reflected negative FITC stain and positive FITC stain, respectively. The positive control (F) had its FITC signal peak in the M2 region, indicating the greatest amount of apoptotic cells in this group compared to others. Even though the peak of fluorescence intensity in graphs of the La-5 treatment group (G) and LH-2 treatment group (H) was shifted more to the right side (higher FITC fluorescence intensity) than the negative control (E), the major proportion of FITC signals in these groups were still in the M1 region.
Figure 3.1: Effect of CFM on *Salmonella* Typhimurium induced apoptosis of polarized HT-29 cells. A-D showed Forward (FSC-H) and Side (SSC-H) light scattering graphs. E-H showed the dot plot of FITC signals intensity. The first lane (A & E) represents negative control (untreated cells); the second lane (B & F) represents positive control (*S. Typhimurium* infected cells); the third lane (C & G) represents La-5 treatment (La-5 pre-incubation and co-incubation during infection); the fourth lane (D & H) represents LH-2 treatment (LH-2 pre-incubation and co-incubation during infection). Light scattering signals indicate morphological change of cells. FL1-H displays the number of FITC positive stain cells. For statistical analysis see Figure 3.12.
The data shown in Figure 3.12 were obtained from the cell number ratio of M2/M1, indicating the percentage of apoptotic cells. This analysis provided a better view of the effect of CFSM on *Salmonella* induced apoptosis in polarized HT-29 cells. Compared with the positive control, very significant differences ($P < 0.01$) were found in cells treated with La-5 and LH-2. Apoptosis of La-5 treated or LH-2 treated groups compared with positive control were dramatically reduced by $76 \pm 2\%$ and $62 \pm 7\%$, respectively. Interestingly, we found La-5 stimulated or LH-2 stimulated groups exhibited a slight reduction in apoptosis compared to the negative control. These findings further supported the results obtained using the LDH assay and demonstrate that CFSM enhanced the health of epithelial cells under normal growing conditions and protected them from *Salmonella* infection.

To further confirm the anti-apoptotic effect of CFSM, cell viability was determined by comparing levels of FITC (FL1-H)/PI (FL2-A) dual stain signals (as shown in Figure 3.13).

As shown in Figure 3.13 FITC and PI dual stains differentiated cell viability. Cell population in the lower left quadrant [FITC(-) and PI(-)] were designated as vital cells; cell populations in the upper left quadrant [FITC(+) and PI(-)] were designated as apoptotic cells. The upper right quadrant represented late apoptotic or necrotic cells, which was merely observed in all the tested groups. For the figures, the positive control (B) consisted of cell population distributed mainly in the upper left quadrant, indicating the presence of more apoptotic cells than other groups. Even though cells treated with La-5 or LH-2 was distributed in the upper left quadrant than negative control, it was obvious that they were significantly lower than the positive control. Cell viability, determined by comparing efficiency of the two stains, suggested that CFSM treatment had a significant anti-apoptotic effect on *Salmonella*-induced apoptosis of polarized HT-29 cells.
Figure 3.12 Apoptotic rate of polarized HT-29 cells and the protective effect of CFSM pre-incubation (24 h) and co-incubation during exposure to Salmonella (1 h) (Flow cytometric TUNEL analysis). La-5 and LH-2 stimulated cells represented cells only pre-incubated with La-5 and LH-2, respectively. La-5 and LH-2 groups represented cells pre-incubated with CFSM and co-incubated with Salmonella during 1 h infection. Negative control represented untreated cells. Positive control represented cells infected with Salmonella for 1 h. Results were obtained after analyzing M2/M1 populations in FL1-H graph (As shown in Fig. 3.10 E-H), displaying in means ± SD of two independent experiments performed in replicate. Bars identified with ** are very significantly different ($P < 0.01$) compared to positive control group.
Figure 3.13 Cell viability by comparing efficiency of FITC (Y-axis)/PI (X-axis) dual stain signals. A: negative control (untreated cells); B: positive control (S. Typhimurium infected cells); C: La-5 treatment (La-5 pre-incubation and co-incubation during *Salmonella* infection); D: LH-2 treatment (LH-2 pre-incubation and co-incubation during *Salmonella* infection). E: cartoon detailing elaboration of quadrant gates of figures A-D, adapted from Jahan-Tigh et al., 2012.
Many apoptotic studies revealed that pathogen induced apoptosis of epithelial cells was a prolonged process. Conversely, our experiment found that S. Typhimurium with MOI of 10 caused up to 84 ± 15% apoptosis of polarized HT-29 cells within 1 h. The differences could be due to using different strains, different MOI, different treatment before fluorescence staining and, most likely, different cell models.

It is speculated that apoptosis is not easily induced in carcinoma derived cells. Kim et al. (1998) claimed that it took 12-18 h for bacterial adhesion, invasion and replication, as well as expression of apoptotic signals to initialize the appearance of apoptosis in HT-29 cells (Kim et al., 1998). Lundberge et al. (1999) revealed that HeLa, Caco-2 and MDCK cells did not show apoptosis after Salmonella infection (MOI 25, 30 min), while 80% of macrophages were not viable under the same conditions, elaborating apoptosis induction from Salmonella infection was more rapid in macrophages than in epithelial cells (Lundberg, Vinatzer, Berdnik, von Gabain, & Baccarini, 1999). A Salmonella-effector associated with phosphoinositide phosphatase activity SigD, also named SopB, may account for the late appearance of apoptosis in epithelial cells compare with macrophages (Knodler et al., 2005). Furthermore, Knodler and Finlay (2001) compared the time for the occurrence of apoptosis among carcinoma HeLa cells and a non-transformed rat intestinal epithelial cell line, IEC-6. They found much faster kinetics of Salmonella induced apoptosis on IEC-6 cells than immortalized carcinoma HeLa cells (Knodler & Finlay, 2001). This suggests that it is harder to show apoptosis in a carcinoma cell line. In our apoptosis assay, the cell line we used was a polarized human colon carcinoma HT-29 cell line, which was grown in Transwell inserts for up to 40 days. The biophysical features of these cells were closer to naïve human intestine epithelial cells. It is speculated that cell polarization could exert a major role on the rate of appearance of apoptosis.

Besides the diverse kinetics of using different strains, which may lead to variations in time to induce
apoptosis, another factor could be the amount of bacteria that were added. It is reported that the apoptotic rate of epithelial cells increased with bacterial inoculum size (Kim et al., 1998). Cerquetti et al. (2008) found more than 80% apoptosis caused by S. Enteritidis with MOI 10 in macrophage RAW 264.7 cells, whereas around 25% apoptosis and less than 10% apoptosis were produced by S. Enteritidis with MOIs of 1 and 0.1, respectively (Cristina Cerquetti, Hovsepian, Sarnacki, & Goren, 2008). Therefore, the amount of bacteria added to cells was a crucial factor to determine time to induce apoptosis. Furthermore, how cells are treated before the fixation step of the assay, together with the procedures for fluorescence staining may also affect the apoptosis timeline. After 1 h infection of S. Dublin, Kim et al. (1998) left cells in the presence of gentamicin for a designated time before fixation. As a result, they found apoptotic cells (cell nuclei stained with Hoechst dye 33258) did not always appear in the presence of Salmonella (GFP expressing). Some apoptotic cells did not contain GFP-labeled Salmonella inside. They hypothesized during apoptosis intracellular Salmonella cells were either killed possibly by entry of gentamicin from culture medium and/or they were released from apoptotic cells. Moreover, they found prostaglandin H synthase-2, which should inhibit apoptosis in both bacteria-infected intestinal epithelial cells and non-infected cells, contractedly increased the numbers of apoptotic cells in both infected and non-infected neighboring epithelial cells after more than 24 h post-infection (Kim et al., 1998). In our study, extracellular Salmonella cells were treated with gentamicin for 1.5 h in total, followed by an instant fixation step. The exact mechanism of transmission of apoptotic signals is yet to be elucidated. From the host perspective, an extended gentamicin step would affect the amount of apoptotic cells to some extent. Instant fixation is crucial to determine the total amount of cells undergoing Salmonella-induced apoptosis.

In recent years, there have been numerous publications indicating that probiotics suppress cellular
apoptosis following infection by a pathogen. Putative mechanisms involved in this suppression effect have been investigated (as described in Chapter 1). Some of the cytokines, such as Interferon INF-γ (Gobbato et al., 2008), Tumor Necrosis Factor TNF-α and Interleukin IL-8 (Hausmann, 2010) are considered to play a central role for triggering apoptosis. Based on previous in vivo and in vitro studies, La-5 and LH-2 CFSM showed an ability to modulate cytokines. Tellez (2009) revealed a dose dependent modulation on cytokines such as IFN-γ and TNF-α in mice fed with a peptidic fraction of LH-2 CFSM (Tellez Garay, 2009). Further study on dendritic cells revealed that levels of the proinflammatory cytokine TNF-α increased significantly \( P < 0.0001 \) in the presence of LH-2 and La-5 CFSM (1:10 dilution) compared to a negative control (PBS treated). However a 1:100 dilution of LH-2 and La-5 CFSM, which is similar to the concentration used in the present study, resulted in a small but insignificant increase in the production of TNF-α by dendritic cells (Elawadli, 2012). All these results could lead to the conclusion that the modulation of cytokines when epithelial cells are stimulated with CFSM may contribute to their anti-apoptotic capacity during Salmonella infection. La-5 or LH-2 CFSMs showed a significant reduction of Salmonella-induced apoptosis on polarized HT-29 cells line.

SRB Assay

As one of the most consumed meats, much attention has been focused on broiler chickens. Chicken meat provides valuable nutrition with relative reasonable price. Unfortunately, it also offers the largest and most vital reservoirs for Salmonella colonization. S. Typhimurium and S. Enteritidis are two main pathogenic strains responsible for human Salmonellosis in the past few years. These two serovars have the ability to asymptotically colonize chickens (Chalghoumi et al., 2009). With the previous
beneficial results in human epithelial cells, we created an in vitro chicken model using LMH chicken hepatoma cells to evaluate the protective efficiency of La-5 and LH-2 CFSM against S. Typhimurium isolated from chicken.

SRB viability results (see Figure 3.14) show a correlation between the CFSM concentration and LMH cell proliferation. For La-5 CFSM, cells treated with 1% (v/v) exhibited higher viability than untreated cells. In terms of LH-2, cells treated with 0.5%, 1%, 1.5% or 3% of LH-2 CFSM showed no statistical significant difference with untreated cells. Further LDH test confirmed that 1% of La-5 (protein concentration 1.0 mg/ml) or 1% of LH-2 (370 μg/ml) was not toxic for LMH cell line (data not shown) and was used in the following experiments.
Figure 3.14 La-5 and LH-2 CFSM toxic dose test on LMH cells proliferation, estimated by SRB colorimetric assay. Cells were plated and after 24 h the medium was replaced with new medium containing different concentrations of La-5 CFSM (A) or LH-2 CFSM (B) and incubated for 24 h. Results are normalized to untreated cells (viability of untreated cells = 100%) and display as means ± SD of two independent experiments performed in triplicate. Bars identified with ** are very significantly different from untreated cells (P < 0.01). NS means there is no significant difference compared to untreated cells (P ≥0.05).
Invasion Assay

Invasion by *Salmonella* results in bacteria internalization and systematic spread (Chalghoumi et al., 2009). Bacterial pathogenicity is determined by the organism’s propensity to colonize and invade the host cells. Therefore, an assay was conducted to evaluate CFSMs regulation of *Salmonella* invasion in the chicken model.

*Salmonella* infection depends on factors such as bacterial strain, duration of exposure to the pathogen, and MOI. Initially, same incubation condition as apoptosis assay (MOI of 10, 1 h infection) was assessed on LMH cell line infected with chicken isolated *Salmonella Typhimurium* DT104 (SA2001-4368 & SA2000-0406). The results are shown in Figure 3.15. Only LMH cells treated with LH-2 CFSM showed a significant reduction and this was limited to the SA2000-0406 isolate. Hence, it may be concluded that *Salmonella* internalization depends on the bacterial strain. Lin et al. (2008) also found that levels of inhibition due to *L. acidophilus* LAP5 differed between strains *S. Choleraesuis* 2a and 26a, and this was probably due to differences in their ability to adhere to and invade cells (Lin, Tsai, Lin, Tsen, & Tsai, 2008).

Afterward, we assessed the regulation of LH-2 CFSM under different infection conditions (shown in Fig. 3.16), e.g., 2 h infection with MOI of 20 using *S. Typhimurium* SA 2000-0406, or 1 h infection with MOIs of 1 or 0.1 using *S. Typhimurium* SA2001-4368. Under these conditions, the ability of *Salmonella* to invade cells was reduced, albeit not significantly, in the presence of LH-2 CFSM. This led to the conclusion that a reduction of *Salmonella* internalization in host cells may be one of the mechanisms enabling CFSM to execute an antagonistic effect against *Salmonella* infection, but it is not the most important factor.
**Figure 3.15** Invasion assay on LMH cell using *Salmonella* Typhimurium DT104 (SA2001-4368 & SA2000-0406) isolated from chicken for 1 h infection with MOI of 10. Positive control: infected cells. La-5: cells pre-incubated with La-5 CFSM and co-incubated *Salmonella* in the presence of La-5 CFSM. LH-2: cells pre-incubated with LH-2 CFSM and co-incubated *Salmonella* in the presence of LH-2 CFSM. Results are normalized to positive control (positive control = 100%) and display as means ± SD of two independent experiments performed in triplicate. The bar identified with * is significantly different ($P < 0.05$) compared to positive control. NS means there is no significant difference compared to positive control ($P \geq 0.05$).

**Figure 3.16** Invasion assay using LMH cells exposed to different infection conditions. Positive control: infected cells. LH-2: cells pre-incubated with LH-2 CFSM and co-incubated *Salmonella* with the presence of LH-2 CFSM. Results are normalized to positive control (positive control = 100%) and display as means ± SD of two independent experiments performed in triplicate. NS means there is no significant difference compared to positive control ($P \geq 0.05$).
The ability to prevent pathogen invasion depends on the specific probiotics and tested pathogens, indicating a high variability. Lin et al. (2008) found both a culture of *L. acidophilus* LAP5 strain and its spent culture supernatant had a strong antagonistic effect on *S. Choleraesuis* infection of Caco-2 cells, of which live probiotic strain showed a stronger antagonistic effect than its spent culture supernatant (Lin et al., 2008). Ingrassia et al. (2005) determined that *E. coli* invasion of differentiated and undifferentiated intestinal epithelial cells was inhibited by pre-incubation (75% and 84%) or co-incubation (43% and 62%) with *L. casei*. A better inhibitory effect on *E. coli* invasion was observed when *L. casei* culture supernatant was added to the incubation medium together with its live cells, and this effect was thought to be associated with the probiotic adhesion improvement to the cells when the supernatant was present (Ingrassia, Leplingard, & Darfeuille-Michaud, 2005). Interestingly, mucus pre-treated with probiotic strains such as *Lactobacillus* (Gueimonde, Jalonen, He, Hiramatsu, & Salminen, 2006; Tuomola, Ouwehand, & Salminen, 1999) or *Bifidobacterium* (Collado, Gueimonde, Hernandez, Sanz, & Salminen, 2005) have been reported to increase the adhesion of pathogenic *E. coli*, *L. monocytogenes* and *S. Typhimurium*. The biological significance of these increases is unknown, but Collado et al. (2007) thought, instead of decrease the pathogens’ adhesive ability, the protective function of these probiotic strains against pathogens might compete for the specific adhesion site(s) and receptors or other factors (Collado, Meriluoto, & Salminen, 2007).

Conclusions

In summary, results demonstrate that bioactive components from *L. acidophilus* (La-5) and *L. helveticus* (LH-2) fermented milk enhanced the epithelial membrane integrity and exerted antagonized effect against *Salmonella* Typhimurium infection. First of all, non-toxic doses of CFSMs were tested.
Trypan blue exclusion and LDH results indicated that 1.5% La-5 or 1% LH-2 CFSM (v/v) was safe on HT-29 cell line; whereas SRB and LDH results showed that 1% La-5 or 1% LH-2 CFSM (v/v) was safe on LMH cell line. Afterward, when HT-29 cells were pre-incubated with non-toxic dose of CFSM and co-incubated during Salmonella infection with the presence of CFSM, Salmonella triggered TEER loss [8 h post-infection: La-5 ($P < 0.05$); LH-2 ($P < 0.01$)], LDH production [La-5 or LH-2 ($P < 0.01$)] and apoptosis [La-5 or LH-2 ($P < 0.01$)] were significantly attenuated. Since Salmonella often colonizes and contaminates chickens asymptomatically, chicken hepatoma LMH cell line together with Salmonella isolates from chicken was utilized as in vitro poultry model. Then the evaluation of CFSMs on Salmonella invasion was carried out under various infection conditions. Results showed CFSMs disruption of Salmonella invasion was highly strain dependent. Therefore, a reduction of Salmonella internalization in host cells may be served as one of the mechanisms, but not the most important factor, endorsing CFSM to perform an antagonistic effect against Salmonella infection.
CHAPTER 4

General Conclusions

The main purpose of this study is to determine the mechanism whereby bioactive components from L. acidophilus or L. helveticus fermented milk (La-5 or LH-2) protect epithelial cells from Salmonella Typhimurium infection. Based on previous in vitro and in vivo studies related to milk fermented by probiotic bacteria performed in our laboratory, it was found that the bioactive components derived from the probiotic bacteria fermentation stimulate the immune system and exert an antagonistic effect against various enteric infections (Chin, 2002; Elawadli, 2012; M. J. Medellín-Peña, 2007; Ng, 2000; Tellez Garay, 2009). It was further hypothesized that the bioactive components from milk fermented with two probiotic bacterial strains (L. acidophilus La-5 and L. helveticus LH-2) could inhibit Salmonella infectivity through modulation of cell membrane integrity and inhibition of Salmonella-induced apoptosis in epithelial cells. To test this hypothesis, the non-toxic dose of La-5 and LH-2 cell free spent medium (CFSM) was determined for application to epithelial cells using the Trypan Blue Exclusion assay, LDH and SRB colorimetric assays. The integrity of the epithelial monolayer was assessed by measuring trans-epithelial electrical resistance (TEER), as well as release of intracellular LDH by a colorimetric assay. Afterward, the inhibitory effect of CFSM on Salmonella induced apoptosis in epithelial cells using the TUNEL assay was determined. Finally, the evaluation of CFSMs on Salmonella invasion was carried out under various infection conditions.

The research was divided in three sections: (1) the preparation of CFSM, (2) an in vitro study using human colon carcinoma HT-29 cells, as a human model, and (3) an in vitro study using chicken hepatoma LMH cells, as a poultry model. L. acidophilus La-5 or L. helveticus LH-2 cell-free spent
medium (CFSM) were prepared from a 48-h fermentation either with whey protein-based medium for La-5 or skim milk for LH-2. The CFSMs were filtered, lyophilized and concentrated (10×). Human colon carcinoma HT-29 cells and chicken hepatoma LMH cells were used as the *in vitro* human intestinal model and the chicken model, respectively. In the human model, HT-29 cells were grown in Transwell permeable inserts for 40 days to allow the cells to become polarized, as polarized cells are more physiologically and biochemically related to native human intestinal cells. Pre-incubation and co-incubation with CFSM during *Salmonella* infection sensibilized and enhanced cell membrane integrity. In TEER measurement, the presence of La-5 (*P* < 0.05) or LH-2 (*P* < 0.01) significantly attenuated the *S.* Typhimurium-induced disruption of the epithelial barrier function at 8 h post-infection. In addition, release of intracellular LDH, as an index of cell membrane damage induced by *Salmonella*, was also attenuated (*P* < 0.01) in the presence of both La-5 or LH-2. To further elaborate the mechanism of the protective action on cells, apoptosis induced by *Salmonella* was investigated. When cells were exposed to CFSM from La-5 or LH-2, significant (*P* < 0.01) anti-apoptotic effects were observed. Interestingly, without *Salmonella* infection, cell membrane integrity was also improved when cells were exposed to La-5 or LH-2 CFSM.

The effects were less pronounced when chicken hepatoma LMH cells were used. Exposure of the LMH cells to CFSMs of La-5 or LH-2 resulted in less than a log₁₀ cycle reduction in the number of internalized *Salmonella* cells. The impact varied depending on the *Salmonella* strain tested. Further work using the chicken cell line needs to be done in order to elucidate whether CFSMs exert similar protective mechanisms against *Salmonella* and whether this offers a promising approach to reduce *Salmonella* carriage in poultry.

Overall, it can be concluded that the inhibitory function of CFSMs on *Salmonella* internalization may
not play a pivotal role in preventing salmonellosis, but the enhancement of cell membrane integrity and anti-apoptotic effect on *Salmonella*-infected cells suggest that bioactive molecules produced by probiotics hold promise as therapeutic and prophylactic agents to combat enteric infections.

Future Research

Encouraged by such *in vitro* results, future work could focus on the anti-microbial effects of CFSM from various LAB strains and how medium composition affects the activity of the bioactives. Moreover, the synergistic effects of diverse bioactive components from different LAB should be investigated to determine if their combinatorial effects offer better protection to the intestine. Furthermore, the stability of the bioactive components present in the CFSMs needs to be investigated. Improvement could be achieved by using protective agents and other formulations e.g., encapsulation material (Jankovic, Sybesma, Phothirath, Ananta, & Mercenier, 2010). Probiotic bacterial genes could be modified to improve proteolytic activity during fermentation in order to acquire a greater array of bioactive components. Along with the previous *in vivo* studies that revealed CFSMs had an antagonistic effect against various enteric pathogens, *in vivo* anti-apoptotic activity of La-5 or LH-2 against *S. Typhimurium* infection as well as their active doses in laboratory animals or in clinical trials need to be assessed. These findings will contribute to providing solid evidence to related government departments, such as Health Canada, for the approval of using these CFSMs as therapeutic supplements in functional foods.
Anderson, R. C., Cookson, A. L., McNabb, W. C., Park, Z., McCann, M. J., Kelly, W. J., & Roy, N. C. (2010). Lactobacillus plantarum MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. *Bmc Microbiology, 10*, 316.


Chalghoumi, R., Thewis, A., Beckers, Y., Marcq, C., Portetelle, D., & Schneider, Y. J. (2009). Adhesion and growth inhibitory effect of chicken egg yolk antibody (IgY) on salmonella...
enterica serovars enteritidis and typhimurium in vitro. *Foodborne Pathogens and Disease, 6*(5), 593-604.


