Antivirulence activities of Bioactive Peptides produced by Lactobacillus helveticus and Lactobacillus acidophilus against Salmonella enterica serovar Typhimurium

by
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ABSTRACT

ANTIVIRULENCE ACTIVITIES OF BIOACTIVE PEPTIDES PRODUCED BY \textit{Lactobacillus helveticus} AND \textit{Lactobacillus acidophilus} AGAINST \textit{Salmonella enterica} SEROVAR TYPHIMURIUM.

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University of Guelph, 2014

During fermentation, \textit{Lactobacillus acidophilus} and \textit{Lactobacillus helveticus} release many small peptides as secondary metabolites. Previous studies have showed the protective effects of these biomolecules against enteric pathogens \textit{in vitro} and \textit{in vivo}. The purpose of the present study is to observe the effects of the bioactive peptides from \textit{Lactobacillus helveticus} and \textit{Lactobacillus acidophilus} on the virulence factors of \textit{Salmonella} Typhimurium and to observe the effects of fermentation conditions on the antivirulence activities of the bioactive peptides from \textit{Lactobacillus acidophilus}.

Cell-free spent media (CFSMs) were prepared from \textit{Lactobacillus helveticus} (LH-2) fermented skim milk and \textit{Lactobacillus acidophilus} (La-5) fermented whey protein based media. Lactate dehydrogenase (LDH) production, which is used as an indicator to cytotoxicity, was assayed in
Salmonella infected RAW 264.7 cells co-incubated with CFSMs. The effects of the CFSMs on the gene expression of Salmonella were analyzed using a two-step RT-qPCR assay. Additionally, the antivirulence effects of the La-5 CFSMs produced under different fermentation conditions were compared and correlated with specific peptides in the La-5 CFSMs.

The LH-2 and La-5 CFSMs significantly decreased (p< 0.05) the cytotoxicity caused by Salmonella infection in RAW 264.7 cells. In terms of gene expression, both LH-2 and La-5 CFSMs showed two-fold down-regulation in sipB and more than six-fold down-regulation in hilA, ssrB, sopD and invA genes expression. The La-5 CFSM produced under strict anaerobic condition with medium rate of agitation and fresh culture inoculation showed highest down-regulation of all the genes tested. The down-regulation of the virulence genes when correlated with reverse phase- HPLC profiles, significant correlation (p<0.01 or p<0.05) was found for few specific peaks.

These results suggest that the bioactive peptides obtained from Lactobacillus helveticus and Lactobacillus acidophilus can protect against cell damage and apoptosis caused by Salmonella Typhimurium infection in vitro. Bioactive peptides obtained from both Lactobacillus species exhibited antivirulence effects against Salmonella Typhimurium. The production method can be optimized to increase the efficacy of antivirulence effect of the peptides.
ACKNOWLEDGEMENT

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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>ATP</td>
<td>Adenosine Tri-phosphate</td>
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<td>BOD</td>
<td>Biological oxygen demand</td>
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<td>CDC</td>
<td>Center for Disease Control</td>
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<td>CFMS</td>
<td>Cell free spent media</td>
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<tr>
<td>Cas</td>
<td>Caseins</td>
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<tr>
<td>EHEC</td>
<td>Enterohemorrhagic E. coli</td>
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<tr>
<td>FAO</td>
<td>Food and Agricultural Organization</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>FPLC</td>
<td>Fast Performance Liquid Chromatography</td>
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<td>GAP</td>
<td>Glyceraldehyde phosphate</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>HP-SEC</td>
<td>High performance-Size Exclusion Chromatography</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IOM</td>
<td>Institute of Medicine</td>
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<tr>
<td>La-5</td>
<td>Lactobacillus acidophilus La-5</td>
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<tr>
<td>LAB</td>
<td>Lactic Acid Bacteria</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
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<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<td>LH-2</td>
<td>Lactobacillus helveticus LH-2</td>
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<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
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<tr>
<td>MRS</td>
<td>DeMan, Rogosa and Sharpe</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide Adenine Dinucleotide (reduced)</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptors</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
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<tr>
<td>PAMP</td>
<td>Pathogen associated molecular patterns</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>RP-HPLC</td>
<td>Reversed-Phase HPLC</td>
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<td>RT-PCR</td>
<td>Reverse transcription-Polymerase Chain Reaction</td>
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<td>SCV</td>
<td><em>Salmonella</em>-containing vacuole</td>
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<td>SEC</td>
<td>Size exclusion chromatography</td>
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<td>SIF</td>
<td><em>Salmonella</em>-induced filaments</td>
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<td>SPI</td>
<td><em>Salmonella</em> pathogenicity island</td>
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<td>SRB</td>
<td>Sulforodhamine B</td>
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<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
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<tr>
<td>TEER</td>
<td>Trans-epithelial electrical resistance</td>
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<tr>
<td>TFA</td>
<td>Trifluoroacidic acid</td>
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<tr>
<td>TLR</td>
<td>Toll-like Receptors</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>TSA</td>
<td>Tripticase Soya Agar</td>
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<tr>
<td>TSB</td>
<td>Tripticase Soya broth</td>
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<tr>
<td>TTSS</td>
<td>Type Three Secretion System</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>α-La</td>
<td>α-lactalbumin</td>
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<td>β-Lg</td>
<td>β-lactoglobulin</td>
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CHAPTER 1

1 Introduction

Many potent pathogens, including *Salmonella* species have developed resistance against major antibiotics used today. *Salmonella enterica* serotypes are among the most important foodborne pathogens causing thousands of hospitalizations and deaths worldwide each year (Scallan, 2011; Hendriksen et al., 2009). So, the management of *Salmonella* infections results in a high economic burden. *Salmonella* infections are particularly important also because of their increasing antimicrobial resistance against a broad range of antibiotics. However, new effective antibiotic development is a very slow and expensive process and so, the discovery of new therapeutic alternatives is urgently needed. Among several other options, targeting bacterial virulence offers a promising approach to inhibit pathogenesis that can be applied clinically to combat the pathogens (Cegelski et al., 2008).

Anti-virulence describes the negative influence on virulence gene expression of pathogenic micro-organisms, which decreases their pathogenicity. *Salmonella enterica* are potent pathogens which cause intestinal as well as systemic infections. *Salmonella* possess two major pathogenicity islands containing virulence genes, which encode distinct type three secretion systems (TTSS). These TTSS produce and inject the effector proteins for cellular invasion and intracellular colonization into host cells. After establishing themselves intracellularly, *Salmonella* cause cell apoptosis and are released to infect other cells or are transferred through the lymphatic system. So, from the primary site of infection i.e. the epithelial cells or the immune cells, they migrate to internal tissues, including the liver and spleen to cause systemic infections.
If the virulence genes of *Salmonella* could be negatively influenced, cellular invasion and establishment in the host could be greatly attenuated, which would prevent disease. In the present study, the biomolecules produced by specific lactic acid bacteria (LAB) were studied for their anti-virulence effects against specific *Salmonella Typhimurium* TTSS genes.

Beside the search for alternative antimicrobial substances, nowadays, there is increasing popularity among people for foods with functional activities. People are looking for more natural foods that provide health benefits beyond energy and nutrients. Thus, it has become a key research priority and a challenge for both the industry and science sectors (Granato et al., 2010). Fermented foods, especially fermented dairy products, are popular foods among all age groups worldwide as well as being a source of many bioactive compounds. Dairy-derived bioactive peptides, which have many functional activities, are being researched to elucidate other potential benefits. In the current study, the protective effects of dairy-derived bioactive peptides against infection and their anti-virulence effects against *Salmonella Typhimurium* were investigated for use as food or feed additives.

Bioactive peptides are defined as specific protein fragments that have a positive impact on physiological functions of the body, which ultimately influence health (Kitts and Weiler, 2003). Milk proteins or milk-derived products such as cheese and yogurt have been used to study the isolation and activity of peptides (Urista et al., 2011). The exploitation of the benefits provided by bioactive components relies upon cost-effective production and isolation. So, the use of whey as a cheaper source of proteins and peptides has been increasing. Fermentation of milk and whey by proteolytic microorganisms such as LAB releases different types of peptides, specific to the species. LAB, which are used widely for the fermentation of dairy products produce peptides with different bioactivities, such as immunomodulatory, anti-inflammatory, hypocholesteremic,
antimicrobial, anti-hypertensive, antithrombotic, mineral-binding, and opioid activities (Fitzgerald & Murray, 2006; Pihlanto, 2013).

Specific *Lactobacillus* species, viz. *L. acidophilus* and *L. helveticus*, which are used in many fermented food as well as designated as probiotics, have highly developed proteolytic systems (Griffiths & Tellez, 2013; Savijoki et al., 2006). These two species were used in this research to ferment milk and whey to obtain bioactive peptides. The peptides obtained from *L. helveticus* decreased *Salmonella* proliferation and colonization in mice (Brovko et al., 2003; Vinderola et al., 2007a; Tellez et al., 2011). The peptides down-regulated virulence gene (*ssrB*) expression, when detected by bioluminescence assay, but did not inhibit the growth of *Salmonella* (Tellez et al., 2011). Tellez et al. (2010) also isolated the peptides from *L. helveticus* fermented milk and found that the bioactive peptides constitute different peptide fractions of casein and whey proteins. Most recently, Peng (2014) showed that the peptides from *L. acidophilus* fermented whey protein and *L. helveticus* fermented milk helped retain tight junction integrity and decreased the rate of apoptosis in a *S. Typhimurium*-infected epithelial cell line.

Similarly, Medellin-Pena et al. (2007) and Zeinhom et al. (2012) observed the effect of the peptides produced by *L. acidophilus* on the virulence genes of enterohemorrhagic *Escherichia coli* O157:H7 (EHEC O157). The clinical manifestations of EHEC O157 infection were significantly less severe when the mice were fed with yoghurt containing *L. acidophilus* produced bioactive peptides and EHEC attachment and colonization were also attenuated (Zeinhom et al., 2012). The peptide fractions from *L. acidophilus* fermented media down-regulated several virulence genes of EHEC O157 and interfered with the quorum sensing systems of the pathogen (Medellin-Pena et al., 2007; Zeinhom et al., 2012); thus decreasing their pathogenicity. Other studies have shown the antivirulence effects of *L. acidophilus* on
Campylobacter jejuni (Mundi et al., 2013) and Clostridium difficile (Yun et al., 2014) and of Bifidobacterium spp. on pathogens such as Salmonella and Campylobacter (Bayoumi & Griffiths, 2012; Mundi et al. 2013). However, these studies utilized semi-defined media, which are costly, to produce the peptides. Whey protein may be a cheaper alternative protein source for the production of bioactive peptides. The bioactive components in whey have potential benefits in terms of nutritional, functional, and bioactive food and pharmaceutical products (Ko & Kwak, 2009). Also if the peptides exhibiting the bioactivities could be characterised, their potential bioactivity and in vivo effects can be studied individually. Identification of bioactive peptides is important to study their mode of action, toxicity and pharmacokinetics in vivo, which opens the possibility for their use in clinical trials. The characterisation of these molecules will also allow their chemical synthesis and further study. Though the previous studies showed protective and antivirulence effects of the bioactive peptides against many enteric pathogens, study of their production from natural sources such as milk and whey is lacking. Also the antivirulence effects of the L. acidophilus and L. helveticus produced peptides against Salmonella Typhimurium have not been studied in detail. This research gap is addressed in the present study.

In the present study, the effects of peptides produced from milk and whey fermented with L. helveticus and L. acidophilus on several virulence genes of Salmonella Typhimurium were studied, along with their protective effects in vitro using a macrophage cell line. The effects of different incubation conditions on the efficacy of La-5 bioactive peptides were further studied to optimize the conditions for L. acidophilus antivirulent peptide production. The peptides in the fractions were analyzed using size exclusion and reverse phase-HPLC and correlated with their antivirulence effects.
2 Literature Review

2.1 Salmonella enterica

Salmonella are gram-negative, facultative intracellular anaerobes with several serotypes capable of causing disease in a wide range of hosts (Owens and Warren, 2014). Diseases due to Salmonella range from mild gastroenteritis to enteric fever, bacteremia and septicemia (Mastroeni and Maskell, 2006) as well as various chronic sequelae (Castillo et al., 2012). Infection with Salmonella may also result in a convalescent lifetime carrier state. As Salmonella can cause infections in a broad host range including animals and humans, they have a huge impact on human and animal health.

Because of their taxonomical similarity, previously allocated Salmonella species are also now grouped into a single species, Salmonella enterica, with different serotypes (Bell & Kyriakides, 2002). More than 2500 serotypes (Coburn et al., 2007; Grassl & Finlay, 2008) of Salmonella have been identified among which the most human pathogens worldwide are Salmonella enterica subspecies enterica serotypes Typhi, Typhimurium, and Choleraesuis (Chambers et al., 2008). The US Centers for Disease Control and Prevention (CDC) (2010) indicated that the most common serotypes in the U.S. are S. Typhimurium, S. Enteritidis, S. Newport, S. Heidelberg, and S. Javiana. Outbreaks associated with many other serotypes have also been reported by CDC (2013b), including Montevideo, Saintpaul etc.

2.1.1 Importance of Salmonella infections

Salmonella has a widespread distribution in the environment and is particularly important because of its prevalence and virulence as well as increasing antimicrobial resistance (Owens &
Salmonella enterica serotypes are among the most important foodborne pathogens causing thousands of hospitalizations and deaths worldwide each year (Scallan, 2011; Hendriksen et al., 2009). According to the estimates of Majowicz et al. (2010), 3.8 million cases of gastroenteritis due to Salmonella species occur globally each year, with 155,000 deaths. This shows the prevalence of Salmonella infections worldwide. In spite of the money invested on research and many international attempts to implement control strategies, the incidence of human salmonellosis in most countries remains high (Wegener et al., 2003).

Every year, Salmonella is estimated to cause illness in about 1.2 million people in the United States, with about 23,000 hospitalizations and 450 deaths (CDC, 2014a). In a study of cases between 2000-2010 in Canada, non-typhoidal Salmonella was found to be the fourth major cause of foodborne infections and the third major among the bacteria after Clostridium perfringens and Campylobacter spp. (Thomas et al., 2013). It was estimated that about 88,000 cases of salmonellosis are recorded each year in Canada. In the U.S., salmonellosis is considered as the most frequently occurring foodborne infection with annual direct medical costs estimated to be $365 million (CDC, 2011a). According to the Director of the CDC, Thomas R. Frieden, “Salmonella accounts for about half of the hospitalizations and deaths among the nine foodborne illnesses CDC tracks through FoodNet” (CDC, 2011b).

As Salmonella infection is transmitted through contaminated food and as various types of foods are associated with Salmonella outbreaks, control of Salmonella has always been a concern for health workers, government health sectors, food industry and its suppliers. Ninety-five percent of cases of Salmonella infection are foodborne and the rest are from direct exposure to animal carriers (Linam and Gerber, 2007). Salmonella have been reported to transfer due to direct contact, most commonly from poultry. Multistate outbreaks of human Salmonella Infantis and
*Salmonella* Newport infections were reported in May 2014, which were linked to live poultry in backyard flocks (CDC, 2014c). Similar outbreaks were also reported in 2013 and 2012 (CDC, 2014d). However, the major source of *Salmonella* infection is food. Because of the ubiquitous nature of *Salmonella*, contamination has been observed in all raw foods whether they are derived from animals, fish or shellfish, eggs, poultry or game birds, fruits, vegetables, cereals, or dairy products. Recent outbreaks have been related to a variety of food products. In 2013/2014, a large outbreak of *Salmonella* Heidelberg infection was reported from 25 states in the US, affecting more than 500 people and was attributed to consumption of “Foster Farms” brand chicken (CDC, 2014a). Several other multistate outbreaks of salmonellosis were reported in 2013, mostly related to food products like chicken and ground beef (CDC, 2014d). *Salmonella* outbreaks have also been linked to fresh produce like mangoes, cantaloupes and sprouts, to peanut butter and pistachios (CDC, 2014d) and to sesame paste (CDC, 2013a). Also dried spices and spice products have been found to be contaminated with several serotypes of *Salmonella* during a survey involving 20,000 samples of spices imported into the US between 2006 and 2009 (Van Doren et al., 2013). The bacterium was most common in coriander, basil, oregano, sesame seeds, pepper, cumin and curry powder. This research was done in the context of three large salmonellosis outbreaks in the United States due to *Salmonella*-contaminated spices/seasonings from 2007-2010 (Van Doren et al., 2013).

*Salmonella* are especially important also due to their ability to cause several debilitating diseases at a very low infectious dose. Enteric fever is a severe and debilitating illness caused by typhoidal *Salmonella* serotypes in humans. Mild gastroenteritis is caused by non-typhoidal *Salmonella*, which generally does not progress to systemic infection. However, depending on the strain and immune status of patients it can develop into severe forms. Up to 40% of
salmonellosis cases in the developing world are accompanied by septicaemia, which results in up to 30% mortality (Old and Threlfall, 1998). Mortality due to Salmonella infections is mainly a health problem in developing countries, and morbidity due to acute Salmonella infections also has important socio-economic impact in industrialized nations (Hansen-Wester & Hensel, 2001). Different serotypes can cause different forms of salmonellosis in specific hosts. For example, serovar Typhimurium causes gastroenteritis in humans and cattle, but causes systemic infection in mice (Grasl & Finlay, 2008). Chronic disorders associated with salmonellosis are arthritis, osteoarthritis, appendicitis, endocarditis, pericarditis, meningitis and peritonitis, among others (Bell & Kyriakides, 2002). Previously, it was believed that the infective dose of salmonellosis in humans was about 100,000 cells, but it has now been shown that in some instances Salmonella can cause disease with an infectious dose of < 10-100 cells (Bell & Kyriakides, 2002). This is particularly evident in immuno-compromised hosts, children and old people and the food sources associated are foods high in fat. D’Aoust et al. (1985) reported an infectious dose for S. Typhimurium of 1-10 in Cheddar cheese and Greenwood & Hooper (1983) reported < 50 S. Napoli cells in a chocolate bar could result in illness.

Antibiotic resistance in Salmonella, which has been observed since the beginning of the 1990s, has caused serious public health problems worldwide (WHO, 2013). Beside the first and second generation antibiotics, Salmonella have developed resistance to clinically important antimicrobial agents such as fluoroquinolones and third-generation cephalosporins (Hur et al., 2012). Cases caused by multiple antibiotic-resistant S. Typhimurium DT 104 have also increased in recent years (Bell & Kyriakides, 2002). Salmonella Typhimurium definitive phage type 104 (DT104) strains harbor a genomic island, called Salmonella genomic island 1 (SGI1), which contains an antibiotic resistance gene cluster conferring resistance to ampicillin,
chloramphenicol, florfenicol, streptomycin, sulfonamides, tetracyclines and may also be resistant to quinolones (Baucheron, 2004). Moreover, many outbreaks associated with multi-drug resistant *Salmonella* have been traced to ground beef and poultry, which may indicate a link between the use of antibiotics in animals and human diseases (WHO, 2014). The *Salmonella* obtained from the recent outbreak associated with “Foster Farm” chicken in the US were found resistant to combinations of different antibiotics such as ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, sulfisoxazole, and tetracycline (CDC, 2014b).

Though pathogens are developing resistance to antibiotics at a very rapid rate, new antibiotic development has become slower and expensive. It can cost up to £1 billion and up to 12 years to take a drug from an initial idea into the market and the problem is also that the drugs will often fail during the development process (Stephens, 2014). Another report by Di Masi et al. (2003) states that antibiotic development initiated today would likely require 10 or more years and an investment of $800 million to $1.7 billion before it appears on the market. Infections that were once easily curable with antibiotics are becoming difficult, even impossible, to treat, and an increasing number of people are suffering severe illness or even dying. According to the Institute of Medicine (IOM) and the US Food and Drug Administration (FDA), only two new classes of antibiotics have been developed in the past 30 years, and resistance to one class emerged even before FDA approved the drug (IOM, 1998; FDA, 2004). Due to these reasons, researchers are looking for alternatives to antibiotics. The present study focused on the potential of bioactive peptides as an alternative to antibiotics as many bioactive compounds obtained from probiotic lactic acid bacteria (LAB) have health beneficial properties, including antimicrobial and antivirulence activities.
Besides the therapeutic use of bioactive peptides, bioactives obtained from probiotics also could be used to control pathogens in animals and animal products. *S.* Typhimurium and *S.* Enteritidis colonize the gut of poultry without causing any symptoms. So, the infected chickens could not be identified when used as a source of meat or if they are producing contaminated eggs (Guard-Petter, 2001). Poultry is the primary source of *Salmonella* infections, so it is important to implement control measures to ensure the production of *Salmonella* free eggs and poultry products. Different interventions and control measures have been introduced or are under study, such as vaccination, use of probiotics etc. Bioactive peptides are considered as promising agents to control pathogens such as *Salmonella* in poultry and other meat-producing animals. So, this research also targets the possible use of bioactives obtained from beneficial LAB (lactic acid bacteria) to control pathogens or attenuate their virulence in poultry products.

2.1.2 Pathogenesis of *Salmonella*

New studies have highlighted the roles of many important genes and *Salmonella* pathogenicity islands (SPIs) in establishing infection in the host. *Salmonella* Typhimurium is capable of causing gastroenteritis and systemic phases of infection in different hosts. It causes localized gastroenteritis in humans and cattle with symptoms such as diarrhea, nausea, vomiting, intestinal cramping and fever within 12 to 72 hours of infection.

*Salmonella* enter the host most commonly through contaminated food and water. The bacteria are able to pass through the acidic environment of the stomach and then colonize the intestine from where they translocate across the intestinal epithelium. Three mechanisms have been identified for the translocation of *Salmonella* through the intestinal epithelium; (1) direct invasion of the intestinal cells, (2) invasion through the special epithelial cells, called the M cells,
associated with Peyer's patches and lymphoid follicles that actively take up particulate matter from the intestinal contents (Jones et al., 1994) and (3) through dendritic cells that extend the protrusions into the intestinal lumen to capture antigens and then present the processed antigens to T cells (Vazquez-Torres et al., 1999; Niess et al., 2005). The entry route of *Salmonella* depends on several factors including the strain and the immune response depends on the entry route. Depending on the portal of entry, *Salmonella* may or may not colonize the intestinal mucosa and Peyer’s patches (Vazquez-Torres et al., 1999). When studied in an invA negative mutant of *Salmonella* with inactivated SPI1 genes, it was found that these bacteria are directly transmigrated outside into the blood and spleen by the phagocytic cells (Vazquez-Torres et al., 1999).

Interaction of *Salmonella* with the epithelium and resident cells leads to the activation of transcription factors in host cells and subsequent production of the proinflammatory factors, cytokines and chemokines. This activates the infiltration of immune cells such as neutrophils, macrophages, dendritic cells, and T and B cells. The inflammatory host response can actually benefit intestinal pathogens such as *Salmonella*, which can survive intracellularly. This decreases the competitive effects of the normal gastrointestinal flora and helps to disseminate the pathogen both inter and intra-cellularly to establish the infection (Grassl and Finlay, 2008).

Bacteria in the intestinal lumen are recognized by their pathogen associated molecular patterns (PAMPs). PAMPs are mainly recognized by the toll-like receptors (TLRs) or nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) of the host cells, which induce the production of chemokines and cytokines. The major subunit of bacterial flagella, flagellin is a major ligand for TLR5 and NLR, Ipaf. The delivery of flagellin into the host cell cytoplasm is dependent upon the TTSS of S. Typhimurium (Miao et al., 2006) and *Salmonella* can control the
expression of flagellin according to their location (Cummings et al., 2006). The expression of flagellin is prominent in *Salmonella* residing is Peyer’s patches, whereas it is suppressed in *Salmonella* after translocation to systemic sites (Cummings et al., 2006). Recognition of cytoplasmic flagellin by Ipaf leads to caspase-1 activation, which, in turn, leads to apoptosis in macrophages (Fink and Cookson, 2006). Thus, the analysis of the pathogenesis of *Salmonella* infections revealed the presence of two prominent virulence characteristics. During interaction with the host, *Salmonella* first infect various cells of the gastrointestinal epithelium, which trigger infiltration of immune cells such as macrophages. *Salmonella* then could be phagocytosed or actively enter the macrophages and replicate intracellularly (Hansen-Wester & Hensel, 2001). *Salmonella* causes apoptosis of macrophages as a mechanism to overcome the host defense and results in enterocolitis. Through the macrophages or dendritic cells, *Salmonella* may disseminate into different systems of the body.

**Salmonella** Pathogenicity Island and *Salmonella* Virulence

Many virulence genes of *Salmonella* are clustered in large multigene chromosome regions termed *Salmonella* pathogenicity islands (SPIs) (Shea et al., 1996). SPI-1 and SPI-2 are two major pathogenicity islands, which encode structurally similar but functionally distinct type three secretion systems (TTSS). These are used by *Salmonella* to inject the virulence proteins, called effectors, into the cytosol of host cells (Hueck, 1998). The importance of SPI-1 and SPI-2 is illustrated by the fact that mutants lacking SPI-1 and SPI-2 were not able to induce gastroenteritis (Hapfelmeier et al., 2004). SPI-1 encoded genes are expressed early in the infection during the intestinal phase of the infection cycle, and its effector molecules actively induce bacterial entry into both phagocytic and nonphagocytic cells of the intestinal mucosa (Galan, 1996; Wallis & Galyov, 2000). The SPI-2 virulence genes were thought to be expressed
after *Salmonella* entered the host cells (Cirillo et al., 1998; Hensel et al., 1998). However, many recent publications have shown their activation before *Salmonella* invade the cells (Brown et al., 2005) and have highlighted their roles in triggering intestinal inflammation in mice and calves (Coombes et al., 2005; Hapfelmeier et al., 2005).

Products of SPI-1 are required for active invasion into various intestinal cells including epithelial and immune cells and also for the disruption of the tight junction of epithelial cells so that *Salmonella* can transit across the epithelium. *Salmonella* modifies tight junctions through the action of four TTSS1 effectors SipA, SopB, SopE, and SopE2, which decrease transepithelial resistance and increase permeability when studied in cell lines (Boyle et al., 2006). These effectors also cause cytoskeletal rearrangement and cause the entry of *Salmonella* in both epithelial cells and macrophages by SPI-1-dependent macropinocytosis (Boyle et al., 2006). Also the SPI-1 type three secreted proteins like SipA, SopB and SopD cause fluid accumulation in the intestine (Hapfelmeier, 2004). The presence of SPI-1 is found essential to trigger inflammation at early stages of infection (day 1 or day 2 post infection) whereas SPI-2 effectors are required in the later phase (day 5 post infection) (Hapfelmeier, 2004). Also this study showed that intracellular replication of *Salmonella* Typhimurium is required for the induction of SPI-2 mediated inflammation (Hapfelmeier, 2004).

SPI-2 expression is generally believed to be required for the phase of infection after the initial invasion. *Salmonella* internalized by either invasion or phagocytosis both induce the formation of a specialized membrane-bound compartment called *Salmonella*-containing vacuole (SCV). The effectors secreted by the TTSS of SPI-2 are translocated across the membrane of SCV. SPI-2 mutant strains show impaired intracellular replication and survival in macrophages. The effector proteins released in the macrophages modify the cellular environment so that *Salmonella*
can multiply and cause programmed cell death and also the systemic spread of *S. Typhimurium* in murine infections (Cirillo et al., 1998; Hensel et al., 1998; Vazquez-Toress et al., 2000). These modifications include massive reorganization of vesicular transport and endosomal systems, suppression of bacterial molecules like flagellin, synthesis of enzymes to resist the oxidative and nitrogenous radicals etc. (Erickson et al., 2003; Rajashekar & Hensel, 2011).

The SPI-2 translocates more than 20 effector proteins into the host cells (Rajashekar & Hensel, 2011). The specific roles of all of these effectors are still not known, however, when studied in mutated strains, the genes *sifA*, *sseF*, *sopD2*, *sseJ* and *pipB2* are the most important (as reviewed in Rajashekar & Hensel, 2011). The product of *sifA* plays a prominent role in the proliferation of bacteria in SCV. It specifically induces the formation of *Salmonella*-induced filaments (SIFs), which are defective in *sifA* strains. The intracellular survival and proliferation of *Salmonella* are dependent upon the formation of extensive tubular structures (SIFs), which are formed in various cell types including epithelial cells and macrophages (Rajashekhar & Hensel, 2011). SIFs are extensions from SCV, which extend throughout the cell and their formation coincides with intracellular replication. Formation of SIF is not efficient in strains that lack other effectors such as PipB2, SopD2, SseF and SseG, though their specific roles are not clear (Guy et al., 2000; Kuhle & Hensel, 2002). Hence, it can be concluded that various effector proteins from SPI2 function cooperatively to exert their effects on the host cell. The roles of SPI-1 and SPI-2 effector proteins in establishing the *Salmonella* infection *in vivo* is represented in Figure 1.1.

There have been some studies on *Salmonella* gene expression that compare *Salmonella* transcriptomes under variable conditions *in vivo*. In macrophages, 42% of the genome showed an altered expression pattern during infection, whereas about 31% of *Salmonella* genes changed expression significantly in epithelial cells compared to cells grown in culture media (Eriksson et
The process of infection requires the expression of a complex group of metabolic and stress-related genes. Microarray analysis of S. Typhimurium gene expression in murine macrophage-like cells revealed that the majority of the SPI-2 genes like ssrA, ssrB ssaG, and allied sifA genes were upregulated (Erickson et al., 2003).

Figure 1.1 Schematic representation of Salmonella infection (Adapted from McGhie et al., 2009).

Salmonella Virulence and Apoptosis of Macrophages

Macrophages are important in the pathogenesis of Salmonella infections. Unlike many other pathogens, Salmonella can survive inside macrophages by forming SCV (Brumell et al., 2002),
where they adapt to survive host defense mechanisms like reactive oxygen species, nitric oxide (NO), antimicrobial peptides etc and replicate. *Salmonella* expresses different virulence factors to deal with this stressful environment (Ernst et al., 1999).

Macrophages are specialized cells which engulf large particles such as bacteria, yeast and dying cells by a process called phagocytosis. Macrophages secrete signaling molecules called cytokines and chemokines for activation of immune reactions (Maassen et al., 2000). They respond to a wide range of inflammatory mediators and play a central role in subsequent immune responses (DeLeo, 2004).

When inside macrophages, *Salmonella* avoid the cell’s antimicrobial defences and proliferate successfully using a specialized protein secretion system encoded in the *Salmonella* pathogenicity island 2 (SPI2). *Salmonella* are also capable of causing apoptosis of macrophages; however, the mechanisms are found to be different under different conditions, namely, rapid TTSS1- dependent macrophage apoptosis, and delayed TTSS2- dependent macrophage apoptosis (Fink and Cookson, 2007). Finally, the bacteria disseminate to surrounding tissues and colonize the liver and spleen via the reticuloendothelial system.

Apoptosis is the process of programmed cell death that may occur in multicellular organisms. Biochemical events characteristic to apoptosis are blebbing inside the cells, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal fragmentation and, finally, death of the cell. Unlike necrosis, which is a form of traumatic cell death, apoptosis produces cell fragments called apoptotic bodies that are quickly removed by professional and nonprofessional phagocytes (Alberts et al., 2008). Hence, a characteristic feature of apoptosis is the lack of
accompanying inflammatory reactions, which could help *Salmonella* to avoid the subsequent immune responses (Platt et al., 1998).

The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly (extrinsic inducers) or intracellularly (intrinsic inducers). Extracellular signals may include toxins (Popov, 2012), hormones, growth factors, nitric oxide (Brune, 2003) or cytokines that must either cross the plasma membrane or transduce to produce a response. These signals may positively or negatively affect apoptosis. Intracellular apoptotic signals are initiated in response to glucocorticoids, heat and radiation, lack of nutrient, hypoxia and increased intracellular calcium concentration (Cotran 1994). Apoptosis is mediated by a special group of enzymes termed caspases (cysteine-aspartate proteases).

In contrast to apoptosis, necrosis is characterized by metabolic collapse, cell swelling and rupture of cell organelles; leading to breakdown of the plasma membrane. The intracellular contents are released into the surrounding tissues and so, the process of necrosis leads to inflammation. In contrast to what was thought before, the process of necrosis also involves specific genetic pathways (Chan et al., 2013). Necrosis can be detected by measurement of lactate dehydrogenase (LDH) enzyme, which is released after cell lysis (Chan et al., 2013). The process of necrosis has not only been observed in cells lysed due to external cell factors, such as toxins or trauma, but also the process of apoptosis could lead to autolytic necrosis when the scavenger cells are not operating (Silva, 2010). The phenomenon, which was termed secondary necrosis by Wyllie et al. (1980), has been observed both *in vivo* in multicellular organisms (Wyllie et al., 1980) and *in vitro* in cell culture (Don et al., 1977).
Various mechanisms have been suggested for cytotoxicity of *Salmonella* towards macrophages (Hueffer & Galan 2004). This cytotoxicity is independent of intracellular replication, since invasive but non-replicative *S. Typhimurium* strains also induced apoptosis in RAW264.7 macrophages (Monack et al., 1996). Macrophage apoptosis is dependent on the SPI-1 TTSS (Chen et al. 1996). Studies have suggested that a SPI-1 secreted effector protein called SipB activates caspase-1, which triggers apoptosis at a rapid rate (Monack, 2000; Leist & Jaattela, 2001; Jarvelainen et al., 2003). This mechanism of apoptosis is also known as pyroptosis, which is different from the apoptosis observed in epithelial cells, as in epithelial cells caspase-3 is responsible for causing rapid cell death (Fink & Cookson, 2005; 2007). Evidence of the importance of caspase-1 in salmonellosis comes from studies using knockout mice, since a 1000-fold lower dose of *Salmonella* was required to lethally infect wild-type mice compared with caspase-1-knockout mice (Monack, 2000). However, caspase-1 knockout macrophages also undergo cell death after longer periods of *Salmonella* infection (Jesenberger et al., 2000). So, according to the studies of Jesenberger et al. (2000) and van der Velden et al. (2000), more than one apoptotic pathway can be activated during *Salmonella* infection of macrophages. Libby et al. (2000) have indicated that the delayed form of cell death requires the TTSS2 effectors SpvB and SseL. This form of cell death is characterized by cell lysis and inflammation, which are among the characteristics of necrosis. As a number of different signaling pathways are engaged in apoptosis, it is obvious that more than one mechanism is used by *Salmonella* to manipulate the host’s immune system and cause macrophage death (Monack et al., 1996).

### 2.1.3 RAW 264.7 cells as Model of Infection

RAW 264.7 cells are an immortalized cell line derived from Abelson leukemia virus-induced tumor cells from BALB/c mice (https://www.atcc.org/products/all/TIB-71.aspx). These cells
behave as macrophages and they maintain many properties of macrophages, including NO production, phagocytosis, extreme sensitivity to TLR agonists and motility. RAW 264.7 cells are commonly used as models of infection for intracellular pathogens such as *Salmonella*. Though they are immune cells responsible for pathogen destruction, *Salmonella* have adapted to an intracellular niche in the macrophage-like RAW 264.7 cells. Macrophages appear to be the preferred site for *Salmonella* replication (Wijburg et al., 2000). These cells have been used to observe the intracellular life of *Salmonella*, host macrophage response to infection, changes in gene expression of host cell and *Salmonella* etc. RAW 264.7 cells are also used to observe immune responses, such as cytokine and chemokine production, by these cells in response to infection. RAW 264.7 cells were used in a previous study by Tellez et al. (2010) to observe the changes in cytokine production after *Salmonella* infection in the presence and absence of bioactive peptides. In the present study, the apoptosis caused by *S. Typhimuum* in RAW 264.7 cells are compared in the presence and absence of bioactive peptides.

**2.2 Bioactive Peptides**

Biologically active or bioactive peptides are specific protein fragments that participate and modulate physiological functions thereby enhancing the overall health of the individual (Kitts & Weiler, 2003). Bioactive peptides were first reported in 1950 when casein-derived phosphorylated peptides enhanced vitamin D-independent calcification in rachitic infants (Hayes et al., 2007). Fitzgerald and Murray (2006) defined bioactive peptides as “peptides with hormone- or drug-like activity that eventually regulate physiological function through binding interactions to specific receptors on target cells leading to induction of physiological responses”.
Bioactive peptides can be obtained from animal protein sources such as milk, egg, gelatin, and fish, or from plant protein sources such as wheat gluten and soy (Korhonen & Pihlanto, 2003). Milk proteins, such as casein, or milk derived products, such as cheese and yoghurt, act as precursors for the formation of some bioactive peptides (Kitts & Weiler, 2003; Urista et al., 2011). Currently, milk whey waste obtained during the production of cheese has also been identified as a protein source from which active peptides could be isolated (Urista et al., 2011). The size of active sequences may vary from two to 20 amino acid residues, and many peptides are known to possess more than one type of bioactivity (Meisel & FitzGerald, 2003). The activity of peptides is based on their inherent amino acid composition and sequence. In milk proteins, a great number of peptide sequences with different bioactivities have been identified in the last decade (Sharma et al., 2011). These bioactive peptides are inactive within the sequence of the parent protein molecule and will be released from precursor proteins either during digestion, or by fermentation of milk with proteolytic starter cultures such as proteolytic lactic acid bacteria (LAB). In addition, bioactive peptides can also be obtained from milk using enzymes derived from micro-organisms or plants (Korhonen, 2009).

### 2.2.1 Sources of Bioactive Peptides: Milk and Whey

Milk is the fluid secretion from mammary glands, which contains an emulsion of fat globules in a colloidal solution of protein. Milk from different species, especially milk from cattle, buffalo, sheep and goats, has been used in the human diet for more than 8,000 years (Fox, 2009) and it has been used industrially in the production of many different dairy products. The majority of milk processed commercially is bovine milk (Fox, 2003). Milk is composed of different types of proteins, lipids and lactose as major constituents and minerals, vitamins, enzymes and hormones etc. as minor constituents.
Whey is the liquid remaining after the milk has curdled and it is a by-product of cheese or casein production. Whey consists of soluble proteins, lactose, minerals, vitamins as well as variable amounts of lactic acid and non-protein nitrogen (Kosikowski, 1979). Depending on the type of the fermentation, the whey produced may be sweet or sour (acidic). The whey obtained from rennet-set cheeses is sweet, pH 5.8 to 6.6, but from acid-set fresh cheeses is acidic, usually pH 4 to 5 (Kosikowski & Mistry, 1997).

Previously, whey from cheese or casein production was considered to be a waste material that was disposed of into rivers and water systems (Fox, 2009). Such disposal is detrimental to aquatic plants and animals since whey contains 6.0 to 7.0% solids and has a biological oxygen demand (BOD) of over 32,000 mg/l (Kosikowski & Mistry, 1997). So, the proper utilization of whey has always been a concern within the industry as well as to governments and environment conservationists.

Whey is an important source of nutrients and a cheap option for various industrial uses. As more and more benefits of whey are known, several new applications of whey have been introduced along with its use as fertilizer and feed. About 50% of cheese whey is treated and transformed into various foods, pharmaceutical products, and other bio-products in the form of liquid whey, powdered whey, WPC (Whey Protein Concentrate) and WPI (Whey Protein Isolates). Whey is now used in different foods and beverages as well as a source of protein, bioactive compounds and functional food (Ko & Kwak, 2009).

**Milk and Whey proteins**

All of the major milk proteins (except serum albumin and immunoglobulins) are synthesized by epithelial cells in the mammary gland from amino acids extracted from the blood. Milk contains
two major types of proteins, casein and whey, as well as trace amounts of other proteins and non-protein nitrogenous components.

**Caseins (Cas)**

In all mammals, milk caseins are a family of phosphoproteins. They exist in milk as complex micelles of the proteins and the mineral calcium phosphate dispersed in aqueous phase. Casein (Cas) makes up about 75% or more of the total protein content of milk and they can be further divided into five groups – the α-s1, α-s2, β, γ, and κ caseins. Casins do not possess a defined secondary and tertiary structure and so are resistant to heat denaturation. After caseins are phosphorylated, calcium (Ca++) binds to the phosphate and form supramolecular structures of caseins called casein micelles (Swaisgood, 1982).

The five different types of casein molecules have distinct molecular composition. The α-s1-casein has two hydrophobic regions containing proline residues, separated by a polar region and the α-s2-casein has concentrated negative charges near the N-terminus and positive charges near the C-terminus. They are found in multi-phosphorylated forms. The β-casein has a highly charged N-terminal region and a hydrophobic C-terminal region. It self associates to form a large polymer at 20°C. The κ-casein has a hydrophobic portion called para-κ-casein, and a hydrophilic portion called κ-casein glycomacropeptide (GMP) (Swaisgood, 1993). κ-Cas acts to stabilize the micelle structure. γ-Cas are C-terminal fragments of β-Cas, which are released by plasmin digestion, mostly while the milk is in the mammary gland (Hurley, 2010).
**Whey Proteins**

About 20% of the total proteins in bovine milk are whey proteins. Whey protein can be obtained after the separation of casein by several methods such as isoelectric precipitation at pH 4.6, salting out with saturated NaCl, ultracentrifugation or rennet coagulation. Commercially the whey protein products are prepared by,

- ultrafiltration of casein or rennet whey and spray drying
- ion-exchange chromatography and spray drying
- demineralization by electrodialysis and ion exchange and thermal evaporation of water etc. (Fox, 2009)

Whey proteins have secondary, tertiary and in most cases, quaternary structures. Whey proteins have many important biological roles, such as carrying calcium, zinc, copper, iron and phosphate ions in the body. They also possess biological activity as an important source of a number of different bioactive peptides (Korhonen et al., 1998).

Whey contains the major proteins β-lactoglobulin and α-lactalbumin as well as several other minor proteins. β-lactoglobulin (β-Lg) comprises more than 50% of the total whey protein and about 2% of total proteins in bovine milk. It is the major whey protein in milk from ruminants and pigs and is very resistant to proteolysis in its native state; this feature suggests that its primary function is not nutritional (Fox, 2009). α-lactalbumin (α-La) makes up about 20% of the protein of bovine whey (3.5% of total milk protein) and is the principal protein in human milk (Fox, 2009). Other proteins in whey are serum albumin and immunoglobulins (Fox, 2009).
Several other minor proteins and non-protein nitrogenous compounds are also found in the milk. One of the important minor proteins is lactoferrin, which is an iron binding protein, which has antibacterial and immunomodulating properties. It is found in high concentrations in human milk (Hurley, 2010).

2.2.2 Roles of Bioactive peptides obtained from milk and whey

Besides providing nutrients, milk and whey are also sources of important peptides, which have specific functions in the body. Peptides can be released from milk and whey by enzymatic activities in vivo, by the enzymes extracted from plants, animals and microorganisms and by the hydrolytic activities of proteolytic microorganisms. Among these various methods, microbial fermentation and proteolysis are important to release bioactive peptides. Lactic acid bacteria (LAB), which are used widely for the fermentation of dairy products, are major producers of bioactive peptides. They are used as starter cultures for the manufacture of numerous dairy products. They produce a variety of peptides from milk proteins during fermentation depending on their proteolytic system. The bioactive peptides produced by these bacteria have different bioactivities, such as immunomodulatory, anti-inflammatory, hypocholesteremic, antimicrobial, anti-hypertensive, antithrombotic, mineral-binding, and opioid activities (Fitzgerald & Murray, 2006; Pihlanto, 2013). Thus, the peptides could elucidate their biological activities in different major organ systems of the body, including the digestive, cardiovascular, nervous, and immune systems. Some examples of milk bioactive peptides with their physiological activities and amino acid sequences are listed in Table 1.1.
### Table 1.1. Examples of bioactive peptides released from milk proteins and their physiological activities
(Korhonen & Pihlanto, 2006; Minervini et al., 2003; Tellez et al., 2010; Juillard et al., 1995)

<table>
<thead>
<tr>
<th>Micro-organisms and/or enzymes used</th>
<th>Precursor proteins</th>
<th>Peptide sequence</th>
<th>Bioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus helveticus</em>, β-Cas*, κ-Cas</td>
<td>VPP; IPP</td>
<td>ACE inhibitor, antihypertensive</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus GG</em> enzymes + β-Cas, αs1-Cas, pepsin &amp; trypsin</td>
<td>TFP; AVPPYPQ R; TTMPLW</td>
<td>ACE inhibitor and immunomodulator</td>
<td></td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> subsp. <em>cremoris</em> FT4, β-Cas, κ-Cas, Many fragments</td>
<td>ACE inhibitor</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. delbrueckii subsp. bulgaricus</em> SS1, κ-Cas</td>
<td>ARHPHPHLSFM</td>
<td>Antioxidative</td>
<td></td>
</tr>
<tr>
<td><em>L. delbrueckii subsp. bulgaricus</em> IFO13953, β-Cas</td>
<td>QELELNPTHQIYPVT QPLAPVHNPISV</td>
<td>Antimicrobial</td>
<td></td>
</tr>
<tr>
<td><em>L. helveticus</em> proteinase, β-Cas</td>
<td>f 148-154, f 145-160, f 143-154</td>
<td>Immunomodulator</td>
<td></td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>f 60-68</td>
<td>Opioid</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em> + <em>L. lactis</em> var. <em>diacetylactis</em>, β-Cas</td>
<td>SLVYP</td>
<td>ACE inhibitor</td>
<td></td>
</tr>
<tr>
<td><em>L. helveticus</em> cell-free extract, Skim milk</td>
<td>VPP; IPP</td>
<td>ACE inhibitor</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.2. Commercial dairy products and ingredients with health/functional claim based on bioactive peptides (Hebert et al, 2010; Korhonen and Pihlanto, 2006).

<table>
<thead>
<tr>
<th>Brand Name</th>
<th>Manufacturer</th>
<th>Bioactive peptides</th>
<th>Health or function claim</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calpis</td>
<td>Calpis Co., Tokyo</td>
<td>IPP, VPP</td>
<td>Antihypertensive</td>
</tr>
<tr>
<td>Evolus</td>
<td>Valio, Helsinki</td>
<td>IPP, VPP</td>
<td>Antihypertensive</td>
</tr>
<tr>
<td>Casein DP</td>
<td>Kanebo Ltd., Tokyo</td>
<td>FFVAPFPEVFGK</td>
<td>Antihypertensive</td>
</tr>
<tr>
<td>PRODIET F200</td>
<td>Ingredia, France</td>
<td>TLGTLGGLLA</td>
<td>Reduction of stress</td>
</tr>
<tr>
<td>Capolac</td>
<td>Arla Foods Ingredients, Sweden</td>
<td>Caseinophosphopeptide</td>
<td>Helps mineral absorption</td>
</tr>
<tr>
<td>BioPURE-GMP</td>
<td>Davisco, USA</td>
<td>k-casein f(106–169)</td>
<td>Anticariogenic, antimicrobial, antithrombotic</td>
</tr>
<tr>
<td>BioZate</td>
<td>Davisco, USA</td>
<td>β-Lactoglobulin fragments</td>
<td>Reduction of blood pressure</td>
</tr>
<tr>
<td>PeptoPro</td>
<td>DSM Food Specialties, Netherlands</td>
<td>Casein derived peptide</td>
<td>Improves athletic performance and muscle recovery</td>
</tr>
<tr>
<td>Vivinal Alpha</td>
<td>Borculo Ingredients (BDI), Netherlands</td>
<td>Whey derived peptide</td>
<td>Aids relaxation and sleep</td>
</tr>
<tr>
<td>Recaldent</td>
<td>Cadbury Adams, USA</td>
<td>Caseinophosphopeptide</td>
<td>Anticariogenic</td>
</tr>
</tbody>
</table>
Based on their physiological activities, some milk peptides have potential for application in functional foods and pharmaceutical products. Several products have already been commercialized by different companies world-wide. For example, “Calpis” is certified as a “food for specialized health use” (FOSHU) in Japan with a health claim on the label (Shimizu, 2002). Cas-derived peptides have been considered for use as dietary supplements, and pharmaceutical preparations (Gobbetti et al., 2002). Some of the peptide products with their health/functional claims are listed in Table 1.2.

One of the widely studied biological activities of milk peptides is their anti-hypertensive activity and it has resulted in a number of commercial products. The anti-hypertensive activity is mediated through the inhibition of angiotensin I-converting enzyme (ACE). In some studies, consumption of milk peptides has led to a decrease in blood cholesterol levels (Hata et al, 1996; Yamamoto et al. 1994; Nakamura et al. 1995). Other important activities associated with fermented milk peptides are their anti-oxidative and anti-inflammatory properties (Kudoh et al., 2001; Qian et al., 2011). Radical scavenging and activities specific to enzymes that control cell oxidative stress have also been shown for milk peptides, and these mechanisms play a central role in human health, in inflammation, hypertension, coronary heart disease, atherosclerosis, cancer and aging (Beermann & Hartung, 2013). Metal-binding and anticariogenic peptides have also been isolated from fermented milk (Tidona et al, 2009; Clare and Swaisgood, 2000). Milk peptides also improve humoral and cell-mediated immune functions (Laffineur et al., 1996; Matar et al., 2001; Ng and Griffiths, 2002; Tellez et al., 2010). The anti-tumor activity of milk peptides was observed by Tidona et al. (2009), Vinderola et al. (2007b) and LeBlanc et al. (2002). According to Teschemacher et al. (1997), numerous peptides present in dairy products have neural activity and they are known as opioid peptides. These opioid peptides are released
during the fermentation of milk (Hebert et al., 2010; Kayser and Meisel, 1996; Gobbetti et al, 2002). Last but not least, some bioactive peptides have antimicrobial and antivirulence activities and they inhibit pathogens or mitigate against pathogenesis both \textit{in vitro} and \textit{in vivo}. Anti-microbial peptides are generally produced by the hydrolysis of casein molecules during fermentation (Hayes et al, 2006; Gueitz et al, 2013). Anti-microbial peptides have also been isolated from different cheese varieties (Rizzello et al, 2005; Lignitto et al, 2012). Major bioactivities of peptides from the fermented milk proteins are listed in Figure 1.2.

![Figure 1.2: Milk derived bioactive peptides and their bioactivities](image)

**Figure 1.2. Milk derived bioactive peptides and their bioactivities** (Adapted from McCarthy, O’Callaghan & O’Brien, 2013)

### 2.3 Probiotics and Lactic Acid Bacteria (LAB)

Probiotics are “live microorganisms, which, when administered in adequate amounts, confer a health benefit to the host” as defined by FAO/WHO (2002). Among various probiotics, LAB are a group of important bacteria, which produce bioactive peptides during fermentation of milk and other media. As they have high proteolytic activities, they release bioactive peptides as
secondary metabolites following degradation of milk proteins such as casein and whey (Barrett et al., 2005). Numerous peptides and peptide fractions having bioactive properties have been isolated from fermented dairy products and the bioactivities generated are specific to the types of enzymes possessed by LAB. Thus, the health properties associated with the fermented products may be LAB strain specific.

2.3.1 Properties of LAB

LAB are a heterogeneous group of bacteria having as a common metabolic property the production of lactic acid as a major end-product. LAB are Gram positive, non-sporulating, catalase negative, acid-tolerant, facultative anaerobic bacteria. They lack the ability to synthesize cytochromes and porphyrins (components of respiratory chains) and therefore cannot generate ATP by creation of a proton gradient. The LAB can only obtain ATP by fermentation, usually of sugars. All LAB grow anaerobically, but unlike most anaerobes, they grow in the presence of O₂ as "aerotolerant anaerobes". Although they lack catalase, they possess superoxide dismutase to detoxify peroxyde radicals (Mozzi et al, 2010; Todar, 2008).

LAB have complex, sometimes very fastidious nutritional requirements for carbohydrates and amino acids (Patrick, 2012). So, their habitats are the areas where the required compounds are abundant, such as grains, green plants, dairy and meat products, fermenting vegetables, and the mucosal surfaces of animals (Rattanachaikunsopon & Phumkhachorn, 2010). LAB can grow at temperatures from 5-45°C and most are tolerant to acidic conditions. Most are free-living or live in beneficial or harmless associations with animals, although some are opportunistic pathogens. They are part of the normal flora of humans in the oral cavity, the intestinal tract and the vagina, where they play a beneficial role (Todar, 2008). LAB are commonly used for fermentation of
different food products including meat, milk and vegetables. The LAB consist of 13 genera that include *Carnobacterium, Enterococcus, Lactococcus, Lactobacillus, Lactosphaera, Leuconostoc, Oenococcus, Pediococcus, ParaLactobacillus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella*, although many other genera of bacteria also produce lactic acid as a primary or secondary end-product of fermentation (Patrick, 2012).

LAB are typical fermentative bacteria, which ferment sugars as the source of carbon and energy. Besides sugar fermentation, they have evolved to perform electrogenic decarboxylations and ATP-forming deaminations. The right balance between sugar fermentation and decarboxylation/deamination ensures buffered environments that facilitate LAB survival and colonization of the GI tract (Pessione, 2012).

Based on sugar fermentation patterns, two broad metabolic categories of LAB exist: homofermentative and heterofermentative. Homofermentative LAB (*Lactococcus, Streptococcus, Pediococcus, Enterococcus*, and some species of *Lactobacillus*) ferment sugars by the Embden-Meyerhof pathway to produce pyruvate, which is converted into lactic acid by lactate dehydrogenase (LDH). Intracellular redox balance is maintained through the oxidation of NADH, concomitant with pyruvate reduction to lactic acid. This process yields two moles of ATP per glucose consumed. Heterofermentative LAB utilize the phosphoketolase pathway (pentose phosphate pathway) to dissimilate sugars. One mole of glucose-6-phosphate is initially dehydrogenated to 6-phosphogluconate and subsequently decarboxylated to yield one mole of CO₂. The resulting pentose-5-phosphate is cleaved into one mole of glyceraldehyde phosphate (GAP) and one mole of acetyl phosphate. GAP is further metabolized to lactate as in homofermentation, with the acetyl phosphate reduced to ethanol via acetyl-CoA and acetaldehyde intermediates. Theoretically, end-products (CO₂, lactate and ethanol) are produced
in equimolar quantities from the catabolism of one mole of glucose. Obligate heterofermentative LAB include *Leuconostoc, Oenococcus, Weissella*, and certain lactobacilli (Mozzi et al., 2010).

### 2.3.2 Genus *Lactobacillus*

*Lactobacillus* spp. are ubiquitous in nature and are also associated with many food products. Many species of this genus are commercially used as starter cultures for production of fermented dairy products, such as cheeses, sour milk and yoghurt. Although some *Lactobacillus* spp. have been recognized as aerotolerant and may utilize oxygen through the enzyme flavoprotein oxidase, others have been identified as strictly anaerobic. The optimum pH for lactobacilli growth is between 5.5 and 5.8 and they have intricate nutritional needs for amino acids, peptides, vitamins, minerals, fatty acids and carbohydrates (Axelsson, 2004). Lactobacilli may be homo or hetero-fermentative. They have many beneficial roles in the body. They are normally found in the mouth, intestinal tract and vagina, where they play a major part in preventing mouth sores and vaginal infections caused by bacteria and yeast. *Lactobacillus* spp. have been used to restore intestinal flora in the digestive tract after antibiotic treatment. Some species such as *L. acidophilus* and *L. rhamnosus* have been approved as dietary supplements by the US FDA. Thus, they are commercially available as probiotics either singly or as a mixture with other probiotic bacteria. Beside other physiological activities, the proteolytic system of *Lactobacillus* has been widely discussed. Because of their developed proteolytic system, they are capable of producing different peptides from the substrate and these peptides have been isolated from fermented milk and other dairy products. Many biological activities are attributed to these peptides. Both *L. acidophilus* and *L. helveticus* have been studied for their proteolytic activities. *L. helveticus* contains a highly sophisticated proteolytic system and produces many bioactive peptides (Tellez et al., 2013; Elfahri et al., 2014)
2.3.3 Proteolytic System of LAB

LAB have a very limited capacity to synthesize amino acids from inorganic nitrogen sources. They are therefore dependent on preformed amino acids being present in the growth medium as a source of nitrogen. Since the quantities of free amino acids present in their environment are not sufficient to support the growth of bacteria to a high cell density, they require a proteolytic system capable of hydrolyzing peptides and proteins in order to obtain essential amino acids. So, most LAB have specific proteolytic activity (Todar, 2008).

LAB have complex proteolytic and peptidolytic systems. These include three components: **proteases** that hydrolyze proteins to oligopeptides; **membrane transport proteins** that catalyze intracellular uptake of oligopeptides and **intracellular peptidases** that cleave the peptides (Konings, 2002). With these sophisticated proteases, either cell membrane bound or extracellular, they can act on the protein source before uptake. The amino acids will be further degraded by strain dependent metabolic pathways to generate the actual volatile compounds responsible for the aroma profile of fermented products (Mozzi et al, 2010). The proteolytic system of LAB, such as *Lactococcus lactis*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, is already well characterised. In addition to cell-wall-bound proteinases, at least 16 peptidases have been identified and genetically characterised from LAB (Christensen et al. 1999). *Lactobacillus helveticus* has been especially reported as a species with very high proteolytic and peptidolytic activity in comparison to other LAB strains; presumably due to the structure of their proteolytic system consisting of proteases, peptidases and a transport system.
2.3.4 Physiological Roles and Application of LAB

Many LAB are found as commensal bacteria in different parts of the body where they have many important roles. The most commonly found LAB in the intestine are lactobacilli, and their presence varies between individuals, with numbers as high as $10^6$ CFU/g faeces (Menard & Heyman, 2006). Due to their resistance to low pH and bile acids, their survival rate in the intestine is high, and their presence has been associated with several advantageous biological functions.

LAB have several physiological roles in the gastrointestinal (GI) tract. Their metabolic products, such as organic acids, fatty acids, hydrogen peroxide, diacetyl, bacteriocins and peptides have antimicrobial effects, causing death or growth inhibition of other bacteria including pathogens (Menard & Heyman, 2006; Klaenhammer, 1988; Bernet-Camard et al., 1997). LAB under normal conditions can contribute to digestion of not fully hydrolyzed proteins in the gut, and based on their types they will release different peptides (Pessione, 2012). Many LAB also have beneficial effects on intestinal integrity. They can physically hinder pathogen binding (Chan et al., 1985; Velraeds et al., 1996) or improve the integrity of the barrier and tight junctions of the epithelial cells (Resta-Lenert & Barrett, 2003). The combination of *Streptococcus thermophilus* and *Lactobacillus acidophilus* caused a significant increase in trans-epithelial electrical resistance (TEER) of intestinal cell (Caco-2) monolayers. TEER detects the permeability of the cell monolayer after their differentiation into polarised cells in permeable filter inserts. Because the tight junction is often rate limiting to paracellular solute movement, an alteration in transepithelial resistance is used as an index of tight junction permeability (Madara et al., 1998). *Lactobacillus casei* GG also has been shown to stabilize the permeability of intestinal cells to macromolecules in suckling rats (Isolauri et al., 1993).
LAB also play an important role in regulating the local immune response in the gut by enhancement of IgA production, cytokine release and down-regulation of inflammation. *L. rhamnosus* GG enhances the secretion of rotavirus-specific IgA (Kaila et al., 1992). A co-culture of *L. casei* and *L. bulgaricus* decreased TNFα production in the mucosa, thus decreasing the inflammation encountered in Crohn’s disease (Menard et al., 2004). Many *in vitro* studies with cell lines show modulation of cytokine release by LAB. For example, when cultured in the presence of *L. sakei*, Caco-2 cells increase their expression of pro-inflammatory chemokines and cytokines (Haller, 2000). As reported by Fuller (2000), orally administered lactobacilli can improve immune status by increasing circulating and local antibody levels, gamma interferon concentration, macrophage activity and the number of natural killer cells. In addition, various lactobacilli have been studied to protect against antibiotic-associated diarrhea (D’Souza et al., 2002). *L. rhamnosus* significantly reduced the risk of nosocomial diarrhea in hospitalized infants (Szajewska et al., 2001). Hence, LAB have a significant effect on the re-establishment of normal gut conditions and improvement of the conditions in case of any disorders.

Another role of LAB is the biotransformation of deleterious compounds in the gastro-intestinal tract to less deleterious compounds such as deconjugation of bile acids, reduction of enzymes associated with conversion of procarcinogens to carcinogens etc. (Gilliland, 1989; Goldin & Gorbach, 1984). Thus, they also have potential anti-cancer activities. LAB may also produce other useful compounds such as vitamins and short chain fatty acids, which are reported to lead to improved health (Pessione, 2012). These beneficial roles of LAB including their antihypertensive activity, anticancer and immunomodulating properties have been associated, in the last decade, with bioactive peptides produced by the LAB themselves or as a result of milk fermentation. During the fermentation of milk, important proteolytic enzymes of LAB release
different types and sizes of peptides from the milk proteins; both casein and whey proteins. These known bio-potentials of LAB have led to their application in products aimed at improving human health. They have been marketed as probiotic live cells or used in fermented milk products.

Though the potential of probiotics has been proved by several studies, there are still many questions to deal with such as efficacy, viability, optimal dose, and method of delivery (Verna & Lucak, 2010). Very few studies have actually documented survival of an administered probiotic as it transits the gut, by means of fecal recovery studies (Verna & Lucak, 2010). If the biogenic products of probiotics can be identified, supply of these products could alleviate the problems related to the delivery of live probiotic bacteria. Hence, the bioactive peptides, which have been discovered as important products of LAB fermentation activities, have high potential for commercialization in place of live probiotic bacteria.

2.4 Characterisation of Peptides by High Performance Liquid Chromatography (HPLC)

Characterisation of peptides in bioactive cell free spent medium produced by Lactobacillus species is highly important. The peptides can be separated and analyzed along with the detection of their biological activity to complete their characterization as bioactive peptides. Different methods used for separation of peptides include salting out with ammonium persulphate and polyethylene glycol, electrophoresis on different supports, column chromatography, high performance liquid chromatography (HPLC), isoelectric focusing, gel filtration, membrane filtration, ultrafiltration and dialysis etc. (Gokavi, 2009). Chemical measurements and analytical techniques are the critical components of the molecular understanding of the biological process.
HPLC is a technique used to separate, identify and quantify the components in a mixture. It is a developed form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster. It also uses a much smaller particle size for the column packing material, which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture. Various modes of HPLC have become central techniques for the characterization of peptides and proteins (http://www.chemguide.co.uk/analysis/chromatography/hplc.html).

HPLC has become a popular analytical tool because of its reproducibility, ease of selectivity and manipulation, and generally high recoveries. The most significant feature is the excellent resolution that can be achieved under a wide range of conditions for very closely related molecules, as well as structurally distinct molecules. Different types or modes of chromatography are based on recognition forces that can be easily manipulated through changes in the elution conditions that are specific for the particular mode of chromatography. Peptides and proteins interact with the chromatographic surface in an orientation specific manner, in which their retention time is determined by the molecular composition of specific contact regions. Based on the specific properties of the molecules to be separated, various chromatographic techniques can be used, including size exclusion, affinity, reverse phase, ion exchange or hydrophobic interaction (Aguilar, 2004).

2.4.1 Reversed-phase high-performance liquid chromatography (RP-HPLC)

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) is the most commonly used HPLC and it has become a widely used, well-established tool for the analysis and
purification of proteins and peptides. Because of its high resolution, RP-HPLC is able to separate polypeptides of nearly identical sequences. Polypeptides differing by a single amino acid residue can often be separated by RP-HPLC, as shown in the case of separation of human and rabbit insulin, which only differ by a methylene group (Rivier & McClintock, 1983).

RP-HPLC involves the separation of molecules on the basis of hydrophobicity. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase (Aguilar, 2004). A schematic diagram showing the binding of a peptide or a protein to a reversed-phase surface is shown in Figure 1.3. Polypeptides adsorb to the hydrophobic surface after entering the column and remain adsorbed until the concentration of organic modifier reaches the critical concentration necessary to cause desorption. They then desorb and interact only slightly with the surface as they elute down the column (Geng & Regnier, 1984). Separation of molecules in RP-HPLC is also dependent on their molecular conformation (Kunitani & Johnson, 1986).

![Figure 1.3. Schematic representation of the binding of (A) a peptide and (B) a protein, to an RP-HPLC silica-based adsorbent. (Adapted from Aguilar, 2004).](image-url)
Gradient elution is usually preferred for RP-HPLC polypeptide separations, even if the gradient is very shallow, i.e., a small change in organic mobile phase concentration per unit time. Gradient elution results in much sharper peaks than isocratic elution, in which the mobile phase composition remains constant throughout the procedure. A separation in which the mobile phase composition is changed during the separation process is described as gradient elution (Snyder & Dolan, 2006).

The RP-HPLC system for the analysis of peptides and proteins usually consists of an \( n \)-alkyl silica-based adsorbent; from which the solutes are eluted with gradients of increasing concentrations of organic solvent such as acetonitrile containing an ionic modifier such as trifluoroacetic acid (TFA) (Aguilar & Hearn, 1996; Mant & Hodges, 1996). The HPLC columns generally are stainless steel tubes filled with small diameter, spherical adsorbent particles, generally composed of silica whose surface has been reacted with silane reagents to make them hydrophobic. Spherical particles of synthetic polymers, such as polystyrene-divinylbenzene can also serve as HPLC adsorbents for polypeptides. The hydrocarbon group forming the hydrophobic phase is usually a linear aliphatic hydrocarbon of eighteen (C18), eight (C8) or four (C4) carbons. C18 columns are generally preferred for peptides and small proteins less than about 5,000 daltons (Wehr, 1984; Handout, Grace Vydac). Various factors can influence the peptide resolution and retention such as column length and diameter, \( n \)-alkyl chain length and ligand density, gradient steepness, flow-rate and temperature. The variations also can be compensated by appropriately modifying the retention coefficients. The retention coefficient is affected by the mobile phase, which consists of organic solvent paired with ionic modifier and TFA-water to TFA-acetonitrile gradient is popularly used for the separation of peptide mixtures. The concentration of ion-pairing reagent such as TFA and the steepness of the gradient of the
organic modifier determine the peptide retention and resolution of the column (Guo et al., 1987). RP-HPLC can be adapted for both analytical and preparative purpose.

2.4.2 High performance-Size Exclusion Chromatography (HP-SEC)

Size exclusion chromatography (SEC) separates molecules based on their size by filtration through a gel. The gel consists of support of the stationary phase containing pores of a specific size distribution. Separation occurs when molecules of different sizes are included or excluded from the pores within the matrix. Small molecules diffuse into the pores and their flow through the column is retarded according to their size, while large molecules do not enter the pores and are eluted in the column's void volume. Consequently, molecules separate based on their size as they pass through the column and are eluted in order of decreasing molecular weight (Mori & Barth, 1999). The stationary phases available for HP-SEC include surface modified silica and organic polymers. For the chromatography of proteins and peptides, silica-based supports with pore diameter in the range of 250 to 300 Å are most commonly used. Resolution also depends upon column efficiency, pore-size distribution, and pore volume. For HP-SEC separation the stationary phase bonded to silica should adequately shield silica to prevent polar and ionic interaction with the peptide samples and should be chemically stable in buffers used in peptide separation and characterization.

HP-SEC which is carried out in a unit with the flow rate controlled by pumps is also known as FPLC (Fast Performance Liquid Chromatography). The flow rate of the solvent is set through computer input and controlled by pumps. The chromatographic bed is composed of gel beads and the sample is introduced into the injector and then carried into the column by the flowing solvent. Once in the column, the sample mixture separates as a result of different components
adhering to or diffusing into the gel. In contrast to HPLC, the buffer pressure used is relatively low in this system.

The AKTA chromatography system is an FPLC system that allows automated protein analysis and purification. Protein separation takes place in a column. Buffers and other liquids are delivered via the system pump, and sample can be applied in different ways (e.g., using a syringe to fill a sample loop or by using a sample pump). Detectors (e.g., UV/Vis absorbance, conductivity, pH) are placed after the column to monitor the separation process. The purified proteins can be collected in a fraction collector.

2.5 Anti-virulence activities of the bioactive peptides

Bioactive peptides produced by probiotic bacteria exert protective effects against infections. Such effects have been observed in cell culture as well as in animal models. *Lactobacillus helveticus* R389-fermented milk and its cell-free fraction were fed to mice, which were subsequently infected with *S. Typhimurium*. The cell-free fraction of milk fermented by *L. helveticus* R389 induced better immune response against *Salmonella Typhimurium* than the fermented milk (Vinderola et al., 2007a). Brovko et al. (2003) showed that both fermented milk and a cell free fraction of the milk, when administered orally to mice prior to infection made them less susceptible to bacterial colonization. Tellez et al. (2011) showed that the severity of *Salmonella* infection in mice was greatly reduced in mice fed with cell-free fractions of milk fermented with *Lactobacillus helveticus*. In the same study, it was also shown that the systemic spread of *Salmonella* to the liver and spleen was low in the mice fed the cell-free fraction. Four novel peptide fragments derived from β-Cas and one from α-La were identified when the cell-free fractions were fractionated by size exclusion chromatography and analyzed by mass
spectrometry with sequences being identified using the NCBI database (Tellez et al., 2010). Three of the peptides shared a common amino-acid sequence within β-casein with slight variation (f 148-154, f 145-160, f 143-154) confirming that the fermented milk products were bioactive peptides derived from β-Cas (Tellez et al., 2010). The protective effect of the bioactive peptides was not due to the direct inhibition of the growth of the pathogen, but rather the peptides were found to down-regulate the ssrB gene from SPI-2 (Tellez et al., 2011).

Other studies have shown that molecules secreted by another probiotic strain of LAB, L. acidophilus La-5 could down-regulate expression of virulence genes in many pathogens. The molecules secreted by L. acidophilus La-5 suppressed expression of genes associated with the major pathogenicity island loci of enterocyte effacement (LEE) 1 and 2 of enterohemorrhagic E. coli O157 (Medellin-Pena et al., 2007). In their study, they detected gene expression by conventional RT-PCR and found that the expression of several virulence genes was significantly down-regulated though the down-regulation was not significant for shiga toxin producing genes, stxA2 and stxB2. Medellin-Pena et al. (2007) showed that the bioactive molecules secreted by La-5 interfered with quorum sensing in E. coli O157:H7. Medellin-Peña (2007) also isolated and identified the peptide sequences in the bioactive fractions of L. acidophilus fermented modified MRS media. The La-5 active fractions were able to protect mice from infection when challenged with enterohemorrhagic Escherichia coli (EHEC) (Medellin-Pena and Griffiths, 2009). In another study, Zeinhom et al. (2012) also reported that the clinical manifestations of EHEC infection were significantly less severe in mice fed with yogurt supplemented with L. acidophilus cell-free supernatant. EHEC attachment and colonization in intestines were attenuated by the molecules. When tested by reverse transcription real-time PCR, these fractions were able to down-regulate several virulence genes of EHEC, including stxB2, qseA, luxS, tir, ler, eaeA, and
hlyB (Medellin-Pena et al., 2007; Zeinhom et al., 2012). The cell extract of *L. acidophilus* GP1B was capable of interfering with quorum sensing in *Clostridium difficile* by decreasing AI-2 production and it also caused the down-regulation of virulence genes of *C. difficile* (Yun et al., 2014). In a similar study by Mundi et al. (2013), cell-free supernatants obtained from the probiotic bacteria *Bifidobacterium longum* and *L. acidophilus* were able to down-regulate the expression of virulence genes of *Campylobacter jejuni*. Das et al. (2013) reported that a cell free supernatant obtained from *L. plantarum* (KSBT 56, isolated from a traditional food product of India) decreased the pathogenicity of *Salmonella in vitro*. They observed down-regulation of *hilA*, which is a major virulence gene of SPI-1 in *Salmonella*. Similarly, down-regulation of virulence genes due to bioactive molecules from other probiotic bacteria has also been observed, which supports the inhibition of virulence as a potential mechanism of protection against pathogens. Bayoumi and Griffiths (2010) detected down-regulation in constructs in which a reporter gene (*lux*) was fused to promoters of two virulence genes from SPI-1 and SPI-2 of *S. Typhimurium* and observed highest down-regulatory effects with molecules obtained from *B. bifidum*. Bayoumi and Griffiths (2012) studied the effects of molecules obtained from *B. bifidum* on the gene expression of *Salmonella Typhimurium* and *E. coli* O157:H7 by qPCR and found down-regulation of several virulence genes in both pathogens. Bayoumi and Griffiths (2012) also observed the protective effects of the *B. bifidum* bioactive extract against the infections with both pathogens using different cell lines. More than 70% reduction in the degree of attachment to HeLa cells was observed for both pathogens and also reduction in the proliferation in macrophage cell line was observed when the *B. bifidum* active fractions were present.

In most of these studies, modified-MRS was used as the culture medium for *Lactobacillus* and cell-free spent medium was prepared by centrifugation and filter-sterilization. However, the cost
of utilization of synthetic media on an industrial scale is high and the cost can be decreased if natural media or by-products of other industries can be utilized. So, the use of milk and whey is a better alternative for cost effective production of bioactive peptides. Though, several bioactivities in milk and whey based peptides have been studied previously, the number of studies on their anti-virulence activities is very limited.

Based on these previous studies, it is proposed to observe the protective effects against *Salmonella* infection of two highly proteolytic probiotic species of *Lactobacillus*; *L. helveticus* and *L. acidophilus* produced biomolecules in a macrophage cell line and to observe their effects on gene expression in *S. Typhimurium* in the present research.

### 2.6 Analysis of Gene Expression by qPCR

Gene expression is the process by which information from a gene is used to synthesize a functional gene product. Even though nearly every cell in an organism's body contains the same set of genes, only a fraction of these genes are used in any given cell at any given time. Also in the single celled prokaryotes, specific sets of genes are expressed at a time depending on the environment and other triggers. So, the expression of genes of a particular cell at a time makes one cell different from another though they have the same DNA. The differentiation of cell types or cell functions at different times is because the cells synthesize and accumulate different sets of mRNA and protein molecules (Alberts et al., 2002).

Gene regulation gives the cell control over structure and function, and is the basis for cellular differentiation, morphogenesis and the versatility and adaptability of any organism. The genetic code stored in DNA in the form of a nucleotide sequence is interpreted during transcription and
the mRNA formed in turn directs synthesis of the proteins which give rise to the organism's phenotype. A cell can change (or regulate) the expression of each of its genes according to the needs of the moment. Gene expression can be regulated at different points; however, for most genes mRNA synthesis is the most important point of control (Alberts et al., 2002).

By measuring gene expression it is possible to quantify the level at which a particular gene is expressed in a cell and it gives an important indication of the metabolic status of the cell at a specific time and its reaction to a set of environmental conditions. In the present research, cells of *Salmonella* Typhimurium were exposed to certain concentrations of bioactive peptides produced by *Lactobacillus* and their effects on gene expression of *Salmonella* were measured. For the measurement of gene expression, the amount of mRNA or proteins is measured at a particular time and there are several methods available to achieve this, such as observation of the expression of a reporter gene, Northern blot, Western blot, fluorescent *in situ* hybridization, reverse transcription qPCR as well as more complex techniques like SAGE (Serial Analysis of Gene Expression), DNA microarray and RNA sequencing (Fryer et al., 2002).

Real time PCR or qPCR is highly popular for gene expression analysis among academic scientists because it is less difficult to perform, has comparatively lower cost, has high specificity, low carry-over of contamination and wide dynamic quantification range (Fryer et al., 2002). In this technique, reverse transcription is followed by quantitative real time PCR. Reverse transcription first generates a single-stranded template, cDNA, from the mRNA. The cDNA template is then amplified in the quantitative step, during which the fluorescence emitted by labeled hybridization probes or intercalating dyes changes as the DNA amplification process progresses. It is different from other PCR techniques because instead of the endpoint detection,
real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e., in real time) (Higuchi, 1993).

The signal of the fluorescent reporter increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. A fixed fluorescence threshold is set significantly above the baseline, known as threshold cycle ($C_T$). The parameter $C_T$ is defined as the cycle number at which the fluorescence emission exceeds the fixed threshold.

The threshold cycle or the $C_T$ value is the cycle at which a significant increase in $\Delta R_n$ is first detected. $\Delta R_n$ is the magnitude of the fluorescence signal generated during the PCR at each time point. The threshold cycle is associated with an exponential increase in concentration of PCR product during the log-linear phase. The slope of the log-linear phase reflects the amplification efficiency ($E$). $E$ can be calculated by the formula: $E = 10^{(-1/\text{slope})}$. The efficiency of the PCR should be 90 - 100% (-3.6 > slope > -3.1) (Pfaffl, 2004). The higher the initial amount of genomic DNA, the sooner accumulated product is detected in the PCR process, and the lower the $C_T$ value. The template quantification can be done by different approaches viz., absolute and relative quantification. Absolute quantification determines the input copy number of the transcript of interest, usually by relating the PCR signal to a standard curve. Relative quantification describes the change in expression of the target gene relative to some reference group, such as an untreated control or a sample at time zero.
So, for gene expression analysis, relative quantification is used. The change in C_T is normalized to the reference gene or housekeeping gene and the amount of template in the treated sample is compared to the untreated control to find out the change in the expression level. A housekeeping gene is typically a constitutive gene that is required for the maintenance of basic cellular function, is found in all cells and is not affected by environmental conditions. Quantifying the relative changes in gene expression using real-time PCR requires certain equations, assumptions, and the testing of these assumptions to properly analyze the data (Livak & Schmittgen, 2001). There are two methods for relative quantification.

In one of the analysis methods, the relative expression ratio (R) of a target gene is calculated based on the efficiency (E) and the deviation in C_T values of an unknown sample versus a control and expressed in comparison to a reference gene.

\[
\text{Ratio} = \left( \frac{E_{target}}{E_{ref}} \right)^{\Delta C_T \text{target} \text{ sample}} / \left( \frac{E_{ref}}{E_{target}} \right)^{\Delta C_T \text{ ref} \text{ sample}}
\]

The ratio of a target gene is expressed in a sample versus a control in comparison to a reference gene. E_{target} is the real time PCR efficiency of target gene transcript and E_{ref} is the real time PCR efficiency for reference gene transcript calculated according to the equation \( E = 10^{-\frac{1}{\text{slope}}} \). \( \Delta C_T \) is the deviation in C_T values between control and sample and is calculated for both target and reference genes (Pfaffl, 2001).

Another method is the Comparative threshold (C_T) method (ddC_T) method. The ddCT method is a convenient way to analyze the relative changes in gene expression from real time quantitative PCR experiments. Using the ddC_T method, the data are presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the untreated control (Livak & Schmittgen, 2001). The purpose of the reference gene is to normalize the PCR for the
amount of RNA added for reverse transcription. Standard house-keeping genes are used as the normalizers and they should be validated to determine that they are not affected by the experimental treatments. The untreated control is used as a calibrator and ddCt signifies the change in gene expression relative to an untreated control (Livak & Schmittgen, 2001). Before using the ddCt method for quantitation, a validation experiment should be performed to demonstrate that PCR efficiencies of target and reference are approximately equal. If both PCR perform equally efficiently across the range of initial template concentrations, it eliminates the need for efficiency correction and this method is more practical and widely used in the relative analysis of gene expression.

The advantages of using the comparative C_T method are that the need for a standard curve and the effect of the dilution error during preparation of standard curves are eliminated. It is expected that the normalizer will have a higher expression level than the target (thus, a smaller C_T value). The calculations for the quantification start with identifying the difference (dC_T) between the C_T values of the target and the normalizer:

\[ dC_T = C_T (target) - C_T (normalizer) \]

This value is calculated for each sample to be quantitated and for the calibrator or untreated controls. The untreated controls should be chosen as the reference (baseline) for each comparison to be made. The comparative ddC_T calculation involves determining the difference between each sample's dC_T and the dC_T of the baseline. Based on whether the transcription of the gene increases or decreases the values are negative or positive, respectively. The last step in quantitation is to transform these values to absolute values. The formula for this (assuming 100% efficiency or doubling of the product at each cycle) is:
Comparative expression level (fold change) = $2^{-\Delta\Delta Ct}$ (Pfaffl, 2004).

For the successful analyses of qPCR results to determine gene expression, utmost care should be taken during the performance of the experiments. The crucial factors which determine the reproducibility and reliability of qPCR results include the RNA extraction, reverse transcription, template purity and concentration, primer design and optimization of PCR reactions, the fluorescent dyes or probes used in the reaction, master-mix for the reaction, cycling conditions, PCR controls etc. (Edwards, 2004). The preparation of intact cellular total RNA or pure mRNA is the first requirement in gene quantification. For successful and reliable diagnostic use, real-time RT-PCR needs high quality, DNA-free, and undegraded RNA. Reverse transcription should be done to ensure good quality and specificity of cDNA. The fluorescent probes or dyes can be used as required and the master mixes should be optimized for each assay. Commercial master-mixes, which are now widely available, simplify the optimisation. The primers are designed using appropriate software or based on the literature and annealing temperatures should be analyzed during optimization. The detection of primer dimer formation is also essential. In addition, the optimum cycling conditions should be determined and should be well evaluated before use for the tests (Edwards, 2004).
1.3 Objectives

Previous studies and findings have provided a basis upon which to further investigate the antivirulence and protective activities of bioactive peptides against various enteric pathogens. Both *Lactobacillus acidophilus* and *L. helveticus* produced peptides that have protective effects against enteric infection *in vitro* and *in vivo*. So, this research was aimed to test the hypothesis that *L. acidophilus* and *L. helveticus* produce peptides in fermented milk and whey that down regulate virulence gene expression by *Salmonella* Typhimurium. The following specific objectives were set to test the hypothesis.

1. To observe the protective effects of peptide extracts from *L. helveticus* and *L. acidophilus* against *Salmonella* Typhimurium infection in RAW 264.7 cells.

2. To analyze the effects of peptide extracts on gene expression of *Salmonella* Typhimurium.

3. To observe the efficacy of antivirulence activity of *L. acidophilus* extracts at different production conditions.

4. To characterize the antivirulent peptides from *L. acidophilus* by size exclusion and reverse phase chromatography.
CHAPTER 2: METHODOLOGY

2.1. Bacterial strains and growth conditions

All bacterial strains used in this study (Table 2.1) were stored at −80 °C in glycerol (25%, v/v; Fisher Scientific, Nepean, Ontario, Canada). All the strains were obtained from the culture collection of the Canadian Research Institute of Food Safety (University of Guelph, Guelph, Canada). Lactobacillus helveticus strain LH-2 and Lactobacillus acidophilus strain La-5 were 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 48h.

Salmonella Typhimurium strain SA1997-0934 phage type DT104 was cultured on 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 the 4 h incubation period, the S. Typhimurium strain was in the mid-exponential phase of growth and the absorbance at OD_{600} was adjusted to 1.50. The bacterial count was approximately 1 × 10^9 CFU (colony forming units)/ml (= 9 log_{10}CFU/ml).

S. Typhimurium ssrB::lux (Bayoumi and Griffiths, 2010) was grown on LB agar and LB broth (BD Bioscience, Mississauga, ON, Canada) supplemented with 50 μg/ml of ampicillin (Sigma, Markham, ON, Canada) (Amp) for both S. Typhimurium constructs.
Table 2.1 The bacterial strains used in the study

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Source</th>
<th>Gram Stain</th>
<th>Growth Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. Typhimurium</em></td>
<td>SA1997-0934</td>
<td>CRIFS*</td>
<td>Negative</td>
<td>TSA/TSB, 37°C</td>
</tr>
<tr>
<td><em>L. helveticus</em></td>
<td>LH-2</td>
<td>CRIFS*</td>
<td>Positive</td>
<td><em>Lactobacillus</em> MRS Agar, 37°C</td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td>La-5</td>
<td>CRIFS*</td>
<td>Positive</td>
<td><em>Lactobacillus</em> MRS Agar, 37°C</td>
</tr>
<tr>
<td><em>S. Typhimurium</em></td>
<td>ssrB::lux</td>
<td>CRIFS*</td>
<td>Negative</td>
<td>LB broth/LB agar + 50µg/ml Amp.</td>
</tr>
</tbody>
</table>

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

2.2. Preparation of CFSMs

Preparation of LH-2 Cell Free Spent Medium (CFSM)

Skim milk powder (Smucker's, Markham, ON, Canada) was reconstituted 10% (w/w) with sterile water. Reconstituted skim milk was heat treated at 95°C for 30 min and then cooled until it reached room temperature. Isolated colonies of the *L. helveticus* strain LH-2 were used to inoculate four tubes of 5 ml heat-treated reconstituted milk, which were then incubated anaerobically with a BBL GasPak system (BD Bioscience, Mississauga, ON, Canada) at 37°C for 24 h. The culture (2.5 ml) was added to 250ml of heat-treated reconstituted skim milk and incubated aerobically at 37°C for 26 h. The fermented milk was centrifuged at 16,000 ×g for 10 min at 15 °C (Avanti J-20 XPI, Beckman Coulter, Canada). The supernatant was collected and sequentially filtered through a 0.7μm pore-size filter (EMD Millipore, Billerica, MA, USA), and then through a 0.45μm pore-size PVDF, sterile syringe filters (Fisher Scientific, Ireland) in order
to remove any bacterial cells present. The cell-free spent media (CFSM) (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 CFSM 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. Before use it was also neutralized using filter-sterilized 5N NaOH (Fisher Scientific) and filtered using 0.45 μm, PVDF, sterile syringe filters (Fisher Scientific, Ireland). Portions of the reconstituted samples were also kept at -20°C. The protein concentration was measured using a Nanodrop 1000 Spectrophotometer (Thermoscientific, Wilmington, DE) using the Protein A280 program in Nanodrop according to the manufacturer’s instructions. The protein concentration of LH-2 CFSM was 22.1 ± 0.1 mg/ml.

**Preparation of La-5 Cell Free Spent Medium (CFSM)**

Chemically defined medium (CDM) was composed of 28 g whey protein isolate (WPI) (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 in a tightly closed jar (static culture) 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 0.7μm pore-size filter (EMD Millipore, Billerica, MA, USA), and then through a 0.45μm pore-size low-protein-binding PVDF, sterile syringe filter (Fisher Scientific, Ireland) in order to remove any bacterial cells present. The cell-free spent media (CFSM) 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 CFSM 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 and neutralized and filtered as described before. Portions of the reconstituted samples were also kept at -20°C until analyzed. The protein concentration was measured by Nanodrop 1000 Spectrophotometer (Thermoscientific, Wilmington, DE) as described above. The protein concentration of La-5 CFSM was 199.5 ± 0.4 mg/ml.

2.3 Protective effects of CFSMs on cell culture against Salmonella infection

Cell Culture and Maintenance

The RAW 264.7 cell 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, GlutaMAX™ Supplement, HEPES, 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 RAW 264.7 cells (12-20 passages) were incubated in a humid atmosphere of 5% CO₂ at 37°C in an incubator (Forma™ Series II3110 Water-Jacketed CO₂ Incubators, Thermo Fisher Scientific, Mississauga, ON, Canada), and grown as a monolayer in 75 cm² flasks (Corning, NY, USA). The cells were passed to a new flask after obtaining 75-80% confluence in the flask using cell scraper (Fisherbrand™ Cell Scrapers, Fisher Scientific, Canada) and a new vial thawed after 20 passages.
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).

Non-toxic doses of CFSMs from La-5 and LH-2 were determined by SRB assay. After seeding 2 × 10^4 RAW264.7 cells/well in a 96-well plate and incubating for 24h at 37°C in a 5% CO₂ incubator for attachment, cells were stimulated with different concentrations of La-5 (0-6%) or LH-2 (0-5%) CFSM, followed by a further 24 h incubation at 37°C. The spent medium was removed and cells were fixed with 50 μl of 50% (w/v) cold 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.
**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, the amount of LDH released following *Salmonella* infection of the RAW 264.7 cell culture was quantified as an index of cell damage 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.

![Figure 2.1 Catalytic function of the Lactate Dehydrogenase (LDH) enzyme and colour reaction.](image)

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Preliminary studies showed that the optimal cell density of RAW 264.7 cells for LDH quantification was $2 \times 10^4$ cells/well in a 96-well plate. Twenty-four hours before *Salmonella* infection, 100μl of RAW 264.7 at a concentration of $2 \times 10^5$ cells/ml were seeded into a 96-well plate, resulting in a seeding density of approximately $2 \times 10^4$ cells/well. The cells were seeded in triplicate for each test, low controls (controls with only the cells with lowest possible production of LDH), high controls (controls in which Cell Lysis solution is added for maximum production of LDH) and experimental controls. Plates were incubated for 24h at 37°C in a 5% CO$_2$ incubator. After the incubation period, the spent medium was replaced with fresh DMEM without serum and antibiotic for low and high controls. Ten microlitres of Cell Lysis solution from the kit was added to high controls. Fresh *S. Typhimurium* DT104 (SA1997-0934; MOI of 10) was added to positive controls and the same bacterium together with the CFSMs from La-5 (1.5%) or LH-2 (1%) were added to the respective test wells. Before using the MOI- 10 and incubation period of 4 h, similar methods were followed to determine optimum MOI and incubation period for the LDH test in *Salmonella* infected RAW 264.7 cells co-incubated with La-5 and LH-2 CFSMs.

In some wells, 100 μl of a non-toxic concentration of La-5 (1.5%) or LH-2 (1%) CFSM (v/v) adjusted in antibiotic and serum-free medium was added in the absence of *Salmonella*. Three wells with cell culture medium only were included as background controls. The background value has to be subtracted from all other values. All the tests were performed in triplicate. The plates were incubated for 4 h at 37°C in a 5% CO$_2$ incubator, after which they 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 (from the kit) for 30 min at
room temperature. 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 using the following equation:

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

Low Control: untreated cells

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

Values of test groups were expressed as % cytotoxicity.

2.4 Evaluation of the effect of LH-2 and La-5 CFSM on the virulence gene expression of *Salmonella Typhimurium*

**RNA Extraction and Reverse Transcription**

One colony of *Salmonella Typhimurium* SA1997-0934 DT104 was inoculated in Luria Bertani broth (LB, BD Bioscience, Mississauga, ON, Canada) and incubated at 37°C for 18 hours with shaking (200 rpm; orbital shaker incubator, New Brunswick Scientific, USA). Fifty microlitres of the culture were transferred to 4.5 µl of fresh LB broth alone or LB broth supplemented with 10% v/v of reconstituted and neutralized LH-2 or La-5 CFSM. The final concentration of LH-2 CFSM in the culture was 2mg/ml and La-5 CFSM was 10mg/ml. They were incubated at 37°C for 4 hours with shaking (200 rpm; orbital shaker incubator, New Brunswick Scientific, USA). After 4 h, when the culture reached an optical density at 600 nm (OD$_{600}$) of 1.5 (mid-exponential phase), 2-ml samples were withdrawn and centrifuged at 16,000 x g (Spectrafuge- 16M, Labnet International, Edison, USA) for 4 min at room temperature (25°C). The pellet was mixed with 2
ml of RNAProtect Bacteria Reagent (Qiagen Inc., Mississauga, Ontario, Canada) and incubated for 5 min. Again, pellet was obtained by centrifuging at 16,000 x g (Spectrafuge- 16M, Labnet International, Edison, USA) for 3 min at room temperature and stored at -20°C for up to 2 weeks or immediately processed to extract RNA. The pellet was resuspended in 20 µl of proteinase K (Qiagen), 200 µl of Tris-EDTA buffer, BioUltra for molecular biology, pH 8.0 (Fluka, Sigma-Aldrich Co., St. Louis, USA) and 60 µl of 20 mg/ml lysozyme for molecular biology (Sigma-Aldrich Co., St. Louis, USA) and incubated at 37°C for 1 h at 450 rpm (Thermomixer, Eppendorf, Hamburg, Germany). RNA was extracted using the RNeasy minikit (Qiagen) according to the manufacturer’s instructions. After RNA extraction, residual DNA was eliminated from each sample using RNase-free DNase (Turbo™ DNase, 2 U/µL, Ambion) and incubated at 37°C for 15 min and treated with 0.5 M molecular grade EDTA at 70°C for 10 min to remove the activities of DNase. The RNA was then purified using the RNeasy MinElute cleanup kit (Qiagen) according to the manufacturer’s instructions and solubilized in 30µl of RNase-free water. The RNA concentration was determined by measuring the absorbance using a NanoDrop 1000 spectrophotometer (Thermoscientific, Wilmington, DE). The RNA quality was verified by measuring the ratio of the absorbance at 260 nm/280 nm and by gel electrophoresis. The purified RNA was used immediately for reverse transcription (RT) PCR using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Burlington, Ontario, Canada). In brief, 1 mg of RNA was reverse transcribed with 0.8 µl of 25× deoxynucleoside triphosphate (100 mM), 1 µl of Multiscribe reverse transcriptase (50 U/ml), 2 µl of 10× random hexamer primers, 2 µl of 10× RT buffer in an adjusted total volume of 20 µl using molecular-grade water (Water, nuclease-free, Thermo Scientific). For each sample, a control without reverse transcriptase was included to confirm the absence of contaminating DNA. Synthesis of cDNA
was performed in a Mastercycler Gradient Thermocycler (Eppendorf, Mississauga, Ontario, Canada) under the following conditions: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and a holding step to 4°C. The cDNA was stored at -20°C.

**Quantitative real-time PCR**

Quantitative PCR was performed using the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Burlington, Ontario, Canada) and SYBR® Select Master Mix (Applied Biosystems, Burlington, Ontario, Canada) according to the manufacturer’s instructions. Components of the master mix in the optimized buffer included: AmpliTaq® Polymerase, UP (UltraPure) engineered with a proprietary hot-start mechanism, SYBR® GreenER™ dye to detect double-stranded DNA, heat-labile uracil-DNA glycosylase (UDG), a dNTP blend of dUTP/dTTP and passive internal reference based on proprietary ROX™ dye.

The genes studied and primers used are documented in Table 2.2. The PCR was performed in a total volume of 20 µl, which contained 10 µl of SYBR® Select Master Mix, 1.6 µl of forward primer (5 µM), 1.6 µl of reverse primer (5 µM), 5 µl of 1/10 diluted cDNA, and 1.8 µl of molecular-grade water with the final primer concentration of 400 nM for all the genes except *16s* and *rpoD*. For these genes, a final primer concentration of 200 nM was used and volumes were 10µl of SYBR® Select Master Mix, 0.8 µl of forward primer (5 µM), 0.8 µl of reverse primer (5 µM), 5 µl of 1/10 diluted cDNA, and 3.4 µl of molecular-grade water. Each PCR was performed in triplicate. PCR conditions were as follows: UDG Activation at 50°C for 2 min and AmpliTaq DNA Polymerase, UP activation at 95°C for 2 min. It was followed by 40 repeated cycles of denaturation, annealing and amplification, at 95°C for 15 s, 54°C for 30 s, and 72°C for 45 s.
Subsequently, a default dissociation curve was performed in the instrument and specific amplicon was verified for the presence of a single melting-temperature peak.

**Table 2.2. Oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Function</th>
<th>Sequences</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssrB</td>
<td>Transcriptional regulator the SPI-2</td>
<td>F: 5’-TGGTTTACACAGCTACCAA-3’&lt;br&gt;R: 5’-GGTCAATGTAACGCTTGTTT-3’</td>
<td>This study</td>
</tr>
<tr>
<td>hilA</td>
<td>Transcriptional regulator of SPI-1</td>
<td>F: 5’-TGTCGGAAGATAAAGAGCAT-3’&lt;br&gt;R: 5’-AAGGAAGTATCGCCAATGTA-3’</td>
<td>This study</td>
</tr>
<tr>
<td>sopD</td>
<td>Secreted effector protein</td>
<td>F: 5’-ATTAATGCCGTAACCTTG-3’&lt;br&gt;R: 5’-CTCTGAAAACGGTGAATAGC-3’</td>
<td>This study</td>
</tr>
<tr>
<td>sipB</td>
<td>proapoptotic protease caspase 1: causes apoptosis</td>
<td>F: 5’-AACTCTTTACCAGGGGATG-3’&lt;br&gt;R: 5’-GGTATTGCTGACTTCCATGG-3’</td>
<td>Asakura et al., 2012</td>
</tr>
<tr>
<td>invA</td>
<td>Required for invasion of cells</td>
<td>F: 5’-GAAATTATCGCCACGTTCGGGCAA-3’&lt;br&gt;R: 5’-TCATCGCACCGTCAAAGGAACC-3’</td>
<td>Rahn et al., 1992</td>
</tr>
<tr>
<td>Gmk</td>
<td>House-Keeping gene</td>
<td>F: 5’-TGGCAGGGGAGGCGTTT-3’&lt;br&gt;R: 5’-GCGCAAGTGCCGTAGTAAT-3’</td>
<td>Botteldoorn et al., 2005</td>
</tr>
<tr>
<td>16s</td>
<td>House-Keeping gene</td>
<td>F: 5’-CAAGTCATCATGGCCCTTAC-3’&lt;br&gt;R: 5’-CGGACTACGACGACTTAT-3’</td>
<td>Asakura et al., 2012</td>
</tr>
<tr>
<td>rpoD</td>
<td>House-Keeping gene</td>
<td>F: 5’-GTGAAATGGGCACTGTGAAGCTG-3’&lt;br&gt;R: 5’-TTCCAGCAGATAGGTAAATGCTTC-3’</td>
<td>Velayudhan et al., 2014</td>
</tr>
</tbody>
</table>
The cycle threshold (C\textsubscript{T}) values were determined with the ViiA software. Standard curves of the C\textsubscript{T} values were prepared for all the genes using triplicate of all the dilutions and biological triplicates. The reaction efficiency \([E = 10 ^ {(-1/Slope)}]\) calculated for each gene varied from 1.93 to 2.05. Three reference or housekeeping genes, \textit{gmk}, \textit{rpoD} and \textit{16s} were included in this study. The transcript levels were normalized to the gene expression of the most stable housekeeping gene for each sample. The relative changes in gene expression were calculated as described previously (Livak & Schmittgen, 2001; Pfaffl, 2001). The samples were analyzed in biological triplicates, and the experiments were performed three times. A relative expression value of more than twofold was considered significant up- or down-regulation (Phongsisay et al., 2007).

2.5 Production of La-5 CFSMs at different fermentation conditions and comparision of their effects on S. Typhimurium virulence genes

After observing the antivirulence effects of La-5 CFSM on the virulence gene of \textit{S. Typhimurium} SA1997-0934, five different batches of La-5 CFSMs were prepared as described in section 2.2 with slight modifications in the inoculation and incubation conditions. The various conditions for their preparation are listed in Table 2.3. To study the effects of the CFSMs on the gene expression of \textit{Salmonella} Typhimurium, the previously mentioned methodology for gene expression in section 2.4 was followed and all of the experiments were done three times with at least two biological replicates.
Table 2.3 Fermentation conditions for different batches of La-5 CFSMs

<table>
<thead>
<tr>
<th>Designation</th>
<th>Inoculum</th>
<th>Incubation</th>
<th>Agitation of culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td>Lyophilized culture*</td>
<td>37°C, 48 h, strictly anaerobic condition</td>
<td>No</td>
</tr>
<tr>
<td>Batch 2</td>
<td>Colonies from fresh culture (48h) MRS plate</td>
<td>37°C, 48 h, in tightly closed jar, static culture</td>
<td>No</td>
</tr>
<tr>
<td>Batch 3</td>
<td>100 ml fermented starter culture in CDM (48h)</td>
<td>37°C, 48 h, strictly @ 150 rpm anaerobic condition</td>
<td></td>
</tr>
<tr>
<td>Batch 4</td>
<td>Pellets from 100ml fermented starter culture in CDM (48h)</td>
<td>37°C, 48 h, strictly @ 150 rpm anaerobic condition</td>
<td></td>
</tr>
<tr>
<td>Batch 5</td>
<td>Colonies from fresh culture (48h) MRS plate</td>
<td>37°C, 48 h, strictly @ 70 rpm anaerobic condition</td>
<td></td>
</tr>
</tbody>
</table>

* The lyophilized culture was obtained from the company “Chr. Hansen”

**Study of effect of La-5 CFSMs on the virulence gene of Salmonella by reporter gene fusion technique**

The antivirulence effects of different batches of La-5 CFSMs were also studied by reporter gene fusion technique. *S. Typhimurium ssrB::lux* construct was used for the assay that contains the plasmid construct of *ssrB*, fused with luxCDABE genes from *Xenorhabdus luminescens* and cloned with an ampicillin resistance gene. The *ssrB::lux* construct strain was subcultured from a vial in LB agar with 50 µg/ml ampicillin (Amp). The luminescent colonies were confirmed with a cooled, slow-scan CCD camera (NightOWL Molecular Imager, EG&G Berthold Technologies, Wildbad, Germany). Single colony was transferred into LB broth supplemented with Amp at a concentration of 50 µg/ml and was grown overnight at 37 °C. The overnight culture was diluted
1:100 with fresh medium with and without supplementation of 10% of all the five batches of La-5 CFSMs making the final concentration of CFSMs as 10mg/ml. Two hundred microliters of each sample was distributed in triplicate into wells of a sterile, opaque 96-well plate (Corning No. 3610, Fisher Scientific Canada, Ottawa, Ontario, Canada) and incubated at 37 °C for four hours with shaking (200 rpm; orbitary shaker incubator, New Brunswick Scientific, USA). Luminescence and cell density were measured after 4 h with a Victor™ Multilabel Counter (Wallac, PerkinElmer Life Sciences Canada, Woodbridge, Ontario, Canada). Luminescence counts were expressed as counts per minute and results presented as RLU/OD\textsubscript{600}.

2.6 Chemical Analysis of La-5 CFSMs by High Performance liquid chromatography (HPLC) Techniques.

**High Performance-Size exclusion chromatography (HP-SEC)**

All chemicals were purchased from Fisher Scientific (Mississauga, ON, Canada).

Five La-5 CFSM samples were used for the observation of size exclusion profiles of peptides. The peptides were fractionated using a Superdex 75 10/300 GL column (GE Healthcare Life Sciences) in an AKTApurifier\textsuperscript{TM} 10 system (GE Healthcare, Uppsala, Sweden). The mobile phase used for the elution was 50mM sodium phosphate (Na\textsubscript{2}HPO\textsubscript{4}) buffer (pH 7) containing 0.15 M NaCl prepared in milliQ water. The buffer was filtered through a 0.45 μm filter (47 mm diameter, Millipore Corp., Fisher Scientific, Mississauga, ON, Canada) and degassed overnight before use. After running filtered milliQ water to wash the system, the column was connected and equilibrated by running milliQ water followed by buffer. For the equilibration of the system, the flow rate was gradually increased from 0.5 ml/min to 1 ml/min. maintaining the system
pressure below the maximum recommended value of 1.8 MPa. All the samples of CFSM were prepared in the buffer at a concentration of 18.0 ± 0.5 mg/ml and filtered using a 0.45 µm syringe filter (Fisher Scientific, Mississauga, ON, Canada). Two hundred microlitres of the samples were injected and eluted at a flow rate of 1ml/min. The duration of the run was 48 min. The absorbance was recorded at 214nm for the detection of peptides and analyzed using the specific protocol set in the AKTApurifier™ system.

**Reverse-phase HPLC**

All the chemicals were purchased from Fisher Scientific (Mississauga, ON, Canada).

Reverse phase HPLC was carried out for all five La-5 CFSM samples using an Ultimate 3000 HPLC system (Thermofisher, Mississauga, ON, Canada). The samples were diluted to 10.0 ± 0.2 mg/ml and filtered using a 0.45 µm Millex-GV filter (Fisher Scientific, Mississauga, ON, Canada). The samples were loaded using a 20 µL loop and detected with a UV detector set at 214 nm. The column used was a Nova-Pak C18 4 µm, 3.9 × 150 mm with a C18 guard column (Waters Ltd., Mississauga, ON, Canada). The sample was eluted with a nonlinear gradient of 0.1% trifluoroacetic acid in filtered (0.45 µm pore size) milliQ water (buffer A) and 0.1% trifluoroacetic acid in 90% acetonitrile and 10% milliQ water (buffer B) at a flow rate of 1 ml/min. Gradient conditions were as follows: buffer B concentration increased from 2% to 70% between 0 and 40 min and increased further to 100% B in 1 min, where it was kept constant for 6 min. Then buffer B concentration decreased to 2% in 1 min and was kept constant for 6 min. The total time of the run was 54 min. The system was controlled and run data were obtained from the Chromoleon7 software connected to the instrument. The integration of peak values was
performed by the software and the values were compared with gene expression results to
determine the location of peaks associated with the antivirulent peptides.

2.6 Statistical Analysis

All experiments were performed at least three times independently (unless otherwise stated) and
results are expressed as mean ± SD. The results were analyzed using IBM SPSS version 21
software. Differences among means were tested for statistical significance using a one-way
ANOVA and subsequent Tukey’s post-hoc test. The correlation between the average peak
heights and gene expression was determined by Pearson’s correlation. Differences at P < 0.05
were considered to be statistically significant.
CHAPTER 3- RESULTS AND DISCUSSION

SRB Assay

The dried cell free spent media (CFSM) obtained from L. helveticus (LH-2) fermented milk and L. acidophilus (La-5) fermented whey protein-based medium were reconstituted in a volume that was 10× less than that of the CFSM and applied to the cell culture of RAW 264.7 cells to observe their protective effects against Salmonella infection. Previous studies have shown that some peptides are toxic to cell lines (Saar et al., 2005; Pushpanathan et al., 2013). Previous in vivo studies using mice fed with a partially purified LH-2 CFSM fraction (F5) also showed that 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 (Tellez et al., 2011). A large dose (>20 μg/ml) also caused macrophage death (Tellez, 2009). Thus, before using the CFSMs on the cell culture, non-toxic doses for RAW 264.7 cells were determined using the sulforhodamine B (SRB) assay. This assay measures the viability of cells, based on the measurement of cellular protein content. After an incubation period, cell monolayers are fixed with trichloroacetic acid and stained for 30 min, after which the excess dye was removed. The protein-bound dye was dissolved in 10 mM Tris base solution and the OD was measured at 560-580nm using a microplate reader. This assay has been widely used as an efficient and highly cost-effective method for screening drug toxicity on different cell lines (Vichai & Kirtikara, 2006). The results are also linear over a 20-fold range of cell numbers and the sensitivity is comparable to those of fluorometric methods (Vichai & Kirtikara, 2006). For the SRB assay, different concentrations (V/V %) of LH-2 and La-5 CFSMs were applied to the RAW cells and
their toxicity was determined relative to untreated cells. Cell viability at different concentrations of CFSM was determined as,

\[
\text{Percentage cell viability} = \frac{OD \text{ of test}}{OD \text{ of control}} \times 100 \%
\]

In the case of LH-2, concentrations of 1%, 2% and 3% showed no significant effect on cell viability \((p \geq 0.05)\) (Figure 3.1 A). RAW 264.7 cells showed highest viability at 1% LH-2 CFSM. In the case of La-5, no significant effect on cell viability was observed for the concentrations of 1 to 4\% \((p \geq 0.05)\) (Figure 3.1 B). Hence, because they did not exert a toxic effect on the macrophage cell line, 1\% LH-2 CFSM (total protein concentration after suspension in cell culture media \(\approx 200 \mu g/ml\)) or 1.5\% La-5 CFSM (total protein concentration after suspension in cell culture media \(\approx 3mg/ml\)) was chosen as the dose in subsequent experiments. The results were also verified by LDH cytotoxicity tests (Figure 3.2).
Figure 3.1 LH-2 and La-5 toxicity test on RAW 264.7 cells, estimated by SRB assay. A: LH-2 treatment; B: La-5 treatment. RAW 264.7 cells seeded in 96-well plate were exposed to the cell culture medium containing different concentrations of LH-2 CFSM or La-5 CFSM and incubated for 24 h. Cell viability was compared to untreated cells and expressed as Cell Viability (%). Results represent the means ± SD of two independent experiments, each performed in triplicate. Bars identified with ** are significantly different from untreated cells ($P < 0.05$). NS means there is no significant difference compared to untreated cells ($P \geq 0.05$).

**LDH Cytotoxicity Test**

Lactate dehydrogenase (LDH) is a cytosolic enzyme that is an indicator of cellular toxicity. Damage to the cell membrane can be quantified by measuring the concentration of LDH released in the cell culture medium. A LDH kit was used for the test, which contains the advanced WST reagent that reacts with NADH to generate a yellow colour. NADH is produced when LDH released from damaged cells oxidizes lactate. The intensity of the colour produced by the reaction correlates directly with the number of cells lysed.

First of all, using the LDH test, 1% LH-2 and 1.5% La-5 CFSMs were verified as non-toxic for cells. The LDH production of cells incubated with subsequent concentrations of CFSM for 24 h was not significantly different from the untreated cells (Figure 3.2).
Figure 3.2 O.D. corresponding to the amount of LDH produced by untreated and CFSM- treated RAW 264.7 cells. The cells were incubated with 1% LH-2 and 1.5% La-5 CFSMs in 96-well plate for 24 hrs. LDH produced by treated cells was compared with the untreated cells by one-way ANOVA and post-hoc Tukey’s test. All results represent the means ± SD of three independent experiments performed with three replications for each. NS represents the values are not significantly different from the control or the untreated cells.

Then optimum cell seeding concentration of RAW 264.7 cells was determined as $2 \times 10^4$ cells/well, which resulted in rapid and reproducible LDH production. Also a multiplicity of infection (MOI, number of bacteria: number of cells) of 10, 50 or 100 for *Salmonella Typhimurium* was compared together with an infection period of 2 or 4 hours to determine optimum conditions for use with the RAW 264.7 cells. Cytotoxicity % was calculated for each group as shown in Figure 3.3.

Figure 3.3 shows that an incubation period of 2h for all the MOIs did not cause the release of a large amount of LDH from the macrophages. Though cytotoxicity was measureable, it was insufficient to allow reliable estimates of the protective effects of CFSMs against *Salmonella* induced cytotoxicity to be obtained. However, when the macrophage cells were exposed to *Salmonella* for 4 h, a large amount of LDH was released at all the MOIs tested. Addition of the
pathogen at an MOI of 50 caused more than 40% cytotoxicity and when the MOI was increased to 100 more than 90% cytotoxicity was observed. Thus, an MOI 10 and an incubation period of 4h were chosen to assess the protective effects of CFSMs against *Salmonella* infection of RAW 264.7 cells in subsequent experiments.

![Graph showing cytotoxicity (%) of RAW 264.7 cells induced by *Salmonella* infection at different MOIs and incubation periods as measured by the release of LDH.](image)

**Figure 3.3.** Cytotoxicity (%) of RAW 264.7 cells induced by *Salmonella* infection at different MOIs and incubation periods as measured by the release of LDH. 2 × 10⁵ cells/ml were seeded in 96-well plate and incubated for 24 h for the cell attachment. LDH production was measured after adding *S. Typhimurium* DT 104 (SA1997-0934) at MOI 10, 50 or 100 and after incubation for 2 h or 4 h. All results represent the means ± SD of two independent experiments performed with three replications for each.

In addition, the protective effect of CFSMs on *Salmonella* infected RAW 264.7 was determined by the LDH test. Previous studies have showed that probiotics or their cell-free supernatants decrease cytotoxicity caused by *Salmonella* infection as detected by the LDH assay (Burkholder & Bhunia, 2009 & Peng, 2014). Therefore, the release of LDH from infected RAW 264.7 cells was compared when they were co-incubated with and without LH-2 or La-5 CFSMs during infection with *Salmonella* Typhimurium. Macrophage cells (100 µl of 2 × 10⁵ cells/ml) were inoculated in 96 well plates. After allowing 24 h for cell attachment, *S. Typhimurium* was added...
at an MOI of 10 in the presence and absence of LH-2 and La-5 CFSMs for 4 h. Figure 3.4 shows the effect of CFSMs on RAW 264.7 cell death caused by *Salmonella* infection. Compared to the untreated cells very significant (p < 0.01) differences were found for both LH-2 and La-5 treated cells. Also the difference in cytotoxicity % was significant (p < 0.05) between LH-2 and La-5 treatments. The cytotoxicity % (as measured by the amount of LDH release) was reduced by 57 ± 3% and 39 ± 8% respectively by LH-2 and La-5 treatments. This significant reduction suggests that *Salmonella* induced cell damage is highly attenuated by the bioactive peptides present in the CFSMs produced by *L. helveticus* and *L. acidophilus*.

**Figure 3.4** Cytotoxicity (%) of RAW 264.7 cells induced by *Salmonella* infection in the presence and absence of LH-2 or La-5 CFSM as measured by the release of LDH. Macrophages (2 × 10^5 cells/ml) were seeded in 96-well plate and incubated for 24 h to allow cell attachment. LDH production was measured after adding *S. Typhimurium* DT 104 (SA1997-0934) at an MOI of 10 and co-incubated with and without LH-2 or La-5 CFSM for 4 h. All results represent the means ± SD of three independent experiments performed with three replications for each. Bars identified with different letters above the standard error bars represent significant difference (p < 0.05).

The results show that cytotoxicity evoked by *Salmonella* invasion of the macrophage cell line was highly alleviated by bioactive components produced by *L. helveticus* and *L. acidophilus*. These results are in agreement with previous studies where the cytoprotective effect of probiotics
or their products against a variety of pathogens have been reported. LDH liberation is an indicator of membrane damage and cell viability. Hence, the reduction in LDH could effectively demonstrate the cytoprotective effects of probiotic bacteria and their products. Burkholder & Bhunia (2009) found that 1h pre-incubation with *L. rhamnosus* GG (LGG) strain significantly attenuated *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). Similarly, Myllyluoma et al. (2008), tested different lactic acid bacteria and their combinations and found that *L. rhamnosus* GG, *L. rhamnosus* Lc705, *P. freudenreichii* subsp. *shermanii* Js, individually and in combination inhibited *Helicobacter pylori*-induced cell membrane leakage as detected by LDH production test.

A protective effect against cell cytotoxicity has also been observed with cell-free preparations from many LAB. *Lactobacillus fermentum*-spent media reduced the cytotoxicity of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in HT29, INT 407 and HEK 293 cells at a concentration of 2.5 μg/ml (Varma et al., 2011). *Lactobacillus delbrueckii* ssp. *bulgaricus* can eliminate *C. difficile*-mediated cytotoxicity when studied in Caco-2 cells. The inhibition of cytotoxicity resulting from the toxins produced by *C. difficile* was also observed when tested with cell free supernatant (CFS) from *L. delbrueckii* ssp. *Bulgaricus*, which suggested that the bacterium releases one or more bioactive component(s) into the CFS (Banarjee, 2009). Peng (2014) showed that cell free spent media (CFSM) from *L. helveticus* and *L. acidophilus* protect against *Salmonella* Typhimurium induced cytotoxicity in an epithelial cell line HT-29. The results obtained in the present study agree with these prior studies.
Different mechanisms have been suggested for the inhibition of cytotoxicity such as competitive exclusion of the pathogens (Burkholder & Bhunia, 2009), immunomodulation of host cells (Myllyluoma et al., 2008), inhibition of proliferation (Varma et al., 2011) or the inhibition of toxin (Kakisu et al., 2013). The mechanism for the protective effect against cytotoxicity caused by the pathogens has not been understood yet. So, in the present study, the effect of the peptides in the CFSMs of *L. helveticus* and *L. acidophilus* on virulence gene expression was studied.

**Evaluation of the effect of LH-2 and La-5 CFSM on the virulence gene expression of *Salmonella Typhimurium***

qPCR is the method of choice for gene expression studies because of its technical feasibility, comparatively low cost, high specificity, low potential for contamination and wide dynamic quantification range (Fryer et al., 2002). In this study, the effects of the peptides on gene expression in *Salmonella Typhimurium* DT 104 strain SA1997-0934 were analyzed by a 2-step RT qPCR method. The LH-2 or La-5 CFSM was added to a broth culture of *Salmonella* Typhimurium to final peptide concentrations of 2mg/ml and 10mg/ml for LH-2 and La-5, respectively. The bacterium was allowed to grow to logarithmic phase and RNA was extracted using an extraction kit and reverse transcribed to cDNA using 1µg RNA for each untreated and treated samples. The cDNA was used for qPCR using standardized conditions and optimized primers. Three reference genes *gmk, rpoD* and 16S and five virulence genes, *ssrB, hilA, sopD, sipB* and *invA*, were used in the analysis. The most stable of the three reference genes was found to be 16S, so it was used to normalize gene expression of the virulence genes as described before (Phongsisay et al., 2007).
The *ssrB* gene is the transcriptional regulator of SPI-2. When activated, the *ssrB* response regulator directly promotes the transcription of multiple genes within SPI-2 (Choi et al., 2010). Similarly, *hilA* is the transcriptional regulator of SPI-1. Environmental conditions such as growth phase, pH, oxygen tension, and osmolarity regulate gene expression of *hilA* which binds to the promoters of SPI-1 operons to activate their transcription (Boddicker, 2003). *SopD* encodes for the effector protein required for the invasion of epithelial cells (Raffatellu, 2005). The SPI-1 effector SipB activates caspase-1 in macrophages, releasing IL-1β and IL-18 and inducing rapid cell death by a mechanism that has features of both apoptosis and necrosis. Caspase-1 is required for *Salmonella* to infect Peyer's patches and disseminate to systemic tissues (Guiney, 2005). InvA is a protein that forms a locus of type three secretion and it causes alterations to the architecture of the microvilli of the epithelial cells to enable invasion (Galan et al., 1992).

The results presented in figure 3.5A show that LH-2 CFSM caused significant down-regulation of all virulence genes studied. The fold-change in gene expression, as determined from $2^{\text{ddCt}}$, indicates significant up-regulation if the value is >2 and significant down-regulation when the value is <-2. The values in between indicate no significant effect from the environment (Phongsisay et al., 2007). The decrease in the expression of *sipB* was significant but moderate with the fold change of -2.74. For all the other genes down-regulation of expression was high (> 8-fold) and was greatest for *sopD* (-16.83).

Similarly, the La-5 CFSM also caused down-regulation of all the 5 virulence genes of *Salmonella* Typhimurium SA1997-0934 (Figure 3.5 B). As was the case with LH-2 CFSM, the down-regulation for *sipB* (-2.45) was significant but lower than for other genes. For other genes, down-regulation of expression was more than 6-fold with the greatest down-regulation for *sopD* (-10.31). When the fold-change of gene expression caused by La-5 CFSM was compared with
that produced by LH-2 CFSM, LH-2 CFSM caused higher down-regulation for most of the genes.

**Figure 3.5** Effect of LH-2 and La-5 CFSM on expression of select virulence genes in *Salmonella* Typhimurium. A: LH-2; B: La-5. *Salmonella* Typhimurium SA1997-0934 was allowed to grow in presence of 2mg/ml of LH-2 or 10mg/ml La-5 CFSM and gene expression was studied by a 2-step RT-qPCR method. The gene expression of five virulence genes affected by LH-2 CFSM was compared with untreated calibrator and normalized to a reference gene. The results were analyzed by the ddCt method to obtain the fold-change compared to untreated *Salmonella* Typhimurium. The results are the mean ± S.D. of three independent studies with replicates for each.
Thus, antivirulence effects were observed for the CFSMs from both species of *Lactobacillus* used in the study. These results are in agreement with several previous studies. Antivirulence effects have been suggested as the mechanism for the protective effect against enteric pathogens exerted by many probiotic bacteria in a number of studies. There is substantial evidence to indicate the protective effects of probiotic bacteria and their metabolic products against enteric pathogens such as *Salmonella* spp. *in vitro* and *in vivo*. The consumption of *Lactobacillus casei* prior to infection decreased the intestinal and joint inflammation triggered by *Salmonella*. *L. casei* drastically diminished *S. enteritidis* invasiveness and shortened splenic persistence of the pathogen (Llana et al., 2013). Bacterial loads recovered at days 2 and 5 from Peyer's patches were 10-fold lower in mice fed with *L. casei*. Similarly, another study showed that *Lactobacillus paracasei* CBA L74 fermented products (both culture medium and fermented milk) could protect against colitis and against *Salmonella Typhimurium* infection *in vivo* in mice (Zagato et al., 2014). Brovko et al. (2003) showed that both *L. helveticus* fermented milk and its cell free fraction, when administered orally to mice prior to infection, made them less susceptible to bacterial colonization. Tellez et al. (2011) showed that the severity of *Salmonella* infection in mice was greatly reduced in mice fed with cell-free fractions of milk fermented with *Lactobacillus helveticus* resulting in lower colonization of spleen and liver. In another study, Zeinhom et al. (2012) reported that the clinical manifestations of enterohemorrhagic *Escherichia coli* (EHEC) infection were significantly less severe in mice fed with yogurt supplemented with *L. acidophilus* La-5 cell-free supernatant. Also the La-5 active fractions were able to protect mice from infection when challenged with EHEC (Medellin-Peña and Griffiths, 2009).

Several studies on the effect of these bioactive molecules on virulence gene expression using constructs in which virulence genes were fused with a *lux* reporter gene as well as qPCR suggest
their antivirulence effect as a potential mechanism to mitigate against enteric infection. Bayoumi and Griffiths (2010) detected down-regulation in constructs in which a reporter gene (lux) was fused to promoters of two virulence genes from SPI-1 and SPI-2 of S. Typhimurium and observed highest down-regulatory effects with molecules obtained from B. bifidum. Bayoumi and Griffiths (2012) also studied the effects of molecules obtained from B. bifidum on gene expression in Salmonella Typhimurium and E. coli O157:H7 by qPCR and found down-regulation of several virulence genes in both pathogens. Antivirulence effects have also been reported for the bioactive molecules produced by L. helveticus and L. acidophilus. The peptides obtained following fermentation of milk by L. helveticus down-regulated the ssrB gene of SPI-2 in S. Typhimurium when detected by reporter gene fusion technique. The molecules secreted by L. acidophilus La-5 suppressed expression of genes associated with the major pathogenicity island loci of enterocyte effacement (LEE) 1 and 2 of enterohemorrhagic E. coli O157 (Medellin-Pena et al., 2007). In their study, they detected gene expression by conventional RT-PCR and found that the expression of several virulence genes was significantly down-regulated. Several virulence genes of EHEC, including stxB2, qseA, luxS, tir, ler, eaeA, and hlyB were down-regulated by La-5-produced bioactive peptides as determined by qPCR (Zeinhom et al., 2012). In a similar study, Bifidobacterium longum and L. acidophilus produced biomolecules that were able to down-regulate the expression of virulence genes of Campylobacter jejuni (Mundi et al., 2013). They found that the CFSMs of Bifidobacterium longum and L. acidophilus were able to down-regulate the expression ciaB (ratio of 22.80 and 25.51, respectively) and flaA (ratio of 27.00 and 25.13, respectively) in Campylobacter jejuni. Similarly, the cell extract of L. acidophilus was capable of causing the down-regulation of virulence genes of C. difficile (Yun et al., 2014). Down-regulation of hilA, which is a major virulence gene of SPI-1 in Salmonella by
cell free supernatant obtained from *L. plantarum* is also reported by Das et al. (2013). Similar to these prior results, the present study confirms that *L. helveticus* and *L. acidophilus* can secrete bioactive peptides in fermented milk and whey that affect the virulence of *Salmonella* without inhibiting their growth, and that these bioactive molecules mainly target virulence genes involved in TTSS so that the colonization and intracellular proliferation of *Salmonella* is highly attenuated.

**Comparison of effects of La-5 CFSMs produced at different fermentation conditions on Salmonella Typhimurium virulence gene expression**

The antivirulence effect on *Salmonella* Typhimurium was observed for La-5 CFSM obtained from whey fermented by *L. acidophilus* La-5 under variable fermentation conditions. The fermentation conditions can affect the type of peptides released and consequently their biological activities. Praveesh et al. (2011) showed that different inoculation and incubation conditions during fermentation can affect the biological activities of the LAB-produced biomolecules in fermented products. Major factors affecting the biological activities included the temperature, incubation period and size of inocula (Praveesh et al., 2011).

In the present study, the fermentation conditions including the type of inoculum and incubation conditions were changed and five different CFSMs were prepared from *L. acidophilus* La-5 fermented whey protein. Batch 1, 3, 4 and 5 were strictly anaerobic and batch 2 was cultured in tightly closed jars. Batches 3, 4 and 5 were agitated during fermentation by shaking at different speeds. Also the inoculants used were different, *viz.*., lyophilized culture for batch 1, colonies from fresh plate for batches 2 and 5 and inoculum as broth culture or pellet for batches 3 and 4, respectively. They were used in a gene expression study with *Salmonella* Typhimurium to
observe if the fermentation conditions affect their antivirulence activities. The results are presented in Figure 3.6. The extents of down-regulation of different genes caused by different batches of CFSM are different. For all the virulence genes tested, batch 5 showed highest down-regulatory effects. It is particularly interesting because the down-regulatory effects are almost double than those caused by other batches. When tested in SPSS by one-way ANOVA and post hoc Tukey’s test, batch-5 CFSM was significantly different ($p < 0.05$) from all other batches for all the genes tested. A significant difference ($p < 0.05$) was also observed between other individual batches for different genes. For the four genes, $ssrB$, $hilA$, $sopD$ and $invA$, batch-1 showed least down-regulation, whereas it is comparable with batch 2, 3 and 4 for the gene $sipB$.

![Figure 3.6](image)

**Figure 3.6** Effects of the five batches of La-5 CFSMs on the virulence gene expression of *Salmonella Typhimurium*. *Salmonella Typhimurium* SA1997-0934 was allowed to grow in presence of 10mg/ml of La-5 CFSMs, which were obtained from La-5 fermented whey protein-based medium under different conditions and gene expression was studied by a 2-step RT-qPCR method. The gene expression results were analyzed to obtain the fold-change compared to untreated *Salmonella Typhimurium*. The results are the mean ± S.D. of three independent studies with replicates for each. Different letters below the standard error bars indicate significant differences based on ANOVA followed by Tukey’s post hoc test ($p < 0.05$) within the same gene.
When compared among all the genes, batch-1 and batch-2 showed similar down-regulatory effects for most of the genes. Similarly, batch-3 and batch-4 showed similar down-regulation for most of the genes and batch-5 showed highest down-regulation for all the genes tested.

These results suggest that the changes in fermentation process have their effects on the antivirulence activities and fermentation conditions could be optimized to obtain potent antivirulent CFSM. The difference in antivirulence activity is due to the difference in types or concentrations of the peptides produced during the fermentation. The types and concentrations of peptides could be correlated with their antivirulence activities.

Previous studies also have indicated that cultural conditions such as temperature, pH, incubation period, inoculum size and shaking greatly affected the growth and production of bioactive metabolites (Bhattacharyya & Jha, 2011). Changes in the environmental conditions alter the chemical and biological profiles and this leads to the difference in metabolite production. The changes could be characterized by chemical analysis methods such as HPLC.

**Study of effect of La-5 CFSMs on the virulence gene of Salmonella by reporter gene fusion technique**

The antivirulence effects of different batches of La-5 CFSMs were also studied using the reporter gene assay. The bioluminescence produced by the S. Typhimurium *ssrB::lux* construct was compared after the bacterium was allowed to grow in the presence or absence of CFSMs. The luminescence produced following incubation of the reporter strain is directly correlated with the expression of *ssrB*. Luminescence is observed after the 4 h incubation with and without the La-5 CFSMs and OD$_{600}$ was measured to determine RLU/OD$_{600}$. Figure 3.7 shows the RLU/OD$_{600}$
ratio of S. Typhimurium ssrB::lux construct when cultured in the presence of 10 mg/ml of 5 different batches of La-5. All five batches of La-5 CFSMs significantly down-regulated the virulence gene expression (p <0.05). The percentage of reduction of gene expression by batch 1 is 9.8 ± 0.9 %, by batch 2 is 10.8 ± 0.7 %, by batch 3 is 15.8 ± 1.4 %, by batch 4 is 18.3 ± 0.3 % and by batch 5 is 22.3 ± 0.4 %. Thus, the highest reduction was observed for batch 5 CFSM and this result is in accordance with the qPCR results for different virulence genes.

![Figure 3.7](image)

**Figure 3.7** Effects of five batches of La-5 CFSMs on bioluminescence activity of S. Typhimurium ssrB::lux reporter construct. Data are the means ± standard deviations derived from two independent replicate trials and expressed as relative light units (RLU) defined as counts/min and adjusted to OD₆₀₀ (RLU/OD₆₀₀). Different letters above the standard error bars indicate significant differences based on ANOVA followed by Tukey’s post hoc test (p < 0.05).

Previous researchers have analyzed the antivirulence effects of peptides by bioluminescence reporter constructs. The peptides derived from *Lactobacillus* spp. and *Bifidobacterium* spp. down-regulated the bioluminescence production suggesting the down-regulation of virulence genes with results similar to our findings (Bayoumi & Griffiths, 2010; 2012; Tellez et al., 2011).
Chemical Analysis of La-5 CFSMs by High Performance-Size exclusion chromatography (HP-SEC) and Reverse phase-High Performance liquid chromatography (RP-HPLC)

As the antivirulence activities of peptides are directly influenced by the types and concentrations of peptides in the active fractions of the La-5 CFSMs, the peptides in the CFSMs were characterized and quantified by HPLC and correlated with the extent of down-regulation caused for the individual genes. High performance-size exclusion chromatography (HP-SEC) and reverse phase-high performance liquid chromatography (RP-HPLC) were carried out to determine differences in the peptides distribution.

Analytical HP-SEC was performed using an AKTA analyzer for all the five La-5 CFSMs at the concentration of 18.0 ± 0.5 mg/ml. The peptides were eluted with sodium phosphate buffer at pH 7. Figure 3.8 illustrates the differences in the elution chromatograms for the five CFSMs at neutral pH and similar elution conditions. Chromatograms for 5 CFSMs from different batches were different based on the peak retention times and areas.

From Figure 3.8, it is apparent that there are two major peaks between 10-13 ml retention volumes. These two peaks represent two whey major whey proteins, β-Lg and α-La (Kristo et al., 2012). However, we are mainly concerned with the peptides obtained from the fermentation of whey proteins. The peaks other than β-Lg and α-La and the peak area (%) obtained for the CFSMs are compiled in Table 3.1.
Figure 3.8 Size-exclusion chromatographs of five batches of La-5 CFSMs obtained at 215 nm. A- batch 1; b- batch 2; C- batches 3 and 4; and D- batch 5. The freeze dried forms of all the five CFSMs were dissolved in sodium phosphate buffer at pH 7 at the concentration of 18.0 ± 0.5 mg/ml and eluted using the same buffer. The analytical SE-chromatography run was performed in an AKTA purifier and the absorbance (mAU) of the eluted material was monitored at 215 nm.
Table 3.1 Areas of differential peaks collected from five batches of La-5 CFSMs obtained by SEC

<table>
<thead>
<tr>
<th></th>
<th>Retention time (13-14 min)</th>
<th>Area (%)</th>
<th>Retention time (15.4-16.8 min)</th>
<th>Area (%)</th>
<th>Retention time (29 min)</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td>13.97</td>
<td>14.93</td>
<td>16.88</td>
<td>20.74</td>
<td>29.18</td>
<td>2.63</td>
</tr>
<tr>
<td>Batch 2</td>
<td>---</td>
<td>---</td>
<td>15.41</td>
<td>14.74</td>
<td>29.12</td>
<td>0.19</td>
</tr>
<tr>
<td>Batch 3</td>
<td>13.89</td>
<td>11.58</td>
<td>15.80</td>
<td>17.60</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Batch 4</td>
<td>13.76</td>
<td>12.91</td>
<td>15.92</td>
<td>13.58</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Batch 5</td>
<td>---</td>
<td>---</td>
<td>16.45</td>
<td>11.17</td>
<td>29.51</td>
<td>0.05</td>
</tr>
</tbody>
</table>

The flow rate in the column used was 1 ml/min, therefore the retention volume = retention time.

“---” refers to the absence of differential peak at that similar retention time.

The five batches of La-5 showed peaks at different retention times that range from 13 to 29 min. All of the five batches of La-5 showed antivirulence activities so the bioactive peptides should be concentrated in the material eluting between 15.4 to 16.8 min. The most peaks were obtained for batch 1 and largest peak area (%) of 20.74% was also found for the peak eluting at a retention volume of 16.8 ml for batch 1. It shows that the greatest size range of peptides is found for batch 1, but the antivirulence activities of batch 1 were comparatively lower than those for the other production batches. SEC separates the proteins and peptides on the basis of their size, so all the peptides with similar size range form the same fraction. This method could not differentiate specific types of peptides on the basis of their function or specific charge. The peak retention time (min) and peak areas (%) of the CFSMs as obtained from SEC could not be directly
correlated with their antivirulence activities. So, the CFSMs were subjected to another analytical chromatographic technique, RP-HPLC.

RP-HPLC separates the peptides on the basis of charge as well as molecular weight. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase (Aguilar, 2004). Polypeptides which differ by a single amino acid residue can often be separated by RP-HPLC (Rivier & McClintock, 1983). So, this technique was used to characterize the peptides in the bioactive fractions of CFSMs. Figure 3.9 shows the chromatograms for the 5 different batches of CFSMs prepared with different inoculation and incubation conditions. The figure only comprises the data obtained from time 0 to 24 min. After 25 min, two major peaks for whey proteins, β-Lg and α-La were found and this is not included in the data.

RP-HPLC profiles of the La-5 CFSMs show that the variation in inoculation and incubation conditions resulted in a variety of types and concentrations of the peptides. This suggests that the variation in antivirulence effects is due to these differences. As all the five CFSMs show antivirulence effects, an attempt was made to correlate the bioactivity particularly with the variation in concentration of specific peptides in the CFSMs.

Before analyzing specific peptides, the chromatogram was divided into 4 min periods and the average of absorbance (mAU) for each time interval was calculated and compared with the antivirulence effect against each specific virulence gene of Salmonella Typhimurium. The average mAU for each time period are presented in Figure 3.10.
Figure 3.9 Reverse phase-HPLC profiles of La-5 CFSMs obtained at 215 nm from retention time 0 min to 24 min. The freeze dried samples of 5 batches of CFSMs were reconstituted as 10.0 ± 0.2 mg/ml and 20 µl of each loaded in Nova-Pak C18 column in RP-HPLC system. The peptides were eluted with a nonlinear gradient of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in 90% acetonitrile/water at a flow rate of 1 mL/min.
Figure 3.10 Average absorbance at 215nm (mAU) for the La-5 CFSM samples eluted within each 4 min interval. The La-5 CFSMs were run in RP-HPLC with a nonlinear gradient of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in 90% acetonitrile/water at a flow rate of 1 mL/min.

Average mAU corresponds to the concentration of peptides within each 4 min range for each batch. Figures 3.9 and 3.10 show that the highest concentration of smaller peptides is found in the batch-1 CFSM. For other batches, more peptides are eluted within the range of 12 to 24 min. There is less variation in concentration of peptides eluted between 12-16 min for batches 3, 4 and 5 and also less variation among batches 2, 3, 4 and 5 for the elution range of 20-24 min. However, the average concentration of peptides eluted between 16 – 24 min is highest for batch-5 compared to all the other batches.

The correlation between the fold-change of gene expression caused by the La-5 CFSMs and the average mAU of the intervals of each four-minute time interval of the reverse phase HPLC data were plotted as scatter plots and analyzed using Pearson’s correlation. The results are presented in Figure 3.11.
A

Fold Change for ssrB

Average mAU

0-4 min
4-8 min
8-12 min
12-16 min
16-20 min*
20-24 min*

B

Fold Change for hilA

Average mAU

0-4min
4-8min
8-12min
12-16min*
16-20min**
20-24min**

C

Fold Change for sopD

Average mAU

0-4min
4-8min
8-12 min
12-16 min
16-20 min*
20-24 min
Figure 3.1 Average absorbance (mAU) of La-5 CFSM batches versus down-regulation (fold-change) caused by the CFSM for each gene: A-ssrB; B- hilA; C- sopD; D- sipB and E- invA. The average mAU is calculated for the time ranges of 4 min from RP-HPLC data. The down-regulation fold-change was calculated for La-5 CFSM treated and untreated Salmonella Typhimurium and normalized to a reference gene. The average mAU and fold-change was correlated by Pearson’s correlation in SPSS and the significant correlations are showed in the scatter plot diagrams. * represents correlation significant at p <0.05 and ** represents correlation significant at p <0.01.

Figure 3.11 shows statistically significant (p <0.05) relationship between the values of average mAU for 16-20 and 20-24 min elution time and down-regulation of ssrB. This suggests that the greater the concentration of peptides that elute between 16-20 min. and 20-24 min., the more is the ssrB gene down-regulated. Similarly, for hilA and invA, the correlation between average mAU and down-regulation is significant for 12-16 min. and 20-24 min (p <0.05) and highly significant for the 16-20 min period (p <0.01). For sopD, the correlation was significant for
column eluate between 16-20 min (p <0.05) but no significant correlation existed between down-regulation for sipB and average mAU.

The correlation between the antivirulence effects on the select virulence gene of *Salmonella* Typhimurium and peptide concentration at different time intervals shows that the bioactive peptides are eluted from the column between 12 to 24 min. However, it does not suggest a particular peptide is involved. Hence, the individual peaks from this range of data were selected and using the software in the instrument, the peak areas were obtained. Some specific peaks from 12-24 min elution time were selected on the basis of antivirulence activities of the five batches of CFSMs. These peaks along with their peak areas are presented in table 3.2. The peak areas were then correlated with the down-regulation for each gene using Pearson’s correlation (Figure 3.12).

**Table 3.2 Area of the specific peaks from RP-HPLC retention time 12-24 min**

<table>
<thead>
<tr>
<th>Peak Retention time (min)</th>
<th>Area (mAU* min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFSM1</td>
</tr>
<tr>
<td>13.8-13.9</td>
<td>1.168</td>
</tr>
<tr>
<td>14.9-15.0</td>
<td>0.47</td>
</tr>
<tr>
<td>15.4-15.6</td>
<td>1.757</td>
</tr>
<tr>
<td>15.9-16.0</td>
<td>2.534</td>
</tr>
<tr>
<td>17.2-17.3</td>
<td>3.307</td>
</tr>
<tr>
<td>20.3-20.8</td>
<td>7.511</td>
</tr>
</tbody>
</table>
Figure 3.12 Peak area (mAU*min) for specific peak versus down-regulation (fold-change) caused by the CFSM for each gene: A- ssrB; B- hilA; C- sopD; D- sipB and E- invA. The peak area is obtained by the integration of RP-HPLC data from the instrument with subtraction of baseline. The down-regulation fold-change was calculated by the ddCt method from the Ct values obtained for La-5 CFSM treated and untreated Salmonella Typhimurium and normalized to a reference gene. The peak area and fold change was analyzed using Pearson’s correlation in SPSS and the significant correlations are shown in the scatter plot diagrams. * represents correlation significant at p <0.05 and ** represents correlation significant at p <0.01.

From figure 3.12, it could be observed that the peak areas of specific peptides have significant correlations with the down-regulation of virulence genes. The fold change of down-regulation
and peak area of the specific eluate at 15.4-15.6 min is significantly correlated (p<0.05) for all the five genes. Similarly, significant correlation was also observed with the peak area of the eluate at 20.3-20.8 min (p<0.01 for ssrB and sopD and p<0.05 for hilA, sipB and invA). Similarly, significant correlations also occurred for the eluate at 14.9-15.0 min (p<0.05 or p<0.01) for the four genes (ssrB, hilA, sopD and invA) and the eluate at 15.9-16.0 min (p<0.05) for the three genes (ssrB, sopD and sipB). This suggests that the antivirulence effect is due to the specific peptides which elute in RP-HPLC column from the retention time of around 14 to 20 min. As the peptides are concentrated in specific small region, they must be similar in their amino acid composition, charge distribution and hydrophobicity. The bioactivity could be due to the synergistic effects of the different specific peptides or specific peptides might have effect on the specific gene expression.

The isolation and purification of bioactive peptides are important for exploration of their physicochemical properties and evaluation of their in vitro and in vivo bioactivities. So, chemical analysis of bioactive peptides has been carried out in many studies. Various peptides from casein and whey proteins have been identified as having different bioactivities in previous studies (Shahidi & Jhong, 2008; Gobetti et al, 2002). Chromatography based on size difference, size-exclusion chromatography and based on hydrophobicity and hydrophilicity, like reversed-phase HPLC, ion-exchange chromatography (IEC) etc. are commonly used techniques for the separation and purification of peptides. HPLC is usually coupled with quantitative/qualitative analyzing equipment such as a UV detector or mass spectrometer (MS) (Shahidi & Jhong, 2008). Tellez Garay (2009) isolated different fractions of peptides from a cell-free supernatant of L. helveticus LH-2 fermented milk using SEC. Five peptides derived from β-casein and α-lactalbumin were identified in the fraction. The fraction which included these five peptides,
showed an immunomodulatory and antagonistic effect against *Salmonella* infection in both *in vitro* and *in vivo* studies (Tellez Garay, 2009). In another study, Medellin-Peña (2007) isolated and identified the peptide sequences in the bioactive fractions of *L. acidophilus* fermented modified MRS media. The CFSM down-regulated the virulence genes as well as interfered with quorum sensing in enterohemorrhagic *E. coli* (EHEC) O157:H7. Tsai et al (2008) identified short peptides in the bioactive fractions of whey fermented by *Streptococcus thermophilus* and *Lactobacillus bulgaricus* and separated by SEC. This fraction of CFSM showed antihypertensive bioactivity both *in vitro* and *in vivo*. SEC was also used by Zhao et al. (2014) to characterize the bioactive molecule with cytomodulatory effect. They found the molecule belonged to a fragment of β-casein (β-casein, f210-219). In another study the antioxidant and cytomodulatory peptides in cheese whey were separated by RP-HPLC and identified by MS (De Simone et al., 2009). RP-HPLC was used to isolate the ACE-inhibitory and antibacterial peptides from *Lactobacillus helveticus* PR4 proteinase-hydrolyzed casein and several fragments from casein showed bioactivity (Minervini et al., 2003). These are a few of the many studies that have used SEC and/or RP-HPLC for the characterization of bioactive peptides derived from milk proteins. These studies also suggest that the peptides have many different bioactivities. In the present study, the peptides obtained from the *L. acidophilus* fermented whey were studied by SE and RP-HPLC and specific peptides from RP-HPLC profiles showed strong possible relationship with their antivirulence effects.

**Conclusions**

In summary, *L. helveticus* LH-2 and *L. acidophilus* La-5 CFSMs obtained from fermented milk and whey exert antivirulence effects against *Salmonella* Typhimurium. They exert a protective
effect against *Salmonella* Typhimurium-induced apoptosis in a macrophage cell line. First of all, non-toxic doses of LH-2 and La-5 CFSMs were tested in RAW 264.7 cells. Results of the SRB assay determined that 1% (v/v) of LH-2 and 1.5% (v/v) of La-5 CFSMs were safe to be applied to RAW 264.7 cells. These concentrations were also verified by LDH cytotoxicity tests. When these concentrations of LH-2 and La-5 CFSMs were applied to *Salmonella* Typhimurium infected RAW 264.7 cells and co-incubated, the production of LDH was significantly reduced (p <0.05), which suggests that the bioactives protect against *Salmonella*-induced membrane damage and apoptosis. The LH-2 and La-5 CFSMs, when applied to *Salmonella* Typhimurium during growth of the organism, resulted in a down-regulation of select virulence gene expression *viz.* ssrB, hilA, sopD, sipB and invA, which form the parts of TTSS of two pathogenicity islands of *Salmonella*, SPI-1 and SPI-2. The gene expression, as analyzed by two-step qPCR, showed more than two-fold down-regulation for all the five genes suggesting significant antivirulence effects. After confirming these significant down-regulatory effects of La-5 CFSM, different batches of La-5 CFSMs were prepared under different fermentation conditions and the influence of fermentation conditions on the antivirulence efficacy was observed. The La-5 CFSMs produced under different fermentation conditions showed different degrees of down-regulation in the virulence genes when tested by two-step qPCR and bioluminescent reporter constructs. The peptide profiles of the CFSMs were observed by size exclusion and reverse phase-HPLC and differences in the profiles were observed. The RP-HPLC data of each of the five CFSMs was correlated with the antivirulence effect on each gene and it suggested that specific peptides that elute between 13 to 20 min during RP-HPLC were significantly correlated with an antivirulence effect on specific virulence gene (p <0.01 or p<0.05). Therefore, the fermentation conditions
could be optimized for *L. acidophilus* La-5 CFSM production to obtain high concentration of the specific peptides and to increase the efficacy of its antivirulence effect.

CHAPTER 4

General Conclusions

The main purpose of this study is to determine the antivirulence effects of bioactive peptides from *L. helveticus* and *L. acidophilus* fermented milk and whey protein against *Salmonella* Typhimurium virulence genes and to observe the efficacy of antivirulence effects under different fermentation conditions. Previous studies have demonstrated the protective effects of bioactive peptides from probiotic bacteria against enteric pathogens *in vitro* and *in vivo* (Medellin-Pena et al., 2007; Bayoumi & Griffiths, 2012; Tellez et al., 2011; Peng, 2014). The peptides also exert a negative influence on the virulence factors of pathogens such as enterohaemorrhagic *E. coli* (EHEC), *Campylobacter jejuni*, *Salmonella* spp. and *Clostridium difficile* (Medellin-Pena et al., 2007; Zeinhom et al., 2012; Bayoumi & Griffiths, 2012; Mundi et al. 2013; Yun et al., 2014). Based on these findings, it is hypothesized that *L. helveticus* (LH-2) or *L. acidophilus* (La-5) ferment milk and whey proteins to produce peptides which protect the cells against apoptosis by down-regulating the virulence gene expression of *Salmonella* Typhimurium and the antivirulence efficacy of the bioactive peptides could be increased by optimizing the fermentation conditions. The research was divided into two major sections: (1) to prepare LH-2 and La-5 CFSMs and observe their effects on the infected RAW 264.7 cells and on the virulence factors of *Salmonella* Typhimurium, and (2) to observe the effects of different fermentation conditions on the antivirulence efficacy of La-5 CFSMs and on their bioactive peptide profiles.
In the first section of research, CFSMs were filtered, lyophilized and concentrated (10×). The non-toxic dose of LH-2 and La-5 cell free spent medium (CFSM) was determined for application to RAW 264.7 cells using the sulforhodamine B (SRB) assay. Both CFSMs protected the cells against *Salmonella*-induced membrane damage, which is indicated by significantly (p <0.05) lowered LDH concentration when the cells were co-incubated with the CFSMs. The effect of LH-2 and La-5 CFSMs on *Salmonella* virulence gene expression was studied using a two-step qPCR method and a two-fold or more than two-fold change in expression when standardized against a house-keeping gene is considered significant (Phongsisay et al., 2007). Both LH-2 and La-5 CFSMs caused no direct effect on the growth of *S. Typhimurium*, but caused significant down-regulation of all the five virulence genes tested viz., *ssrB*, *hilA*, *sopD*, *invA* and *sipB*. These virulence genes are parts of the TTSS, which is required for cellular invasion, proliferation inside the cells and for apoptosis. So, this study confirms that *L. helveticus* and *L. acidophilus* can secrete bioactive peptides in fermented milk and whey that affect the virulence of *Salmonella* without inhibiting their growth, and that these bioactive molecules mainly target virulence genes involved in TTSS, which could be the mechanism of protection of the bioactive peptides against infection.

In the second part of the research, different batches of La-5 CFSMs from fermented whey protein were prepared with different inoculation and incubation conditions. Then, the effects of different batches of CFSMs on virulence gene expression of *Salmonella Typhimurium* were evaluated using a two step qPCR and bioluminescent reporter gene assays. All of the five batches showed antivirulence effects to different extents. When compared for all the individual gene expression analyses, batch-5 CFSM was found significantly different (p < 0.05) from all other batches for all the genes tested and a significant difference (p <0.05) also occurred between other individual
batches for specific genes. In general, the highest antivirulence activity was exhibited by batch-5 CFSM and lowest by the batch-1 CFSM. This suggests that the inoculation of fresh culture and strict anaerobic incubation with medium rate of agitation optimizes the production of antivirulent peptides in La-5 fermented whey protein. Finally, the peptide profiles in the five batches of CFSMs were chemically characterized using HPLC techniques. Size exclusion chromatography of the CFSMs revealed three to five peaks including the peaks for the two whey proteins, β-lactoglobulin and α-lactalbumin. Reverse-Phase HPLC of the five batches of CFSMs produced peak profiles with different peak areas and some specific peak areas were significantly correlated with down-regulation for individual genes (p <0.01 or p <0.05). Thus, the efficacy of the antivirulence activity is dependent on the concentration of specific peptides present in the cell free spent medium obtained from *L. acidophilus* La-5 fermented whey proteins. This also suggests that the fermentation conditions could be optimized to increase the yield of the specific peptides.

So, overall it has been demonstrated that the CFSMs produced by two probiotic species of *Lactobacillus*, *L. acidophilus* and *L. helveticus* contained antivirulent peptides, which inhibit gene expression in *Salmonella enteric* serotype Typhimurium though the CFSMs show no direct inhibition on growth of the pathogen. An antivirulence activity has been considered as a therapeutic alternative instead of antibiotics or other antimicrobials (Cegelski et al., 2008), so these CFSMs have promising application in the present context of scarcity of antibiotic drugs against enteric pathogens.
Future Work

Based on these findings, many future research directions can be recommended to further prove or utilize the antivirulence activities of the bioactive peptides in *L. helveticus* and *L. acidophilus* fermented milk and whey proteins. As whey is a cheaper option obtained as a by-product of cheese production, its further utilization as a substrate for fermentation could be assessed for both *L. helveticus* and *L. acidophilus*. Both species of *Lactobacillus* produce a pronounced antivirulence effect against *Salmonella* Typhimurium, so the synergistic effects of both the species should be investigated to determine if their combined effects offer better antivirulence and protective activity.

The virulence factor expression could differ when the bacteria are cultured *in vivo*. In macrophages, 42% of the genome showed an altered expression pattern during infection, whereas about 31% of *Salmonella* genes changed expression significantly in epithelial cells compared to cells grown in culture medium (Eriksson et al., 2003; Hebrard et al., 2011). So, the effect of the bioactive peptides on gene expression of *Salmonella* isolated from infected cell cultures could provide a more realistic picture of what happens in the intestine. Moreover, determination of their effective doses in cell cultures, animal and clinical trials and toxicity tests are highly important before their application. These data are essential to obtain the approval of regulatory authorities for utilizing these CFSMs as therapeutic supplements or food and feed additives. Also important is the stability of the bioactive components. The bioactive peptides in the CFSMs must be in a protected form until they reach the target cells or tissues. Further studies are required for this.
The active peptides of the CFSMs as established by the size exclusion and reverse phase HPL analyses could be fractionated using preparative HPLC techniques and their antimicrobial efficacy studied. If the peptide sequence of the active fractions could be established, they could be further assessed as well as chemically synthesized.
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