Analysis of Peptide Production by *Lactobacillus* Species and Evaluation of Their Antihypertensive and Immunomodulatory Activities

by

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ABSTRACT

Cardiovascular disease (CVD) is the leading cause of death among Canadian adults. Research has demonstrated an inverse relationship between the consumption of fermented dairy products and a decreased risk of CVD due to lactic acid bacteria used in the fermentation process which liberate small bioactive peptides from larger milk proteins (eg. casein). We observed that supplementation with 0.1% casein significantly increased the growth rate of *L. helveticus* R0389 and *L. rhamnosus* R0011 and increased the ACE-inhibitory activity of their secreted peptide fractions. Peptide-containing supernatants of *L. rhamnosus* R0011 show comparable ACE inhibition to known antihypertensive peptides, VPP and IPP. Supernatants of milk ferments induced the production of the regulatory cytokine, IL-10, by THP-1 monocytes. Novel antihypertensive and immunomodulatory activities of individually synthesized peptides were also reported. By investigating the relationship between these bioactive properties, we can improve upon the use of probiotic organisms to confer maximal health benefits to Canadians.

Keywords: lactobacillus, lactic acid bacteria, bioactive peptides, ACE inhibition, immunomodulatory, antihypertensive

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LIST OF ABBREVIATIONS

ACE – Angiotensin converting enzyme

ATP – Adenosine triphosphate

BSA – Bovine serum albumin

CEP – Cell envelope proteinase

CVD – Cardiovascular disease

DBP – Diastolic blood pressure

DC – Dendritic cell

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbant assay

FAO - Food and Agriculture Organization of the United Nations

FBS – Fetal bovine serum

GI - Gastrointestinal

HCl – Hydrochloric acid

HPLC – High performance liquid chromatography

HRP – Horseradish peroxidase

IL-6 – Interleukin-6

IL-8 – Interleukin-8

IL-10 – Interleukin-10

LAB – Lactic acid bacteria

LDL – Low density lipoprotein

LPS – Lipopolysaccharide

MRS – de Man Rogosa Sharpe

RPMI – Roswell Park Memorial Institute medium

PBS – Phosphate buffered saline

PMS – N-methyl dibenzopyrazine methyl sulfate or Phenazine methosulfate

Prt – Proteinase

RAS – Renin angiotensin system

SBP – Systolic blood pressure

SHR – Spontaneously hypertensive rat

TFA – Trifluoroacetic acid

THP-1 – Human monocytic cell line derived from an acute monocytic leukemia patient

TMB – 3,3’,5,5’-tetramethylbenzidine

TLC – Thin layer chromatography

TLR – Toll-like receptor

XTT – 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt

1 INTRODUCTION

1.1 Atherosclerosis

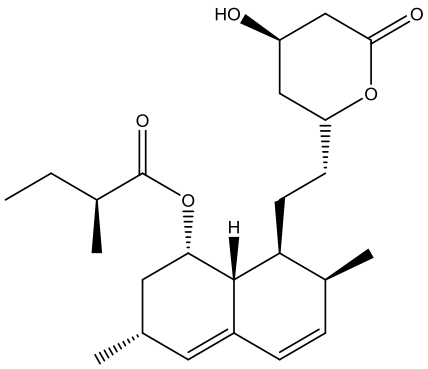
Atherosclerosis is a chronic inflammatory disease that affects millions of people around the world, and accounts for billions of dollars in health care costs every year. It is also a primary cause of cardiovascular disease (CVD) which, in the past decade, has become one of the leading causes of death around the world (FAO/WHO, 2001). Atherosclerosis is characterized by the build-up and rupturing of plaques that form along the arterial wall. These plaques are composed primarily of foam cells, immune cells, endothelial cells, smooth muscle cells and lipids. Although the mechanism responsible for the initiation of plaque formation is not completely understood, it has been recently accepted that the activation of the immune system plays an important role in the pathogenesis of atherosclerosis (Galkina & Ley, 2009; Lundberg & Hansson, 2010; Andersson *et al.*, 2010).

As mentioned previously, the exact cause of atherosclerosis is not known. However, it is understood that damage to the endothelial wall may be one mechanism of initiation of the disease. One example of this damage is oxidized low density lipoproteins (LDL) which can be retained on the arterial wall, leading to activation of both the innate and adaptive immune systems and the recruitment of macrophages, T-cells, dendritic cells (DCs) followed by various other immune cells (Lundberg & Hansson, 2010). Macrophages are of particular interest in the study of the atherosclerotic progression since they are believed to be one of the first inflammatory cells to respond to the development of the disease; they play a role in lipid retention, expression of toll-like receptors (TLRs), and in the production of pro-inflammatory cytokines. These pro-inflammatory cytokines have also been shown

to have multiple key roles in the atherogenic process (Galkina & Ley, 2009). Macrophages will engulf the fatty material to form foam cells which will begin to accumulate as fatty streaks in the wall of the artery. A harder capsule layer then begins to form around the fatty streaks – the initial step in plaque formation. With incoming macrophages, these foam cells will continue to form and grow and will eventually rupture, releasing oxidized cholesterol into the artery, recruiting additional white blood cells, and further contributing to plaque formation (Galkina & Ley, 2009). This accumulation of immune cells, and the rupturing of foam cells causes further buildup in the intima of the blood vessel leading to narrowing of the artery, decreased blood flow, and increased blood pressure.

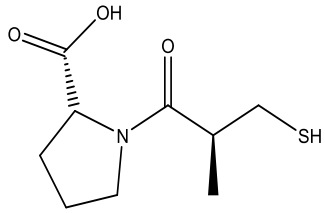
Various factors have been shown to increase an individual's risk of atherosclerosis including high blood pressure, smoking, diabetes, and high blood cholesterol levels. High-fat diets will also lead to elevated levels of LDL, further increasing the risk of atherosclerosis which can ultimately lead to a heart attack or stroke. Current treatments for atherosclerosis include the administration of drugs meant to regulate blood pressure and cholesterol levels such as statins (Figure 1A). Statins inhibit a particular reductase enzyme crucial for cholesterol synthesis and are able to directly and indirectly lower LDL levels and control vascular inflammation and thrombosis, leading to a decreased risk of CVD (Galkina & Ley, 2009). Statins also exhibit immunomodulatory effects, causing impaired leukocyte activation and migration (Andersson *et al.,* 2010). A variety of synthetic angiotensin converting enzyme (ACE) inhibitors also exist to treat hypertension including captopril, lisinopril, and enalapril (Figure 1B). However, like statins, these drugs have been reported to cause numerous side effects such as hypotension, reduced renal function, cough, and rashes (Piepho, 2000; Phelan and Kerins, 2011).

(A)

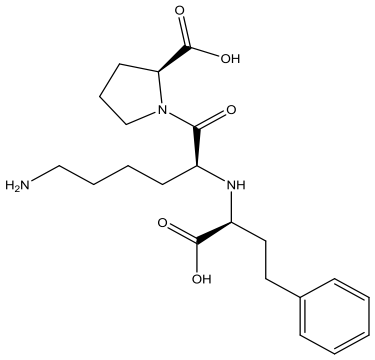


lovastatin

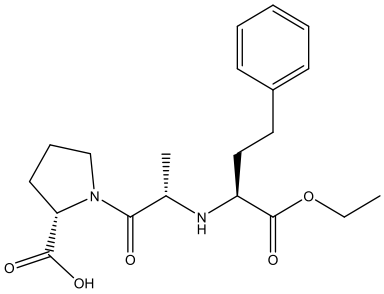
(B)



captopril



lisinopril



enalapril

Figure 1 - Examples of drugs available for the inhibition of cholesterol synthesis such as statins, (A) or ACE inhibitors used for the treatment of hypertension (B). (A) – lovastatin, the first statin to be marketed, isolated from *Aspergillus terreus*; (B) – ACE inhibitors captopril, lisinopril, and enalapril. Structures were drawn using ChemBioDraw Ultra 13.0.

Although medications are available for patients with atherosclerosis, focus has recently been on the prevention of the disease via dietary and lifestyle modifications. Recently, it has been demonstrated that a dietary approach may reduce the risk of CVD development. Clinical studies have established a relationship between hypertension and

diet; implementation of modified dietary and lifestyle habits reduces blood pressure (Huang *et al*., 2013). Any reduction in blood pressure, no matter how small, can be incredibly effective in reducing one's risk of developing CVD. According to Collins *et al.* (1994) a reduction in systolic blood pressure (SBP) of 10 – 12 mmHg and in diastolic blood pressure (DBP) of 5 mmHg may reduce the risk of stroke, coronary heart disease and all-cause mortality by 40, 16 and 13%, respectively. Therefore, changes in diet or lifestyle, such as increased physical activity, weight reduction, smoking cessation, and potentially the consumption of functional foods or bioactive compound-containing products, could be sufficient to lower blood pressure in individuals with mild hypertension to prevent disease.

1.2 Fermented Dairy Products

There is evidence that consumption of low-fat dairy products, specifically fermented dairy products, can lower blood pressure and reduce hypertension, decreasing risk factors associated with the onset of CVD (Lamarche, 2008; Sonestedt *et al.*, 2011). Although the exact role of fermented dairy products in the regulation of risk factors associated with CVD is not fully understood, the probiotic bacteria used in the fermentation process are thought to be responsible for much of the benefit provided by these fermented milk products. During fermentation, lactic acid bacteria convert lactose to various fermentation products, including lactic acid and acetic acid, and break down large milk proteins, both of which contribute to flavour development as well as cheese maturation in the production of these fermented dairy products (Haug *et al.*, 2007; Tidona *et al.*, 2009). The organic acids produced during fermentation can also aid in the absorption of various minerals, including iron and calcium (Haug *et al*., 2007). For these reasons, an interest in dietary prevention

has emerged in recent years, not only to reduce blood cholesterol levels, but to regulate the immune and inflammatory responses associated with atherosclerosis.

Milk proteins play an important role in the benefits conferred by fermented dairy products. Bovine milk contains approximately 32 g/L of protein, 80% of which is comprised of casein proteins. The remaining 20% of milk protein is comprised mostly of whey proteins which include primarily α-lactalbumin and β-lactoglobulin, as well as lactoferrins, immunoglobulins and bovine serum albumin (Marshall, 2004; Jakala *et al.*, 2010). Although whey proteins are also used as a popular dietary supplement, and are proposed to also possess antimicrobial, antihypertensive, immunomodulatory and other bioactive properties (Marshall, 2004), the focus of most studies, including this thesis project, is on the more abundant casein proteins. Since caseins are the predominant protein found in milk, the majority of milk-derived peptides are liberated from casein proteins; therefore the investigation of caseins can provide a broader view of the different peptide sequences liberated during milk fermentation and their bioactive effects. The properties of caseins are also quite different from those of whey proteins; the coagulation of caseins in the intestinal tract increases hydrolysis as opposed to the fast acting soluble whey proteins that are quickly passed through the intestines, providing less opportunity for absorption of liberated peptides in the small intestine compared to caseins (Marshall, 2004). Caseins are phosphoproteins that form micelles (~ 0.04 to 0.3 μM in diameter) in milk that will coagulate at pH 4.6. Casein proteins are comprised of three types of casein (α_{s1}- and α_{s2}-casein, β-casein and κ-casein). Caseins increase the efficiency of calcium and phosphate transport in milk products (Griffiths and Tellez, 2013) and provide a substrate for the liberation of bioactive peptides through bacterial proteolysis during fermentation or

gastrointestinal digestion. Not only does the fermentation process allow for the liberation of bioactive peptides through microbial proteolysis (see section 1.3.2), but the fermentation of milk with lactic acid bacteria, specifically *Lactobacillus helveticus*, is thought to influence the rate and pattern of subsequent release of peptides during digestion (Matar *et al.*, 1996).

1.3 Lactic Acid Bacteria

Lactic acid bacteria (LAB) are gram-positive bacteria, producing lactic acid as their major or sole product of carbohydrate fermentation. They are predominantly used in the food industry as starter strains for the fermentation of a wide variety of dairy products including yogurts and cheeses. Their resistance to acidic environments allows them to survive the fermentation process while most other microorganisms will not. In addition to their release of lactic acid, they produce various anti-microbial compounds such as bacteriocins, lantibiotics, peptides, and fatty acids that prevent the growth of pathogenic or spoilage bacteria during food processing (Zendo, 2013; Hayes *et al.*, 2006). The most common species of LAB used in the fermentation industry include *Streptococcus thermophilus, Lactococcus lactis, Lactobacillus helveticus*, and *Lactobacillus bulgaricus*.

1.3.1 Lactobacillus

The *Lactobacillus* genus is often used as a starter strain in the food industry during fermentation. Lactobacilli species can be divided amongst three groups: Group I lactobacilli are obligate homofermenters, producing lactic acid as their sole product of fermentation. Group II are also homofermentative, however they can produce more oxidized fermentation products if O_2 is present. Group III lactobacilli are

heterofermentative, and produce CO_2 and ethanol in addition to lactic acid. Both strains used throughout this thesis work are Group I homofermentative LAB.

Homofermentative lactobacilli will typically convert glucose into two molecules of pyruvate through glycolysis, which are then converted into two molecules of lactate. Heterofermentative LAB, however, utilize the pentose-phosphate pathway for the breakdown of glucose, releasing CO_2, and leading to the production of lactate and ethanol from xylulose-5-phosphate. Many homofermentative *Lactobacillus* species are considered probiotic organisms. Probiotics are characterized as microorganisms that confer some health benefit to their host. The mechanisms underlying the health benefits of many *Lactobacillus* species have not yet been fully elucidated. However, it is believed that they act to protect against other microorganisms in the gut, strengthen the host immune system, maintain epithelial barrier function, and regulate pro-inflammatory responses (Bermudez-Brito *et al*., 2012). Their production of lactic acid also prevents the growth of many pathogenic microorganisms either in the gut or during food processing (Bermudez-Brito *et al.*, 2012).

1.3.2 Proteolytic Activity of LAB

Lactobacilli are nutritionally fastidious organisms and therefore their proteolytic system is crucial to both their growth in milk as well as to flavour development in various dairy products. Due to the inability of many *Lactobacillus* species to synthesize a majority of the amino acids that they require, they must rely on their proteolytic system, consisting of multiple proteinases, peptide transport systems and peptidases, in order to digest the larger milk proteins, transport the oligopeptides into the cell, and break them down into individual amino acids, respectively (Griffiths and Tellez, 2013; Savijoki *et al.*, 2006).

Lactobacillus helveticus is an example of the very fastidious LAB and has been shown to have the highest proteolytic activity among various LAB species (Yamamoto *et al.*, 1994; Beganovic *et al.*, 2013; Genay *et al.*, 2009). Although not as extensively studied, certain strains of *L. rhamnosus* may also be capable of liberating bioactive peptides from larger proteins in their growth environment. The most highly characterized strain of *L. rhamnosus, L. rhamnosus* GG, has been shown to produce two soluble proteins, p40 and p75, that are able to prevent cytokine-induced epithelial damage and apoptosis (Yan *et al.*, 2007). Few studies have been conducted to investigate the secreted products of *L. rhamnosus* species such as peptides or other low molecular weight (< 10 kDa) compounds. However, one study by Yang *et al.* (2014) showed that the supernatant of *L. rhamnosus* GR-1 in de Man Rogosa Sharpe (MRS) medium was able to reduce inflammation by preventing the lipopolysaccharide (LPS)-induced production of pro-inflammatory cytokines in the maternal plasma of pregnant CD-1 mice. While the proteolytic systems of *L. helveticus* species have been more extensively characterized, few studies have investigated the peptides liberated by strains of *L. rhamnosus* and their bioactive properties compared to those produced by the highly characterized probiotic *L. helveticus*. However many species of lactobacilli possess similar proteolytic enzymes; it would therefore benefit the field of probiotics to direct more attention towards less characterized *Lactobacillus* species and the potential health benefits they may confer.

Lactobacillus species are equipped with a variety of cell-envelope proteinases (CEPs) which act to break down larger milk proteins in order to supply the bacteria with the amino acids required for growth. These CEPs will degrade larger milk proteins, such

as casein, to produce smaller peptides which are taken up into the cell and further broken down by a variety of intracellular peptidases.

1.3.2.1 Cell Envelope Proteinases

A variety of CEPs have been identified in a select number of LAB species. Expression of CEPs will vary not only between LAB species, but even between strains of the same species. *Lactobacillus* species will typically express between 1 and 4 CEPs which include PrtH, PrtH2, PrtH3, PrtH4 (Griffiths and Tellez, 2013; Savijoki *et al*., 2006) and the more recently discovered PrtH5 (Smeianov *et al.*, 2007). CEPs are synthesized as inactive pre-proteinases; maturation to an active proteinase often occurs with the cleavage of a portion of the N-terminal sequence, a process which requires the aid of an additional PrtM chaperone protein (Smeianov *et al.*, 2007; Beganovic *et al.*, 2013). Many experiments have been performed to characterize the different CEPs expressed by different *L. helveticus* strains (Broadbent *et al*., 2013; Martin-Hernandez *et al*., 1994; Ono *et al*., 1997; Pederson *et al*., 1999). Genay *et al.* (2009) found that *prtH2* was more prevalent than *prtH* in a variety of *L. helveticus* strains. However when Broadbent *et al.* (2011) included an additional two proteinase genes in their comparison, they found *prtH3* to be most commonly expressed among *helveticus* strains. The genome of *L. helveticus* R0052 revealed only one *prt* gene encoding for PrtH4 (Tompkins *et al*., 2012) and that of *L. helveticus* H10 contains two proteinase encoding genes (Zhao *et al.*, 2011). *L. helveticus* CNRZ 32 is the only *helveticus* strain known to date to carry genes encoding for four distinct CEPs (PrtH, PrtH2, PrtH3, and PrtH4) (Broadbent *et al.*, 2013). Not only does each strain express a different combination of proteinases, but the expression of those CEPs is dependent on the strain's growth medium and the protein/peptide concentration within their growth environment.

Various genomic and microarray experiments have provided evidence of upregulation of particular genes that encode for CEPs, as well as genes for other proteins involved in peptide transport and degradation, in milk (high in protein, low in amino acids) as compared to the complex amino acid rich MRS broth typically used for the propagation of lactobacilli, or even in the gut (O'Sullivan *et al*., 2009; Smeianov *et al.*, 2007) An increase in growth rate is also observed when *Lactobacillus* species are grown in a medium with additional supplementation of casein. The increase in substrate concentration increases the expression of proteinase-encoding genes, leading to quicker protein degradation, and rapid growth. The opposite is observed when the growth medium is supplemented with an excess of peptides or casein hydrolysate. Since the organism is provided with the oligopeptides and amino acids it requires, the expression of *prt* genes, and the growth rate decreases (Wakai *et al*., 2012; Hebert *et al*., 2000). Although the proteolytic system of *Lactobacillus* is quite efficient at liberating peptides from larger milk proteins, according to Foucaud & Juillard (2000), only approximately 25% of those peptides are transported into the cell, leaving the rest to be found in the extracellular environment.

1.3.2.2 Peptide Transport Systems

Peptides that are required to supply the cells with the necessary amino acids are transported into the cell through specific peptide transport systems, the most common being the oligopeptide Opp transport system. The Opp transporter is a pentameric protein complex consisting of a substrate-binding lipoprotein (OppA), two integral membrane proteins (OppB and OppC), and two membrane-bound cytoplasmic adenosine triphosphate (ATP)-binding subunits (OppD and OppF) (Beganovic *et al.*, 2013). The sequence of *L. helveticus* DPC4571 revealed genes encoding for the Opp transport system, but also for the

di- and tri-peptide transport systems, Dpp and DtpT, respectively (Callanan *et al.*, 2008). Other *L. helveticus* strains, such as H10, have genes that encode for only two of the three transport systems, Opp and DtpT (Zhao *et al.*, 2011). Each *Lactobacillus* species expresses substrate specific transport systems, however a preference for hydrophobic basic peptide substrates has been observed (Juillard *et al.*, 1998). Once transported into the cell, the peptides are then further broken down by intracellular peptidases to release their individual amino acids.

1.3.2.3 Intracellular Peptidases

A variety of aminopeptidases (pepN, pepR, pepT, pepT2) and endopeptidases (pepE, pepO, pepO2) have been identified in *Lactobacillus* species when grown in milk (Savijoki *et al.*, 2006). Additional peptidases were found to be present when grown in both milk and MRS broth including pepC, pepD, pepF (Smeianov *et al.*, 2007). Broadbent *et al.* (2011) identified upwards of 20 peptidase genes encoded by *L. helveticus* CNRZ32. A large variety of peptidases are encoded by different strains of *L. helveticus* and their expression may be dependent on the environment in which they are grown (Wakai *et al.*, 2013). It was observed that particular antihypertensive peptides, previously known to be produced by certain LAB species, were not released from casein cleaved by a purified CEP from *L. helveticus* (Yamamoto *et al.*, 1993).

Various peptidases such as pepR in *L. helveticus* and *L. rhamnosus* have been specifically identified as playing a crucial role in the formation of bioactive peptides from longer peptide sequences (Shao *et al.*, 1997; Varmanen *et al.*, 1998). Ueno *et al.* (2004) also identified a peptidase in another *helveticus* strain responsible for the cleavage of oligopeptides to form the antihypertensive peptides, VPP and IPP.

1.3.3 Bioactive Peptides

Dairy products fermented with LAB have been shown to reduce the risk of CVD; therefore the elucidation of their mechanism of bioactivity is of great interest (Lamarche, 2008; Sonestedt *et al.*, 2011). Various *Lactobacillus* species are used in the fermentation of milk products, each with a unique set of enzymes that aid in the release of bioactive components from the milk proteins. Many dietary proteins have been found to contain encrypted functional peptide sequences that are released during the gastrointestinal breakdown of these proteins or by microbial proteolysis during food processing. These peptides, of approximately 2 to 20 amino acids long, are thought to provide benefits to the cardiovascular, immune, digestive, and nervous systems (Meisel, 1997; Tidona *et al.*, 2009; Korhonen and Pihlanto, 2006). Following digestion, peptides can either be absorbed through the intestine to enter the bloodstream intact, causing systemic effects, or can interact directly with intestinal cells producing local effects in the GI tract (Erdmann, 2008). Increased research surrounding functional foods and dietary approaches may lead to cost effective alternatives for lowering the risk of CVD and other chronic diseases.

Bioactive peptides can be found in a variety of dietary products and other animal or plant sources, however dairy products have the most abundant source of food-derived bioactive peptides (Moller *et al.*, 2008). Bioactive peptides can be released from larger proteins through enzymatic proteolysis, gastrointestinal digestion or during food processing (Kohornen and Pihlanto, 2006; Aluko, 2015). Milk and fermented dairy products are rich in larger proteins, such as caseins, which act as a substrate for the proteolytic enzymes of LAB in order to release bioactive peptides. This results in a higher concentration of peptides and amino acids in fermented milks, compared to non-fermented

milks. Bioactive peptides released from the whey fraction have also been identified (reviewed in Marshall *et al*., 2004), however this study focuses predominantly on casein-derived bioactive peptides. The release of bioactive peptides from milk proteins is largely dependent on both fermentation time as well as on the bacterial strain for fermentation (Jensen, 2009). These casein-derived bioactive peptides have a wide variety of activities including antimicrobial, antihypertensive, antioxidant, and immunomodulatory properties (Tidona *et al.*, 2009; Silva and Malcata, 2005). The two bioactive properties of interest in this study were the antihypertensive and immunomodulating properties of casein-derived peptides.

1.3.3.1 Antihypertensive Peptides

Of the knowledge surrounding bioactive peptides, those with antihypertensive properties, along with immunomodulating peptides, are most favourable for the incorporation into food products to provide a particular health benefit (Meisel & Bockelmann, 1999). Most peptides classified as being antihypertensive elicit their effect through the inhibition of the angiotensin converting enzyme (ACE). ACE, a carboxy-terminal dipeptidyl exopeptidase, is a part of the renin angiotensin system (RAS) and plays an important role in the regulation of blood pressure by cleaving the carboxy-terminal His-Leu of the decapeptide angiotensin I to form the octapeptide angiotensin II, a vasopressor (Unger, 2002; Turner and Hooper, 2002). ACE also inhibits the synthesis of the vasodilator, bradykinin, collectively increasing blood pressure (Figure 2A). ACE is located on the surface of vascular endothelial cells in multiple organs including the brain, heart, lungs, and intestine (Turner and Hooper, 2002). Human somatic ACE (~ 140 kDa) contains two functionally active domains; the C-terminal domain is typically involved in blood

pressure regulation and the N-terminal domain is involved in hematopoietic stem cell differentiation and proliferation (Phelan and Kerins, 2011). Inhibitors of ACE are frequently used to reduce blood pressure in hypertensive individuals. Natesh *et al.* first published the crystal structure of the human testicular ACE enzyme in 2003, complexed to one of the most commonly used synthetic inhibitors, lisinopril. ACE inhibitors bind to both catalytic domains of the enzyme but have greater affinity for, and inhibitory activity against, the C-terminal domain (Natesh *et al.*, 2003). Recent studies have demonstrated the strong influence of peptide sequence and structure on ACE-inhibitory activity. Binding of inhibitors to ACE seems to be highly dependent on the C-terminal tripeptide sequence of the substrate (FitzGerald *et al.*, 2000). Early on, Cheung *et al.* (1980) demonstrated the importance of the C-terminal dipeptide residues and suggested that ACE preferred substrates with more hydrophobic residues at the C-terminal positions with peptides containing C-terminal tryptophan, tyrosine, phenylalanine, and proline residues being most effective at binding the enzyme. Inhibitors of ACE will prevent production of the vasopressor, angiotensin I and inhibit the degradation of bradykinin, collectively resulting in reduced blood pressure (Figure 2B).

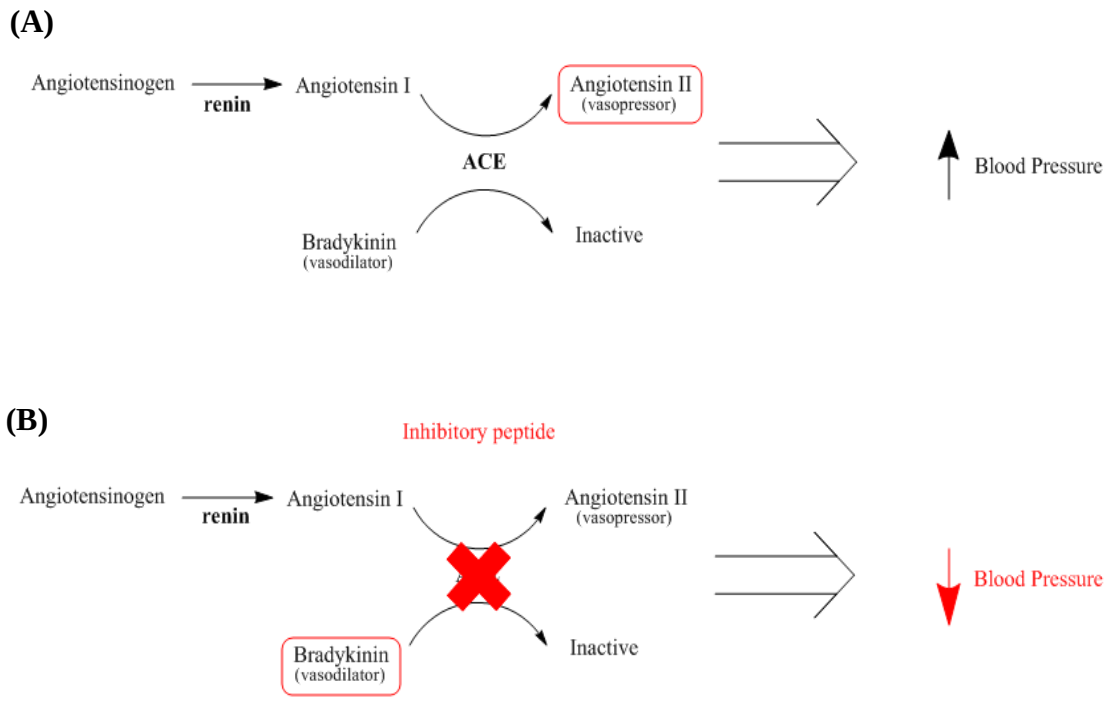


Figure 2 - Function of angiotensin I converting enzyme (ACE) in the renin angiotensin system (RAS) and its role in the regulation of blood pressure (A); The role of ACE-inhibiting peptides to prevent the production of angiotensin II and the metabolism of bradykinin to reduce blood pressure (B).

The ACE inhibition of various peptides and fermented milk products *in vitro* has been studied quite extensively. ACE inhibitory properties of peptides can be measured *in vitro* using a hippuryl-L-histidyl-L-leucine (HHL) substrate which is cleaved by ACE to form hippuric acid and L-histidyl-L-leucine. Liberated hippuric acid can be measured spectrophotometrically as an indication of ACE activity (Cushman and Cheung, 1971). Two previously identified ACE inhibiting peptides, VPP and IPP, have been shown to reduce blood pressure in spontaneously hypertensive rats (SHR) as well as in hypertensive human subjects with an IC_{50} of 5 and 9 μM, respectively (Nakamura *et al.*, 1995a; Yamamoto *et al.*, 1994). However, apart from these two tripeptides, many antihypertensive peptides have been identified and characterized in *in vitro* experiments, but have not been

able to retain ACE-inhibitory activity *in vivo* (Erdmann *et al.*, 2008). The difficulty in establishing a relationship between the two is a result of two main factors: the bioavailability of these peptides in circulation has yet to be fully investigated, and peptides which show bioactivity *in vitro* may undergo proteolytic digestion in the gastrointestinal tract, rendering them inactive. The opposite can also occur in which peptides which show no bioactivity *in vitro* can be cleaved during digestion to form a bioactive peptide with antihypertensive properties. This can make it difficult to identify and characterize peptides with antihypertensive properties both *in vitro* and *in vivo*. Manso *et al.* (2003) and Savoie *et al.* (2005) showed that IPP could be liberated from casein proteins in the gastrointestinal (GI) tract by simulating GI fluids using the digestive enzymes trypsin, pancreatin and pepsin. As mentioned previously, the binding of a substrate to ACE is highly dependent on its amino acid sequence and secondary structure; the same has been found for the stability of a peptide *in vivo*. Through multiple GI digestion studies, Mizuno *et al.* (2004) were able to show that some peptides, particularly those containing C-terminal Pro and Pro-Pro residues, were stable under a simulated gastrointestinal environment. Interestingly, C-terminal proline residues were also shown to enhance bioavailability of peptides (Vermeirssen, 2004). Foltz *et al*. (2007) were able to detect significantly increased levels of both IPP (897 ± 157 pmol/L) and LPP (152 ± 85 pmol/L), compared to the placebo control (555 ± 80 pmol/L and 96 ± 34 pmol/L, respectively) in the plasma of individuals who consumed a lactotripeptide (LTP)-enriched yogurt beverage containing eight ACE inhibitory peptides, including IPP. Jauhiainen *et al.* (2007) also found evidence for the absorption of IPP from the GI tract into the bloodstream after a single oral dose by using radiolabelled tripeptides to visualize their absorption, distribution and excretion. Many

clinical studies have also demonstrated the hypotensive effects of treatments with lactotripeptides; these studies have reported decreases in SBP of 3 mmHg (de Leeuw *et al.,* 2009), 6 mmHg (Turpeinen *et al.*, 2009), up to 16 mmHg (Nakamura *et al.*, 2009).

Milk fermented with *L. helveticus* has also been shown to have antihypertensive properties, and higher ACE-inhibitory and proteinase activity than milks fermented with other LAB species (Yamamoto *et al*., 1994). These fermented milks were also found to contain the VPP and IPP peptides. When milk was fermented with a proteinase-deficient variant of the same *L. helveticus* strain, the antihypertensive effects were no longer observed. It is therefore presumed that these particular ACE-inhibiting peptides are produced as a result of the proteolytic activity of *L. helveticus* (Yamamoto *et al.*, 1994). Interestingly, pure tripeptides seem to have less of an antihypertensive effect than the milk products containing them (Sipola *et al.*, 2001; Jauhiainen *et al.*, 2005). It was thought that minerals such as calcium and potassium present in the milk may contribute to the bioactivity, however Jauhiainen *et al.* (2005) found that minerals alone were not able to attenuate the increase in blood pressure as effectively as fermented milk products. These products may, however, help to improve the bioavailability of these peptides compared to administering them in other media (Jakala, 2010). VPP and IPP have already been incorporated into food products in certain countries around the world and are currently available to the public. Calpis (by Calpis Co., Japan) is a commercially available soft drink product that consists of milk fermented with *L. helveticus* CP790 and *S. cerevisiae* containing VPP and IPP. Evolus (by Valio, Finland) is another milk product, fermented with *L. helveticus* LBK-16H containing IPP. C12 (by DMV International, The Netherlands), another available product, is a casein hydrolysate that contains the peptide

FFVAPFEVFGK. All of these products have been shown to have antihypertensive effects in spontaneously hypertensive rats as well as in mildly hypertensive individuals (Tidona *et al.*, 2009; FitzGerald *et al.*, 2004).

The proteolytic mechanisms responsible for the activation of many antihypertensive peptides still remain unknown; however it has been suggested that they may require further activation after entering the digestive tract. Maeno *et al.* (1996) isolated a heptapeptide (KVLPVPQ) from casein hydrolyzed by purified CEPs. The heptapeptide showed little ACE-inhibitory activity, however after digestion with pancreatin, a hexapeptide was generated (KVLPVP) with high ACE-inhibitory activity. Although many of these peptides are characterized as antihypertensive due to their ACE-inhibitory activity, that is only one of many mechanisms of bio-activation responsible for the antihypertensive properties of milk-derived peptides. While recent clinical studies, such as those described above, report hypotensive effects by treatment with lactotripeptides, many clinical studies have failed to show ACE inhibiting effects with these putative antihypertensive peptides (Wuerzner *et al.*, 2009; Engberink *et al.*, 2008) therefore there must be additional mechanisms responsible for the blood pressure lowering effects observed (Jakala, 2010). Other studies have also found treatment with lactotripeptide-containing products results in either no significant effect on SBP or DBP, or in only short-term reduction of blood pressure (2 mmHg) (Engberink *et al.*, 2008; van der Zander *et al.*, 2008). Although the results of *in vitro* experiments regarding the benefits of antihypertensive peptides are quite consistent, *in vivo* results, including human experiments, are more contradictory. Outcomes of these studies seem to be highly dependent on dosage as well as duration of treatment (Nakamura *et al.*, 1995b). This suggests a need for more standardized methodology and

investigative methods regarding antihypertensive effects of peptide-containing products on humans.

Antihypertensive peptides studied *in vivo* also have little to no effect on normotensive subjects, meaning they do not exert an acute hypotensive effect (Erdmann, 2008; FitzGerald *et al*., 2004). Therefore, while ACE inhibitory peptides cannot be administered as a solitary treatment to individuals with mild hypertension as a low cost alternative to synthetic drugs, they could potentially be used as a preventive measure to decrease the risk of developing hypertension.

1.3.3.2 Immunomodulatory Peptides

Lactobacilli, as well as their products secreted during fermentation, have a large impact on the host immune system and can activate both the innate and, indirectly, the adaptive immune systems by binding to pattern recognition receptors (PRRs) which recognize conserved molecular structures of bacteria (or viruses) known as microbe associated molecular patterns (MAMPs), a term recently adapted from the previously used pathogen-associated molecular patterns (PAMPs) since not all of these organisms are pathogenic (Wells, 2011). PRRs include nucleotide oligomerization domain (NOD)-like receptors (NLRs) and the highly characterized toll-like receptors (TLRs). There are 10 known TLRs in humans and 12 in mice (Kawai, 2010). Human TLRs 1, 2, 4, 5, 6, and 11 are expressed on the surface of immune cells and recognize mainly microbial membrane components. TLR4, specifically, is responsible for the recognition of bacterial lipopolysaccharide (LPS), with the help of LPS-binding protein and CD14. These interactions will initiate signal transduction by recruiting intracellular adaptor proteins such as myeloid differentiation primary response gene 88 (Myd88), toll-interleukin 1 receptor

(TIR) domain-containing receptor (TIRAP) and TIR domain-containing adaptor-inducing interferon β (TRIF) leading to the activation of mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB is a transcription factor that, when inactivated, is found in the cytosol bound to the inhibitory protein IκBα. TLR signaling pathways lead to the phosphorylation of IκBα, allowing NF-κB to translocate into the nucleus, inducing the transcription of multiple pro-inflammatory genes (Figure 3). In intestinal epithelial cells (IECs), activated NF-κB can lead to the production of chemokines and cytokines including various interleukin proteins and tumor necrosis factors.

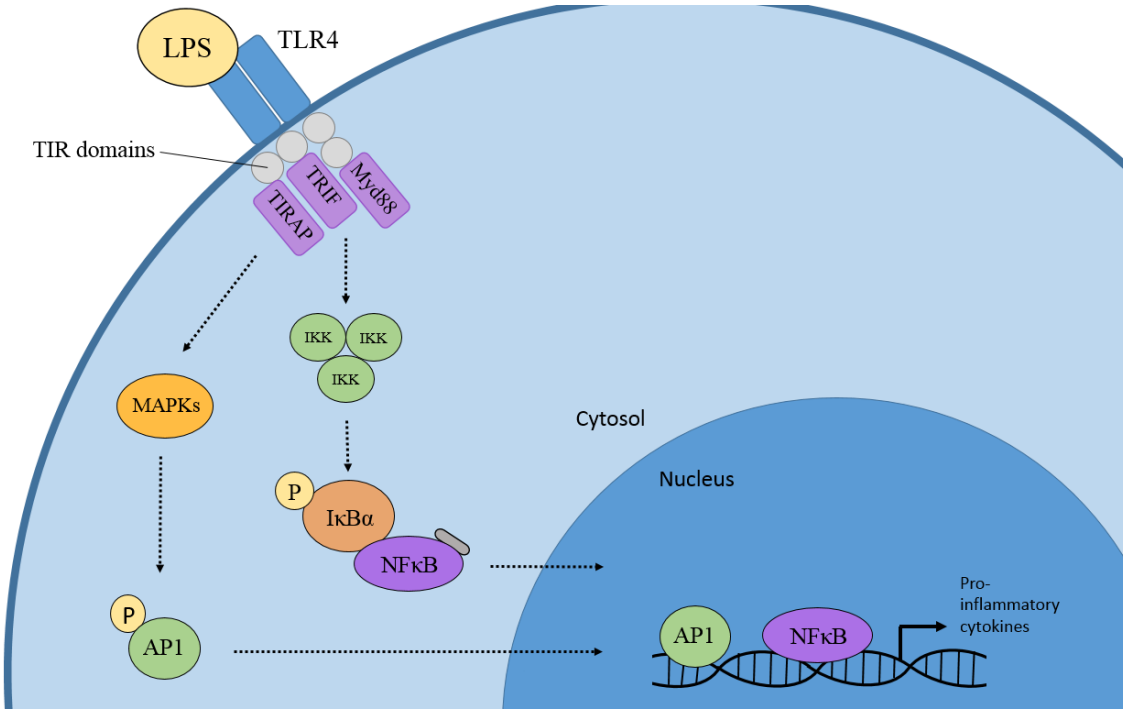


Figure 3 – Simplified scheme of TLR4 signaling pathway leading to the production of pro-inflammatory cytokines.

Some probiotics can suppress intestinal inflammation by down-regulating the expression of TLRs, inhibiting TNF-α via secreted metabolites, and inhibiting NF-κB signaling in enterocytes (Gomez-Llorente *et al.*, 2003). Certain cell wall components of lactobacilli can potentially signal through binding to TLR2. TLR2 is thought to interact with lipoproteins as well as lipoteichoic acids on the surface of gram-positive bacteria (Well *et al*., 2011).

Many *in vitro* experiments have been conducted to assess the immunomodulation of lactobacilli on various types of immune cells including human monocyte derived dendritic cells, and human peripheral blood mononuclear cells (PBMCs). Vissers *et al.* (2010) and Christensen *et al.* (2002) have both confirmed the ability of lactobacilli to modulate cytokine production in human PBMCs and DCs and have demonstrated that cytokine production and immune response to lactobacilli can vary greatly between both species and strain when analyzing for induction of IL-10, IL-12, IL-1β, IL-6 and TNF-α.

IL-10 production is typically measured when assessing immunomodulation by probiotics or bioactive peptides because it is an anti-inflammatory cytokine and suppresses IL-12 production and, consequently, IFN-γ production, favoring a regulatory immune response (Wells, 2011). Foligne *et al.* (2007) provide evidence that immune profiles observed *in vitro* (especially IL-10 and IL-12) can in fact be predictive of their immunomodulatory properties *in vivo*.

In vivo experiments in which mice or rats were fed with milk fermented with *L. helveticus* showed both suppression and activation of the immune system. Some argue that activation of the immune system would protect the host against infection. Many studies look for lactobacilli or LAB-derived peptides that stimulate an immune response

suggesting it confers a protective advantage to the host (LeBlanc *et al.*, 2002; Tellez *et al.*, 2010; Vinderola *et al.*, 2007). Jensen *et al.* (2015) reported an increase in cytokine production and NFκB activation in THP-1 monocytes in response to *L. reuteri*; they report *L. rhamnosus* GG along with *L. plantarum* having the lowest immunostimulating effects. However inducing an inflammatory response could also have potentially detrimental effects to other body systems. Therefore, in this thesis, we aimed to look for peptides that would decrease the production of inflammatory cytokines, and increase that of regulatory cytokines as an indication of beneficial immunomodulating properties. Although there is a lack of correlation between the *in vitro* and *in vivo* response of many lactobacilli strains, certain studies have identified certain strains that are able to induce high levels of IL-10 and low levels of IL-12 or TNF-α, simulating a favorable regulatory response, in immune cells and that can provide significant immune protection in 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice (Foligne *et al.*, 2007; Peran *et al.*, 2005).

Non-bacterial components present in milks fermented with these probiotic organisms may also play a role in the immunomodulating effects of fermented dairy products. Vinderola *et al.* (2007) investigated the non-bacterial fractions of milk fermented with *L. helveticus* R389 and their effect on cytokine production in IECs, the gut lamina propria, and in the lumen of BALB/c mice. Although they were looking for immune stimulation and observed an induction of IL-6, a pro-inflammatory cytokine, this still demonstrates the need to assess the immune effects of both the bacterial and non-bacterial components of fermented dairy products. Although many different mechanisms have been proposed, the mechanisms responsible for *in vivo* immunomodulation by probiotics are not yet known and therefore investigating the interaction of these bacteria and bacterial

components with a variety of immune cells can lead us to better understand how they might confer the observed benefits, in hopes of using the administration of probiotics to treat a variety of inflammatory diseases.

By investigating the immunomodulatory effects of these bioactive peptides liberated by lactic acid bacteria during the production of fermented dairy products in combination with their ACE inhibitory properties we can identify additional benefits to these antihypertensive peptides and further elucidate their interactions within the body system. The lowering of blood pressure through the inhibition of ACE can help to reduce the risk of developing many cardiovascular diseases. However, the onset of atherosclerosis in particular is highly dependent on and regulated by the immune system and therefore a better understanding is required of the interactions between probiotics or bioactive peptides and our immune cells. Therefore, by investigating the relationship between the ability of casein-derived peptides found in fermented dairy products to reduce hypertension and the role that they play in regulating the immune system, we can further improve upon the use of these probiotic organisms and their secreted products in the food industry to confer the maximal health benefits to Canadian consumers.

2 METHODOLOGY

2.1 Materials

All reagents used were purchased from Sigma Aldrich Co. (St. Louis, MO, USA) unless otherwise stated.

2.2 Bacterial and Cell Culture Growth Conditions

The two strains of lactic acid bacteria used throughout this project, *Lactobacillus helveticus* R0389 and *Lactobacillus rhamnosus* R0011, were obtained as a frozen stock, kept at -80^{o}C, from the Green-Johnson Lab at UOIT. Both strains were acquired from Lallemand-Institut Rosell (Montreal, QC, Canada). For the entirety of this project, both strains were grown in Difco^{TM} de Man Rogosa Sharpe (MRS) broth (BD Diagnostic Systems, Sparks, MD, USA) at 37^{o}C under agitation (200 – 220 rpm) unless otherwise stated.

The THP-1 human monocytic cell line (ATCC #TIB-202) was used for immunological studies. THP-1 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 growth medium (supplemented with 10% fetal bovine serum (FBS) or calf serum, 0.05 mg/mL gentamicin, and 0.05 mM β-mercaptoethanol) at 37^{o}C with 5% CO_2. Cells were sub-cultured every 3 to 4 days.

2.3 Growth Analysis & Kinetics

Growth curves of both *Lactobacillus* strains were performed and their growth rate and doubling times were calculated when grown in different media. MRS medium was first supplemented with 0.1% (w/v) casein in order to assess any change in growth rate and subsequently compare the peptide profiles of each strain to cultures grown in MRS. Casein is one the major substrates of the proteolytic systems of LAB when used in the fermentation

industry and we therefore hypothesized that casein supplementation would increase growth rate and increase peptide production during growth. A second supplementation with 0.01% sodium formate (NaCOOH) was performed. *Streptococcus thermophilus* and *Lactobacillus bulgaricus* are commonly used as a mixed-culture in fermentation and are thought to stimulate each other's growth. *S. thermophilus* provides *L. bulgaricus* with formic acid, folic acid and carbon dioxide (Sieuwarts *et al*., 2010). We therefore investigated the potential of sodium formate supplementation to stimulate the growth of *L. helveticus* R0389 and *L. rhamnosus* R0011. Overnight cultures (50 mL) of *L. helveticus* R0389 and *L. rhamnosus* R0011 were centrifuged at 3,000 *x g* for 20 minutes at room temperature. The supernatant was discarded and cells were re-suspended in 10 mL of either MRS, RPMI, MRS supplemented with 0.1% w/v casein (BioShop, Burlington, ON, Canada), or MRS supplemented with 0.01% w/v sodium formate (Sigma). Cells re-suspended in RPMI were washed twice in RPMI prior to re-suspension. Cultures were then diluted 1:20 in fresh medium to an approximate starting OD_{600nm} of 0.2. Two hundred microliters of the diluted cultures were transferred into each well in one row of a 96-well plate. In addition, 200 µL of each medium was added to each well of one row of a 96-well plate to serve as a control. The 96-well plates, along with the 1:20 diluted liquid cultures, were incubated at 37°C under agitation (200-220 rpm) for 24 hours. OD_{600nm} measurements were recorded every 30 minutes for 24 hours using an xMark™ microplate spectrophotometer (Bio-Rad, Mississauga, ON, Canada) and viable counts were determined at various time points throughout the 24 hour growth period by the spread plate method on MRS agar. Spread plates were incubated at 37°C and colony forming units (CFUs) were counted after 3 days and the CFUs were calculated using the following equation:

CFU = (Average # of colonies from each plate) x (reciprocal of dilution) x (reciprocal of plated dilution)

The growth rate and doubling times were calculated for each strain grown in MRS, MRS supplemented with 0.1% (w/v) casein, and MRS supplemented with 0.01% (w/v) sodium formate. The growth rate constant (μ) was calculated using the following equation:

$$\ln(N_t) - \ln(N_0) = \mu(t - t_0)$$

Where N_0 and N_t are the OD_{600nm} values of the culture at two time points, t_0 and t respectively, in the log phase of growth. For our calculations, t_0 = 1 hour and t = 3 hours. The doubling time (g) of each culture was calculated using the following equation:

$$g = ((t - t_0) / (\ln 2)/\mu)$$

2.4 Fermentation

Following growth rate analysis in complex MRS medium, *Lactobacillus* strains were used to ferment milk and their growth and peptide production was assessed. Overnight cultures (50 mL) of *L. helveticus* R0389 and *L. rhamnosus* R0011 in MRS were centrifuged at 3,000 x *g* for 20 minutes at room temperature. The supernatant was discarded and cells were re-suspended in an equal volume of Neilson® 3.25% homogenized milk. Re-suspended cells were added at 16.7% (v/v) to fresh 3.25% homogenized milk. Acidified controls were prepared by adding 0.4% (v/v) D/L-lactic acid to fresh 3.25% milk. Non-acidified controls consisted of 3.25% milk subjected to identical conditions as fermenting samples. All samples and controls were incubated at 37°C under agitation (200 – 220 rpm) for 3, 4, 5, or 6 days.

2.5 Isolation and Collection of Secreted Peptides

2.5.1 Peptides Secreted During Growth in MRS

Overnight cultures of *Lactobacillus helveticus* R0389 and *Lactobacillus rhamnosus* R0011 grown in MRS were centrifuged at 3,000 x *g* for 20 minutes at room temperature and cells were re-suspended in either MRS or MRS supplemented with 0.1% (w/v) casein (BioShop) and diluted to a starting OD_{600nm} of approximately 0.3. After 6, 12, or 24 hours of growth, cells were centrifuged and the cell-free supernatant was collected. To precipitate proteins and peptides, two volumes of ice cold acetone were added to the supernatant solutions. Precipitated supernatant solutions were centrifuged at 3,000 x *g* for 15 minutes at 4°C. The supernatant was discarded and protein pellets were dried and re-suspended in approximately two times the pellet volume with protein buffer solution composed of 25% (v/v) glycerol, 1 mM EDTA, 10 mM Tris-HCl in H_2O). Re-suspended pellets were transferred into Amicon centrifugal filter units (Millipore) with a molecular weight cut-off (MWCO) of either 3 or 10 kDa and centrifuged at 3,000 x *g* for 30 minutes at 4°C. The filtrate was collected and stored in 1.5 mL microcentrifuge tubes at -20°C for further analysis. Filtered protein solutions were quantified via Bradford assay (Bradford, 1976) using bovine serum albumin (BSA, Sigma Aldrich) as a standard. OD_{600nm} and pH measurements of cultures grown in MRS or MRS supplemented with 0.1% casein were recorded at 1, 6, 12, and 24 hours during growth.

2.5.2 Peptides Secreted During Growth in Milk

Inoculated milk samples (prepared as described above) were centrifuged after 3, 4, 5, or 6 days of fermentation at 3,000 x *g* for 20 minutes at 4°C. The supernatant was collected in fresh 50 mL screw capped tubes. To the supernatant solutions, approximately

two volumes of ice cold acetone were added, and samples were allowed to precipitate overnight at -20^{o}C. Precipitated supernatant solutions were centrifuged at 3,000 *x g* for 15 minutes at 4^{o}C and protein pellets were re-suspended in protein buffer solution. Re-suspended pellets were then filtered (<10 kDa) as described above and quantified via Bradford assay (Bradford, 1976) using BSA as a standard.

2.6 Peptide Separation

2.6.1 Protein Gel Electrophoresis

Quantified, filtered and unfiltered supernatant solutions were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a pre-cast Mini-PROTEAN® TGX™ 4-20% tris-glycine or a pre-cast Mini-PROTEAN® 16.5% or 10-20% Tris-Tricine polyacrylamide gel (Bio-Rad).

Sample buffer (5 X) for glycine gels consisting of 60 mM Tris-HCl (pH 6.8), 25% (v/v) glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, and 0.1% bromophenol blue in H_2O and was used to resuspend 5 µg of supernatant protein sample. Ten microliters of protein sample in sample buffer were loaded into each sample well of the gel. Empty sample lanes were loaded with 10 µL of sample buffer and 0.5 or 1 µL of protein ladder was loaded into the first and last lanes of the gel. For glycine gels, the Precision Plus Protein™ Unstained Standard was used; molecular weight marker proteins include a mixture of ten *Strep*-tagged recombinant proteins (10 – 250 kDa). A running buffer consisting of 25 mM Tris, 192 mM glycine and 0.1% SDS was used and the gel was run at 125 V for approximately 1 hour.

Sample buffer (1X) for tricine gels was prepared by adding 20 µL of β-mercaptoethanol to 980 µL of 10X sample buffer consisting of 200 mM Tris-HCl (pH 6.8), 40% glycerol, 2% SDS, and 0.04% Coomassie Blue and was used to dissolve 5 µg of

filtered (< 10 kDa) supernatant protein sample. Polypeptide SDS-PAGE standards (Bio-Rad), a mixture of six proteins, was diluted 1:20 in tricine sample buffer. Standards and sample solutions in sample buffer were then heated to 95^{o}C for 5 minutes. Ten microliters of protein sample, and either 5 or 10 µL of polypeptide standard, in sample buffer were loaded into each well of the gel. Empty sample lanes were loaded with 10 µL of sample buffer. A running buffer consisting of 100 mM Tris (pH 8.3), 100 mM tricine, and 0.1% SDS was used and the gel was run at 100 V for approximately 2 hours.

Protein gels were either stained using silver (Celis *et al.*, 2006) or with Coomassie Blue (Meyer and Lambert, 1965). For silver staining, gels were left overnight in a fixative solution of 50% ethanol, 12% acetic acid, and 0.05% formaldehyde in water. Gels were then washed with 20% ethanol and submerged in a sensitizing solution comprised of 0.02% (w/v) sodium thiosulfate ($Na_2S_2O_3$) in water for 2 minutes. Gels were washed in deionized water and submerged in a cold staining solution comprised of 0.2% (w/v) silver nitrate ($AgNO_3$) and 0.076% (v/v) formaldehyde in water, in the dark, shaking at 4^{o}C for 20 minutes. Gels were once again washed with deionized water and briefly rinsed with a developing solution comprised of 6% (w/v) sodium carbonate (Na_2CO_3), 0.0004% (w/v) sodium thiosulfate ($Na_2S_2O_3$), and 0.05% formaldehyde in water until protein bands are developed. The reaction was terminated by the addition of 12% acetic acid. For Coomassie staining, gels were submerged in 0.1% (w/v) Coomassie G-250, 40% (v/v) methanol, 10% (v/v) acetic acid in water for one hour and then in the de-staining solution comprised of 5% (v/v) methanol, 7% (v/v) acetic acid in water overnight. Stained protein gels, illuminated on a light box, were photographed using a Canon Rebel T1i camera.

2.6.2 Thin Layer Chromatography (TLC)

To accompany the protein gel electrophoresis results, supernatant protein samples were also separated by thin layer chromatography (TLC). Acetone precipitated, filtered (< 10 kDa) supernatant solutions were spotted onto either silica or cellulose TLC plates (Analtech, Newark, DE, USA). Both mobile and stationary phases were altered to optimize separation (See Table 4 in Results). All TLC plates were stained with ninhydrin spray reagent (Sigma Aldrich).

2.6.3 High Performance Liquid Chromatography (HPLC)

Since any peptides present in the cell-free supernatant solution are likely present in very low concentrations, separation via high performance liquid chromatography was performed in the hopes of obtaining improved resolution and separation of peptide components in supernatants of *Lactobacillus* cultures grown in MRS and 3.25% milk. Acetone precipitated, filtered (< 3 or 10 kDa) supernatant solutions from MRS medium grown cultures or milk ferments of both *L. helveticus* R0389 and *L. rhamnosus* R0011 were filtered through Millex GV 0.22 µM, 13 mm filter unit (Millipore) into 1.5 mL Prominence HPLC vials (Shimadzu, Laval, QC, Canada). An acetylated decapeptide standard mixture (Ac-RGXXGLGLGK, where XX is GG,AG,VG,VV or AG) (Thermo Scientific, Rockford, IL, USA) and a non-acetylated peptide standard mixture containing equal masses of each of GY, VYV, YGGFM, YGGFL, DRVYIHPF (Sigma Aldrich) were also analyzed by HPLC to aid in the optimization of chromatographic conditions for sample solutions. All samples (10 µL injection volume) were analyzed on a Shimadzu Prominence HPLC equipped with an apHera^{TM} C18 Polymer column (15 cm x 4.6 mm x 5 µM) and a photodiode array (PDA) detector using a mobile phase of acetonitrile with 0.25%

trifluoroacetic acid (TFA) at a flow rate of 0.8 mL/min. Multiple run conditions were tested in order to obtain optimal separation (Table 1)

Table 1 – Solvent gradient and run time of HPLC experiments conducted

Solvent Gradient (Acetonitrile + 0.25% TFA)	**Run Time (minutes)**	**Flow Rate***
30%	20	N/A
20 to 70%	60	+10% / 10 min
70 to 20%	60	-10% / 10 min
90 to 30%	35	-10% / 5 min
80 to 30%	100	-10% / 20 min
5 to 30%	60	+5% / 10 min
70 to 30%	25	-10% / 5 min
30 to 70%	25	+10% / 5 min
90 to 30%	12	-10% / 2 min
30 to 50%	20	+5% / 5 min
30 to 90%	60	+10% / 10 min
30 to 80%	10	+10% / 2 min
80 to 30%	10	-10% / 2 min
70 to 50%	25	-2% / 2 min + 5 min

*The change in solvent (% change in acetonitrile) over the listed time increments

All HPLC data was analyzed using the LCsolution software by Shimadzu Scientific Instruments.

2.7 Synthesized Peptides

A list of previously identified (and published) bioactive peptides derived from the sequence of β-casein, liberated either through microbial fermentation or by enzymatic digestion, possessing a variety of bioactive properties was compiled. Twelve of the previously identified peptides were selected to be synthesized and assessed for their antihypertensive and immunomodulatory properties. The twelve peptides included the highly characterized and studied antihypertensive VPP and IPP. The remaining ten peptides had either been identified as part of a larger bioactive fraction and their bioactivities had not been individually investigated, or had been assessed for either

antihypertensive or immunomodulatory effects (or other bioactive properties) but not both. Synthesized peptides were obtained from Bio Basic Inc. (Markham, ON, Canada) (Table 2). Stock solutions (4.8 mg/mL) of synthesized peptides were prepared by dissolving peptides in 500 µL of sterile, deionized H_2O. From these, working peptide solutions of 19 µg/mL were then prepared and used in order to assess inhibition of the angiotensin converting enzyme (ACE) and to treat THP-1s at varying concentrations (See APPENDIX A for peptide structures and properties).

Table 2 – Synthesized peptide sequences and origin

ID#	Peptide Sequence	Protein Origin	MW (g/mol)	Reference
P1	VPP	β-CN (84-86)	311.38	Nakamura *et al.*, 1995a
P2	IPP	β-CN (74-76)	325.41	
P3	HQPHQPLPPTVMFPPQ	β-CN (145-160)	1851.18	Tellez *et al*., 2010
P4	HQPHQPLPPT	β-CN (145-154)	1151.3	
P5	WMHQPHQPLPPT	β-CN (143-154)	1468.71	
P6	LYQEPVLGPVR	β-CN (192-202)	1270.51	
P7	LDQWLCEK	β-CN (115-122)	1034.2	
P8	YP	α_{s1}-CN, β-CN, κ-CN	278.31	Yamamoto *et al.*, 1999
P9	PGPIPN	β-CN (63-68)	593.69	Boutrou *et al*., 2013
P10	FFVAP	α_{s1}-CN (23-27)	579.7	Yamamoto *et al.*, 1997
P11	KVLPVP	β-CN (169-174)	651.85	Maeno *et al.*, 1996
P12	KVLPVPQ	β-CN (169-175)	779.99	

2.8 Angiotensin Converting Enzyme (ACE) Inhibition Assay

ACE inhibitory activity was assessed using the method of Cushman and Cheung (1971). The hippuryl-L-histidyl-L-leucine (HHL) substrate and angiotensin converting enzyme (ACE) from rabbit lung were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). The HHL buffer (3.8 mM) was prepared by dissolving the substrate in 0.1 M sodium borate buffer with 0.3 M NaCl (pH 8.3). Peptides were diluted to a working concentration

of 19 µg/mL and 30 µL of 19 µg/mL peptide solution was incubated at 37ºC in 200 µL of the HHL buffer for 5 minutes. Following incubation, 20 µL of 0.1 U/mL ACE was added to the reaction and incubated at 37ºC for 30 minutes. 250 µL of 1N HCl was added to stop the reaction and 1.5 mL ethyl acetate was added to each reaction tube to extract the liberated hippuric acid. Samples were centrifuged at 800 x *g* for 15 min and 300 µL of the top organic layer was transferred to a fresh microcentrifuge tube and placed in a water bath at 65ºC to evaporate the ethyl acetate. The extracted hippuric acid was then dissolved in 1 mL dH_2O. Samples were transferred in triplicate to a UV transparent 96-well plate and the absorbance at 228 nm was recorded. Percent ACE inhibition was calculated using the following formula,

$$[(B - A) / (B - C)] \times 100\ \%$$

Where A is the optical density in the presence of ACE and the potential ACE inhibitor, B is the optical density without an inhibitor, and C is the optical density without ACE.

2.9 Treatment of THP-1 with Peptide Samples

In order to assess the immunomodulating effects of secreted peptides from *Lactobacillus* strains, a human monocytic cell line derived from an acute monocytic leukemia patient (THP-1) was exposed to varying concentrations of cell-free supernatant solutions filtered with molecular weight cut off (MWCO) of 3 or 10 kDa. Cells were either co-incubated with supernatant samples and lipopolysaccharide (LPS) to assess their ability to attenuate or diminish LPS-induced cytokine production, or were exposed to only the supernatant solutions (without LPS) in order to assess the cytokine profiles of THP-1 cells induced by cell-free supernatants of *Lactobacillus* cultures in a non-challenged state.

For THP-1 cell treatments, 100µL of THP-1 cells were seeded in a 96-well plate at 1 x 10^6 cells/mL and exposed to 100 µL of peptide sample diluted to various concentrations in THP-1 challenge medium (RPMI 1640 with 10% FBS) with or without 125 ng/mL of lipopolysaccharide (LPS) from *E. coli* O26:B6 (Sigma Aldrich); the final THP-1 concentration was 5 x 10^5 cells/mL. Cells stimulated with LPS were either co-treated with 125 ng/mL LPS and varying concentrations of peptide-containing sample solutions for 24 hours or were pre-incubated with the sample solution for 17 hours, followed by the addition of 125 ng/mL LPS and incubated for an additional 7 hours. Three technical replicates were performed for each treatment. Controls included no cell controls (with and without 125 ng/mL LPS), no sample controls (100 µL of 1 x 10^6 cells and 100 µL challenge medium with and without 125 ng/mL LPS). All 96-well plates were incubated for a total of 24 hours at 37°C with 5% CO_2. After 24 hours, the plate was centrifuged at 300 x *g* for 10 minutes, and the supernatant (140 µL) was transferred to a fresh 96-well plate and stored at -80°C for further analysis.

2.10 Cell Viability Assay

Cell viability of the THP-1 cells post-treatment was assessed via the 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) viability assay. After the supernatant of the treated cells was removed, 15 µL of XTT reagent (with 125 µM N-methyl dibenzopyrazine methyl sulfate (PMS)) was added to each well of the 96-well plate and the plate was incubated at 37°C with 5% CO_2 for 2 hours. After 2 hours, a Synergy HT microplate reader (Bio-Tek Instrumentation Inc., Winooski, VT, USA) was used to read the absorbance of the treated cells at 450 and 650 nm. The $\Delta OD_{650\text{-}450}$ was recorded. Cells supplemented with RPMI challenge medium (no stimulant or sample) were

designated as the blank sample (100% viable); viability of all sample treated cells were normalized to the medium control.

2.11 Measurement of Cytokine Production by Enzyme Linked Immunosorbant Assays

Production of pro-inflammatory cytokines IL-8 and IL-6, and regulatory cytokine IL-10 was measured via enzyme linked immunosorbant assays (ELISAs). All reagents and buffers were prepared according the Human CXCL8/IL-8 Kit, Human IL-10, or Human IL-6 procedures (reagent compositions listed below) (R&D, Minneapolis, MN, USA). Half well 96-well plates were used for ELISAs. The following solutions were prepared and used for all ELISAs preformed: phosphate buffered saline (PBS) comprised of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2PO_4, 1.5 mM KH_2PO_4 (pH 7.4), wash buffer comprised of 0.005% Tween 20 in PBS, 3,3',5,5'-tetramethylbenzidine (TMB) as the substrate solution and a stop solution of 2 N H_2SO_4.

2.11.1 IL-8

50 μL of 4.0 μg/mL of the mouse anti-human IL-8 capture antibody was added to each well of the 96-well plate and stored at 4°C overnight. The capture antibody was then removed and the plate was washed three times with the IL-8 wash buffer. 150 μL of blocking buffer (1% BSA in PBS) was added to each well and the plate was incubated at room temperature for 1 hour. The plate was then washed three times with the IL-8 wash buffer. Two-fold serial dilutions of recombinant human IL-8 standard were prepared in reagent diluent (0.1% BSA, 0.05% Tween 20 in PBS) to create a seven point standard curve with standard concentrations ranging from 0 to 2000 pg/mL. 50 μL of standard and sample solutions were added to corresponding wells and the plate was incubated for 2 hours at room temperature. Sample solutions (THP-1 supernatants) were diluted, often 1/5, to

obtain concentrations within range of the standard curve. The plate was then washed three times with IL-8 wash buffer. The detection antibody was prepared to a working concentration of 20 ng/mL, and 50 µL was added to each well. The plate was then incubated for another 2 hours at room temperature and then washed three times with IL-8 wash buffer. 50 µL of the diluted horseradish peroxidase (HRP)-streptavidin was added to each well and the plate was incubated for 20 minutes in the dark and then washed three times with the IL-8 wash buffer. 50 µL of the substrate solution (TMB) was then added to each well and the plate was again incubated for 20 minutes in the dark. Following the 20 minute incubation period, 50 µL of the stop solution (2N H_2SO_4) was added to each well and the absorbance was measured at 450 nm.

2.11.2 IL-10

50 µL of the Human IL-10 capture antibody was added to each well of the 96-well plate and stored at 4°C overnight. The capture antibody was then removed and the plate was washed four times with the wash buffer. 100 µL of reagent diluent (1% BSA in PBS) was added to each well and the plate was incubated at room temperature for 1 hour on a plate shaker at 200 rpm. The plate was then washed four times with the wash buffer. Two-fold serial dilutions of human IL-10 standard were prepared to create an eight point standard curve with standard concentrations ranging from 0 to 250 pg/mL. 50 µL of standard and sample solutions were added to corresponding wells and the plate was incubated for 2 hours at room temperature at 200 rpm. The plate was then washed four times with wash buffer. The detection antibody was prepared to a working concentration of 20 ng/mL, and 50 µL was added to each well. The plate was then incubated for 1 hour at room temperature (200 rpm) and then washed four times with wash buffer. 50 µL of the

diluted HRP-streptavidin was added to each well and the plate was incubated for 30 minutes at room temperature (200 rpm) and then washed four times with the wash buffer. 50 µL of the substrate solution (TMB) was then added to each well and the plate was again incubated for 30 minutes in the dark. Following the 30 minute incubation, 50 µL of the stop solution (2N H_2SO_4) was added to each well and the absorbance was measured at 450 nm.

2.11.3 IL-6

150 µL of 2.0 µg/mL mouse anti-human IL-6 capture antibody was added to each well of the 96-well plate and stored overnight at 4°C. The capture antibody was then removed and the plate was washed three times with the wash buffer. 150 µL of reagent diluent (1% BSA in PBS) was added to each well and the plate was incubated at room temperature for 1 hour. The plate was then washed three times with the wash buffer. Two-fold serial dilutions of recombinant human IL-6 standard were prepared to create an eight point standard curve with standard concentrations ranging from 0 to 600 pg/mL. 50 µL of standard and sample solutions were added to corresponding wells and the plate was incubated at room temperature for 2 hours. The plate was then washed three times with the wash buffer. The detection antibody was prepared to a working concentration of 50 ng/mL, and 50 µL was added to each well. The plate was then incubated at room temperature for 2 hours followed by three washes with the wash buffer. 50 µL of the diluted streptavidin-HRP was added to each well and the plate was incubated for 20 minutes in the dark. The plate was then washed three times with the wash buffer, 50 µL of the substrate solution (TMB) was added to each well and the plate was incubated for another 20 minutes in the

dark. 25 µL of stop solution (2 N H_2SO_4) was added to each well and the absorbance was measured at 450 nm.

2.12 Statistical Analysis

Statistical analysis was conducted using Microsoft Excel. Significant differences amongst samples were assessed by analysis of variance (ANOVA) with replication. Significant differences between two sample groups were analyzed by t-test, with a significance of $p < 0.05$ unless otherwise stated. All error bars represent standard deviation values. All data was graphed using Prism by GraphPad Software, Inc. (La Jolla, CA, USA).

3 RESULTS AND DISCUSSION

3.1 Growth Curves and Growth Rate Analysis

The growth of both *L. helveticus* R0389 and *L. rhamnosus* R0011 was assessed in various media. RPMI 1640, the medium used for all THP-1 cell treatments, was first used to assess growth of both strains to investigate the potential to use RPMI as an alternative to the complex, protein rich MRS medium. Although other studies have shown that bacterial propagation in RPMI is possible (Kanangat *et al.*, 1999), neither strain appeared to grow very well in RPMI; therefore this medium was not used for the propagation of bacterial cells (Figure 4).

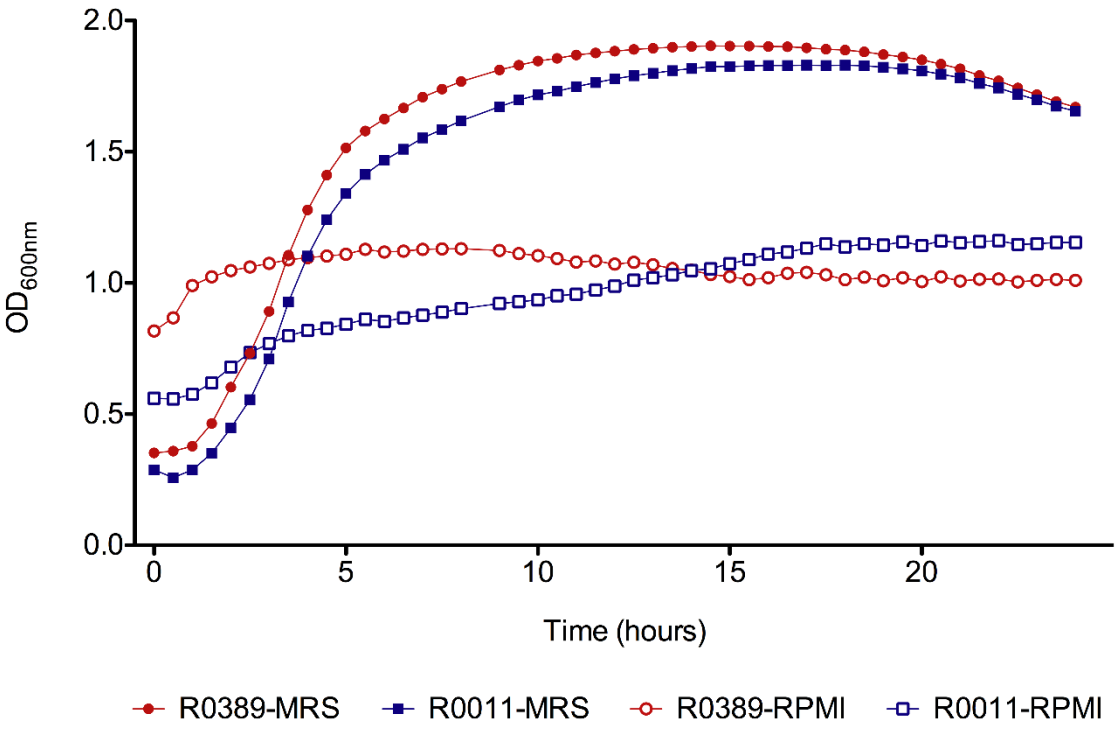


Figure 4 - Growth of *L. helveticus* R0389 and *L. rhamnosus* R0011 in MRS or RPMI medium for 14 hours. Each data point is the mean of 12 replicate values.

The growth of both *L. helveticus* R0389 and *L. rhamnosus* R0011 in medium supplemented with either 0.1% (w/v) casein or 0.01% (w/v) sodium formate (NaCOOH) was compared to growth in non-supplemented MRS medium (Figure 5). All cultures reached stationary phase in approximately 10 hours, however OD_{600nm} values of cultures grown in supplemented medium do not decrease, even after 24 hours. The growth rates and doubling times of each strain in supplemented medium were calculated and compared to those of MRS-grown cultures (Table 3). Supplementation of MRS with either casein (0.1% w/v) or sodium formate (0.01% w/v) significantly increased the growth rate of both *L. helveticus* R0389 and *L. rhamnosus* R0011 when compared to the MRS-grown cultures ($p < 0.05$).

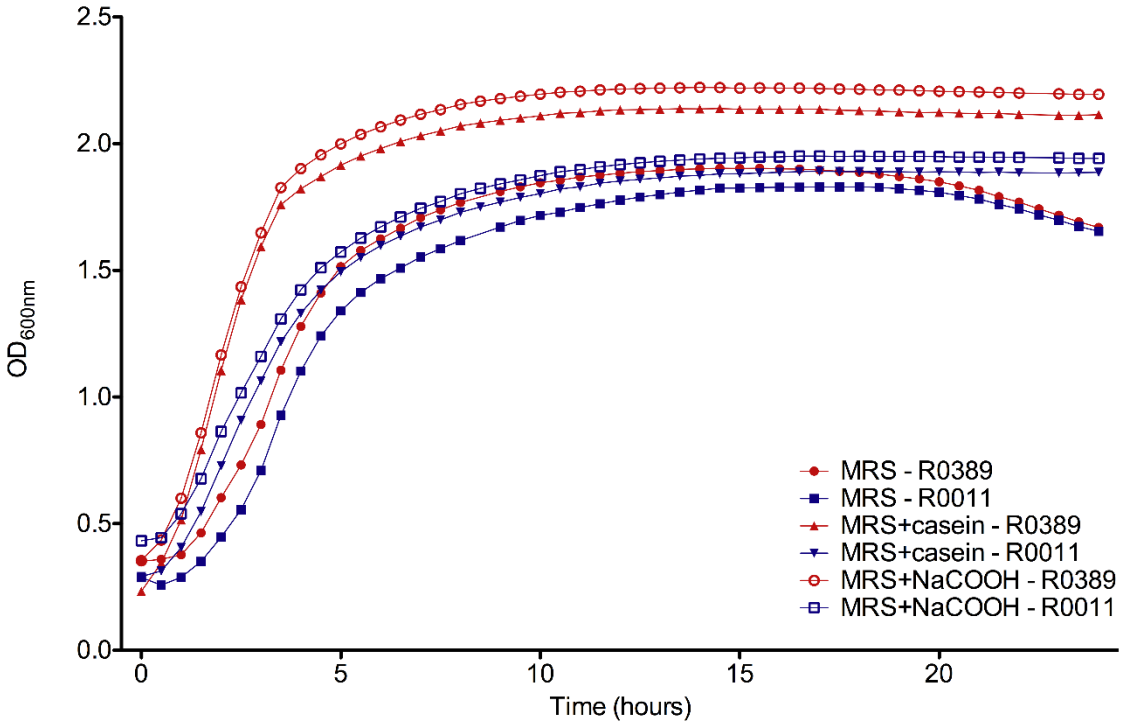


Figure 5 - Growth curves of *L. helveticus* R0389 and *L. rhamnosus* R0011 in MRS, MRS supplemented with 0.1% (w/v) casein or MRS supplemented with 0.01% (w/v) sodium formate over 24 hours. Each data point is the mean of 12 replicate values.

Table 3 - Growth rate constant ($hour^{-1}$) and doubling time (hours) of *L. helveticus* R0389 and *L. rhamnosus* R0011 grown in MRS or in MRS supplemented with either 0.1% (w/v) casein or 0.01% (w/v) sodium formate.

R0389			R0011		
Medium	Growth Rate ($hour^{-1}$)	Doubling Time (hours)	Medium	Growth Rate ($hour^{-1}$)	Doubling Time (hours)
MRS	0.430 ± 0.010	1.239 ± 0.029	MRS	0.451 ± 0.021	1.300 ± 0.060
MRS + casein	0.567 ± 0.017	1.636 ± 0.049	MRS + casein	0.484 ± 0.021	1.395 ± 0.060
MRS + NaCOOH	0.505 ± 0.022	1.457 ± 0.063	MRS + NaCOOH	0.383 ± 0.011	1.105 ± 0.031

3.2 Peptide Separation

The protein profiles of supernatant solutions from *L. helveticus* R0389 and *L. rhamnosus* R0011 cultures grown in MRS or MRS supplemented with 0.1% (w/v) casein or from milk fermented with each strain were analyzed by SDS-PAGE. Gradient (4 – 20%) glycine gels were first used to visualize protein present in precipitated, filtered and non-filtered, supernatant solutions of milk fermented with *L. helveticus* R0389 or *L. rhamnosus* R0011. Figure 6 shows unfiltered supernatant solutions from acidified or non-acidified controls and from milk ferments of both strains collected at multiple time points throughout fermentation. Protein profiles of *L. helveticus* R0389 and *L. rhamnosus* R0011 ferment supernatant solutions observed by gel electrophoresis are noticeably different than those of the acidified and non-acidified controls. All samples (including the acidified and non-acidified controls) contain the same prominent protein bands corresponding to proteins of approximately 14 kDa and slightly larger than 50 kDa. These bands could possibly correspond to two soluble milk proteins in the whey fraction of milk, α-lactalbumin (14 kDa) and the heavy chain of immunoglobin G (IgG) (~ 55 kDa). Casein proteins are typically between 25 and 32 kDa; however, no bands in this size range were observed. Two

bands of approximately 60 and 75 kDa are present in all sample lanes but not in either of the control samples. Yan *et al.* (2007) reported that *L. rhamnosus* GG produced two soluble proteins with immunomodulating properties, one of 75 kDa and the other 40 kDa. Supernatants collected from milk ferments of *L. helveticus* R0389 and *L. rhamnosus* R0011 seem to contain proteins between 17 and 30 kDa, the presence of which seems to differ between fermentation times. Jovanovic *et al.* (2007) identified two dominant complexes of 71 and 141 kDa and suggest they could be soluble chemical complexes of κ-casein, β-lactoglobulin, and α-lactalbumin.

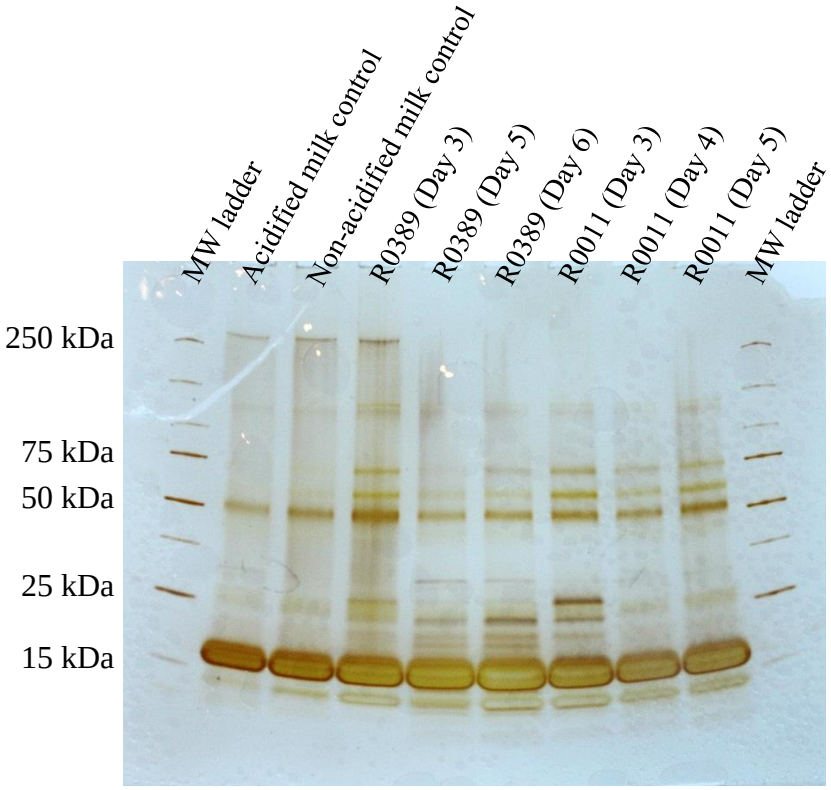


Figure 6 – Protein profiles of precipitated (non-filtered) cell-free supernatants of acidified and non-acidified controls and ferments of *L. helveticus* R0389 and *L. rhamnosus* R0011 collected after 3 to 6 days of fermentation. Aliquots of 5 µg of protein were loaded into each well of a 4 – 20% glycine polyacrylamide gel and silver stained. Molecular weight ladder contains a mixture of ten Strep-tagged recombinant proteins of 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa.

When the same *L. helveticus* R0389 supernatant solutions from above were electrophoresed on a 16.5% tricine gel, meant for separation of smaller molecules, there was an increase in the resolution of < 10 kDa components with each day of fermentation for filtered supernatants of *L. helveticus* R0389 that are not present in either of the control supernatant solutions (Figure 7).

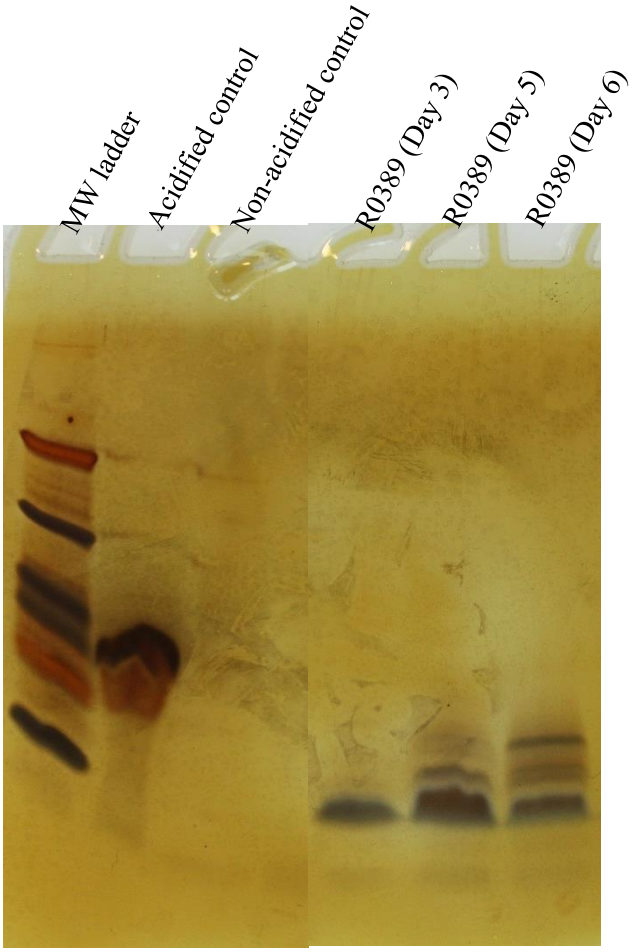


Figure 7 – Protein profiles of acetone precipitated, filtered (< 10 kDa) cell-free supernatants of acidified and non-acidified controls and ferments of *L. helveticus* R0389 and *L. rhamnosus* R0011 collected after 3, 5 and 6 days of fermentation. Aliquots of 5 µg of protein were electrophoresed on a 16.5% tricine polyacrylamide gel and silver stained. MW ladder contains six proteins of 1.4, 3.4, 6.5, 14.4, 16.9, and 26.6 kDa.

Multiple protein and peptide separation methods were utilized in order to separate the components present in each of the supernatant fractions, but also to compare the protein and peptide profiles of each strain. Supernatant solutions were analyzed on both silica and cellulose TLC plates with a variety of solvents to obtain the optimal separation and resolution (Table 4). Although most of the conditions tested resulted in poor separation and limited resolution, silica plates provided better separation than cellulose plates and the solvent that provided the best resolution was ethyl acetate:n-propanol:acetic acid:water (4:2:2:1) with 0.25% TFA (Figure 8A).

Table 4 – Run conditions (stationary and mobile phase) of TLC experiments

Plate (stationary phase)	**Mobile Phase**	**% TFA**	**Results (Separation/Resolution)**
Silica	Butanol:acetic acid:water (3:1:1)	N/A	No Separation
	Ethyl acetate:pyridine:water (10:4:3)	N/A	Poor
	Methanol:DCM (1:1)	N/A	Poor
	100% MeOH	N/A	Poor
	Acetonitrile	0.1%	Little/No Separation
		0.25%	Fair Separation, Poor Resolution
		0.4%	Fair Separation, Poor Resolution
	Ethyl acetate:n-propanol:acetic acid:water (4:2:2:1)	N/A	Fair Separation, Poor Resolution
		0.25%	Good
	Chloroform:ethyl acetate:formic acid (8:1:1)	0.25%	No separation
2D (Silica)	Solvent A: ethyl acetate:n-propanol:acetic acid:water (4:2:2:1) Solvent B: Acetonitrile + 0.25% TFA		Poor
Cellulose	Ethyl acetate:n-propanol:acetic acid	0.25%	Poor

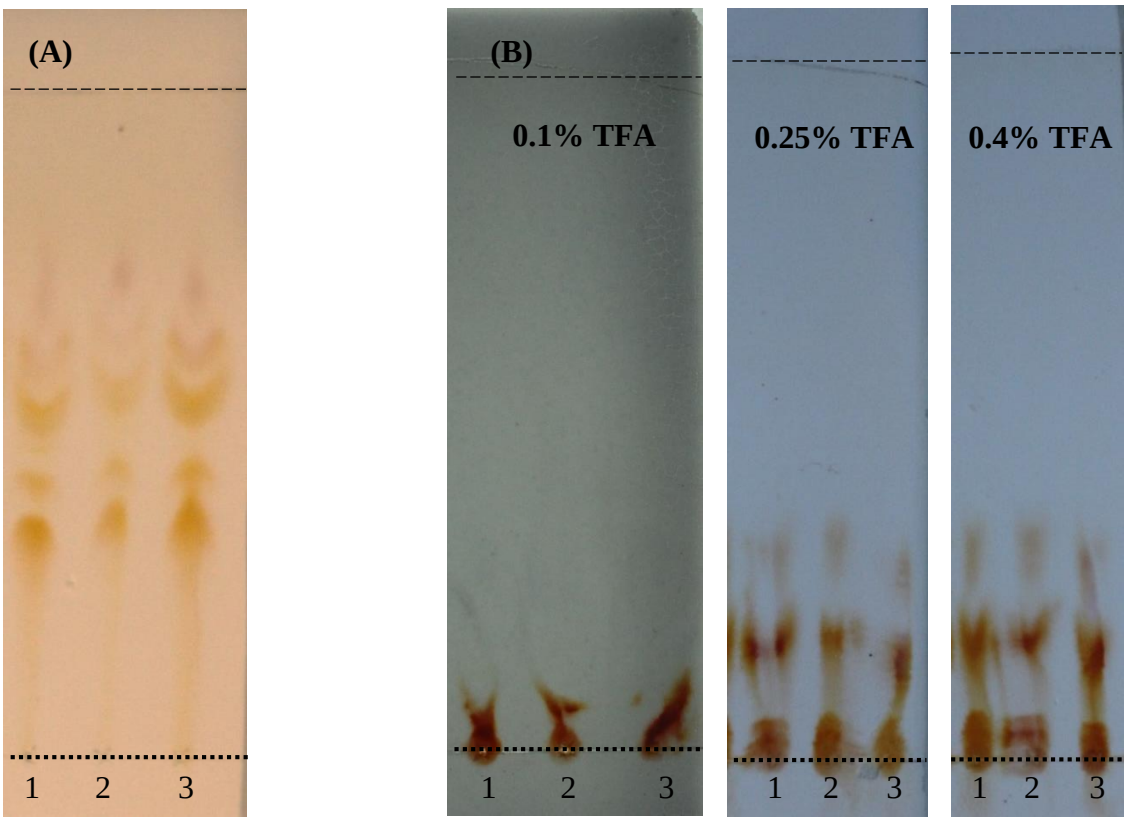


Figure 8 – Protein profiles of acetone precipitated, filtered (<10 kDa) cell-free supernatants of MRS supplemented with 0. 1% (w/v) casein (lane 1) or cultures of *L. helveticus* R0389 (lane 2) and *L. rhamnosus* R0011 (lane 2) grown in MRS supplemented with 0.1% (w/v) casein. 0.5 µL of each sample were separated on silica plates with ethyl acetate:n-propanol:acetic acid:water (4:2:2:1) with 0.25% TFA (A) or acetonitrile with 0. 1, 0.25 or 0.4% TFA (B). TLC plates were stained with ninhydrin.

When acetonitrile was used as a solvent for TLC separation, it was observed that 0.25% TFA provided increased resolution (compared to 0.1%). Increasing the concentration of TFA to 0.4% did not improve resolution compared to 0.25% therefore 0.25% TFA was selected to use for HPLC analysis rather than 0.1% (Figure 8B).

Multiple conditions and solvent gradients were tested to achieve optimal peptide separation with HPLC. A solvent of 30% acetonitrile and a run time of 20 minutes provided the best separation of the non-acetylated peptide standard mixture. Unfortunately, the acetylated peptide standards (Thermo Scientific) could not be visualized via HPLC under

any conditions tested. Supernatant solutions were not reproducibly separated via HPLC; even when samples were concentrated, little chromatographic signal was detected. Peptide fractions of ferment cell-free supernatant solutions were not reproducibly separated via HPLC and resolution was quite poor with all solvent systems used.

3.3 ACE Inhibition

HHL acts as a substrate for ACE and the liberation of hippuric acid can therefore be measured to provide an indication of enzymatic activity; a decrease in absorbance in the presence of an inhibitor solution is inversely proportional to its inhibitory activity (Cushman and Cheung, 1971).

Due to an observed increase in growth rate of lactobacilli grown in MRS medium supplemented with 0.1% (w/v) casein, the cell-free supernatant (precipitated and filtered) of *L. helveticus* R0389 and *L. rhamnosus* R0011 cultures grown in casein-supplemented media were assessed for their abilities to inhibit ACE. A two-way ANOVA, with replication, showed significant differences in percent ACE inhibition between sample types ($p < 0.01$). Further statistical analysis revealed a significant increase in percent ACE inhibition of fractions collected at 12 hours of growth in casein supplemented media, for *L. helveticus* R0389 and *L. rhamnosus* R0011 ($p=0.029$ and 0.004, respectively), compared to those collected from the supernatant of MRS-grown cultures without supplementation (Figure 9). Since the addition of casein to the growth environment of *L. helveticus* R0389 and *L. rhamnosus* R0011 significantly increased the growth rate as well as ACE inhibitory properties of the secreted peptide fraction of both strains, it suggests that an increase in growth rate, likely indicative of increased proteolytic activity, leads to an increase in the bioactive peptides produced during growth. Oddly, the supernatants collected from 6 hour

cultures in MRS had the highest ACE inhibitory activity, whereas ACE inhibitory activity of supernatants collected from cultures in MRS supplemented with 0.1% casein increased throughout growth.

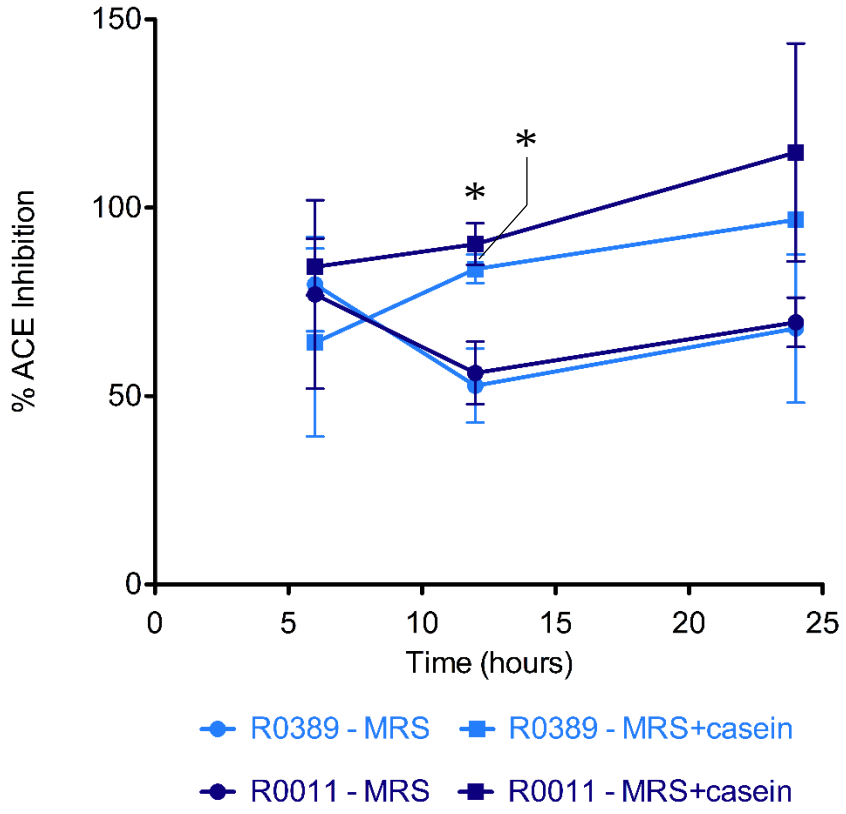


Figure 9 – Mean percent ACE inhibition (± SD) of <10 kDa peptide fractions (1 µg/mL) isolated from the cell-free supernatant of *L. helveticus* R0389 or *L. rhamnosus* R0011 cultures grown in MRS or MRS with 0.1% (w/v) casein over 24 hours (n=3). * indicates a significant difference in ACE inhibition between the casein supplemented sample and the same strain grown in MRS at the same time point ($p < 0.05$).

Preliminary analysis conducted to determine the level of ACE inhibition of peptide fractions isolated from 3.25% milk fermented with *Lactobacillus helveticus* R0389 and *Lactobacillus rhamnosus* R0011 revealed inhibitory properties comparable to the known

tri-peptide ACE inhibitor, IPP. The ACE inhibition assay was therefore performed in triplicate with the peptide fractions (< 10 kDa) obtained from the fermentation of 3.25% milk. ACE inhibition values were all found to be greater than 40%. However upon further investigation, it was discovered that the protein buffer solution in which peptides were re-suspended showed some ACE inhibitory activity. Therefore, filtered ferment peptide fractions (Day 3 – 6) previously tested were acetone precipitated and re-suspended in dH_2O and ACE inhibition was re-evaluated in triplicate. A two-way analysis of variance (ANOVA), with replication, indicates that fermentation time has a significant effect on percent ACE inhibition ($p = 0.004$). In addition, t-tests revealed significant differences between the ACE inhibition of peptide fractions collected from *L. rhamnosus* R0011 fermented cultures after 6 days and the corresponding acidified and non-acidified controls ($p = 0.035$ and 0.011, respectively) (Figure 10). Filtered supernatant solutions collected at each time point from acidified and non-acidified controls retained ACE inhibitory activity of approximately 40%. ACE inhibitory activity of *L. helveticus* R0389 increased from the third to fourth day of fermentation to a maximum of 63.5 ± 0.3% before decreasing over the fifth and sixth day of fermentation. This mimics the results of Meisel *et al.* (1997) in which they observed an increase in ACE inhibitory activity of low molecular weight peptides from several ripened cheeses as proteolysis developed; however ACE inhibition begins to decrease once cheese ripening exceeded a certain level. In contrast, ACE inhibitory activity of supernatants collected from *L. rhamnosus* R0011 ferments continued to increase throughout fermentation. Donkor *et al.* (2007) also found that concentrations in the microgram range of VPP and IPP from yogurt cultures produced with various LAB strains were able to inhibit ACE. However, due to the limitations of absorption and

bioavailability *in vivo*, many studies regarding the administration of bioactive peptides use milligram quantities in order to observe a significant decrease in blood pressure *in vivo* (Sipola *et al.*, 2002; de Leeuw *et al.*, 2009; Foltz *et al.*, 2007; Matsui *et al.*, 2002a,b). The lowest dose of bioactive peptides or peptide-containing products shown to be effective in reducing blood pressure in humans was 3.07 mg of lactotripeptides per day which resulted in a decrease of 3 mmHg after 8 weeks of administration (Sano *et al.*, 2005).

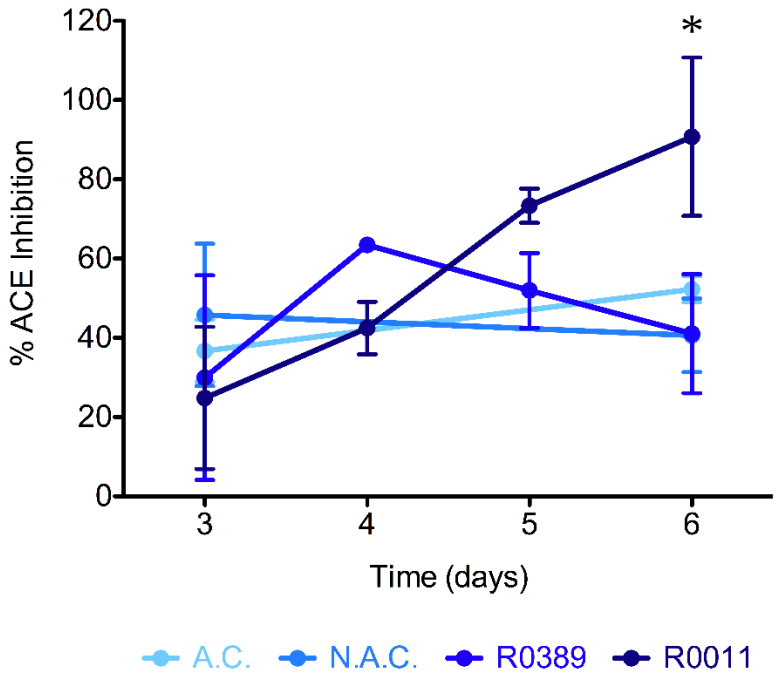


Figure 10 – Mean percent ACE inhibition (± SD) of <10 kDa peptide fractions (1 µg/mL) in dH_2O isolated from the cell-free supernatant of 3.25% milk fermented with *L. helveticus* R0389 or *L. rhamnosus* R0011 for 3, 4, 5, or 6 days, or from acidified (A.C.) and non-acidified (N.A.C.) controls (n=3). * indicates a significant difference between the sample group and both control groups at the same time point ($p < 0.05$).

When compared to the known ACE inhibitors, VPP and IPP, ferment supernatant samples show comparative percent inhibition values (Figure 11). When compared to VPP, the percent inhibition of peptide fractions from day 4, 5, and 6 ferments of *L. helveticus*

R0389 and from day 5 and 6 ferments of *L. rhamnosus* R0011 were not significantly different ($p < 0.05$). When compared to IPP, the percent inhibition of peptide fractions collected from ferments of both strains at each of the time points were not significantly different.

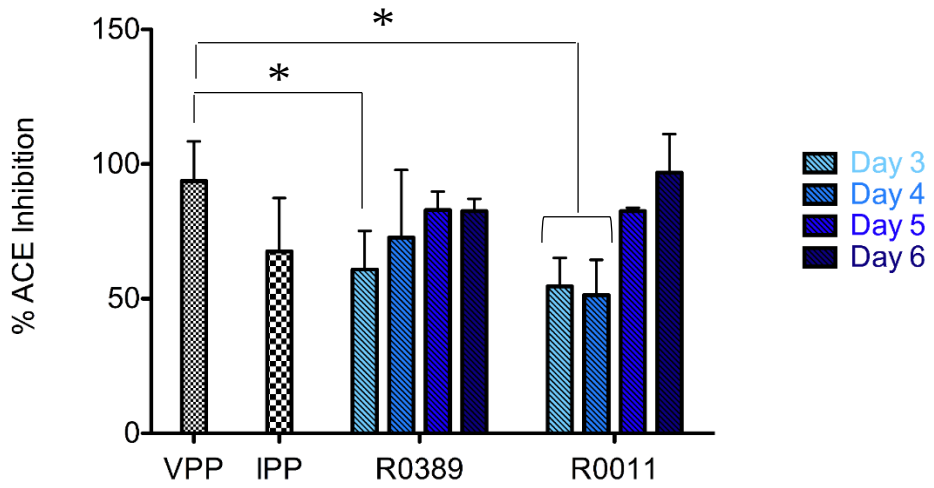


Figure 11 - Mean percent ACE inhibition (± SD) of <10 kDa peptide fractions (1 µg/mL) in dH_2O isolated from the cell-free supernatant of 3.25% milk fermented with *L. helveticus* R0389 or *L. rhamnosus* R0011 for 3, 4, 5, or 6 days compared to percent inhibition of VPP and IPP (n=3).* indicates a significant difference between samples ($p < 0.05$).

In order to further narrow down the bioactive components within the supernatant solutions, filtered milk ferment supernatant samples (< 10 kDa) from *L. helveticus* R0389 or *L. rhamnosus* R0011, and acidified and non-acidified controls, were filtered through centrifugal filter tubes with a MWCO of 3 kDa and assessed for their ACE inhibiting potential. The ACE inhibitory activity of both acidified and non-acidified controls increased with increasing fermentation time to reach percent inhibition values similar to those seen from the < 10 kDa fractions of the same acidified and non-acidified control

supernatants (approx. 50 – 60%). Milk ferment samples of both strains however, showed lower inhibition overall when compared to their corresponding < 10 kDa fractions, with *L. helveticus* R0389 supernatants from day 3, 4, and 5 ferments showing no inhibition at all (Figure 12). When fermentation begins, larger milk proteins are hydrolyzed by proteinases on the surface of the cell (Savijoki *et al.*, 2006). Further breakdown of these resulting polypeptides is then required to produce the small < 20 amino acid long bioactive peptides. Therefore it is expected that it would take longer for smaller peptides (< 3 kDa) to accumulate in the extracellular environment. This could potentially explain the lessened (or lack of) activity compared to the < 10 kDa peptide fractions.

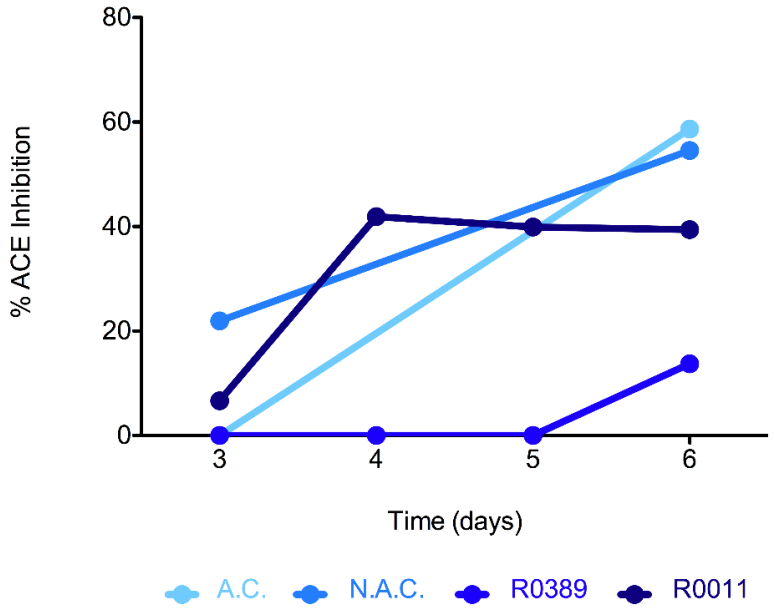


Figure 12 - Percent ACE inhibition of < 3 kDa peptide fractions (1 µg/mL) in dH_2O isolated from the cell-free supernatant of 3.25% milk fermented with *L. helveticus* R0389 or *L. rhamnosus* R0011 for 3, 4, 5, or 6 days, or from acidified (A.C.) and non-acidified (N.A.C.) controls. Each value is an average of three technical replicates of a single biological replicate.

Twelve casein-derived peptides (Table 2) were selected based on literature searches of previously identified bioactive peptides, some of which have been shown to possess certain bioactive properties but which have not been tested for others, or have been

identified as part of a larger bioactive fraction but their bioactivity had yet to be individually tested. These twelve peptides were synthesized and individually assessed for their ability to inhibit ACE. The tripeptides, VPP and IPP, are known ACE inhibitors (Nakamura, 1995a) and displayed the highest ACE inhibition (94% ± 15% and 68% ± 20%, respectively) (Figure 13). Notably, the hexapeptide KVLPVP showed the next highest percent ACE inhibition (65% ± 16%) (Figure 13). The addition of glutamine to the C-terminal end of the hexapeptide abolishes all ACE inhibitory activity, similar to the results seen by Maeno *et al.* (1996). Peptides 3 through 7 (HQPQHPLPPTVMFPPQ, HQPQHPLPPT, WMHQPHQPLPPT, LYQEPVLGPVR, and LDQWLCEK) were identified by Tellez *et al*. in 2010 as part of a larger peptide fraction that displayed immunomodulatory properties (increased IL-6, IL-1β, TNF-α, and nitric oxide (NO) production) in RAW264.7 cells. However, until this study, the bioactive properties of these five peptides had yet to be tested individually, and their ability to inhibit ACE had not been previously investigated at all. From the ACE assays conducted in this study, all five peptides show very similar ACE inhibition (between 23% and 30%). Peptide sequences, those of C-terminal residues in particular, have been shown to play an important role in the bioactive effects of milk-derived peptides. (FitzGerald *et al.* 2000; Cheung *et al.*, 1980). We observed that each of the synthesized peptides with terminal Pro or Pro-Pro residues possessed the highest levels of ACE inhibition (Figure 13).

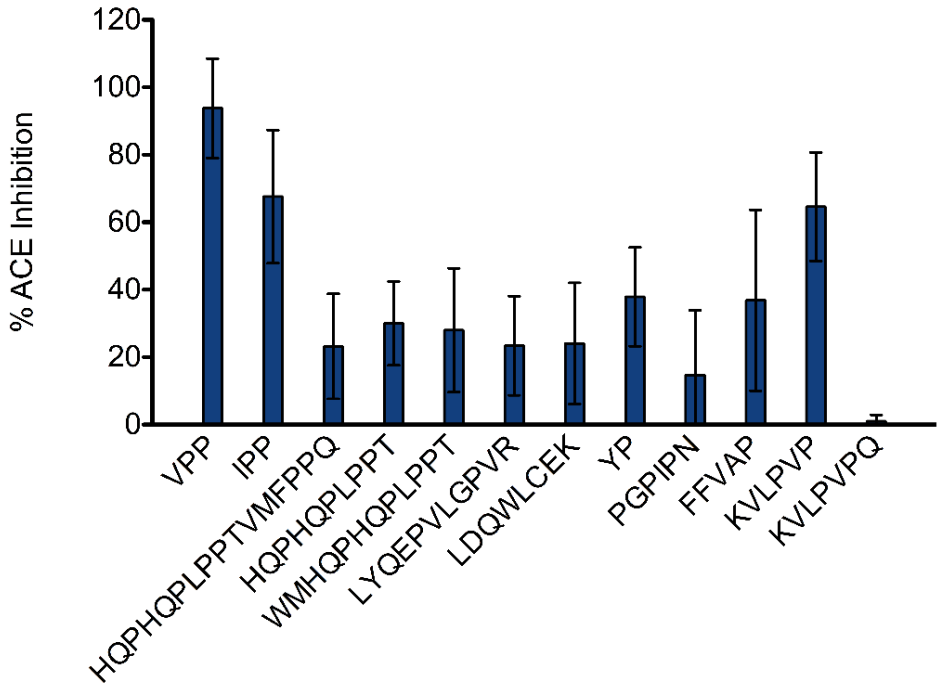


Figure 13 – Mean percent ACE inhibition (± SD) of 1 µg/mL solutions of synthesized peptides (n=3).

3.4 THP-1 Treatments

3.4.1 Ferment-Derived Peptide Fractions

Preliminary cell treatments were conducted in which cells were co-challenged with 125 ng/mL LPS and a range of concentrations of supernatant solutions from 48 hour ferment cultures of *L. helveticus* R0389 and *L. rhamnosus* R0011 as well as those from acidified and non-acidified controls filtered through Millex HV filters (13 mm, 0.45 µM). After 24 hours, cell viability was assessed with the XTT viability assay. THP-1 cells all remained above 80% viable for all but the highest concentration used (Figure 14).

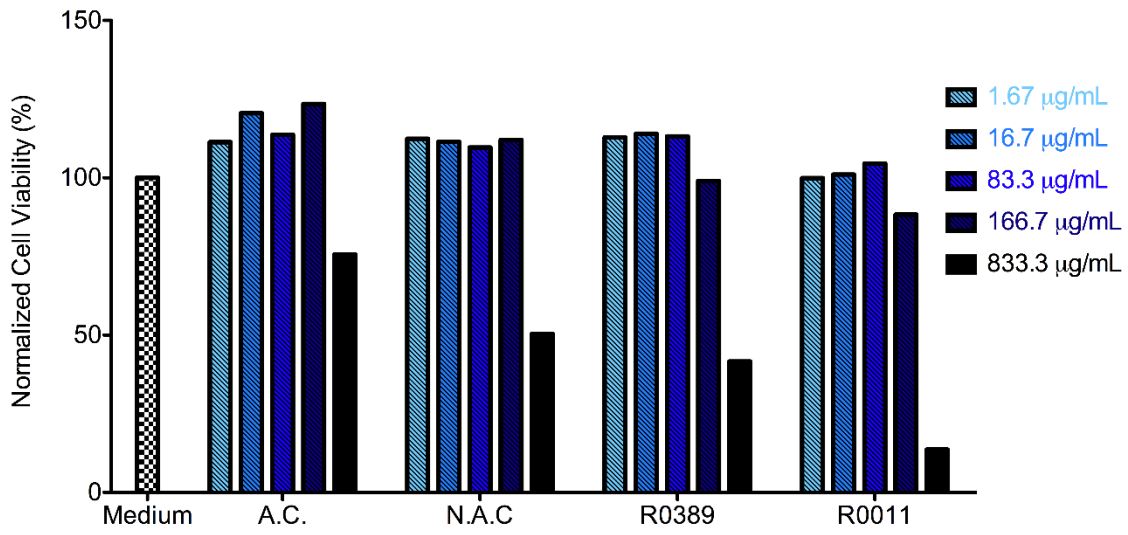


Figure 14 – Normalized viability of THP-1 cells treated for 24 hours with varying concentrations (1.67 to 833.3 µg/mL) of filtered (0.45 µm) ferment supernatant solutions from 48 hour cultures of *L. helveticus* R0389, *L. rhamnosus* R0011 or acidified (A.C.) and non-acidified (N.A.C.) controls as determined by the XTT cell viability assay. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS. Each value shown is an average of three technical replicates of a single biological replicate. Percent viability was normalized to the medium control.

Supernatant solutions were collected from milk cultures after at least 3 days, and up to 6 days, post-fermentation since protein and growth analysis indicated that bacterial growth, and therefore proteolytic activity, was optimal between these time points. Fermentation times of less than 3 days resulted in very low levels of secreted products as not enough time had elapsed to allow for bacterial proteolysis to occur. Lower concentrations, in the ng to low µg/mL range, of the precipitated and filtered (<10 kDa) supernatant of *L. helveticus* R0389 ferments, and acidified and non-acidified controls, fermented for 3 to 6 days, were used to co-challenge THP-1 cells with LPS for 24 hours. In this case, cells retained at least 80% viability with all concentrations used (Figure 15).

The supernatant of the treated THP-1s was collected and production of IL-8 was measured via ELISA (Figure 16).

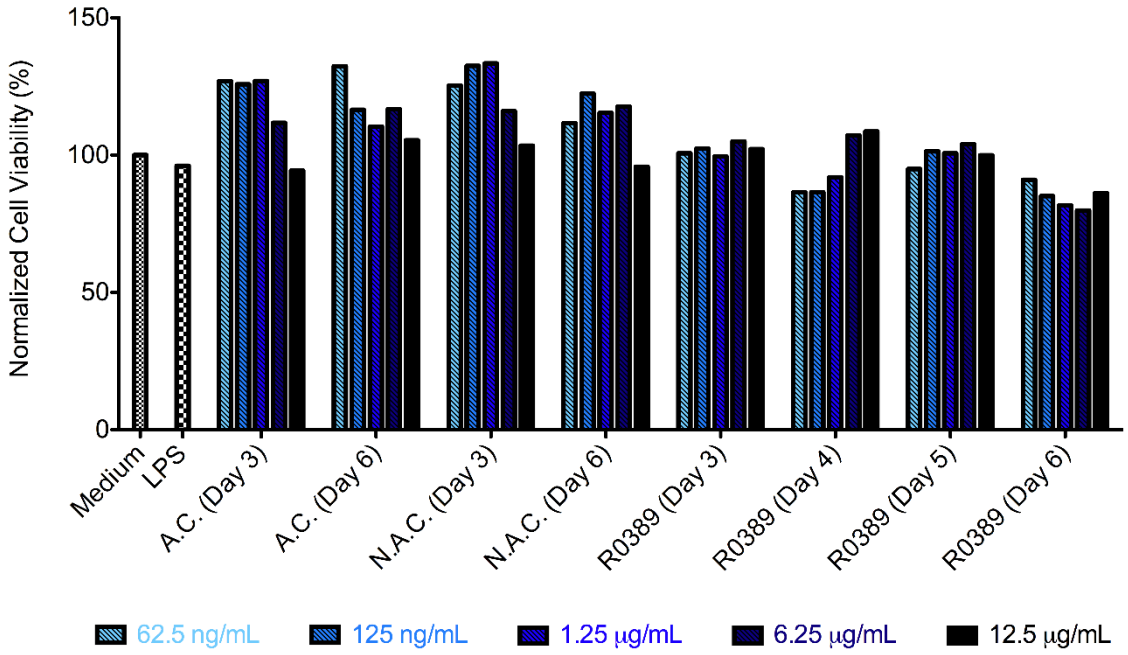


Figure 15 – Normalized viability of THP-1 cells treated for 24 hours with varying concentrations (62.5 ng/mL to 12.5 μg/mL) of acetone precipitated, filtered (< 10 kDa) supernatant solutions of *L. helveticus* R0389, *L. rhamnosus* R0011 or acidified (A.C.) and non-acidified (N.A.C.) controls collected after 3 to 6 days of fermentation as determined by the XTT cell viability assay. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS. Each value is an average of three technical replicates of a single biological replicate. Percent viability was normalized to the medium control.

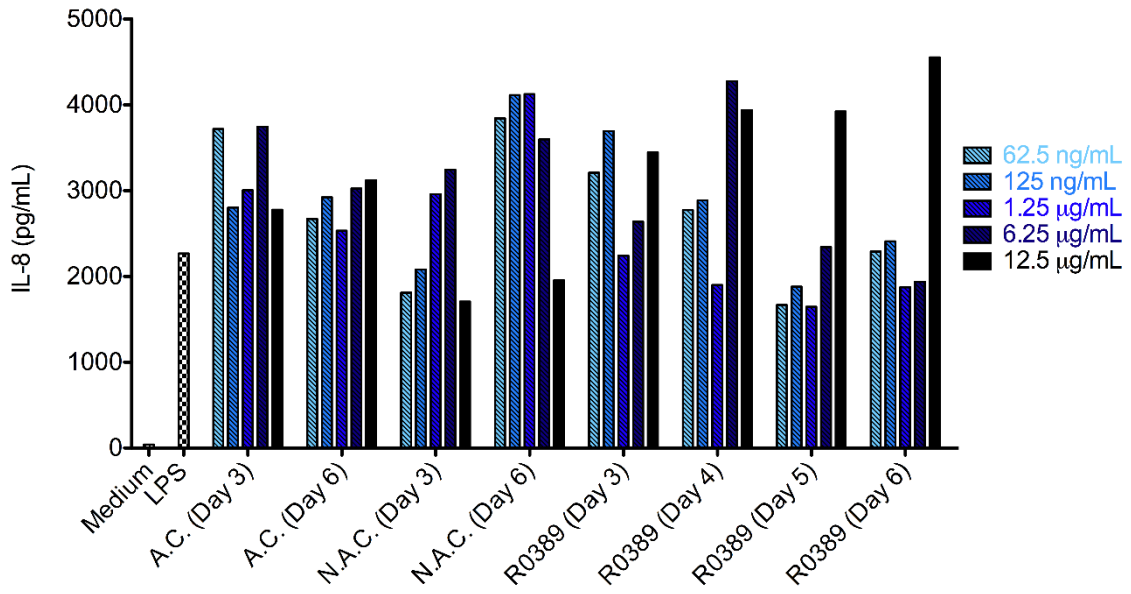


Figure 16 - IL-8 production by THP-1 cells stimulated for 24 hours with 125 ng/mL LPS and varying concentrations (62.5 ng/mL to 12.5 µg/mL) of filtered (< 10kDa) supernatant solutions collected from *L. helveticus* R0389 ferments after 3 to 6 days of fermentation as well as from acidified (A.C.) and non-acidified (N.A.C.) controls. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS. Unlike all other ELISA data presented, this ELISA was performed using a Human IL-8 kit from Invitrogen. All other ELISAs were performed using a Human IL-8 kit from R&D (n = 1).

Since higher peptide concentrations (> 10 µg/mL) seemed to have more of a pro-inflammatory effect, as reflected by IL-8 production by THP-1 cells, cells were subsequently treated with the filtered ferment supernatant solutions (<10 kDa) of *L. helveticus* R0389 and *L rhamnosus* R0011, and acidified and non-acidified controls, but in even lower concentrations (ng and pg range). Viability of THP-1 cells treated with all concentrations of peptide fractions remained above 80% (Figure 17) and IL-8 levels of cells treated with supernatant solutions dropped to below those of the LPS-treated cells (Figure 18). Although most of the ferment supernatant solutions were able to reduce IL-8 levels to below those of the LPS-stimulated control, so did both the acidified and non-

acidified controls and it was therefore more likely a result of the reduced concentrations of peptide sample used rather than an LAB-specific effect.

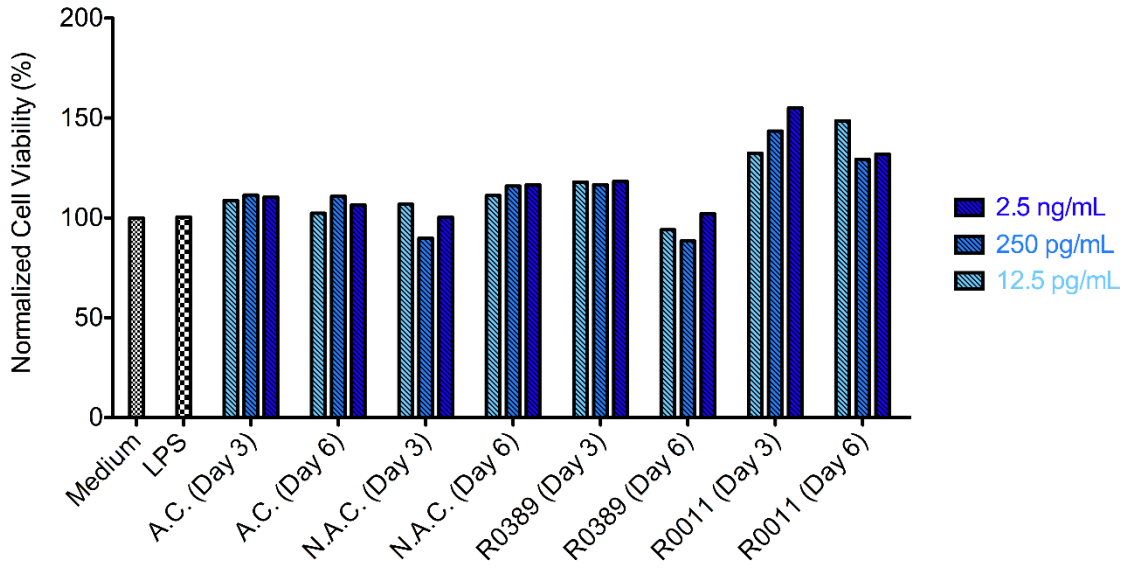


Figure 17 – Normalized viability of THP-1 cells treated for 24 hours with varying concentrations (12.5 pg/mL to 2.5 ng/mL) of acetone precipitated, filtered (< 10 kDa) supernatant solutions of *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments, and acidified (A.C.) and non-acidified (N.A.C.) controls, collected after 3 or 6 days of fermentation as determined by the XTT cell viability assay. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS. LPS. Each value is an average of three technical replicates of a single biological replicate. Percent viability was normalized to the medium control.

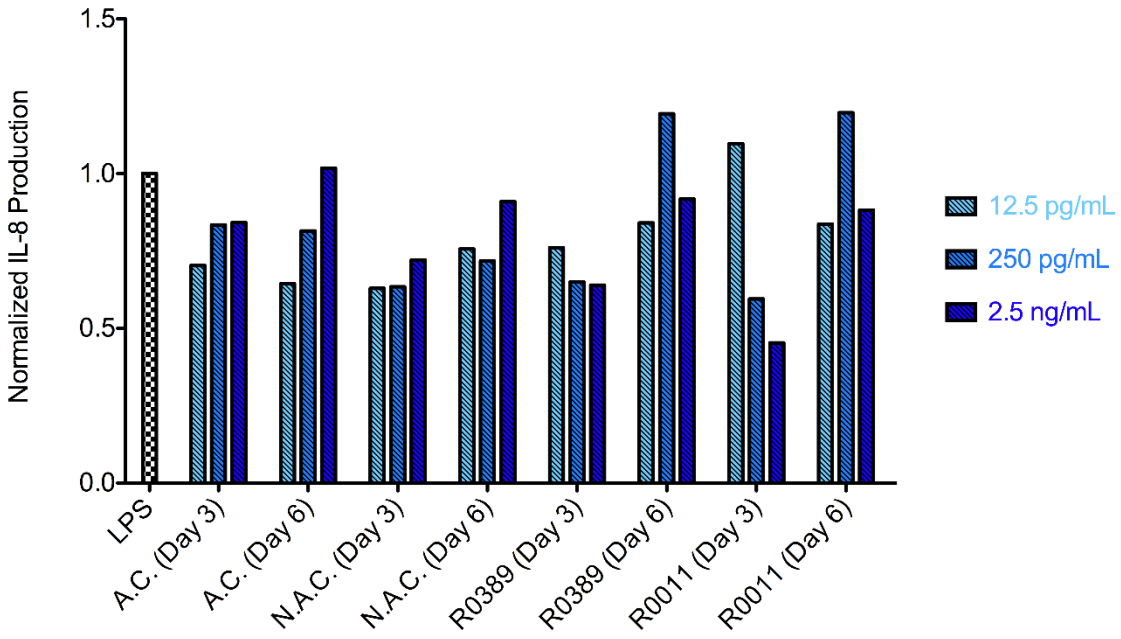


Figure 18 – Normalized IL-8 production by THP-1 cells stimulated for 24 hours with 125 ng/mL LPS and varying concentrations of filtered (< 10 kDa) supernatant solutions from *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments, and acidified (A.C.) and non-acidified (N.A.C.) controls, collected after 3 and 6 days of fermentation. LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS. IL-8 levels were normalized against those of the LPS-stimulated control (n =1).

Based on the above preliminary analyses, filtered supernatant solutions (< 10 kDa) from three biological replicates of *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments and acidified/non-acidified controls, collected after 3, 4, or 5 days of fermentation, were added to THP-1 cells at 2.5 ng/mL. Monocytic THP-1 cells maintained 100% cell viability (Figure 19) compared to the untreated control. Acidified control (Day 3) and supernatant solutions collected from milk ferments of both *L. helveticus* R0389 and *L. rhamnosus* R0011 after 4 days of fermentation significantly increased cell viability values ($p < 0.05$) compared to the untreated control, suggesting these ferment samples can induce an increased rate of growth or proliferation of the THP-1 cells. None of the samples were able to significantly reduce IL-8 production when compared to the LPS-stimulated control.

There were no significant differences in the levels of IL-8 production by cells treated with supernatant solutions of either LAB strain compared to the respective acidified and non-acidified controls (Figure 20). Although our filtered supernatant solutions from milk ferments were not able to reduce the LPS-induced levels of IL-8, the measurement of additional cytokines may be required to observe any protective effect against LPS-induced inflammation. Yang *et al.* (2014) also investigated the anti-inflammatory properties of *L. rhamnosus* supernatant collected from MRS-grown cultures of *L. rhamnosus* GR-1. It is expected that cultures grown in milk, as opposed to MRS, would have increased proteolytic activity, and therefore a higher production of bioactive peptides. However their supernatant solutions were able to reduce LPS-induced levels of pro-inflammatory markers including IL-6. IL-1β, and TNF-α in the maternal placenta of pregnant mice. Thus, investigating the effect on production of additional cytokines and pro-inflammatory markers by milk ferment supernatants could provide a better understanding of the immune response to these peptide supernatant solutions.

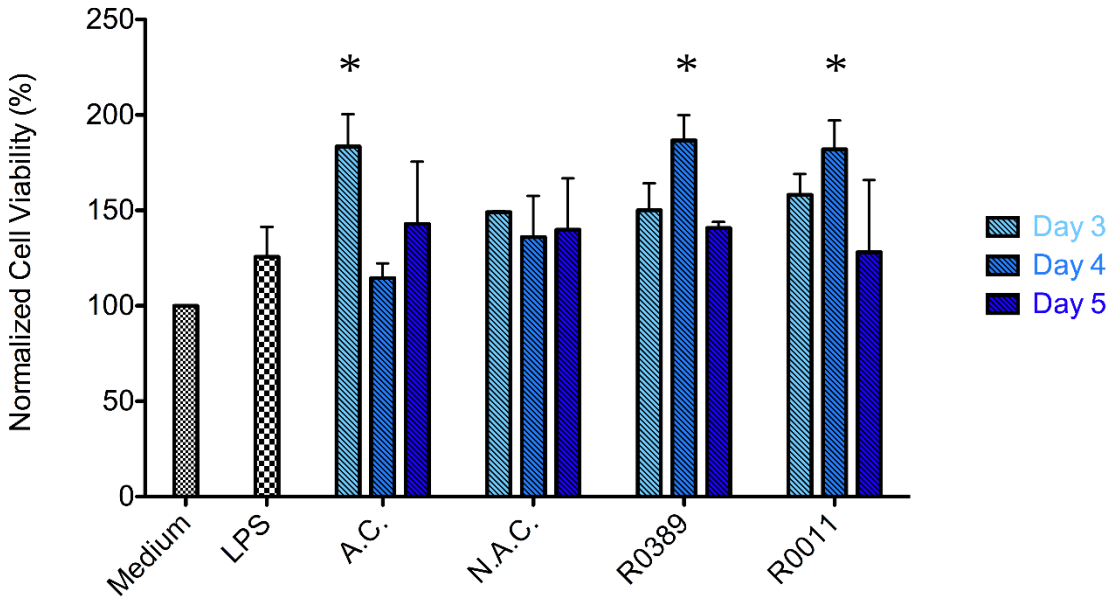


Figure 19 – Normalized cell viability (± SD) of THP-1 cells treated for 24 hours with acetone precipitated, filtered (< 10 kDa) supernatant solutions (2.5 ng/mL) of *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments, and acidified (A.C.) and non-acidified (N.A.C.) controls, collected after 3, 4, and 5 days of fermentation as determined by the XTT cell viability assay. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS. Each value is the average of three technical replicates obtained from the three biological replicates. * indicates a significant difference in cell viability compared to the LPS-treated control ($p < 0.05$). Percent viability was normalized to the medium control.

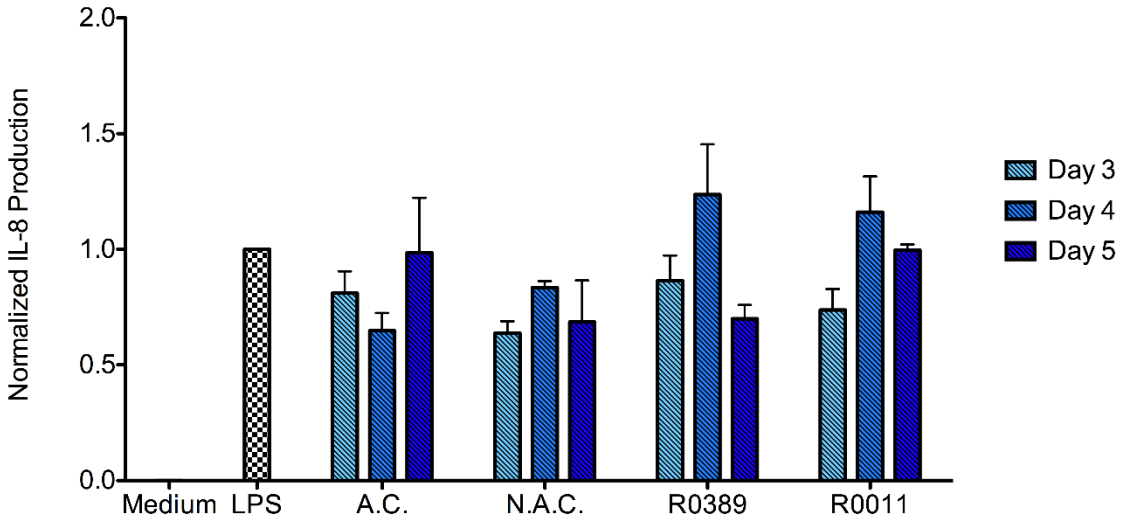


Figure 20 - Normalized IL-8 production (± SD) by THP-1 cells treated for 24 hours with 125 ng/mL LPS and acetone precipitated, filtered (<10 kDa) supernatant solutions (2.5 ng/mL) from *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments after 3, 4, and 5 days as well as an acidified (A.C.) and non-acidified (N.A.C.) control. IL-8 levels were normalized against those of the LPS-stimulated control (n = 3).

The above analyses were all conducted using filtered (< 10 kDa) supernatant samples from milk ferment samples. In order to further assess the effect of the components of these supernatant fractions, they were filtered a second time to retain only components less than 3 kDa in size. Preliminary treatments of THP-1 cells were repeated with the < 3 kDa fractions of the cell-free supernatant solutions of *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments after 3, 4, or 5 days post-fermentation. Cell viability was retained at all three concentrations analyzed (Figure 21). Interestingly, the highest concentration of the < 3 kDa fraction of the ferments from both *L. helveticus* R0389 and *L. rhamnosus* R0011 (1 µg/mL) showed the largest decrease in IL-8 production compared to the LPS-stimulated control (Figure 22). Therefore, further analysis was performed with 1 µg/mL solutions of filtered (< 3 kDa) cell-free supernatant from three biological replicates

of *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments and acidified/non-acidified controls. Again, cell viability remained above 80% of the control (Figure 23). Unfortunately, any reduction in IL-8 production observed from the preliminary ELISA was not observed when the experiment was repeated with the biological replicates of all four ferment samples; none of the treatments were able to significantly reduce LPS-induced IL-8 production ($p < 0.05$) (Figure 24).

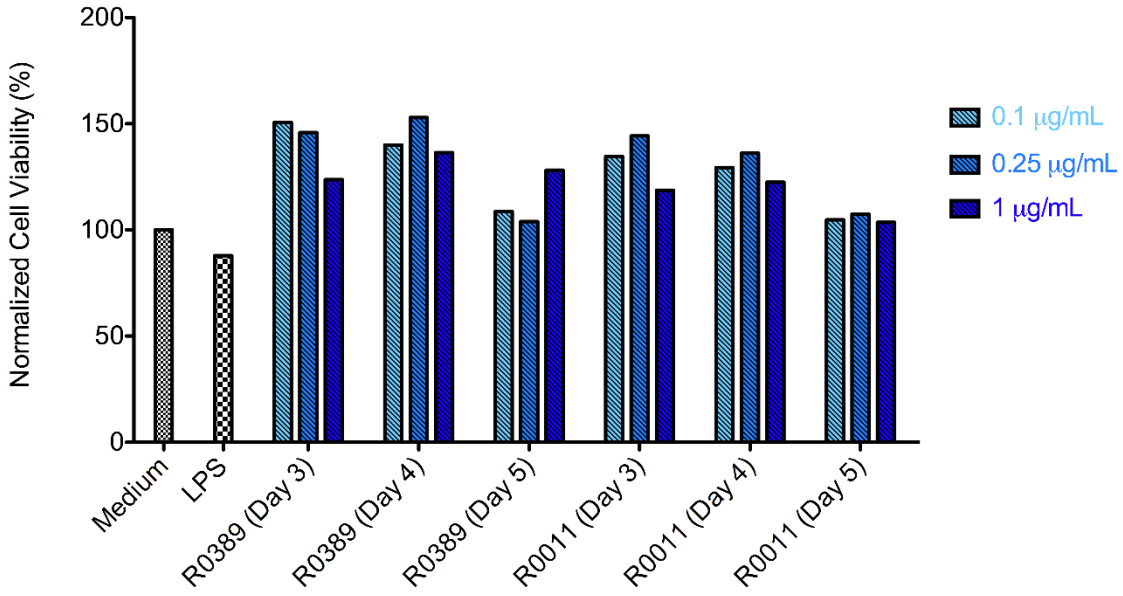


Figure 21 – Normalized cell viability of THP-1 cells treated for 24 hours with varying concentrations (0.1 to 1 µg/mL) of acetone precipitated, filtered (< 3 kDa) supernatant solutions of *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments collected after 3, 4, and 5 days of fermentation as determined by the XTT cell viability assay. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS. Each value is the average of three technical replicates of a single biological replicate.

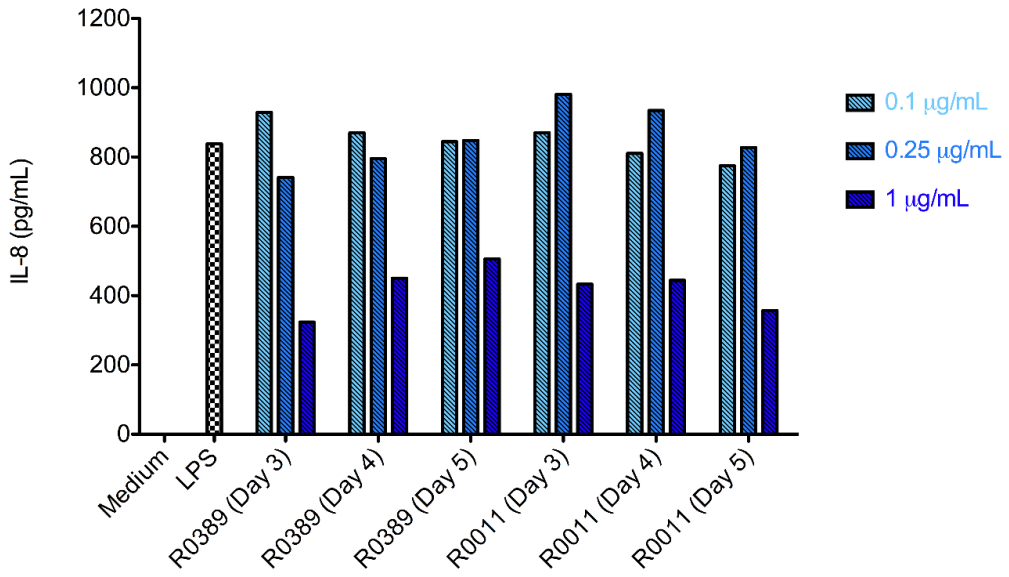


Figure 22 - IL-8 production by THP-1 cells treated for 24 hours with 125 ng/mL LPS and various concentrations (0.1 to 1 µg/mL) of filtered (< 3 kDa) supernatant solutions from *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments collected after 3, 4, and 5 days. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS (n = 1).

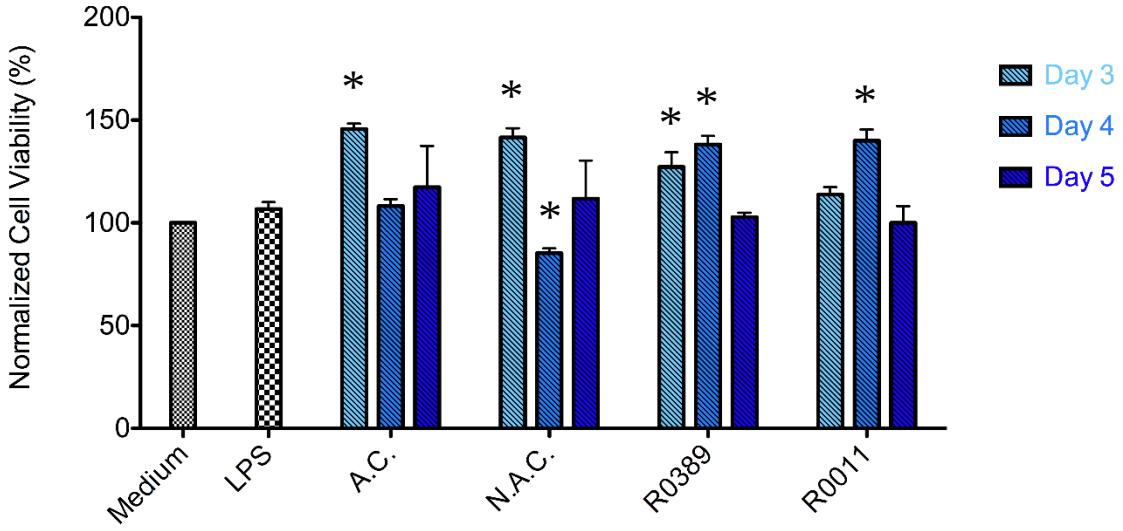


Figure 23 – Normalized cell viability (± SD) of THP-1 cells treated for 24 hours with acetone precipitated, filtered (< 3 kDa) supernatant solutions (1 µg/mL) of *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments, and acidified (A.C.) and non-acidified (N.A.C) controls, collected after 3, 4, and 5 days of fermentation as determined by the XTT cell viability assay. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS (n = 3). Percent viability was normalized to the medium control.

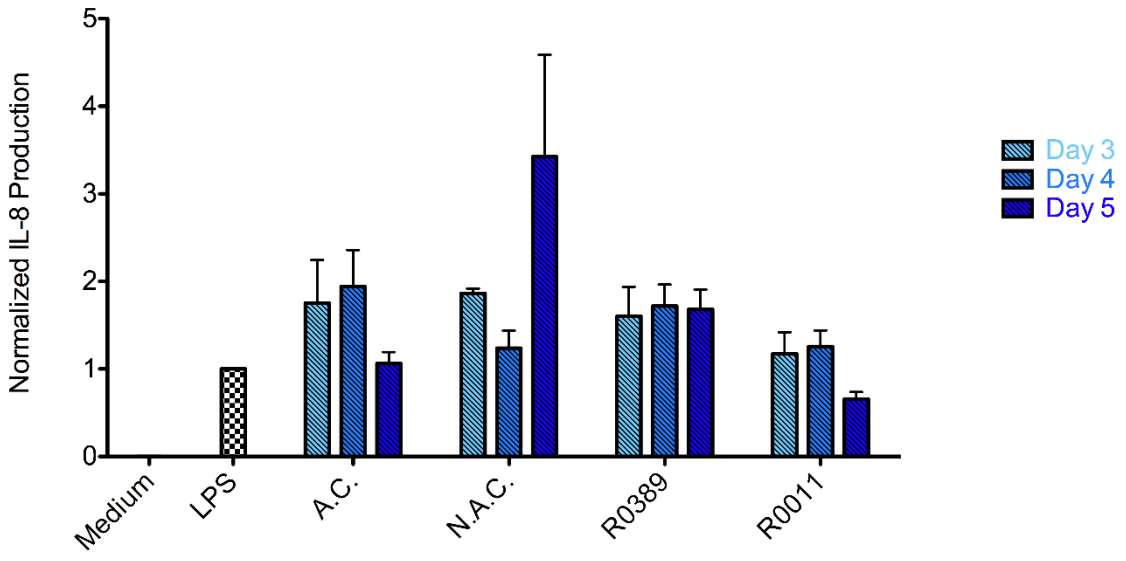


Figure 24 - Normalized IL-8 production (± SD) by THP-1 cells treated for 24 hours with 125 ng/mL LPS and 1 µg/mL filtered (<3 kDa) supernatant solutions from *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments, and acidified (A.C.) and non-acidified (N.A.C.) controls, after 3, 4, and 5 days of fermentation. IL-8 levels were normalized against those of the LPS-stimulated control (n = 3).

In all 24 hour treatments, the cell-free supernatant solutions from both *L. helveticus* R0389 and *L. rhamnosus* R0011 were not able to effectively and reproducibly prevent the LPS-stimulated induction of IL-8 production by THP-1 cells. Monocytic cells are typically responsible for inducing an inflammatory response in the presence of foreign compounds or pathogens. Therefore it was postulated that increasing the time of exposure of the THP-1 cells to the peptide samples may allow the inflammatory cells to reach a more steady, and potentially regulatory, state. To test this, the co-incubation time was increased from 24 hours to 48 hours. After 48 hours, the viability of the cells for all samples remained above 80% when normalized to the medium control (Figure 25). Only cells treated with the supernatant of the non-acidified control collected after 4 days resulted in significantly decreased viability compared to the LPS-stimulated control. The preliminary ELISA of the

cells treated for 48 hours however showed very low levels of IL-8 for peptide-treated cells as well as for the LPS-stimulated control when compared to the IL-8 concentrations typically observed from 24 hour treatments. Therefore, although the levels of IL-8 produced from the sample treated cells were incredibly low, so were those of the cells treated with LPS alone (Figure 26). A 4 hour co-incubation of the THP-1 cells was also conducted with LPS and the supernatant solutions of *L. helveticus* R0389 and *L. rhamnosus* R0011 and both the acidified and non-acidified controls, however it was not long enough for any detectable IL-8 to be produced from any of the LPS or sample treated cells. For these reasons, all subsequent cell treatments were carried out for 24 hours.

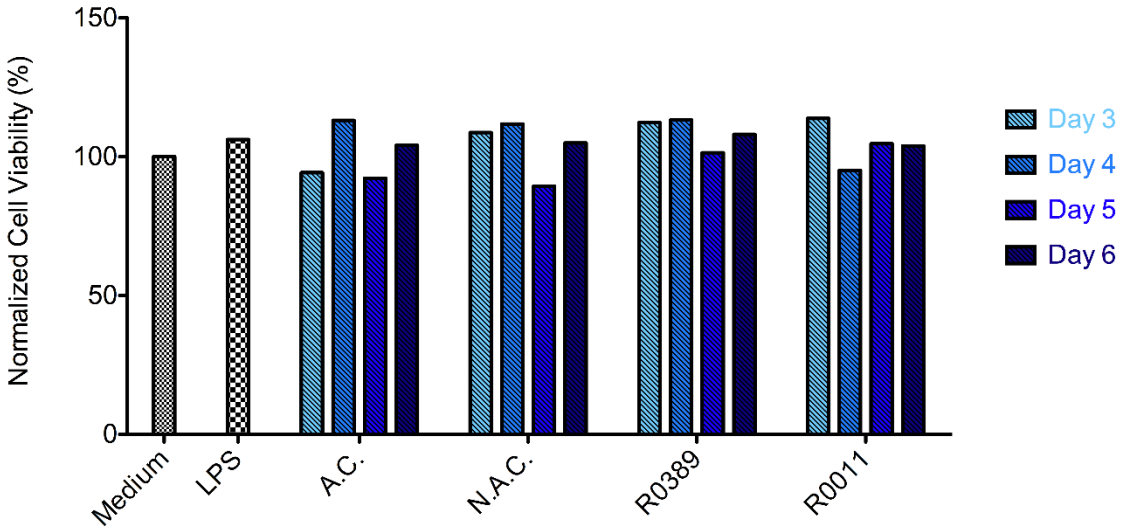


Figure 25 – Normalized cell viability of THP-1 cells treated for 48 hours with acetone precipitated, filtered (< 10 kDa) supernatant solutions (2.5 ng/mL) of *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments, and acidified (A.C.) and non-acidified (N.A.C.) controls, collected after 3, 4, 5, and 6 days of fermentation as determined by the XTT cell viability assay. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS. Each value is the average of three technical replicates of a single biological replicate. Percent viability was normalized to the medium control.

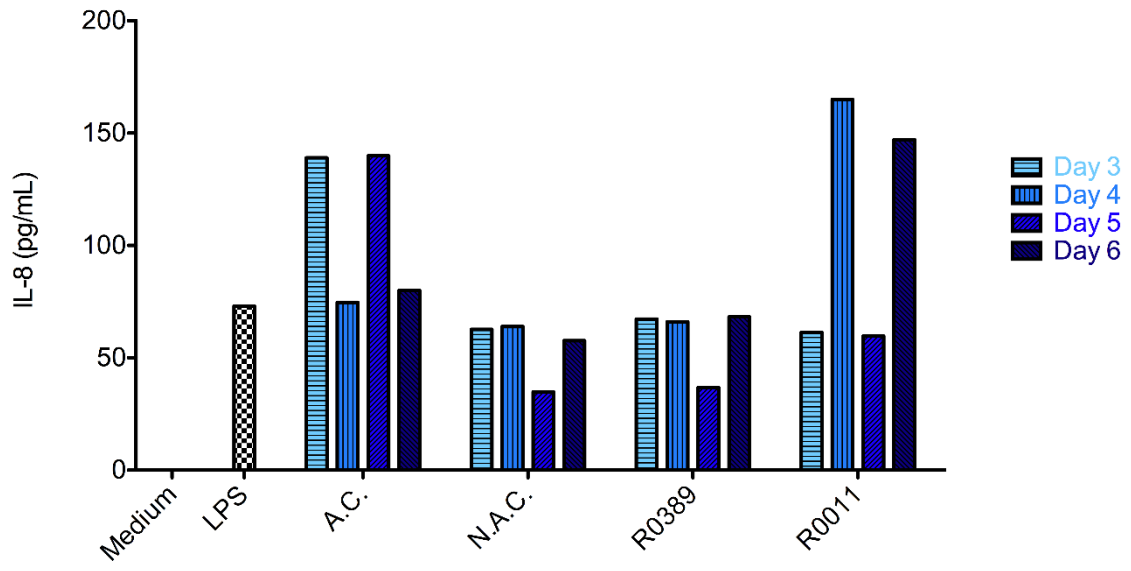


Figure 26 - IL-8 production by THP-1 cells treated for 48 hours with 125 ng/mL LPS and 2.5 ng/mL filtered (<10 kDa) supernatant solutions from *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments, and acidified (A.C.) and non-acidified (N.A.C.) controls, collected after 3, 4, 5, and 6 days of fermentation (n = 1).

One additional set of preliminary treatments was conducted in which THP-1 cells were pre-incubated for 17 hours with filtered peptide samples (< 10 kDa) from *L. helveticus* R0389 and *L. rhamnosus* R0011 supernatants, as well as from acidified and non-acidified controls, collected after 3, 4 or 5 days of fermentation. After the initial 17 hour incubation, 125 ng/mL LPS was added as an inflammatory stimulant to sample wells and the cells were incubated for another 7 hours. Cell viability for all treatments (LPS and supernatant samples) was greatly increased compared to the medium control (Figure 27). Pre-incubation of the monocytes with the peptide solutions prior to LPS stimulation was not able to prevent the LPS-induced IL-8 production (Figure 28). The protein buffer was also

assayed for its potential ability to induce IL-8 production, however no IL-8 was produced by THP-1 cells treated with the buffer solution alone.

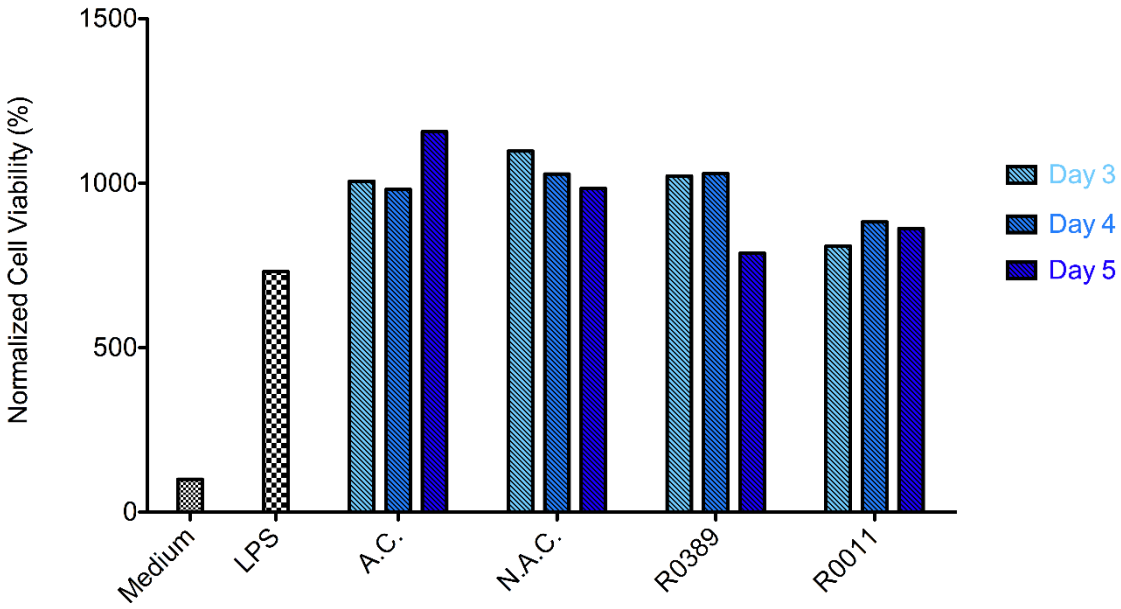


Figure 27 – Normalized cell viability of THP-1 cells pre-incubated for 17 hours with acetone precipitated, filtered (< 10 kDa) supernatant solutions (2.5 ng/mL) of *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments, and acidified (A.C.) and non-acidified (N.A.C.) controls, collected after 3, 4, 5, and 6 days of fermentation followed by the addition of LPS and incubation for an additional 7 hours as determined by the XTT cell viability assay. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells treated with RPMI 1640 medium with 10% FBS for 17 hours, and then stimulated with 125 ng/mL LPS for 7 hours. Each value is the average of three technical replicates of a single biological replicate.

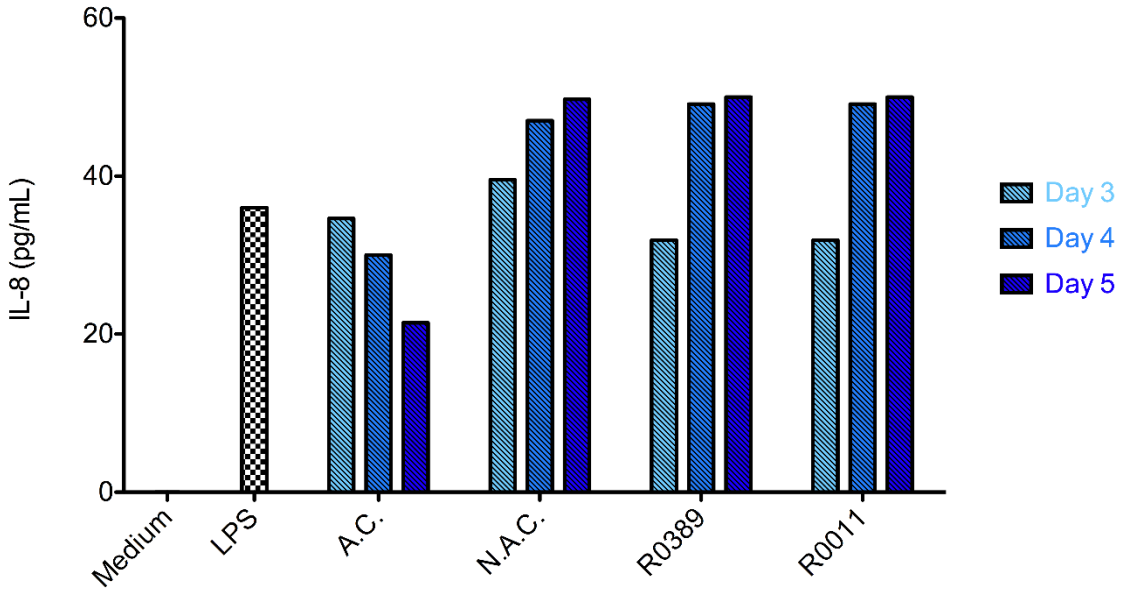


Figure 28 - IL-8 production by THP-1 cells pre-incubated with 2.5 ng/mL filtered (<10 kDa) supernatant solutions from *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments as well as acidified (A.C.) and non-acidified (N.A.C.) controls after 3, 4, and 5 days for 17 hours, followed by the addition of 125 ng/mL LPS and incubation for an additional 7 hours. Medium – THP-1 cells treated for 24 hours with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells treated for 17 hours with RPMI 1640 medium with 10% FBS followed by stimulation with 125 ng/mL LPS for 7 hours (n = 1).

Certain bacterial cell-surface components of lactobacilli, in addition to their secreted products, have been shown to possess bioactive properties through their interaction with immune cells (Wells, 2011). In order to investigate the effects of the bacterial fraction of the ferments, THP-1 cells were also co-challenged with LPS and decreasing volumes of the whole ferment culture solutions after 6 days of fermentation; a dramatic increase in IL-8 production compared to LPS-stimulated cells was observed by ELISA (Figure 29). The whole ferment cultures induced IL-8 production, with IL-8 levels increasing to levels far higher than those of the LPS-stimulated control.

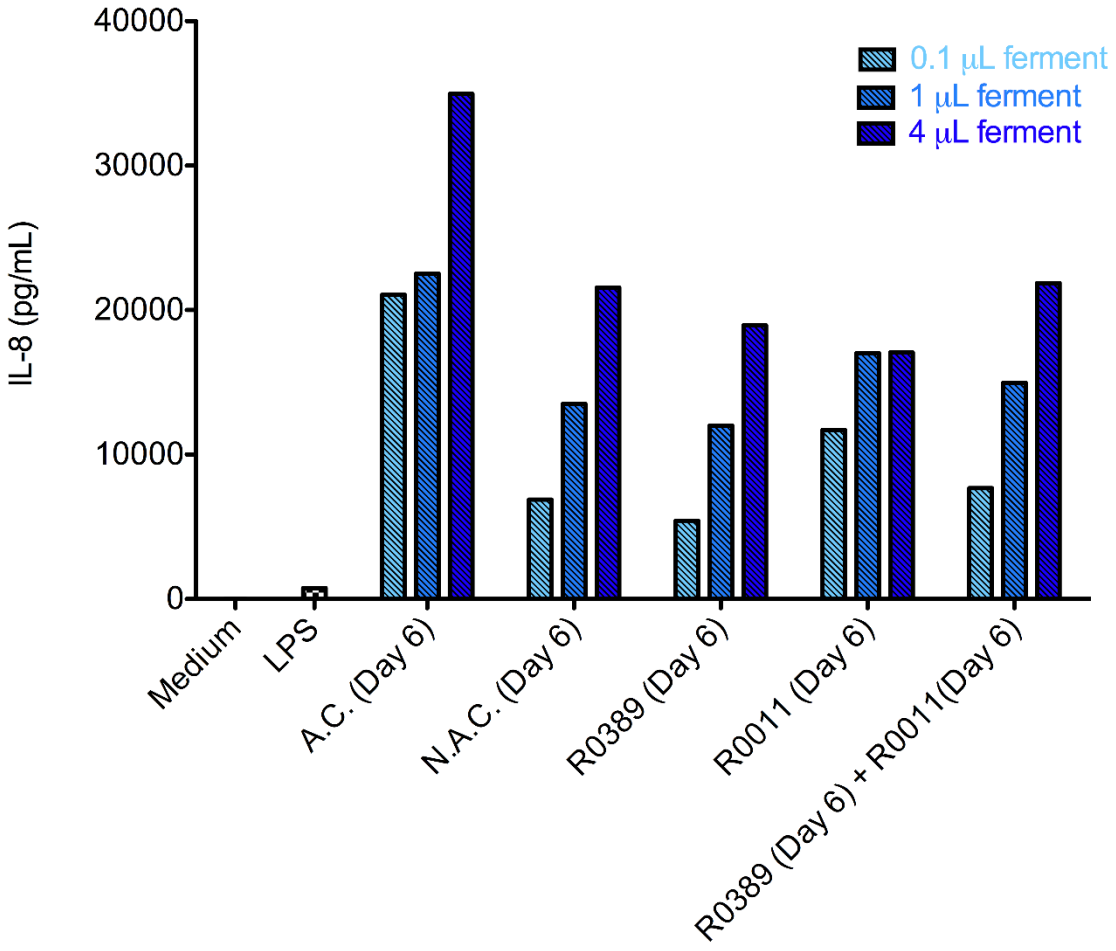


Figure 29 - IL-8 production by THP-1 cells co-treated with 3.25% milk fermented with *L. helveticus* R0389 and/or *L. rhamnosus* R0011, or an acidified (A.C.) and non-acidified (N.A.C.) control after 6 days of fermentation, and 125 ng/mL LPS. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS (n = 1).

Finally, filtered supernatant solutions (< 10 kDa) from three biological replicates of *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments, or acidified and non-acidified controls, were used to treat THP-1 monocytes in the absence of a pro-inflammatory stimulant (LPS). Oddly, viability of the THP-1s following this treatment decreased (Figure 30). However, ELISAs revealed that cells treated with all samples, in the absence of a pro-

inflammatory stimulant, showed very low IL-8 production, comparable to that of the medium control (Figure 31).

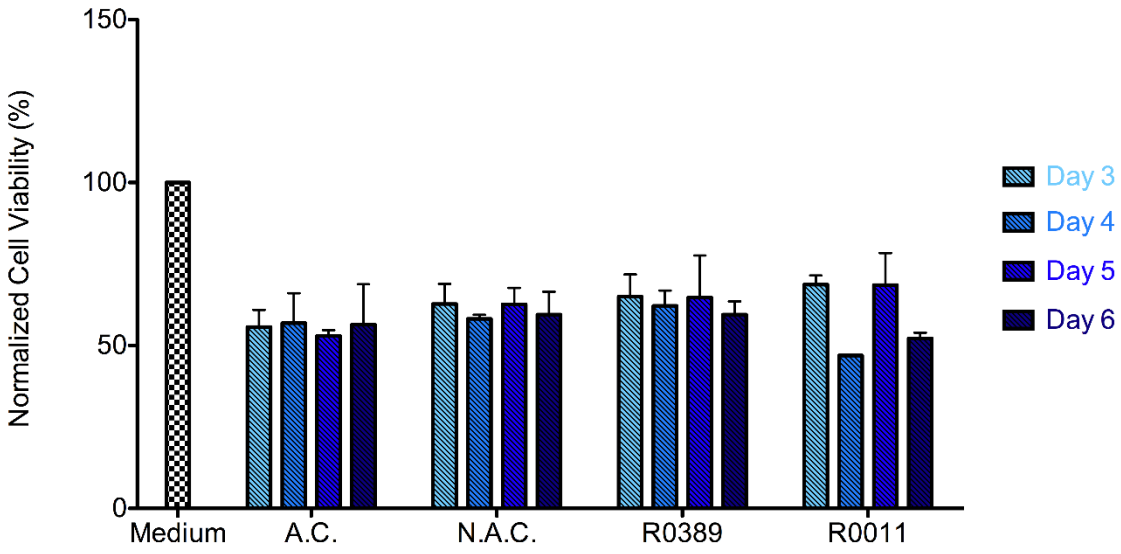


Figure 30 – Normalized cell viability (± SD) of THP-1 cells treated for 24 hours with acetone precipitated, filtered (< 10 kDa) supernatant solutions (2.5 ng/mL) *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments, and acidified (A.C.) and non-acidified (N.A.C.) controls, collected after 3, 4, 5 and 6 days of fermentation as determined by the XTT cell viability assay. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS (n=2). Percent viability was normalized to the medium control.

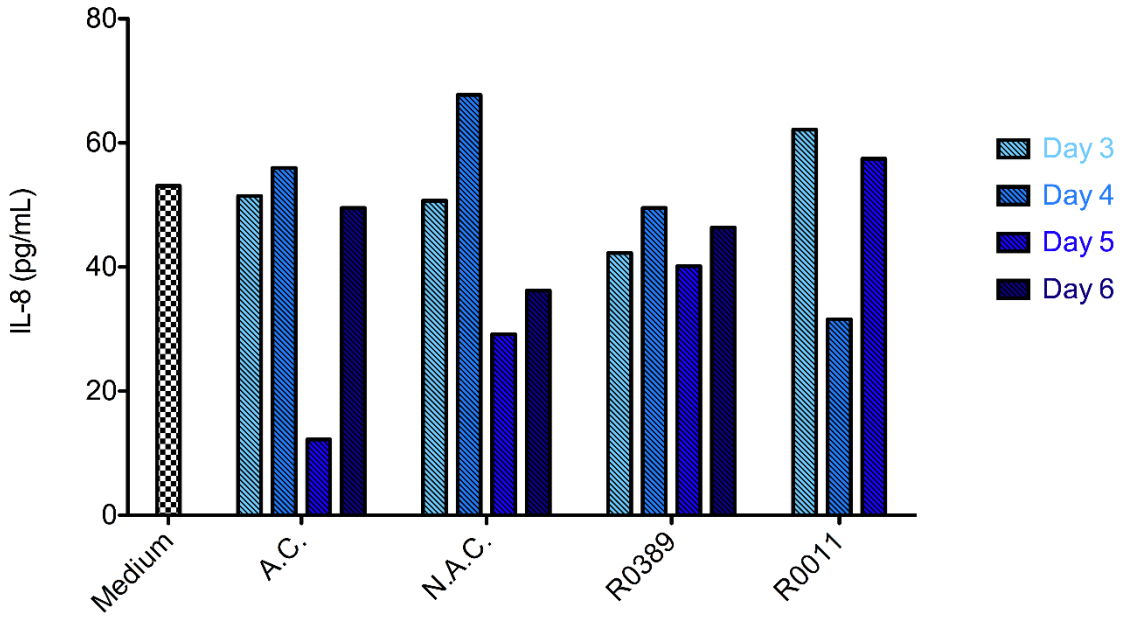


Figure 31 - IL-8 production by THP-1 cells treated for 24 hours with filtered (<10 kDa) supernatant solutions (2.5 ng/mL) from *L. helveticus* R0389 and *L. rhamnosus* R0011 ferments, and acidified (A.C.) and non-acidified (N.A.C.) controls, collected after 3, 4, and 5 days (No LPS). Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS (n = 1).

3.4.2 Synthesized Peptides

Twelve peptides derived from the β-casein sequence were synthesized (Table 2) and used to treat THP-1 monocytes in order to assess their effect on pro-inflammatory and regulatory cytokine production. VPP and IPP were identified by Nakamura *et al.* (1995a) in milk fermented with *L. helveticus* and *S. cerevisiae* and are potent ACE inhibitors (IC_{50} = 9 and 5 µM, respectively). Nakamura *et al.* (1995a) also reported their antihypertensive ability *in vivo*; administration of 1.6 mg/kg of VPP and 1.0 mg/kg of IPP were able to lower SBP in SHR by 20 and 15 mmHg, respectively. Although they are commonly used to supplement dietary products, additional research regarding the immune response induced by these two tripeptides individually is required. Five of the twelve synthesized peptides were identified by Tellez *et al.* (2010) as part of a larger immunomodulatory fraction.

Tellez *et al.* (2010) reported immune stimulation, characterized by an increase in IL-6, I-1β, and TNF-α production, in RAW264.7 cells. However, they observed macrophage cell death at peptide concentrations higher than 20 µg/mL. We therefore decided to further investigate the effects of these five peptides on the immune response by assessing their effect on cytokine production by THP-1 monocytes. Four of the selected peptides, YP, FFVAP, KVLPVP, and KVLPVP, have all been identified as ACE-inhibitory peptides in the literature, however their immunomodulating properties had yet to be investigated (Yamamoto *et al.*, 1999; Yamamoto *et al.*, 1997; Maeno *et al.*, 1996). Since atherosclerosis is characterized as an inflammatory disease, we now know the large role of the immune system in the progression of this disease. Characterizing the multiple bioactive properties of these peptides, including the effects on the immune cell response, can help to further the understanding of the mechanisms of action responsible for the health benefits *in vivo*. Therefore cells were exposed, for 24 hours, to each individual peptide, with and without 125 ng/mL LPS. Cell viability for both treatments remained above 80% (Figure 32 and Figure 33, respectively). None of the synthesized peptides were able to significantly reduce the amount of IL-8 produced compared to the LPS-stimulated levels at any of the concentrations tested; IL-8 production increased with increasing concentrations of peptides (Figure 34). However, without stimulation with LPS, peptides induced minimal amounts of IL-8 on their own (Figure 35). This could indicate that, without existing inflammatory stimuli, they are not able to induce inflammation through the production of IL-8.

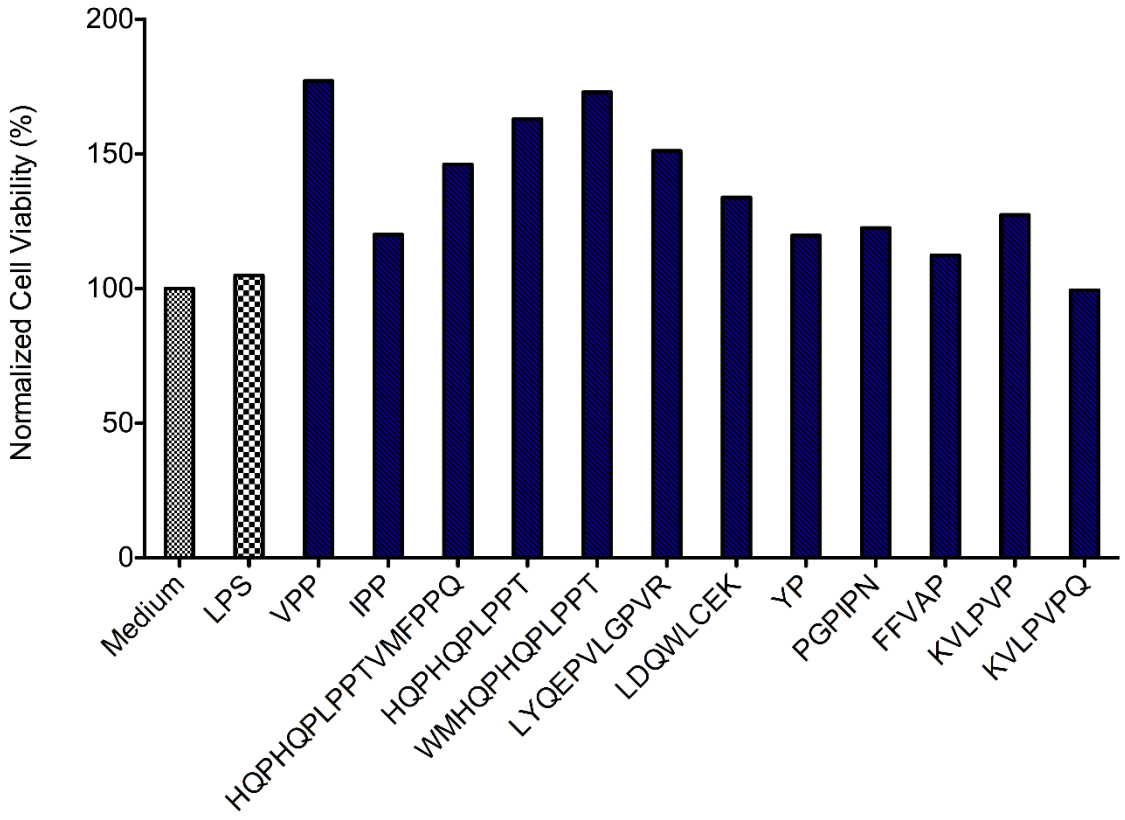


Figure 32 – Normalized cell viability of THP-1 cells treated for 24 hours with 125 ng/mL LPS and 1 µg/mL synthesized peptides as determined by the XTT cell viability assay. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS. Percent viability was normalized to the medium control (n = 1).

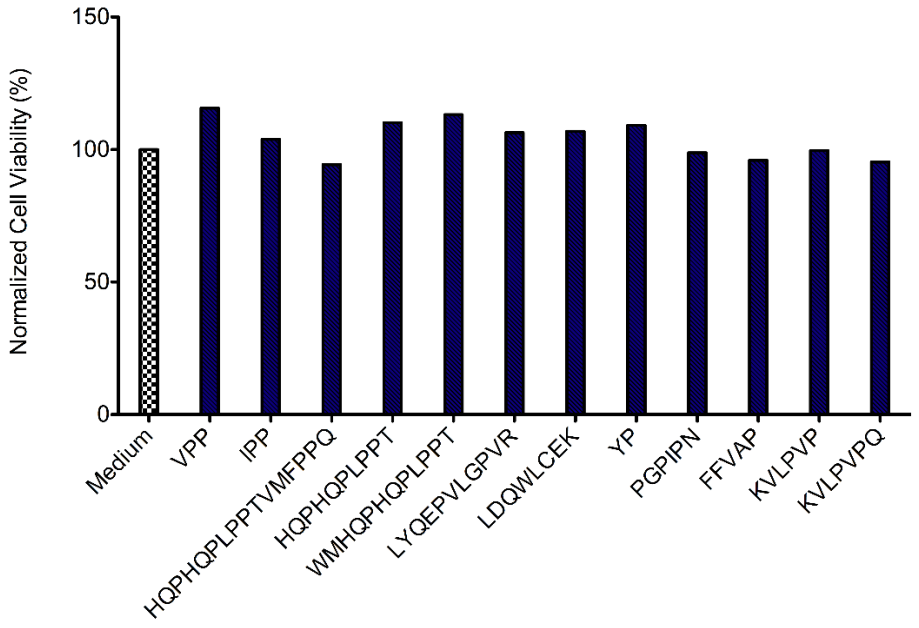


Figure 33 – Normalized cell viability of THP-1 cells treated for 24 hours with 1 µg/mL synthesized peptides (without LPS) as determined by the XTT cell viability assay. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS. Percent viability was normalized to the medium control (n = 1).

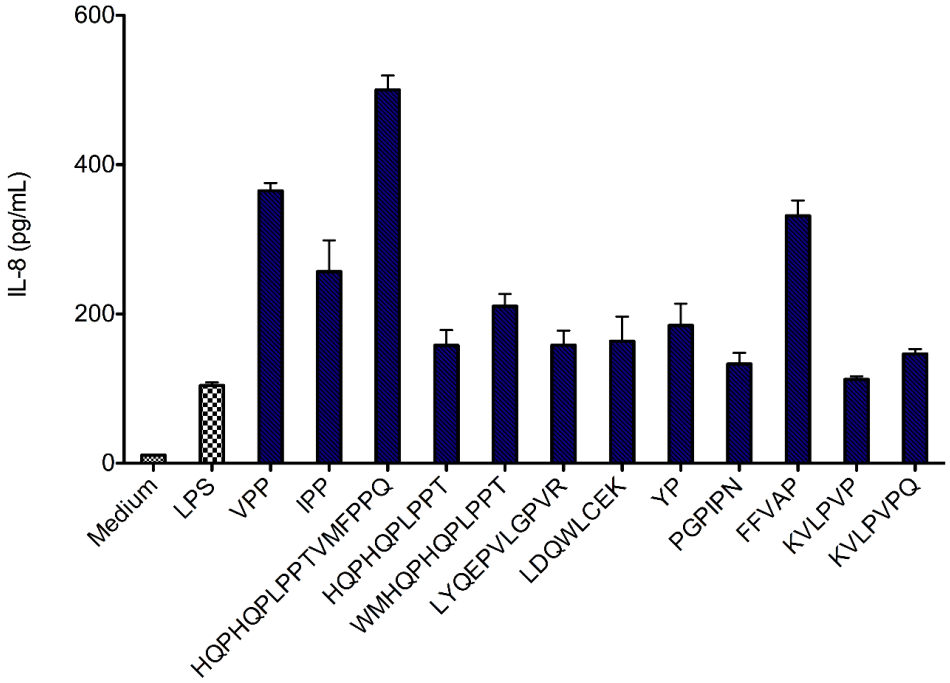


Figure 34 - IL-8 production (± SD) by THP-1 cells treated for 24 hours with 125 ng/mL LPS and 1 µg/mL synthesized peptides. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS (n = 1).

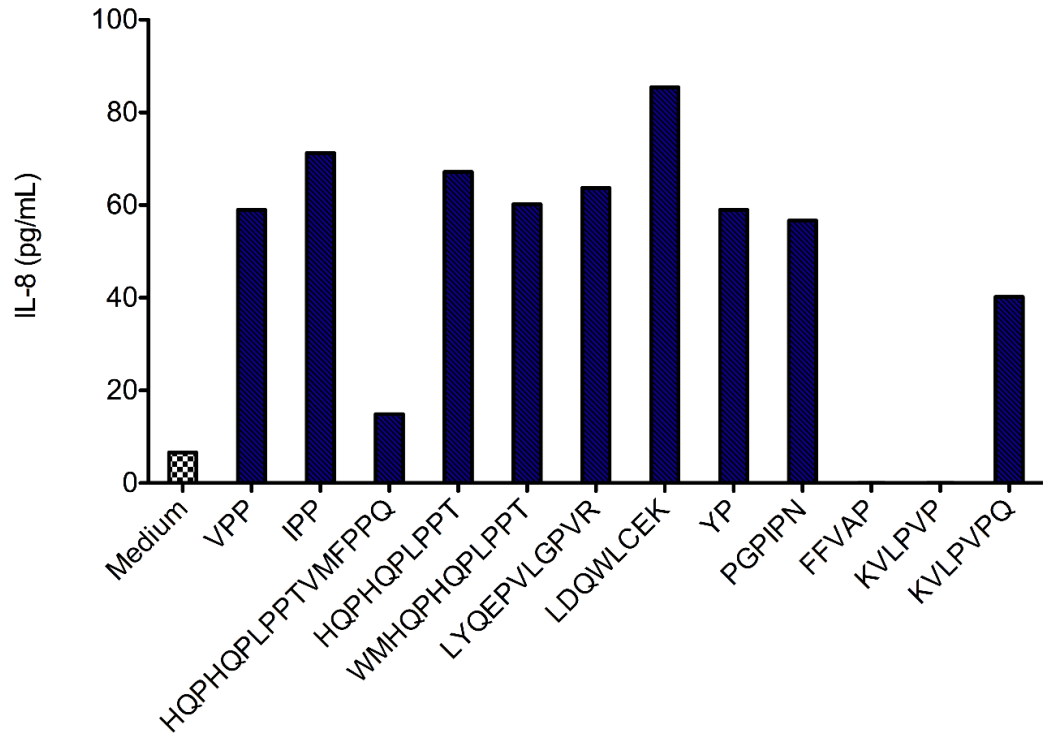


Figure 35 - IL-8 production by THP-1 cells treated for 24 hours with 1 µg/mL synthesized peptides (without LPS). Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS (n = 1).

IL-10 production is often an indication of the regulatory or anti-inflammatory properties of probiotics or bioactive peptides (Wells, 2011). IL-10 may also greatly influence the inflammatory process within atherosclerotic lesions through potent deactivating properties on both macrophages and T cells (Mallat *et al.*, 1999). Therefore, the production of IL-10, a regulatory cytokine, by THP-1 cells was also investigated when treated with various concentrations of the synthesized peptides. In both the presence and absence of the inflammatory stimulant (LPS) a majority of the synthesized peptides, at a concentration of 1 µg/mL, were able to induce the production of the regulatory IL-10 with levels increasing in the absence of LPS; IPP treated cells resulted in the highest production of IL-10 (Figure 36 and Figure 37). Increased concentrations (5 and 10 µg/mL) of three of the twelve

peptides (VPP, IPP, and KVLPVP) were also used to treat THP-1s. In this case, IL-10 production seemed to increase with increasing peptide concentration for VPP and KVLPVP in the presence and absence of LPS. IL-10 levels remained relatively steady, compared to 1 µg/mL levels for cells co-incubated with IPP and LPS, and dropped at higher concentrations for cells exposed to IPP (no LPS) (Figure 38 and Figure 39). The induction of IL-10 production by the THP-1 monocytes indicates a potentially regulatory response to each of the peptides administered.

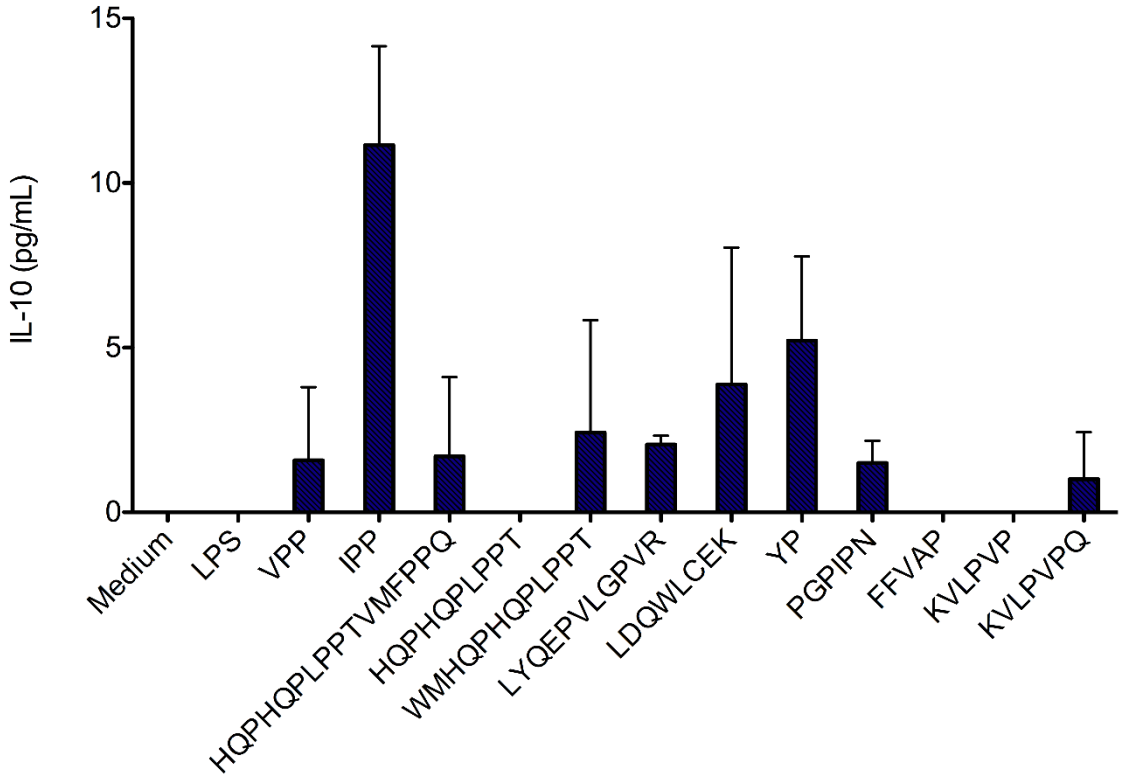


Figure 36 - IL-10 production (± SD) by THP-1 cells treated for 24 hours with 125 ng/mL LPS and 1 µg/mL synthesized peptides. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS (n = 2).

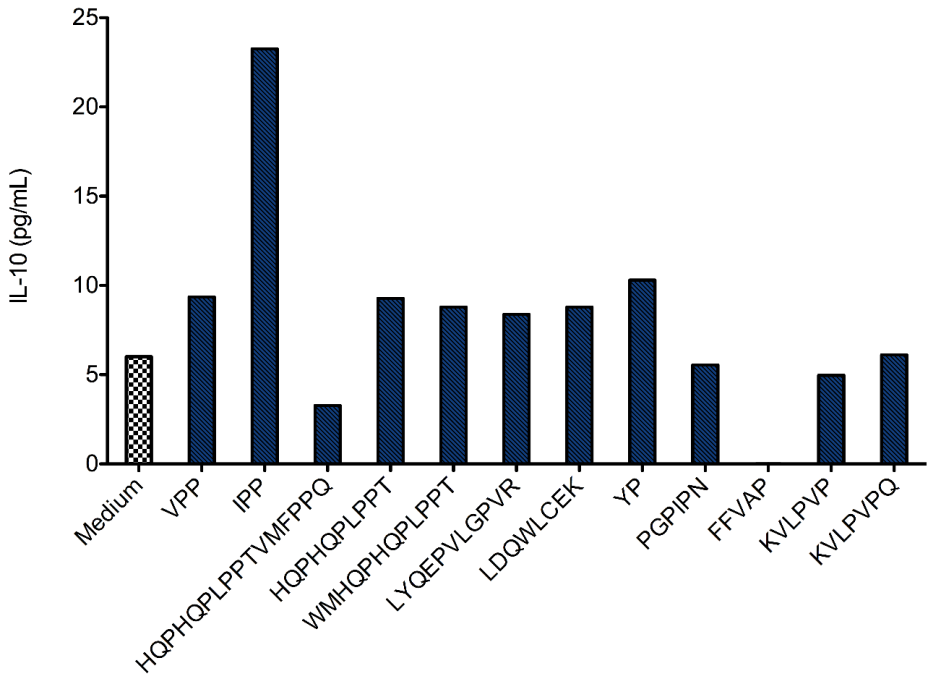


Figure 37 - IL-10 production by THP-1 cells treated for 24 hours with 1 µg/mL synthesized peptides (without LPS). Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS (n = 1).

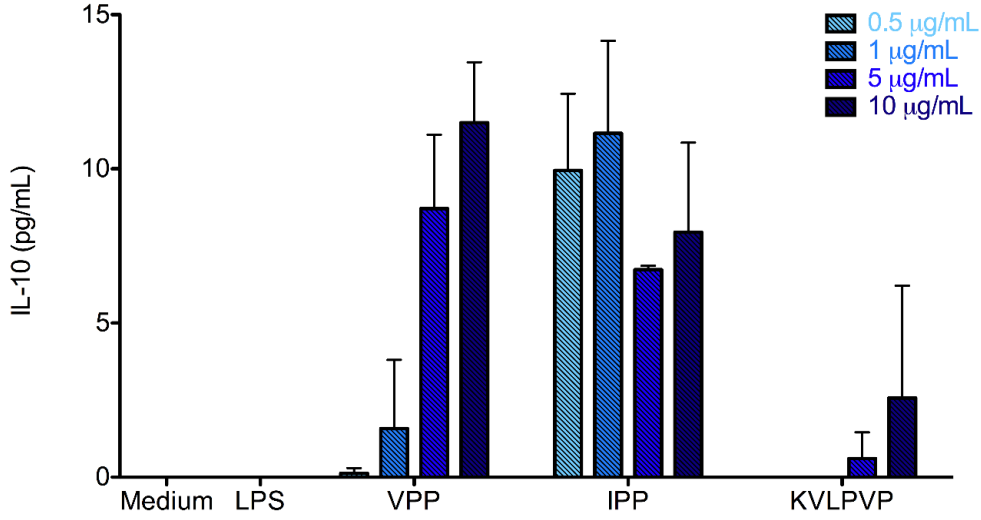


Figure 38 - IL-10 production (± SD) by THP-1 cells treated for 24 hours with 125 ng/mL LPS and 0.5, 1, 5 or 10 µg/mL of VPP, IPP, or KVLPVP. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS (n = 2).

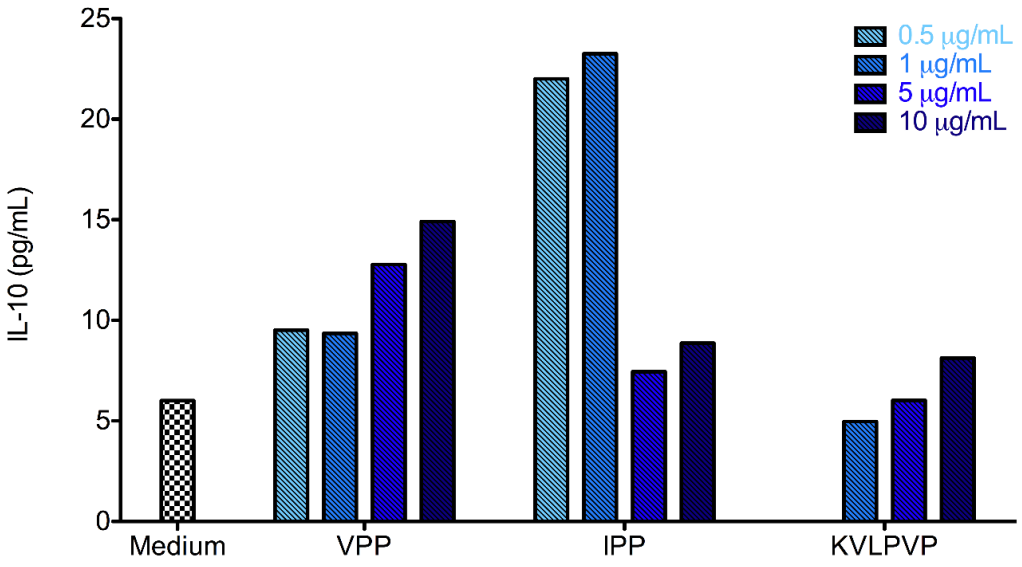


Figure 39 - IL-10 production by THP-1 cells treated for 24 hours with 0.5, 1, 5 or 10 µg/mL of VPP, IPP, or KVLPVP. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS (n = 1).

The effect of the synthesized peptides on the production of IL-6, another pro-inflammatory cytokine, by THP-1 cells was also investigated. Cells were exposed to 1 µg/mL of all twelve synthesized peptides as well as 0.5, 5, and 10 µg/mL solution of VPP, IPP, and KVLPVP (without LPS). At concentrations of 0.5 and 1 µg/mL, THP-1s did not produce any detectable IL-6. At 5 µg/mL, of the three peptides tested, only KVLPVP induced the production of low levels of IL-6 (36.70 ± 8.83 pg/mL). As seen with IL-10, increasing the concentration of VPP, IPP, and KVLPVP to 10 µg/mL further increased IL-6 production to 88.39 ± 4.30 pg/mL, 78.85 ± 1.15 pg/mL and 102.24 ± 18.76 pg/mL, respectively. Therefore it seems that, at low enough concentrations, these peptides will not elicit an inflammatory response through the production of pro-inflammatory cytokines. However, increasing peptide concentration leads to an increase in cytokine production (both regulatory and inflammatory).

4 CONCLUSIONS AND FUTURE DIRECTIONS

The aim of this study was to investigate the antihypertensive and immunomodulatory effects of casein-derived peptides. It was observed that supplementation of MRS medium with either 0.1% casein, an abundant milk protein, or 0.01% sodium formate, a compound known to stimulate the growth of *L. bulgaricus* in co-culture with *S. thermophilus*, increased the growth rate and doubling time of *L. helveticus* R0389 and *L. rhamnosus*. Both strains of lactobacilli were also used to ferment 3.25% milk to investigate the variety of casein-derived peptides liberated by each strain and to identify bioactive properties of *Lactobacillus* peptide fractions. Peptide analysis by polyacrylamide gel electrophoresis revealed unique protein and peptide bands present in milk ferment samples fermented with either *L. helveticus* R0389 or *L. rhamnosus* R0011.

Further elucidation is required in order to identify the specific bioactive compounds within each milk ferment sample. We have shown that the 3 to 10 kDa fraction of the cell-free supernatant of milk fermented with two strains of lactic acid bacteria confers more bioactivity than < 3 kDa or unfiltered supernatant samples; however, multiple components within each of those fractions could be contributing to bioactivity. Fractions should be treated with peptidases, to confirm that any bioactivity observed is solely due to peptides present. Lipase treatments of fractions of different MWCOs can also provide insight into the role of lipids in the antihypertensive and immunomodulating properties of peptide fractions from milk ferments cultured with LAB.

Many other bacterial components can be assessed for their role in conferring benefits to consumers of fermented milk products. Surface layer proteins, cell wall components of gram-positive bacteria (lipoproteins, lipoteichoic acids, and peptidoglycan) or lipolysis

products can also be investigated for their impact on immune parameters or for their potential role in the reduction of hypertension. Despite the extensive research surrounding the bioactive properties of fermented dairy products and the contribution of lactic acid bacteria, little is known regarding the mechanisms responsible for both antihypertensive and immunomodulatory effects observed. Further investigation into the interaction of lactobacilli and the peptides liberated in the fermentation process with various immune cells, as well as intestinal epithelial, and vascular endothelial cells can aid towards elucidating the mechanisms responsible for the benefits of these probiotic organisms in the production of fermented dairy products.

The benefits of using probiotic organisms such as lactobacilli as a treatment or preventive measure for the development of mild hypertension has been well established, however the effect of these bacteria and their secreted products on the immune response still has yet to provide consistent and definitive results. Further investigation into the immunomodulatory effects of fermented dairy products and the probiotic organisms used in their production can supplement the knowledge of their ability to regulate blood pressure; together this information can help to improve upon the use of probiotics in commercially available dietary products or can provide an accepted, cost-effective alternative to synthetic drugs to treat a variety of chronic inflammatory diseases, including atherosclerosis.

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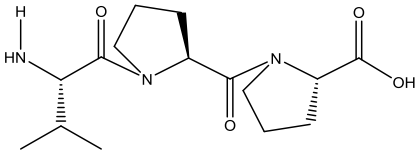
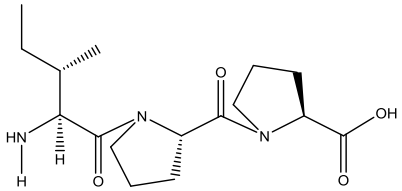
6 APPENDIX A

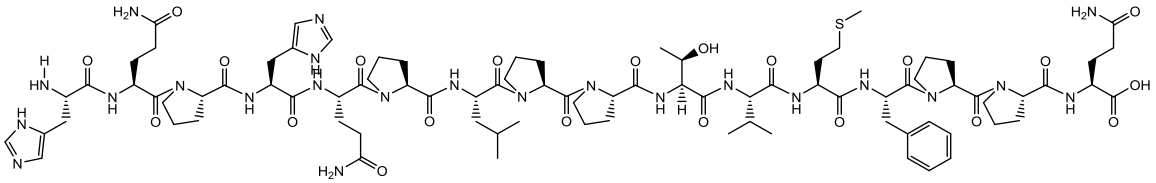
Table 5 – Structures and properties of synthesized peptides



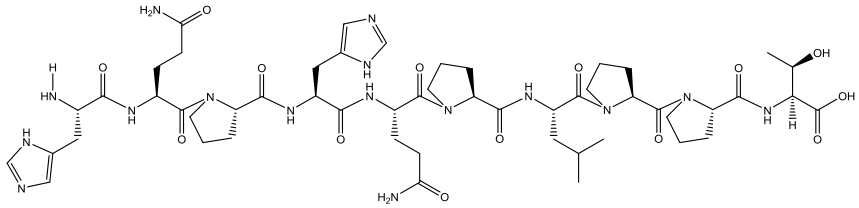
P1: VPP, 311.38 g/mol
Protein origin: β-casein (84-86)
Net Charge: 0 (Neutral), pI: 6.01,
Hydrophobicity: +7.72 Kcal/mol



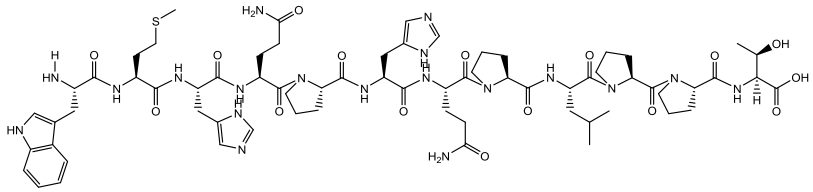
P2: IPP, 325.41 g/mol
Protein origin: β-casein (74-76)
Net Charge: 0 (Neutral), pI: 6.01,
Hydrophobicity: +7.06 Kcal/mol



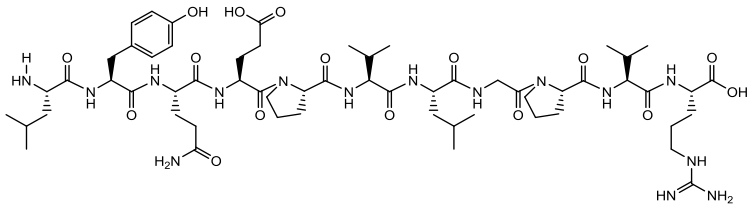
P3: HQPHQPPLPPTVMFPPQ, 1851.18 g/mol
Protein origin: β-casein (145-160)
Net Charge: +2 (Basic), pI: 8.0, Hydrophobicity: +11.87 Kcal/mol



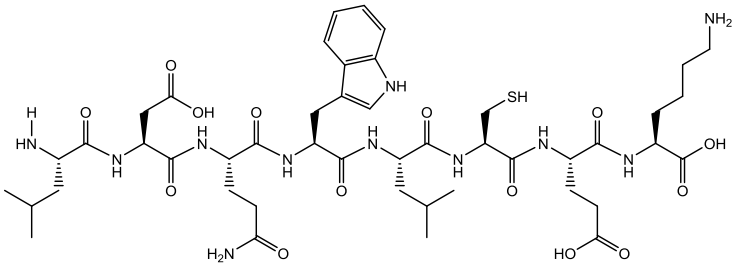
P4: HQPHQPPLPPT, 1151.3 g/mol
Protein origin: β-casein (145-154)
Net Charge: +2 (Basic), pI: 8.0, Hydrophobicity: +13.66 Kcal/mol



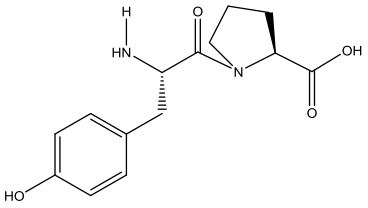
P5: WMHQPHQPLPPT, 1468.71 g/mol
Protein origin: β-casein (143-154)
Net Charge: +2 (Basic), pI: 8.0, Hydrophobicity: +10.90 Kcal/mol



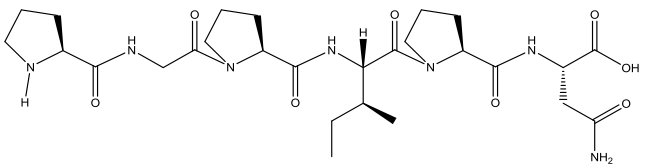
P6: LYQEPVLGPVR, 1270.51 g/mol
Protein origin: β-casein (192-202)
Net Charge: 0 (Neutral), pI: 6.89, Hydrophobicity: +11.41 Kcal/mol



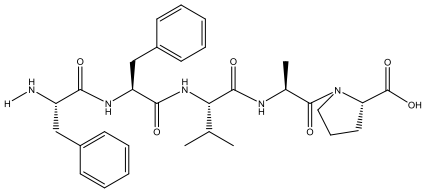
P7: LDQWLCEK, 1034.2 g/mol
Protein origin: β-casein (115-122)
Net Charge: -1 (Acidic), pI: 4.07, Hydrophobicity: +14.13 Kcal/mol



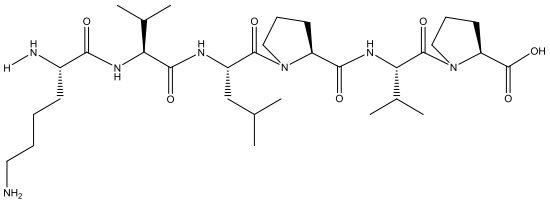
P8: YP, 278.31 g/mol
Protein origin: α_{s1}/β/κ-casein
Net Charge: 0 (Neutral), pI: 5.93, Hydrophobicity: +7.33 Kcal/mol



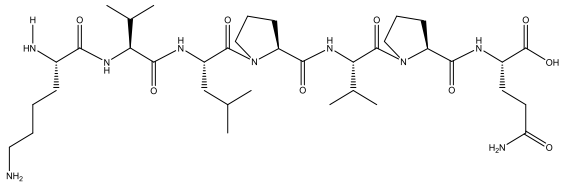
P9: PGPIPN, 593.69 g/mol
Protein origin: β-casein (63-68)
Net Charge: 0 (Neutral), pI: 6.01, Hydrophobicity: +9.20 Kcal/mol



P10: FFVAP, 579.70 g/mol
Protein origin: β-casein (23-27)
Net Charge: 0 (Neutral), pI: 6.01, Hydrophobicity: +4.66 Kcal/mol



P11: KVLPVP, 651.85 g/mol
Protein origin: β-casein (169-174)
Net Charge: +1 (Basic), pI: 10.1, Hydrophobicity: +8.81 Kcal/mol



P12: KVLPVPQ, 779.99 g/mol
Protein origin: β-casein (169-175)
Net Charge: +1 (Basic), pI: 10.1, Hydrophobicity: +9.58 Kcal/mol

*Peptide structures drawn using ChemBioDraw Ultra 13.0. Peptide characteristics were obtained from PepDraw by Tulane University (hydrophobicity values) (http://www.tulane.edu/~biochem/WW/PepDraw/) and Peptide property calculator by Innovagen (pI) (http://www.innovagen.com/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp)

7 APPENDIX B

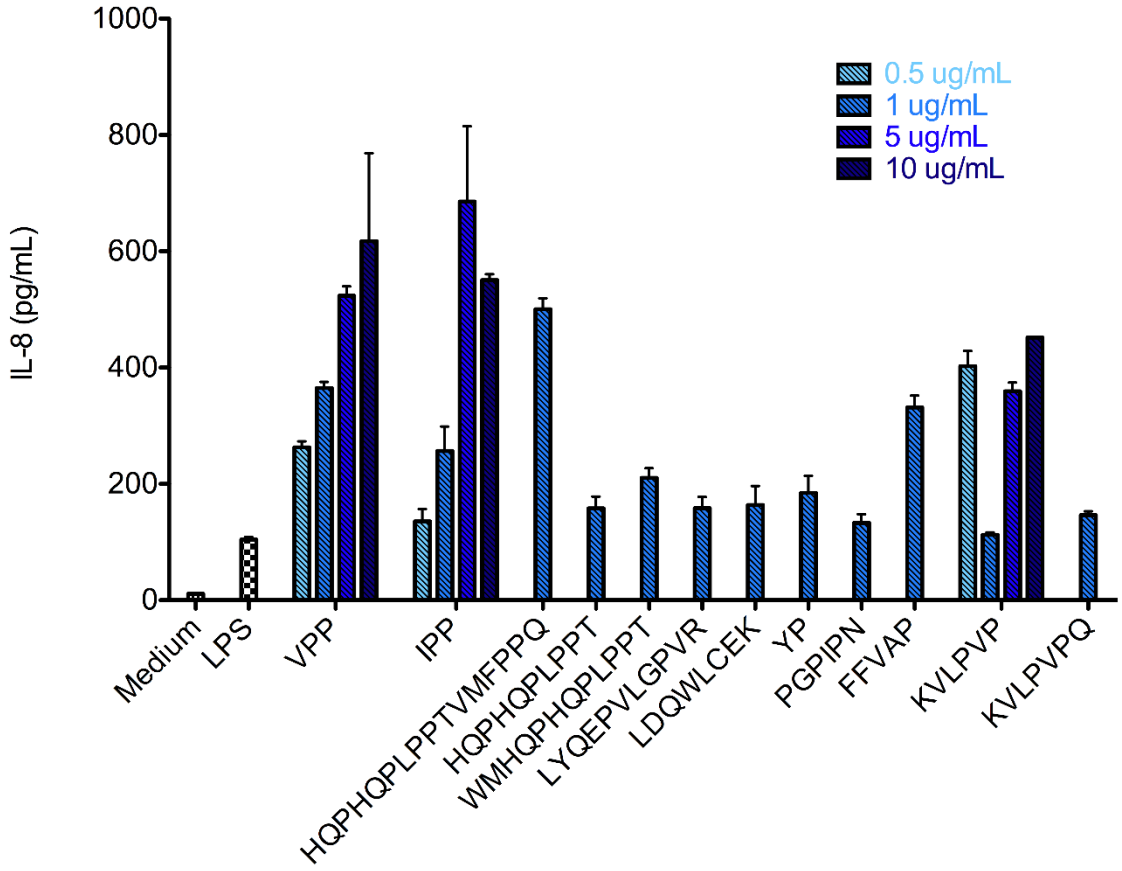


Figure 40 – IL-8 production (± SD) by THP-1 cells treated for 24 hours with 125 ng/mL LPS and 0.5, 1, 5 or 10 µg/mL synthesized peptides. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS.

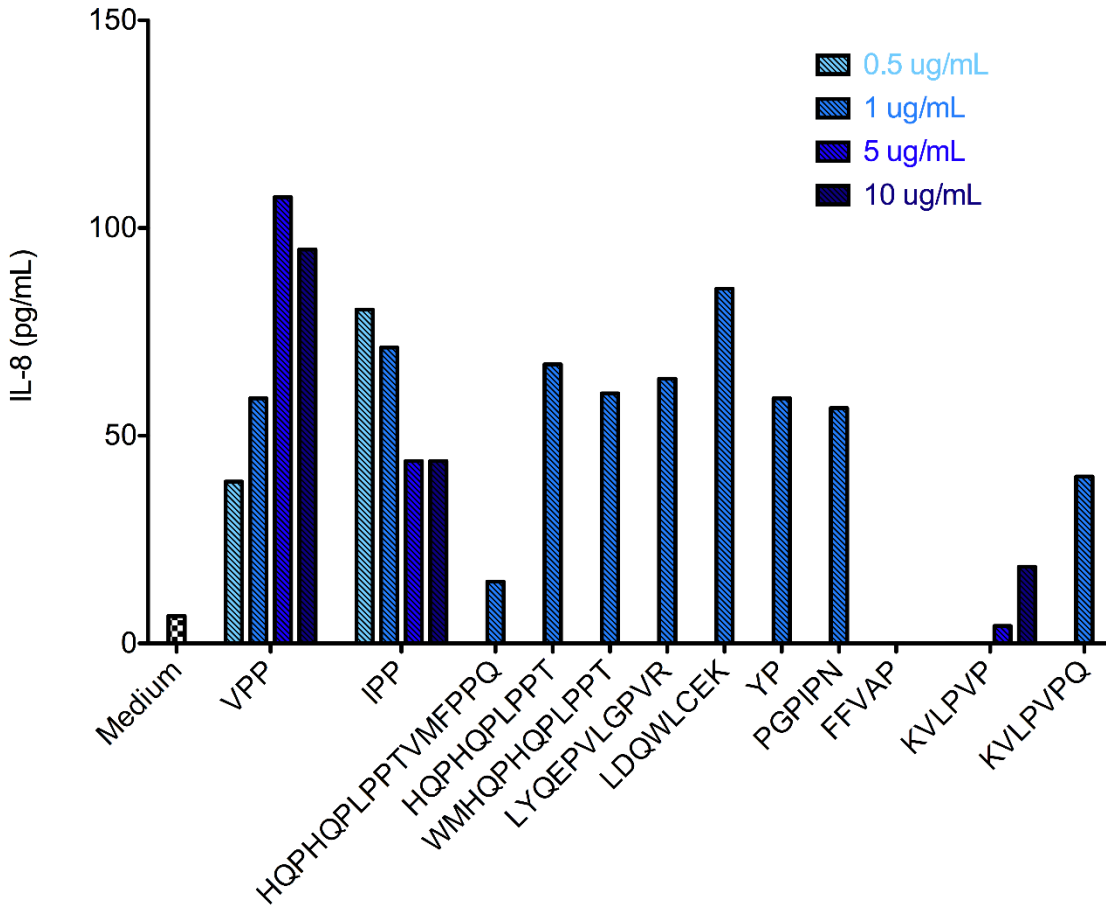


Figure 41 - IL-8 production by THP-1 cells treated for 24 hours with 0.5, 1, 5 or 10 µg/mL synthesized peptides (without LPS). Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS.

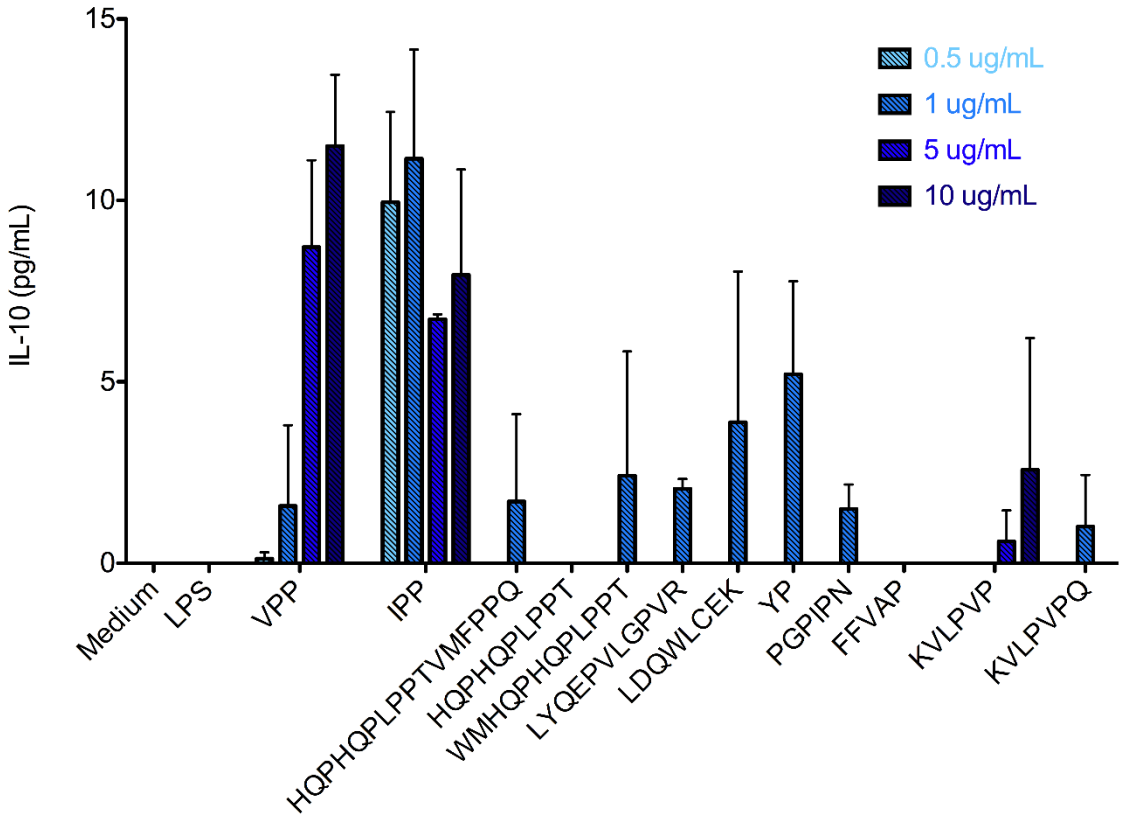


Figure 42 - IL-10 production (± SD) by THP-1 cells treated for 24 hours with 125 ng/mL LPS and 0.5, 1, 5 or 10 µg/mL synthesized peptides. Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS; LPS – THP-1 cells stimulated with 125 ng/mL LPS in RPMI 1640 medium with 10% FBS.

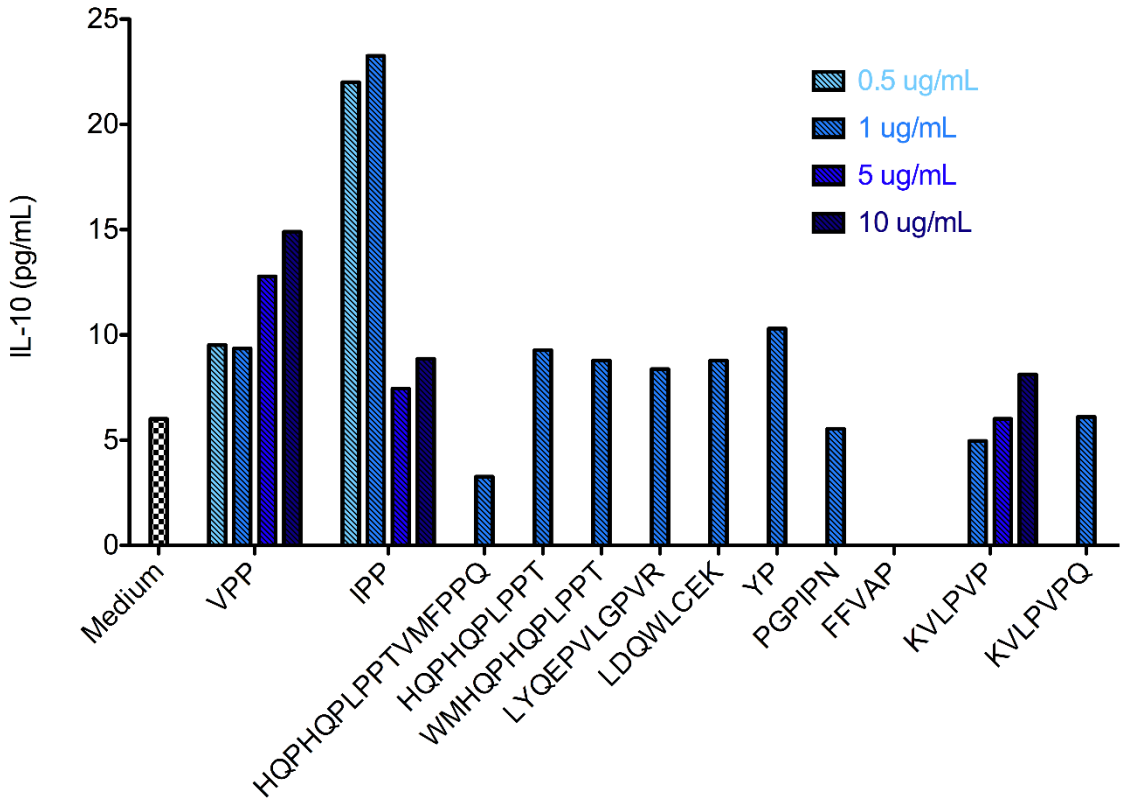


Figure 43 - IL-10 production by THP-1 cells treated for 24 hours with 0.5, 1, 5 or 10 µg/mL synthesized peptides (without LPS). Medium – THP-1 cells treated with RPMI 1640 medium with 10% FBS.