# Examining Effects of Milk Ferment Components on Endothelial Cell Signaling in Pro-Inflammatory Pathways

By:

Kyle R.E. Dobby

A Thesis Submitted in Partial Fulfillment of the Requirements of the Degree of

Masters in Applied Bioscience

In

The Faculty of Science

Applied Bioscience MSc.

University of Ontario Institute of Technology

August 2015

# Abstract

Atherosclerosis is a complex, multifactorial, inflammatory disease involving cholesterol, endothelial cells, smooth muscle cells, macrophages and platelets. Atherosclerosis, and the associated cardiovascular diseases it contributes to, has been a growing health care and economic concern over the last 50 years. Bioactive peptides found in fermented milk have been shown to have cardio and athero-protective effects, but their mechanism has not been fully elucidated. The objective of this study was to test individual components of milk fermentation and macrophage conditioning in order to determine how fermented milk bioactive peptides best exert their effects. This study shows that protein secretions from bacteria, whole and fermented milk ferments and supernatants from macrophages conditioned with fermented milk do not down-regulate inflammatory activity in cultured endothelial cells, however moderate anti-inflammatory nitric oxide (NO) production by cultured endothelial cells was seen with peptides derived from the milk β -casein sequence, warranting further study.



*Keywords*: atherosclerosis, cardiovascular disease, endothelial cells, bioactive peptides, fermented milk, β -casein

# Acknowledgments

First, I would very much like to thank my supervisor, Dr. Holly Jones-Taggart. Thank you for giving me the opportunity to work on such a compelling and rewarding project, and thank you for your constant support and encouragement through all the struggles and successes of my research and writing. Having her to guide me through this process made what could have been an entirely frustrating experience a positive and completely rewarding one. I would not have been able to succeed as I have without her, and for that I will always be grateful.

I wish to also extend a tremendous amount of gratitude to the other members of my committee, Dr. Julia Green-Johnson and Dr. Janice Strap. Your insight into the different aspects of my research, your willingness to always help and your kindness and support was integral to my success here.

As well, I would like to also thank my fellow graduate students. They have always been incredibly supportive, welcoming and empathetic, and they have made my time here at UOIT vastly more enjoyable. Relying on each other during the more stressful periods of our research made the hard times easier and the good times better.

Finally I wish to thank my family and friends, and most importantly my partner and son, for their constant, almost unending, love and support. Without their comfort, encouragement, and belief in my abilities during the long hours and late nights I would

not have been able to succeed at all. Their support has meant the world, and I dedicate all I have achieved to them.

**List of Abbreviations**

AC – Acidified Control

ACE – Angiotensin Converting Enzyme

ANOVA – Analysis of Variance

apoB – Apoprotein B

ATCC – American Type Culture Collection

BP – Blood Pressure

C- Celsius

C - Cysteine

CCL2 – Chemokine (C-C Motif) Ligand 2

CD54 – Cluster of Differentiation 54

CDC – Centres for Disease Control and Prevention

CFU – Colony Formulating Unit

cm - Centimeter

$CO_2$ – Carbon Dioxide

CVD – Cardiovascular Disease

DMSO – Dimethyl Sulfoxide

E – Glutamic Acid

E-selectin – Endothelial Selectin

EDTA – Ethylene diamine tetra acetic acid

ELISA – Enzyme-Linked Immunosorbent Assay

F - Phenylalanine

FBS – Fetal Bovine Serum

FCS – Fetal Calf Serum

FFA – Free Fatty Acids

g – Gravity

GALT – Gut Associated Lymphoid Tissue

H – Histidine

$H_2SO_4$ – Sulfuric Acid

HDL – High Density Lipoprotein

HOMA – Homeostatic Model Assessment

HRP – Horseradish Peroxidase

HUVEC – Human Umbilical Vein Endothelial Cell

I - Isoleucine

IL-6 – Interleukin 6

IL-8 – Interleukin 8

ICAM-1 – Intercellular Adhesion Molecule -1

ICAM-2 – Intercellular Adhesion Molecule 2

IEC – Intestinal Epithelial Cell

IgG – Immunoglobulin G

IPP – Isoleucyl-Prolyl-Proline

K - Lysine

kDa – Kilodalton

L – Leucine

LA – Lactic Acid

LDL – Low Density Lipoprotein

LFA-1 – Lymphocyte Function-Associated Antigen – 1

M - Methionine

MAPK – Mitogen-Activated Protein Kinases

MCP-1 – Monocyte chemoattractant Protein – 1

MCSF – Macrophage Colony Stimulating factor

mL – Milliliter

mM – millimolar

MMP – Matrix Metalloproteinase

MRS – de Man, Rogosa and Sharpe agar

N - Asparagine

ng - nanograms

NO – Nitric Oxide

$O_2$ – Oxygen

oxLDL – Oxidized Low Density Liporotein

P – Proline

P-selectin – Platelet Selectin

PAI-1 – Plasminogen Activator Inhibitor-1

PE – Phycoerythrin

PBS – Phosphate Buffered Saline

PMA – phorbol-12-myrisate-13-acetate

Q - Glutamine

R&D – Research and Development

RANTES – Regulated on Activation, Normal T Cell Expressed and Secreted

RPMI – Roswell Park Memorial Institute

sICAM-1 – Soluble Intercellular Adhesion Molecule – 1

T - Tyrosine

TG – Triglycerides

TMB – Tetramethylbenzidine

TNF- α – Tumor Necrosis Factor Alpha

µg – microgram

uL – microliter

UV – Ultraviolet

V - Valine

VCAM-1 – Vascular Cell Adhesion Molecule – 1

VPP – Valyl-Prolyl-Proline

W - Tryptophan

w/v – Weight by volume

Y – Tyrosine

**Table of Contents**

**List of Figures**

**List of Tables:**

# 1 - Introduction

## 1.1 - Cardiovascular Disease

According to the Public Health Agency of Canada, cardiovascular disease (CVD) is a term that refers to multiple diseases of the heart and circulatory system of the lungs, the brain, kidneys or other parts of the body (Public Health Agency of Canada, 2013). The most recent findings from the Canadian Heart and Stroke Foundation found that 1.3 million people in Canada suffer from chronic CVD or atherosclerosis, with another 300,000 affected by the symptoms of stroke (Dai, Bancej et al. 2009).

Cardiovascular disease can, generally, be divided into 6 categories (Public Health Agency of Canada, 2013). First and foremost, the most common sub-type of CVD in Canada and other industrialized nations is ischemic heart disease. Ischemia presents itself when blood can no longer circulate properly to the heart muscle. The lack of blood supply manifests from a blockage created through a thromboembolism or atherosclerotic plaque buildup. Symptoms generally present themselves through angina (acute chest pain) or dyspnea (shortness of breath). Complete blockage can result in insufficient oxygen leading to necrosis and myocardial infarction, most commonly referred to as a "heart attack". The second subtype is known as cerebrovascular disease which can lead to a stroke. Strokes occur when the blockage occurs in the brain, instead of cardiac muscle. Due to the lack of oxygenation to the brain, symptoms can be far reaching and severe, depending on the area of the brain affected (Macciocchi et al. 1998). Blockage of less than 60 minutes (Albers et al. 2002) is referred to as a "transient ischemic attack." The term "Stroke" is only applied when the blockage is complete and

the damage that results is severe and long term, with the most devastating results arising when blocked blood vessels burst (Fleming et al. 1999). The third subtype, and last one directly related to issues with circulation, is peripheral vascular disease. This disease most typically causes issues in circulation in the legs, manifesting itself as leg pain, and is due to blockage associated with thrombosis or atherosclerotic build up. Left untreated the issues can progress to ischemia, potentially ending in ulceration, necrosis and gangrene, especially in diabetics (Edmonds et al. 2006). The latter three types of cardiovascular disease, while important, do not directly relate to our research. They consist of heart failure, rheumatic heart disease and congenital heart disease. They deal mostly with issues of heart muscle proper, as opposed to the circulation supplying them.

Of those affected, approximately 70,000 people every year die due to CVD complications, or 1 every 7 minutes (Statistics Canada, 2011). In Canada alone, the combination of disease prevalence and mortality rate lead to an egregious economic burden of over $20.9 billion (Conference Board of Canada, 2010). Since 1952, the cardiovascular death rate has declined more than 75% largely due to research advances in surgical procedures, drug therapies, and prevention efforts (Statistics Canada, 2011c). This however, has not completely eliminated the risk associated with CVD. In fact as of 2011, CVD accounted for 29% of all deaths in Canada, 28% of all male deaths and 29.7% of all female deaths (Stats Canada, 2011c). As ours and other countries continue to advance towards a more CVD prone modern lifestyle consisting of sedentary behaviour and high fat diets, it is thought that rates of CVD and atherosclerosis will increase drastically (O'Keefe Jr and Cordain, 2004). In fact it is predicted that by 2020 CVD and

atherosclerosis will be the highest disease burden in most of the developed nations around the world. (Jamison et al. 2006)

## 1.2 – Obesity and Risk Factors for Cardiovascular Disease

Obesity is a complex multifactorial disease that develops from an interaction of genotype and environment. Among the factors that make obesity such a major health concern are the risk factors that promote the onset of atherosclerosis and cardiovascular disease. While atherosclerosis, CVD and their precursor, endothelial dysfunction, are not unique or specific only to individuals with obesity, there are risk factors present in obesity that more readily promote the initiation of a dysfunctional endothelium, thereby also promoting the progression of atherosclerosis. These factors include atherogenic dyslipidemia (increased triglycerides and LDL and decreased HDL levels), insulin resistance, a pro-inflammatory state and a prothrombotic state (Grundy, 2002). Dyslipidemia occurs because of the increased consumption and overconsumption of a diet high in saturated fats. Several studies have shown that high levels of triglyceride associated with visceral obesity and type 2 diabetes mellitus lead to an increase in very-low density protein and apoprotein B (apoB) containing lipoprotein particles (Lewis et al. 1995). There is a positive correlation between levels of apoB in the blood, waist circumference, insulin resistance and cardiovascular disease risk (Lemieux et al. 2000). Individuals who are obese or are at risk with the metabolic syndrome tend to, though not always, consume high fat/ high sugar diet, summarily being supplied with both external sources of glucose and free fatty acids (FFA) and internal FFA that are being released from their adipocytes (Jensen et al. 1989; Bjorntorp et al. 1969). This

increase in FFA concentration in the blood interferes with the ability of skeletal muscle to respond to insulin and uptake glucose, as FFA competes with glucose as an energy substrate (Boden et al. 1991). This leads to increased levels of both glucose and insulin in the blood, conditions known as hyperglycemia and hyperinsulinemia respectively.

Hyperinsulinemia, and the increased concentrations of insulin that are its namesake, have the ability to alter endothelial function in a pro-inflammatory manner (Festa et al. 2000). These increased insulin levels lead to endothelial dysfunction, altered fatty lipid deposition, onset of the atherosclerotic process and stimulated proliferation of vascular smooth muscle (Arcaro et al. 2001; Sidossis et al. 1999; Abhijit et al. 2013). Each of these factors acting together are what produce the disease known as atherosclerosis. As well as damaging the endothelium, insulin, through the MAPK signaling pathway, can cause endothelial cells to express plasminogen activator inhibitor-1 (PAI-1) and cell adhesion molecules such as Vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (Madonna et al. 2004).

ICAM-1 and VCAM-1 are similar in the sense that they are both adhesion molecules found on the surface of the endothelial lining when endothelial cells are exposed to pro-inflammatory conditions. In the case of atherosclerotic progression, inflammation of the endothelium works on a microscopic level. Damage occurs due to modulations in the shear stress applied to a vascular branch point or through exposure to oxidized low-density lipoproteins (oxLDL) (Libby et al. 2002). This damage causes monocytes and lymphocytes, the harbingers of inflammation, to crowd to the damaged endothelium in the same way they would as if there was a physical injury, hence the definition of

atherosclerosis being an inflammatory disease. VCAM-1 and ICAM-1 bind directly to molecules on monocytes and leukocytes to continue the inflammatory signaling through the cells and facilitate movement of leukocytes and monocytes through the endothelium where they become trapped and begin production of the fatty streak (Barriero et al. 2002). This inflamed, adhesive, pro-atherogenic or "sticky" dysfunctional endothelium is required for formation of the fatty streak and, later, progression of atherosclerosis.

The sum total of these risk factors promoting obesity, diabetes and cardiovascular disease are typically referred to as the "metabolic syndrome" (Fulop et al. 2006). The metabolic syndrome is a complex of interrelated factors for cardiovascular disease (CVD) and diabetes. These factors include dysglycemia, raised blood pressure (BP), elevated triglyceride levels, low high-density lipoprotein (HDL) cholesterol levels and obesity, particularly central adiposity (Alberti et al. 2009). Importantly, it has been suggested, and in many cases shown, that obesity, diabetes, or simply the overconsumption of "unhealthy foods" leads to the inflammatory processes of atherosclerosis (McGill et al. 2002; Fekete et al. 2000; Pirro et al. 2001). The high amounts of triglyceride in the blood lead to increased amounts of low and very low density lipoprotein which are then available to be oxidized into LDL. The high amounts of glucose and the insulin released to deal with these high glucose levels can damage the endothelial lining of the blood vessels, causing an immediate and potentially insidious inflammatory response (Ling et al. 2003). As this damage continues, the endothelium

becomes more and more dysfunctional finally resulting in the onset of the atherosclerotic process.

## 1.3 - Atherosclerosis

Initially believed to be a disease solely associated with an increase in serum lipids, it has actually been shown that atherosclerosis is inflammatory in origin (Epstein and Ross 1999). This brought into question what the causative factors that lead to the disease's progression might be. However, understanding that atherosclerosis is no longer solely associated with serum lipids should not in any way diminish their importance (Libby, 2006). Though not necessarily integral to the *initiation* of atherosclerotic processes, the increased levels and eventual modification of serum LDL is vital to the progression of atherosclerosis through their role in foam cell development (Aldrovandi and O'Donnell 2013).

To begin progression towards atherosclerosis the endothelium must become dysfunctional enough to allow leukocytes to invade past the endothelial barrier and initiate plaque buildup (Libby et al. 2002). Endothelial dysfunction is the primary process that leads to the initiation of the atherosclerotic plaque. Healthy endothelium is smooth, mediating the flow of blood and its constituent cells throughout the body. The barrier created by the endothelial wall, consisting of the endothelial cells separating the lumen of the vessel from the smooth muscle cells that control vessel diameter, forms a semipermeable membrane that mediates diffusion and diapedesis of circulating leukocytes (Muller et al. 2014). The endothelial cells press against each other and are

joined through the use of tight junctions, adherens and gap junctions (Yuan and Rigor, 2012). These junctional complexes stop most material, making exceptions for small proteins and leukocytes like macrophages and dendritic cells. Since our circulatory system is one of such intricate balance, it stands to reason that the permeability of the endothelium and the smooth muscle beneath it each have a role to play during physiologic stress. In fact, for every increase and decrease in BP, there is a physiologic reaction seen in the vessels. Besides overall survival, strict regulation of the forces acting upon the endothelial cells allows our cells to maintain their homeostatic balance. It is the imbalance of these forces that leads to dysfunction.

In order to maintain ideal homeostatic conditions, including the release of regulatory cytokines, our blood vessels must be exposed to laminar flow. Laminar flow is a phenomenon seen in liquids moving through a smooth, unobstructed tube. The liquid moves in layers of varying speeds, from fastest in the centre of the tube and slowing as it moves closer to the vessel wall. Laminar flow imparts a certain amount of shear stress upon the endothelium that causes constitutive release of homeostatic molecules, such as the vasodilator nitric oxide (NO) (Malek et al. 1999) which, among other things, acts to suppress the activation of inflammatory transcription factors. Without the suppressive effects of NO, the endothelium begins to produce the adhesion molecules ICAM-1, VCAM-1, e-selectin and p-selectin (De Caterina et al. 1995). These act to cause firm adhesion of leukocytes, in the case of VCAM-1 and ICAM-1, or mediate leukocyte rolling along the endothelium, in the case of e-selectin and p-selectin as well as causing the release of many inflammatory cytokines including interleukin 6 (IL-6), interleukin 8

(IL-8), macrophage colony stimulating factor (MCSF), monocyte chemoattractant protein 1 (MCP-1) and regulated on activation, normal T cell expressed and secreted (RANTES) (Libby, 2006). Respectively, the interleukins (IL-6 & IL-8) mediate overall inflammatory cell signaling, MCSF stimulates macrophage recruitment, MCP-1 attracts monocytes to inflammatory sites and RANTES activates and recruits T-l ymphocytes. When BP rises or when flow reaches natural branch points present in the circulatory system, laminar flow is interrupted and turbulence becomes present within the artery. This turbulence causes oscillating increases and decreases in BP and as such abrogates the natural production and release of NO (Malek et al. 1999). NO is an anti-inflammatory vasodilator that acts to protect endothelial cells when the cells come under stress. Decreasing the levels of NO being released by the endothelial cells is a major contributing factor to the initiation and progression of endothelial dysfunction and atherosclerosis (Matthys and Bult, 1997; Schachinger et al. 2000). The up-regulation of inflammatory cytokines and adhesion molecules causes the endothelium to morph from a smooth passage, mediating laminar flow, to a sticky trap, causing circulating leukocytes to adhere to the surface of the endothelium and make their way inside the vessel wall. Transient accumulations of leukocytes within the walls of vessels at sites of turbulent flow and endothelial dysfunction lead to the production of the hallmark of atherosclerosis, the fatty streak (Libby et al. 2002).

Through normal metabolic processing, free radicals (substances with unpaired valence electrons that have an oxidizing effect), are produced by the body and serve to react with other substances and remove valence electrons (Fang et al. 2002). When this

process occurs on an LDL molecule, the LDL becomes activated into the inflammatory ox-LDL molecule; this is the version of LDL that macrophages react to and consume, a process that turns them into foam cells (Stancu et al. 2012). An increase in consumption of fatty foods leads to an increase in serum triglyceride and LDL levels. This increase leads to an increased availability of LDL to be oxidized by the free radicals, leading eventually to an increase in foam cell production and accumulation. As ox-LDL molecules and monocytes circulate through our bodies, they both create and are attracted to the site of endothelial dysfunction (Valente et al. 2014). Ox-LDL promotes endothelial dysfunction and endothelial cytoskeletal remodelling, allowing for gaps to form in the endothelial barrier (Chouinard et al. 2008). It is through these gaps that ox-LDL infiltrate the blood vessels and begin to accumulate. Monocytes, on the other hand are attracted by the inflammatory cytokines released by the endothelial cells (Libby, 2006). Upon arriving at the site of dysfunction the monocytes stick to and roll along the endothelium, rather than bouncing off (Pober and Sessa, 2007). This rolling is mediated by the presence of E and P selectin; E-selectin and P-Selectin are membrane glycoproteins that are commonly expressed on cells that are affected by cytokines (Leeuwenberg et al. 1992; Frenette et al. 1995). As they populate the endothelial surface, the blood vessels becomes sticky allowing travelling leukocytes to roll along the vessel wall. From there the leukocytes then interact with VCAM-1 and ICAM-1 which act as anchors on the luminal surface and allow for firm adhesion (Sima et al. 2009). Once firmly adhered, the leukocytes then are able to move into the vessel wall through a process known as diapedesis. Activation of the endothelial cells also causes reformation of the actin

cytoskeleton leading to cell contraction. This contraction creates enough of a gap between cells that leukocytes and LDL are allowed to infiltrate into and underneath the dysfunctional endothelium. Once they have infiltrated the endothelial barrier, the now trapped monocytes differentiate into macrophages that begin to release signaling cytokines that attract other monocytes to the sticky endothelium, facilitating further infiltration (Wang et al. 1988). Using scavenger receptors on their surface, these sub-endothelial macrophages engulf ox-LDL, becoming activated foam cells, characterized by the creation of lipid droplets within the macrophages (Gerrity et al. 1981). The initial accumulation of foam cells under the endothelial barrier is known as the fatty streak, the hallmark of the atherosclerotic process.

## 1.4 - Markers of Endothelial Dysfunction

### *1.4.1 - Adhesion Molecules: ICAM-1*

Intercellular Adhesion Molecule-1 (ICAM-1/CD54) is an adhesion molecule that plays an important role in leukocyte adhesion to the endothelium. During inflammation, e-selectin and p-selectin membrane glycoproteins levels increase causing the leukocytes that would normally bounce against the vessel wall to roll along it instead. In a similar fashion, expression of ICAM-1, which is present only at low levels during normal function, increases during inflammation (Hubbard and Rothlein, 2000). While rolling, the leukocytes come into contact with ICAM-1 which functions to firmly adhere the leukocytes to endothelial cells and mediate the movement of cells into the vessel walls. This molecule plays an important role in endothelial dysfunction, formation of the fatty streak and finally development of atherosclerosis (Lawson and Wolf, 2009).

### *1.4.2 - Adhesion Molecules: ICAM-2*

Intercellular Adhesion Molecule-2 (ICAM-2/CD102) is an adhesion molecule that plays an important role in regular systemic immunity. ICAM-2 is readily expressed on endothelial cells and functions as a receptor for lymphocyte function-associated antigen 1 (LFA-1), much the same way as ICAM-1, on leukocytes, mediating their movement from the blood vessel lumen through the endothelial barrier (diapedesis) in non-inflammatory conditions. ICAM-2 functions in much the same way as ICAM-1 but functions during the resting state and is typically unaffected by the presence of pro-inflammatory mediators (de Fougerolles et al. 1991). E-selectin and p-selectin mediate leukocyte rolling along the endothelial barrier until binding of LFA-1 to ICAM-2 causes firm adhesion of the leukocytes to the endothelial barrier before mediating their entry into the vessel wall (Halai et al. 2014; Vanier and Nielsen, 2000)

### *1.4.3 - Cytokines: MCSF*

Macrophage colony stimulating factor (MCSF) is both a hematopoetic growth factor and inflammatory cytokine with important roles in hematopoiesis, angiogenesis, pregnancy, inflammation, bone growth and remodeling. With regards to endothelial function, MCSF is released by endothelial and smooth muscle cells in order to attract macrophages to the site of injury. MCSF is also readily expressed by macrophages and is an important inflammatory cytokine that differentiates monocytes that have been called to the site of inflammation into macrophages (Palucka et al. 2015). It also allows these macrophages to develop further into foam cells, which are the primary cell type present in the so called ‘fatty streak’ (Fixe and Praloran, 1998)

*1.4.4 - Cytokines: sICAM-1*

Soluble ICAM-1 is the same as ICAM-1, except it has been cleaved from the cell surface (Champagne et al. 1998). Though the actual function of sICAM-1 has yet to be fully elucidated, it is associated with a diagnosis of CVD or atherosclerosis as the levels in the blood are always elevated in those with disease (Ridker et al. 1998). It can exist in dimeric, monomeric or truncated forms, the latter two having less binding affinity for LFA-1 (Meyer et al. 1995; Jun et al. 2001). sICAM-1's ability to bind to LFA-1 might have designated it as a potential therapy in blocking the adhesion of LFA-1 to membrane bound ICAM-1; however it has been found that sICAM-1 activates inflammatory cascades (Lawson and Wolf, 2009*)*. sICAM-1's exact effects require more research, and therefore its overall role in atherosclerosis and cardiovascular disease has yet to be revealed.

*1.4.5 - Chemokines: MCP-1*

Monocyte Chemoattractant Protein – 1 (MCP-1) is a key chemokine involved in the inflammatory response. It is released by many different cell types in response to many types of stimuli including oxidative stress and other cytokines. MCP-1 is very important in leukocyte recruitment to areas of inflammation and is abundantly released from endothelial cells (Rollins et al, 1990). Its relation to atherosclerosis was demonstrated through genetic ablation of the CCL2 gene, which encodes this protein, with the resultant mice having a marked reduction in fatty streak formation (Deshmane et al. 2009).

## 1.5 - Anti-Inflammatory Effects of Milk Ferments

It has been shown that consumption of fermented milk products has a suppressive effect on cardiovascular disease and atherosclerosis (Sonestedt et al. 2011), and research conducted at UOIT has shown that fermented milk products cause a decrease in inflammatory cytokine production in treated gut epithelial cells (Wagar et al, 2009). As milk products are fermented by lactobacilli, many potentially bioactive compounds are released. It is through these bioactive compounds that fermented milk products may exert either a direct or an indirect effect upon the body, leading to a noticeable decrease in CVD risk (Panagiotakos et al. 2010). These compounds can include specific bioactive proteins, peptides, lipids and carbohydrates, which will each act within the body to elicit different biological responses. A list of known bioactive compounds was compiled by Young W. Park (2009), with their known biological activity. During the course of our investigation we were attempting to elucidate novel bioactive peptides and as such peptides will be the only molecules discussed. Peptides are defined as a compound containing two or more amino acids bonded in sequence (The Concise Oxford Dictionary, 1991).

A cohort study undertaken by Sonestedt et al. (2011) which looked at 26,445 individuals “without a history of myocardial infarction, stroke and diabetes” found an association between cardiovascular disease and dairy product consumption and observed effects of fermented milk products on CVD risk. Subjects self-reported, using an extensive questionnaire, which included information on lifestyle, socioeconomic and demographic factors; medication and diet supplement use; and previous/current

diseases (Sonestedt et al. 2011). Sample groups were divided based first on alcohol consumption (consumers vs. zero-consumers) and then again into gender specific quintiles based on their self-reported dietary intakes. Over the course of a 12 year follow up, 2520 CVD events occurred out of the 26,445 individuals originally surveyed. Due to the access investigators had to self-reported diet and lifestyle information, they could, as never before, determine how dietary consumption of non-fermented milk, fermented milk, cheese, butter and cream relates to CVD risk factors. To do this they looked at blood pressure (diastolic and systolic), triglycerides (TG), high density lipoprotein (HDL-C), low density lipoprotein (LDL-C) and homeostatic model assessment (HOMA) index, which is a method of estimating insulin sensitivity, in 4535 individuals who were a further subset of the initial study group. What they noticed during this study is that consumption of unfermented milk caused significant increases in all of the above risk factors except HDL-C, which saw a decrease. This is an issue as HDL-C is associated with good heart health. They then looked at the risk factors of individuals after consumption of fermented milk as well as cheese, butter and cream. It was shown that in the individuals that consumed fermented dairy products, there was a significant decrease in the risk factors associated with CVD and atherosclerosis, as well as a significant increase in the levels of HDL. Therefore, it was suggested that consumption of fermented dairy products, not their unfermented precursor, is inversely correlated to CVD risk. Interestingly, it was shown that, as compared to non-fermented milk, consumption of cheese, butter and cream showed many of the same beneficial effects as fermented milk. Since we typically associate cheese, butter and cream with an unhealthy diet, as

they are typically higher in saturated fats, this addresses the fact that these dairy products have beneficial cardio-protective effects and their complete dietary effects cannot be assessed by their levels of a single nutrient (in this case, saturated fatty acid).

Corroborating this information, Wagar et al. (2009) presented a study in which they treated the intestinal epithelial cell line (IEC) HT-29 with fermented dairy products and looked at the alterations in the expression and release of the inflammatory cytokine, IL-8. The cells were treated with either 0, 1.6 ng/mL, or 160 ng/mL of LPS to induce IL-8 production and $5x10^4$, $5x10^6$ and $5x10^8$ –CFU/mL of L. *rhamnosus* ferments to observe the effects on gut epithelial cells. A significant decrease in IL-8 levels was reported compared to cell controls at 3 different effective concentrations. Every concentration served to decrease the IL-8 levels in the HT29 IEC being treated. (Wagar et al. 2009).

Given that consumption of fermented dairy products has a reported inverse correlation to CVD and atherosclerosis and that their presence serves to abrogate inflammatory cytokine release at the level of the intestinal epithelium, the proposed next step is to explore the possible systemic effects of bioactive peptides from fermented milks on the endothelial cells of the blood vessels and propose a mechanism whereby fermented milk consumption would decrease CVD risk.

## 1.6 - Bioactive Peptides

Milk proteins consist of 80% casein and 20% whey. Whey is the liquid remaining following the precipitation and removal of milk casein during cheese making (Siso,

1996). Whey protein, while historically considered a waste product or used as animal feed, has gained a lot of traction in its use as a fitness supplement when concentrated and isolated from the other proteins in the liquid suspension. On the other hand, casein proteins make up a majority of the dry matter protein weight within milk. When looking at the potential of dairy products to exert a protective/anti-inflammatory effect it is important to assess the effects of both whey and casein in order to construct a possible mechanism of milk's, and it is associated dairy products, action within the body. Whole whey and casein protein have both been associated with promoting cardiovascular health and in fact seem to both have similar effects on blood pressure, with whey protein also showing potential serum lipid lowering effects (Phelan and Kerins,; Fekete et al. 2013). Gender, diet, bacterial strain, experimental method and exact temperature can impact whether or not peptides will have a biological effect (Fuente et al. 2002). Certain cheeses exert a cardio-protective effect only in women, while showing no effect in men, and peptides that show anti-inflammatory or protective effects in *vitro* might have no effect in *vivo* and *vice versa* (Fekete et al. 2013; Sonestdedt et al. 2011). While it has been shown that whey and casein have beneficial cardio protective and blood pressure lowering effects, and while it seems that a few studies have made progress in discovering their method of action, the studies are not as strong as they could be due to small sample sizes (Fekete et al. 2013).

Though the action of bioactive peptides has yet to be completely elucidated, there are at least 3 separate proposed mechanisms by which it is thought that certain peptide fractions may exert their effects. The first proposed mechanism is through ACE

inhibition (Donkor et al. 2007; Stressler et al. 2013). Angiotensin Converting Enzyme (ACE) is an enzyme found in the blood that converts angiotensinogen to angiotensin I and then to angiotensin II, a potent vasoconstrictor. The precursor protein, Angiotensinogen, is converted to angiotensin I when acted upon by renin. Though angiotensin I has no real biological activity, it plays an incredibly important role in the body: precursor to angiotensin II. Angiotensin II is created when angiotensin converting enzyme removes two C-terminal residues from angiotensin I. This removal changes a benign protein into an incredibly potent vasoconstrictor. Its primary method of action is to cause the tonicity of the vessels themselves to change, while also promoting the release of aldosterone which acts to increase blood pressure by increasing the resorption of sodium and water by the kidney. ACE also has functions in the production of bradykinin; bradykinin is a potent vasodilator that functions to decrease the tonicity of the vessels by causing release of prostacyclin and nitric oxide (Palmer et al. 1987). ACE acts to remove C-terminal dipeptides from bradykinin, similar to its action upon angiotensin II, thereby inactivating it and blocking its vasodilatory role. Through this action angiotensin converting enzyme also acts to promote vasoconstriction and an increase in blood pressure. ACE inhibition, then, negates vasoconstrictive actions while promoting the release of bradykinin and NO. Studies have suggested that milk ferment peptides may exert cardioprotective effects through have ACE inhibitory activity (Donkor et al. 2007; Fekete et al. 2013; Papadimitriou et al. 2007).

The kinds of novel peptides that are released in the process of fermenting milk are entirely dependent on the kind of bacteria used (Nakamura et al. 1994; Pihlanto et

al. 2009). There are two peptides produced by *Lactobacillus helveticus* and *Saccharomyces cerevisiae* that have routinely been shown to have ACE inhibitory activity and to play a role in the increased release of NO; these peptides are the lactotripeptides Isoleucyl-Prolyl-Proline (IPP) and Valyl-Prolyl-Proline (VPP) (Nakamura et al. 1995). These peptides have been shown to have ACE inhibitory activity and therefore decrease the conversion of angiotensin I to angiotensin II as well as prevent the breakdown of bradykinin (Nakamura et al. 1994). By doing so these peptides act in a similar fashion to ACE inhibitory drugs and protect and promote the release of NO. In fact Hirota et al. (2011) reported that IPP and VPP both showed effects on NO production within cultured endothelial cells and isolated rat aorta when compared to control. By promoting the release of NO, these peptides may increase vascular health by decreasing blood pressure and vascular tonicity. In addition, these bioactive peptides may increase the release of anti-inflammatory endothelial mediators (Hirota et al. 2011). By directly affecting the release of NO, specific bioactive peptides may have a mechanism to not only interfere with the effect of pro-atherogenic cytokines and adhesion molecules (such as angiotensin 2, ICAM-1 and MCP-1) but also have a way to protect cells in the vessels of healthy individuals, through the release of anti-inflammatory cytokines. Unfortunately, IPP and VPP have poor bioavailability when digested and while providing an excellent model for the potency of milk derived peptides, they make poor functional food supplements (Wuerzner et al. 2009). Further identification of novel peptides that may be incorporated into foods (making them functional foods) in order to assist in heart healthy lifestyle choices, is necessary in order to fully realize this promising result.

The other proposed functions of bioactive peptides is in interference with thrombus formation (Qian et al. 1995). It has been shown by Qian et al. that certain peptide fractions can interfere with thrombin-induced platelet aggregation. Thrombin is part of the final steps of the coagulation cascade, right before the production of fibrin allows for the formation of a stable clot. Inhibition of this process causes the clot to fall apart and therefore prevent less of a threat of clot formation and further damage caused by its disengaging from the vascular wall. There have also been reports (Haverstick et al. 1985) that certain peptide sequences can interfere with fibrinogen binding and platelet aggregation.

## 1.7 - Research Objectives

### *1.7.1 - Hypothesis*

Given that there is a reported inverse correlation between CVD risk and fermented dairy product consumption, it is hypothesized that fermented dairy products have the ability to reduce atherogenic inflammatory immune activity through the production of bioactive peptides and their action on endothelial cells. This thesis describes an investigation into the anti-atherogenic effects of fermented dairy products on endothelial cell responses, directly through exposure to novel bioactive peptides and whole and filtered milk ferment, and indirectly, through conditioned macrophage supernatant. Studying endothelial responses to exposure to these different aspects of the fermentation process will allow us to understand at which point during fermentation these bioactive peptides best exert their effects (i.e. directly through ferment, or

through ferment acting on macrophages which then may release anti-inflammatory compounds).

### *1.7.2 - Objectives*

The objective of my research project was to elucidate cellular and molecular mechanisms for the anti-atherogenic effects of bacterial secretome, whole and filtered milk ferment, conditioned macrophage supernatant and novel bioactive peptides on cultured primary human umbilical vein endothelial cells (HUVEC).

The goal of my research was to identify any protective effects these compounds may have on blood vessel endothelium. In order to do this, we tested the effects of the milk ferments in many different ways on Human Umbilical Vein Endothelial Cells (HUVEC) to determine immunomodulatory and anti-inflammatory effects. The long term objective of this research is the characterization of peptides that may have systemic benefits in terms of alleviating CVD and atherosclerosis is part of a heart healthy diet. This would provide an accessible route for people to intake compounds in their diet that will assist them in disease prevention, management or rehabilitation in compliment to traditional approaches.

# 2 – Methods

## 2.1 – Media

The primary cell line of Human Umbilical Vein Endothelial Cells, HUVECs (Cedarlane), were grown, cultured and maintained in MCDB 131 media. The media was adapted from the human microvascular endothelial cell (HMEC) culture protocol from the Centres for Disease Control and Prevention (CDC). Initially the bottled MCDB 131 media was supplemented with 10 mM L-glutamine (Gibco), 10% fetal bovine serum (Sigma), 1% penicillin/streptomycin (Pen/Strep) (Thermo), 1 μg/mL hydrocortisone (Sigma) and 10 ng/mL Endothelial Growth Factor (Sigma). Unfortunately, as the HUVECs on average require culture in larger vessels in order to achieve the required cell density for flow cytometric analysis, the amount of media that was necessary made this initial formulation expensive. We replaced the Bottled GIBCO MCDB 131 with powdered MCDB 131 Sigma media. This also came pre-supplemented with the correct concentration of L-glutamine. FBS was also replaced with iron supplemented fetal calf serum (FCS). The new supplemented media was tested by flow cytometry, both at rest and during activation, to ensure that its addition would cause no detrimental effects on the HUVECs.

## 2.2 – Cell culture flasks

The HUVEC cell line is an adherent cell line with a few special requirements. HUVEC cell lines are seeded in 75$cm^2$ Corning cell culture flasks and passaged into 25 $cm^2$ cell culture flasks, 6-well plates or 0.32 $cm^2$ 96-well plates. Due to their adherent nature HUVECs tear during passaging unless the culture flasks are treated with a 10

μg/cm$^2$ collagen base-layer first. Collagen was dissolved in 0.1 M acetic acid before further dilution in phosphate buffered saline (PBS) to a volume capable of coating the bottom of the cell culture flask (6mL for T-75, 1.5mL for T-25, 600 μL for 6 well plate and 100 μL for 96 well plate). This ensures there are no dry spots forming on the cell culture flask and allows for an even monolayer to form. Collagen coated flasks were then allowed to incubate at 37$^o$C and 5% $CO_2$ for ~ 2 hrs before being placed in a biosafety cabinet, under UV radiation overnight, to sterilize. Following overnight sterilization flasks can either be washed with PBS for use in cell culture or have additional PBS added for storage at 4$^o$C.

### 2.3 – Culture

Following recommendations set in place by the American type culture collection (ATCC), HUVECs were seeded at a density of 5000 Cells/cm$^2$ in order to propagate. Seeding at this density should lead to a confluent monolayer in approximately 8 days. Before culture, flasks were incubated for 30 minutes with cell culture media in order to ensure optimal growth. HUVECs were either seeded from frozen cell stock or after completion of the passaging protocol described below. Following either method of seeding, cells were kept at 37$^o$C at 5% $CO_2$ at all times, except for when media was to be changed. Spent media was removed every other day and replaced with fresh, supplemented MCDB 131 media that was first warmed in a water bath up to 37$^o$C for 1 hour before being added to the cells.

### *2.3.1 – Initial Culture from Frozen Stocks*

HUVEC cell cultures were either stored in a freezer at -80$^{o}$C or in liquid nitrogen, depending on the available storage space. Freezing at either temperature caused no noticeable alterations to cell viability of biomarker presentation. Cells cannot be seeded into well plates directly from frozen; they were first grown in 75 cm$^2$ collagen coated cell culture flasks and passaged into the plates at the appropriate density. Upon removal from frozen containment, cell stock vials were wiped down with ethanol or isopropanol, in order to ensure sterility, and opened slightly (~1/4 turn) in a biosafety cabinet in order to relieve pressure before retightening for thawing. The vial was thawed quickly in a 37$^{o}$C water bath, without completely submerging the entire vial, again to ensure sterility by not allowing the water near the opening of the cryovial. Great care was taken to immediately remove the vial from the water bath the instant the last remnants of ice have melted, as leaving the vial in the water bath for too long can lead to less than optimal culture results. Cells were re-suspended in the cryovial to ensure all cells have been removed from the vial. Before pipetting the required amount of cells into the cell culture flasks. Media must be changed the following day in order to remove any harmful traces of DMSO from the cells. The cryovial was not centrifuged to pellet the cells out of the DMSO storage solution, as centrifuging at that stage would have been more harmful to the viability of the HUVECs than leaving them with trace amounts of DMSO overnight in culture.

### *2.3.2 – Passaging*

Media was completely removed from each culture flask and cells were rinsed with PBS to ensure that no excess media remained that would neutralize the trypsin in the trypsin/EDTA (Thermo) mixture or the Accutase (Sigma) prior to passaging. Trypsin (Thermo) was an ideal choice when removal of the cells is required for experimentation as it required a shorter incubation time, but the harshness of the chemical was not well suited for cell passaging use. Accutase (Sigma), while taking longer, was much gentler, which made it the ideal choice for passaging the delicate HUVECs. A 2 mL volume of either trypsin or Accutase for every 25 $cm^2$ of the cell culture flask was added to initiate the detachment of cells. For trypsin; 2mL was added for 2 minutes at 37$^oC$ before being neutralized with MCDB 131 supplemented media. Neutralized cells can then be transferred into sterile Falcon tubes (Greiner Bio-One) and centrifuged at 300xg for 10 minutes in order to remove dead cells and create a pellet of live ones. Pellets were re-suspended in 1 mL culture media and 50 µL was removed for the trypan blue cell counting protocol described below. The alternative passaging methodology involved using Accutase. One inconvenience of Accutase was that it takes approximately 8x longer than trypsin to dissociate the cells from the culture flask, but the benefit was that it was gentler on the cells and required no neutralization step. Accutase was pipetted onto the PBS rinsed tissue culture flask in the same amounts as the trypsin method described above. Rather than 2 minutes at 37$^oC$, though, the Accutase was left on the cells for 15 minutes up to an hour at room temperature. After ~15 minutes, the flasks were checked to assure that dissociation from the cell culture flask had begun.

Specifically the cells became round, compact, and much more separate, while still clinging to the bottom of the flask. The tissue culture flasks were then struck to dislodge the cells and the entire suspension was transferred into clean Falcon tubes. Again, 50 μL of the suspension was taken and used for Trypan blue exclusion assays. The 50 μL of cell suspension was mixed thoroughly with Trypan Blue and ~15 μL of the final mix was pipetted into a hemocytometer. All cells were counted, but a second tally of blue (dead) cells was kept in order to determine viability. Using the Trypan Blue exclusion assay cells were counted and seeded into prepared collagen coated flasks at an appropriate amount to match the suggested 5000 cells/$cm^2$ seeding density. Seeding at higher densities was, however, possible in order to form a monolayer more quickly.

## 2.4 – Collagen Type I preparation protocol

### *2.4.1 – Sigma Procedure*

Type I collagen l arrived from Sigma as a fibrous bundle for reconstitution. In order to dissolve it properly, 0.1 M acetic acid was added to the collagen bundle to achieve a final working concentration of collagen solution of 0.1% (w/v). Once added, the mixture was allowed to stir for 1-3 hours at room temperature until completely dissolved. Once dissolved, the collagen was moved to a glass container with a layer of cholorform on the bottom and allowed to sit overnight for use beginning the following day. For our applications, 10 μg/$cm^2$ of collagen solution per cell culture flask was optimal. If necessary, the collagen solution could be spread out with a cell scraper in order to ensure that collagen coats evenly across the plates and flasks. The collagen

solution was allowed to bind to the plates and flasks for several hours at room temperature, 37°C or overnight at 2-8°C. In order to ensure that the flasks remain sterile, the collagen coated flasks were placed into a biosafety cabinet overnight, under UV light. Before addition of media, coated flasks and plates were washed with either culture grade water or PBS before the introduction of media for one final incubation prior to the addition of cells for culturing. Additional flasks were stored in enough PBS to cover the flask and left at 4°C until ready to use.

### *2.4.2 – Millepore*

Millepore collagen solutions came pre-made at a higher 2.92 mg/mL concentration. Therefore, the solution was diluted to a working concentration in PBS for dispensing 10 $\mu g/cm^2$ of collagen to completely coat the flask/plate, leaving no dry spots at all. Once the collagen solution has been spread, the flasks were incubated at room temperature for 10-30 minutes. The solution was removed and the flasks were rinsed with PBS before adding media or cells. As above, flasks could be stored at 4°C with bottoms coated in PBS to prevent drying until they were necessary to use.

## **2.5 – Cryopreservation**

Cell lines, ideally, should be frozen down at low passage numbers in order to maximize useful cell life. HUVEC cells are typically only viable for use in experimentation from passage numbers 2-5. Cells were passaged as noted above, before freezing down approximately 1 mL of cell suspension in each cryovial, at $10^6$ cells/mL suspended in medium. Cryovials were clearly labelled with the name of the cell line, the passage

number, the approximate number of cells in the vial, the date and the name of the individual performing the freezing protocol. 100 µL of dimethyl sulfoxide (DMSO) was pipetted to the bottom of the vial, followed by 900 µL of cell suspension from the aforementioned passaging protocol. The mixture was re-suspended once, followed by a quick addition of 300 µL of FBS to the top of the solution, with no resuspension. Cryovials were then capped and placed into a -80$^{o}$C freezer in a specialized cryocontainer for at least 24 hrs, to slowly cool the cell suspensions, before placing the vials into a separate container in the -80$^{o}$C freezer or liquid nitrogen for long term storage.

### 2.6 – HUVEC Activation with TNF-α

For experimentation, it was necessary to determine how best to simulate inflammatory mediator production in the HUVEC culture. Different concentrations of TNF-α were titrated out to determine a concentration that would activate the cells without causing cell death. Concentrations of 1 ng/mL, 10 ng/mL, 25 ng/mL and 50 ng TNF-α were tested for 4, 8, 16, 24, 48 and 72 hours in order to determine the best way to activate the cells. To create these dilutions a TNF-α stock of 10,000 ng/mL was dissolved into the appropriate amount of media, depending on the flask size.

### 2.7 – Ferment, Secretome and Supernatant

Bacterial secretome, milk ferment and conditioned macrophage supernatant was obtained from Dr. Julia Green Johnson's lab and diluted into medium for titration. To create bacterial secretome, *L. rhamnosus* bacterial cells were grown in de

Man, Rogosa and Sharpe agar (MRS) in order to ensure that the cells reach maximal density before being moved into Roswell Park Memorial Institute (RPMI) medium to culture for 23 hours. Following this incubation period, the bacterial suspension was then spun down in a centrifuge in order to remove the bacterial cells from the medium. This "cell-free" medium is then filtered to remove all peptides 10 kilodaltons (kDa) or larger as it has been shown by Dr. Julia Green-Johnson's lab that it was the smaller peptides (i.e. <10 kDa) that exerted the anti-inflammatory/ beneficial effect on gut epithelial cells. This allowed for the testing of untreated *L*. *rhamnosus* protein secretions on endothelial cell biomarker production.

Store bought 3.25% homogenized milk was used to prepare the milk ferments. The milk was exposed to $1x10^8$ CFU/mL of L. *rhamnosus* and allowed to incubate, with a non-fermented milk control and a lactic acid acidified milk control, at 37$^oC$ until the fermenting milk reached a pH of 4.7+/- 0.1. For our purposes, it was necessary to have the milk ferment filtered in order to remove the L. *rhamnosus* as we were unsure of the potential detrimental effects that direct exposure to the bacteria would have on our cells. Additionally, if the acidified controls required further acidification, HCl was added in order to decrease the pH to match the L. *rhamnosus* fermented milk.

Macrophage supernatant was acquired by removing the conditioned medium produced by type 0 macrophages during culture with milk ferments. Monocytes were differentiated into type 0, or resting, macrophages by exposing THP-1 cells to 100ng/mL of phorbol-12-myrisate-13-acetate (PMA) for 48 hours at 37$^oC$ and 5% $CO_2$. Following the differentiation, THP-1 cells became adherent, allowing easy addition and removal of

media and *L. rhamnosus* ferment. Whole and filtered ferment was added to the now adherent THP-1 macrophages for 24 hours before being removed and stored for later use. Dilutions of 1/10, 1/100 and 1/1000 of the secretome, ferment, and supernatant and 1/25 of the supernatant were diluted into culture media for analysis of cell response.

### 2.8 – Incubation Conditions

Incubations with each of the secretome, ferment, supernatant and peptide interventions and TNF-α were investigated as either a pre-incubation, post-incubation or co-incubation. Pre-incubation involved intervention incubation prior to the addition of TNF-α, post-incubation involved activating the cells prior exposing it to the compounds and the co-incubation involved exposing the cells to the intervention and the TNF-α simultaneously. It was important to do this in order to determine if the protective effects of the secretome, ferment, supernatant to peptides acted in either a protective, interfering or restorative fashion. This was important to determine how the interventions acted biologically in the presence of an inflammatory event.

Prior to incubation with TNF-α, bacterial secretome, milk ferment or macrophage supernatant, HUVEC cells were grown for a minimum of 8 days in supplemented MCDB 131 medium. An estimated confluency of >80% was necessary in order to maintain cell density necessary for optimal execution of the flow cytometry protocol. Population and growth viability in media (both FBS supplemented and FCS supplemented) was assessed

through the Trypan Blue Exclusion assay explained above. Optimum culture conditions were achieved when media was changed every Monday, Wednesday and Friday.

Table 1: Description of Incubation Conditions

| | First 24 or 72 hours (Whole macrophage supernatant only) | Second 24 hours |
|---|---|---|
| Pre-Incubation:<br>Incubation first with bacterial secretome, L. *rhamnosus* fermented milk, or macrophage supernatant followed by incubation with TNF- α diluted into supplemented MCDB131 media | Dilutions of:<br>- 1/10, 1/100, and 1/1000 of bacterial secretome<br>- 1/100, 1/1000 of L. rhamnosus fermented milk<br>- 1/25, 1/100 of whole and filtered fermented milk respectively<br>Diluted in supplemented MCDB131 media | 10ng/mL TNF- α diluted in media from 10000ng/mL TNF- α stock solution |
| Post Incubation:<br>Incubation first with TNF- α diluted into supplemented MCDB 131 media followed by incubation with bacterial secretome, L. *rhamnosus* fermented milk or macrophage supernatant | 10ng/mL TNF- α diluted in media from 10000ng/mL TNF- α stock solution | Dilutions of:<br>- 1/10, 1/100, and 1/1000 of bacterial secretome<br>- 1/100, 1/1000 of L. rhamnosus fermented milk<br>- 1/25, 1/100 of whole and filtered fermented milk respectively<br>Diluted in supplemented MCDB131 media |
| Co-Incubation:<br>Simultaneous incubation with TNF-α and bacterial secretome, L. *rhamnosus* fermented milk or macrophage supernatant diluted into supplemented MCDB 131 media | Dilutions of:<br>- 1/10, 1/100, and 1/1000 of bacterial secretome<br>- 1/100, 1/1000 of L. rhamnosus fermented milk<br>- 1/25, 1/100 of whole and filtered fermented milk respectively<br>As well as<br>- 10ng/mL TNF- α from 10000ng/mL stock<br>Diluted in supplemented MCDB131 media | Dilutions of:<br>- 1/10, 1/100, and 1/1000 of bacterial secretome<br>- 1/100, 1/1000 of L. rhamnosus fermented milk<br>- 1/25, 1/100 of whole and filtered fermented milk respectively<br>As well as<br>- 10ng/mL TNF- α from 10000ng/mL stock<br>Diluted in supplemented MCDB131 media |

## 2.9 – Peptide Incubations

We received synthesized peptides from the lab of Dr. Janice Strap at concentrations of 100 μg/mL. Peptide fractions were diluted into MCDB 131 supplemented medium. Final concentrations of 3 μg/mL and 10 μg/mL were used for each peptide tested.

Table 2: List of peptides synthesized from β-casein and used in experimental exposures to endothelial cells.

| | Peptide | MW (g/mol) | |
|---|---|---|---|
| P1 | VPP | 311.38 | Nakamura, 1995 |
| P2 | IPP | 325.41 | Nakamura, 1995 |
| P3 | HQPHQPLPPTVMFPPQ | 1851.18 | Tellez, 2010 |
| P4 | HQPHQPLPPT | 1151.3 | Tellez, 2010 |
| P5 | WMHQPHQPLPPT | 1468.71 | Tellez, 2010 |
| P6 | LYQEPVLGPVR | 1270.51 | Tellez, 2010 |
| P7 | LDQWLCEK | 1034.2 | Tellez, 2010 |
| P8 | YP | 278.31 | Yamamoto, 1999 |
| P9 | PGPIPN | 593.69 | Boutrou, 2013; Migliore-Samour 1989 |
| P10 | FFVAP | 579.7 | |
| P11 | KVLPVP | 651.85 | |
| P12 | KVLPVPQ | 779.99 | |

## 2.10 – Cell Viability

Cell viability was assayed using the Trypan Blue cell exclusion assay both before experiments began (typically when preparing new flasks, during passaging) and

following cell challenge. Following cell dissociation from the flask during the passaging protocol (used both to seed new flasks and test for viability), 50 μL of cell suspension was transferred to a micro-centrifuge tube and mixed with 50 μL of Trypan Blue. This mixture was resuspended by pipetting up and down approximately 6 times to ensure thorough mixing. 15 μL of the Trypan Blue suspension was added to a hemocytometer, and cells were counted through the use of an inverted microscope. All cells were counted in the 4 corner and 1 centre square of the hemocytometer and cell viability was estimated by subtracting the number of blue (dead) cells from the total number of cells counted, and then dividing this new number by the total number of cells and multiplying by 100% (T-D = Y, Y/T x 100% = viability). As long as viability was 85% or higher, experiments continued.

### 2.11 – ELISA Protocol

The following protocol was used for the MCSF, MCP-1 and sICAM-1 ELISA antibody from R&D. Plates were prepared by diluting the capture antibody, whose concentration will change depending on the antibody used in PBS (360 μg/mL to 2.0 μg/mL for MCSF, 120 μg/mL to 1.0 μg/mL for MCP-1, 720 μg/mL to 4.0 μg/mL for sICAM-1). This capture antibody dilution was then used to coat a 96-well microplate with 100 μL per well before the plate was placed in the fridge overnight to allow the capture antibody to adhere to the plate. The following morning, the plate was thoroughly shaken into the sink to remove excess liquid, prior to each well then being washed three times using R&D wash buffer formulation (0.05% Tween20 in PBS, pH 7.2-7.4) to ensure that no unbound antibody remained in the plate. Following the final wash step, plates were then inverted

against paper towel placed on the lab bench in order to completely remove the wash buffer from the plate. 100 μL (full well plate) or 50 μL (half well plate) of standards (MCSF, MCP-1 or sICAM-1) and samples were placed in the plate, in triplicate, following the pre-arranged "96 well plate map". After the addition of standards and samples, the plate was allowed to incubate at room temperature for 2 hours in order to allow the cytokines, chemokines and biomarkers of interest to bind to the capture antibody in the plate. Following the 2 hr room temperature incubation the plate was, again, washed three times using the R&D wash buffer and dried. The once again dry plate was then incubated with the detection antibody, which was diluted in reagent diluent to the concentration required by each of the three respective kits (36 μg/mL to 200 ng/mL for MCSF, 3.0 μg/mL to 50 ng/mL for MCP-1, 18 μg/mL to 100 ng/mL for sICAM-1). 100 μL (full well plate) or 50 μL (half well plate) of the detection antibody suspension was placed in each of the 96 wells, and the plate was then incubated at room temperature for a further 2 hours. After a third washing/drying step, 100 μL (full well plate) or 50 μL (half well plate) of HRP/streptavidin, which is the secondary antibody used to capture and cleave the substrate, diluted in reagent diluent, was added to each of the wells, put in a drawer in order to limit its exposure to light, and left to incubate for 20 minutes at room temperature. Following this incubation step, the final washing/drying step was carried out and followed by the addition of 100 μL (full well plate) or 50 μL (half well plate) of TMB, the substrate that will cause the color change. The final incubation was also carried out in a drawer for 20 minutes at room temperature, followed by 100 μL (full well plate) or 50 μL (half well plate) of 1.8N $H_2SO_4$ in order to stop the reaction and

activate the color change. The plates were then read in a plate reader at 450 nm wavelength. R&D protocols call for the use of a "full well plate" and therefore full volumes of antibody and samples, ½ well plates can, in some cases, provide better, more accurate results.

### 2.12 – Flow Cytometry

Experimental incubations, as listed above, were carried out in 25 $cm^2$ tissue culture flasks in order to ensure sufficient cell population of ~$10^6$ cells for successful flow cytometric analysis. Cells were first removed from the flasks using either of the two passaging methods mentioned above. For this particular application, passaging with trypsin was especially useful due to the rapid dissociation of the HUVECs from the tissue culture flasks. After the neutralization and centrifugation step (section 2.3.2), the media was aspirated from the Falcon tube and the cell pellet was resuspended in 1mL of PBS. Two microcentrifugation tubes are prepared for each experimental population to be used for both the detection antibody and for the control antibody. The detection antibody used was ICAM-1 (CD54) and the control was immunoglobulin G (IgG) conjugated with phycoerythrin (PE), the fluorescent molecule used for detection. Due to the large size of HUVEC cells, they were dehydrated to ensure their fit into the needle of the GUAVA flow cytometer. In each tube 40 μL of formaldehyde (final concentration of 2-4%) was added to 500 μL of cell suspension in PBS in order to fix surface proteins. HUVEC cells were then allowed to sit in the PBS/formaldehyde mixture for 10 minutes at 37°C, followed by 1 minute on ice. Before cell permeabilization, the fixation mixture was removed from the cells through centrifugation. Cells were spun in a LabNet international

micro-centrifuge at 300x*g* for 10 minutes at 4$^{o}$C and carefully aspirated so as not to disturb the pellet. Following their careful aspiration, cells were re-suspended in 90 μL of 100% ice cold methanol mixed with 10 μL of PBS and chilled on ice for 30 minutes. The tubes were then placed in a -20$^{o}$C freezer overnight in order to fully permeabilize and shrink HUVEC cells. The following day, the methanol was removed from the cells by microcentrifugation in the aforementioned centrifuge at 4$^{o}$C at 300x*g* for 10 minutes. Incubation buffer was then prepared by mixing 2 mL of FBS, 2 mg of BSA and 38 mL of PBS together, vortexing thoroughly to ensure that all the BSA has been removed and placed on ice until required. Following the first spin down step after permeabilization, the cell tubes were kept on ice at all times. While being careful not to disrupt the pellets, the permeabilization mixture was removed from each microcentrifuge tube and the remaining pellets were resuspended in 1mL of incubation buffer described previously. This "wash step" of centrifuging and resuspending in incubation buffer was repeated twice more for a total of 3 washes. After aspirating the final wash step, each pellet was resuspended in 100 μL of the incubation buffer to block for 10 minutes. Cells were combined with either the detection antibody or the control antibody at the appropriate dilutions noted on their respective anti-body data sheets, and the cells were thoroughly resuspended by pipetting. The detection antibodies are conjugated to a fluorophore that is light sensitive, thus care was taken to minimize exposure to light  the tubes were then placed back on ice before being placed into a dark cupboard for 1 hour at room temperature for the antibodies to bind appropriately. Following the required incubation time, the cells were washed 3x as mentioned above, washing with reagent diluent and

centrifugation before finally being suspended in 500 μL of PBS. This PBS suspension was then analyzed using the GUAVA flow cytometer.

### 2.13 – Nitric Oxide Assay

The Cayman Chemical Nitrate/Nitrite assay kit allows for measurement of total nitric oxide production by causing a color change in the presence of the NO metabolites nitrate and nitrite. Prior to the initiation of the Nitric Oxide assay, a 96 well plate was incubated with 12 peptide fractions, synthesized from the sequence of β-casein, gifted to us from Dr. Janice Strap's lab. 96-well plates have 0.32 $cm^2$ of available growth area which allowed for a population density of HUVEC amounting to approximately 12,800 cells on average per well. Reagents were prepared as per the instructions of the Cayman Chemical Nitrate/Nitrite Assay kit. Plate maps were laid out prior to the initiation of any peptide addition into the media. In the blank well, 200 μL of Assay buffer or milliQ water was added, with no other reagents being added for the duration of the Assay. 80 μL of media from peptide conditioned HUVEC cells was placed in the 96 well assay plate, in triplicate, as well as the prepared standard, in triplicate. 10 μL of nitrate reductase enzyme cofactor mixture was placed in each well, followed by 10 μL of nitrate reductase. The plate was then covered with an adhesive strip and left to incubate, at room temperature, for 2 hours. After the required incubation time, the adhesive strip was removed and 50 μL of Greiss Reagent 1 (Sulfanilic Acid) was added to each well followed by 50 μL of Greiss Reagent 2 (Azo Dye). After the addition of the Azo compound, a pink colour should form in any wells that contain nitrate or nitrite. The plates were allowed

to sit for 10 minutes for the colour to develop and then read at 540nm using a plate reader (Bio-Tek).

### 2.14 – Statistical Analysis

Statistical analysis was performed on any tests repeated in triplicate. Indexed one way ANOVA tests were performed using the statistical analysis program SigmaPlot.

# 3.0- Results

## 3.1 – Optimization of HUVEC culture and experimental conditions:

Human Umbilical Vein Endothelial Cells (HUVEC) are primary endothelial cells taken directly from umbilical veins. In order to ensure that they would be suitable for repeated testing, the cell culture protocol was optimized. Discovery of adequate culture conditions (i.e. media supplementation and collagen coating) allowed for the reliable culture of the HUVEC to ensure that tests carried across passages would not only be usable, but biologically relevant.

Determining optimal conditions for activation of the HUVEC was important to allow analysis of the biomarkers of inflammation. A baseline biomarker expression was established in order to analyze the secretome, ferments, supernatant or peptide's effects (appendix 1). A balance between activation and cell viability was necessary to establish, as too much TNF-α, while activating the cells, would have reduced the number of viable cells, while too little would have produced such a small effect that effects due to the ferment components would have been difficult to analyze. Exposure to 10 μg/mL of TNF-α for 24 hours was determined to be optimal for HUVEC activation (appendix 1)

## 3.2 – Bacterial Secretome

Bacterial secretome dilutions were adopted from Julia Green Johnson's laboratory to match those that had down-regulatory effects on IL-8 production by the HT-29 IECs. It was decided, though, that lower concentrations be tested to examine lower effect limits. Effects of bacterial secretome incubation on TNF-α activated HUVEC

cells were assayed using both R&D immune-linked enzyme assay and GUAVA immuno-fluorescent cytometry assay. Cells were assayed for expression of cytokines and chemokines macrophage colony stimulating factor (MCSF), monocyte chemoattractant protein 1 (MCP-1) and soluble intercellular adhesion molecule 1 (sICAM-1) as well as the surface markers intercellular adhesion molecule 1 (ICAM-1) and intercellular adhesion molecule 2 (ICAM-2). sICAM-1 pre-incubation experiments and MCP-1 co-incubation experiments were performed in triplicate and statistically analyzed through one way ANOVA using the SigmaPlot statistical analysis program, while MCSF and MCP-1 pre-incubations were performed in duplicate. Conditioned media was removed every 24 hours during the pre-incubation and post-incubation experiments in order to assess the individual effects of TNF-α and bacterial secretome dilutions on the HUVECs expression of inflammatory biomarkers and after 48 hours for co-incubation experiments. Removed media was stored in a -80$^{o}$C freezer for at least 24 hours prior to use in the R&D Enzyme Linked immunosorbent assay kits (ELISA). Figure 1 (Fig.1) represents MCSF expression levels for 24 hours of bacterial secretome pre-incubation plus an additional 24 hour incubation with 10ng/mL TNF-α. There was a significant increase in the level of the inflammatory biomarker MCSF produced following activation with 10ng/mL TNF-α however, there was no change in MCSF levels seen with secretome pre-incubation compared to acidified control incubation. This pattern was also seen in that levels of sICAM-1 produced by HUVEC did not decrease with the secretome pre-incubation (Fig.2). The MCP-1 levels produced by HUVEC cells were not different between any of the incubations before or after the addition of 10 ng/mL of TNF-α (Fig. 3). This effect was

seen again for MCP-1 in the secretome co-incubation, as the levels of MCP-1 shown by the cells were not significantly different between any of the incubation conditions. Therefore for MCP-1 and sICAM-1, at least, incubation of HUVEC cells with bacterial secretome did not significantly alter the levels of inflammatory cytokines produced by HUVEC cells when activated compared to controls (figs 3-4). Although we were not able to statistically analyze MCSF and MCP-1 secretome pre-incubation experiments, it is suggested that the same pattern is mirrored in these results.

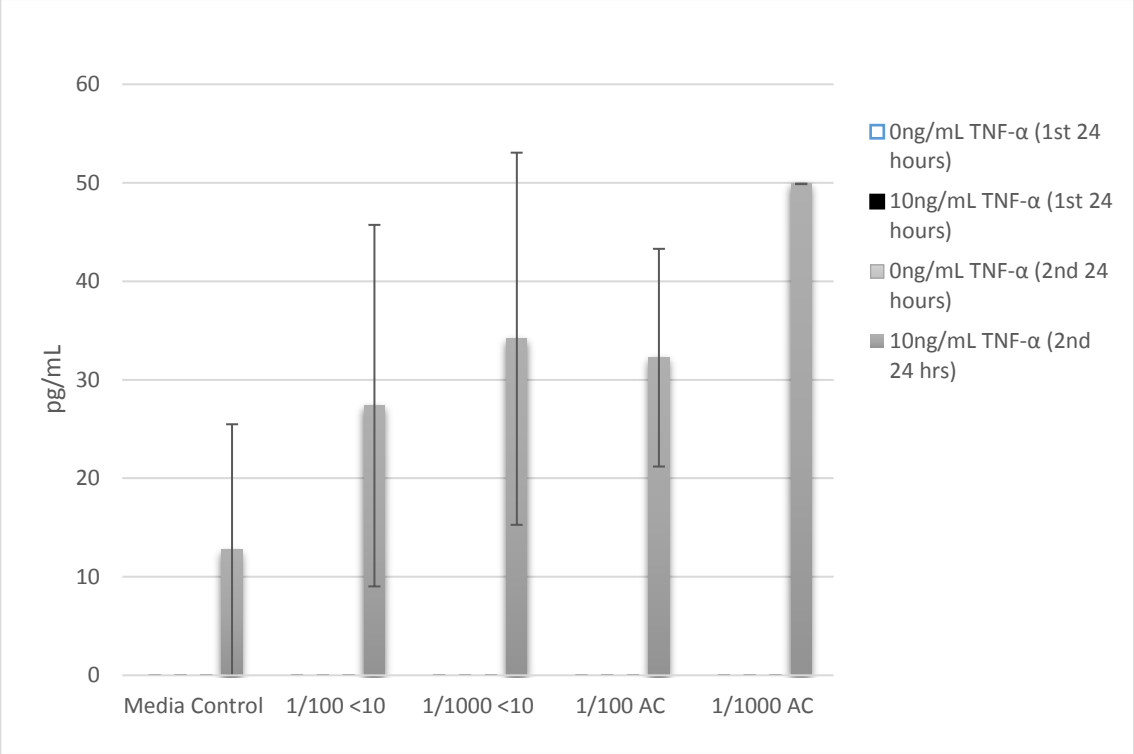



**Fig 1**: ELISA analysis of the immunomodulatory effects of bacterial secretome pre-incubation on production of MCSF by HUVEC. For all treatments including media control, HUVEC cells were seeded at a density of 5000 cells/cm$^2$ into collagen coated, tissue culture treated 6 well plates (Falcon) and allowed to grow to a confluent monolayer over 8 days. Confluent wells containing HUVEC cells were incubated for 24 hours using either plain supplemented MCDB 131 media, 1/100 or 1/1000 dilutions of bacterial secretome, filtered to be <10kDa (<10), or acidified lactic acid control (AC) diluted into 3 mL of MCDB131 media. Following the first 24 hour incubation, the secretome supplemented media was replaced with 3 mL of MCDB131 media supplemented with 10 ng/mL TNF-α diluted from a 10,000 ng/mL TNF-α stock. Experiments were repeated in duplicate and therefore statistical analysis was not possible.

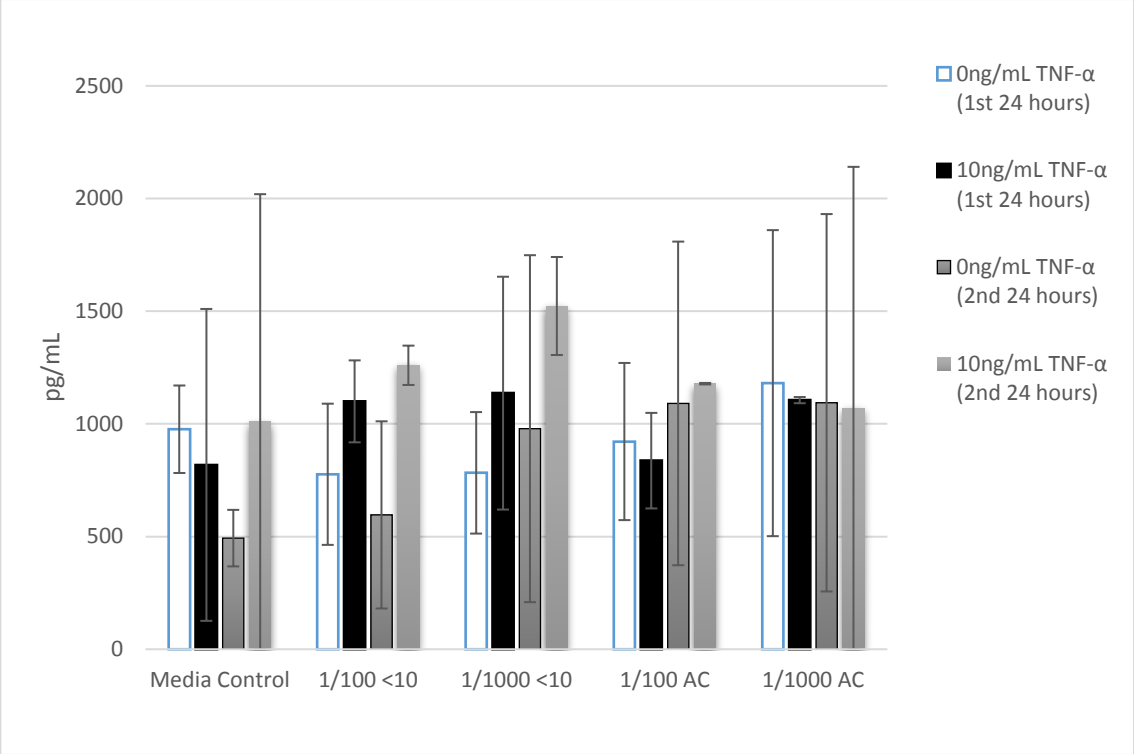



**Fig 2**: ELISA analysis of the immunomodulatory effects of bacterial secretome pre-incubation on production of MCP-1 by HUVEC. For all treatments, including media controls, HUVEC cells were seeded at a density of 5000 cells/cm$^2$ into collagen coated, tissue culture treated 6 well plates (Falcon) and allowed to grow to a confluent monolayer over 8 days. Confluent wells containing HUVEC cells were incubated for 24 hours using either plain supplemented MCDB 131 media, 1/100 or 1/1000 dilutions of bacterial secretome, filtered to be <10kDa (<10) or acidified lactic acid control (AC) diluted into 3 mL of MCDB131 media. Following the first 24 hour incubation, the secretome supplemented media was replaced with 3 mL of MCDB131 media supplemented with 10 ng/mL TNF-α diluted from a 10,000 ng/mL TNF-α stock. Experiments were repeated in duplicate and therefore statistical analysis was not possible.

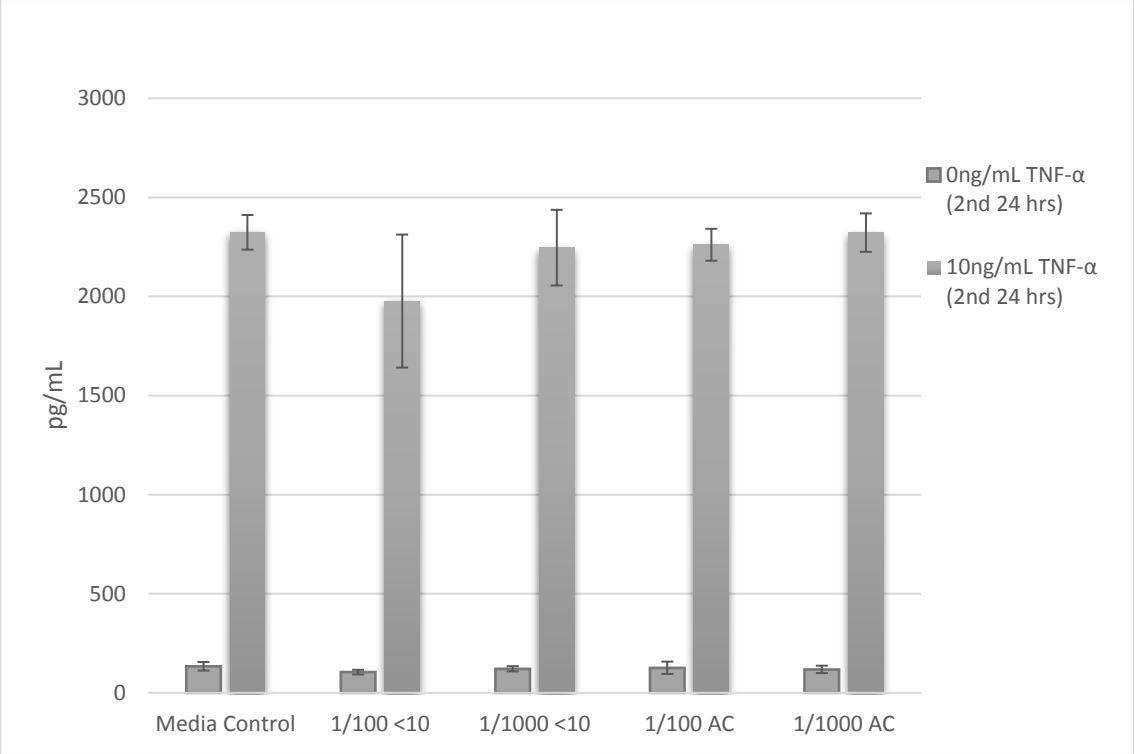



**Fig 3**: ELISA analysis of the immunomodulatory effects of bacterial secretome pre-incubation on production of sICAM-1 by HUVEC. For all treatments, including media controls, HUVEC cells were seeded at a density of 5000 cells/cm$^2$ into collagen coated, tissue culture treated 6 well plates (Falcon) and allowed to grow to a confluent monolayer over 8 days. Confluent wells containing HUVEC cells were incubated for 24 hours using either plain supplemented MCDB 131 media, 1/100 or 1/1000 dilutions of bacterial secretome, filtered to be <10 kDa (<10), or acidified lactic acid control (AC) diluted into 3 mL of MCDB131 medium. Following the first 24 hour incubation, the secretome supplemented media was replaced with 3 mL of MCDB131 media supplemented with 10 ng/mL TNF-α diluted from a 10,000 ng/mL TNF-α stock. Experiments were repeated in triplicate and tested for significance using the SigmaPlot statistical analysis program. No significant differences were seen.

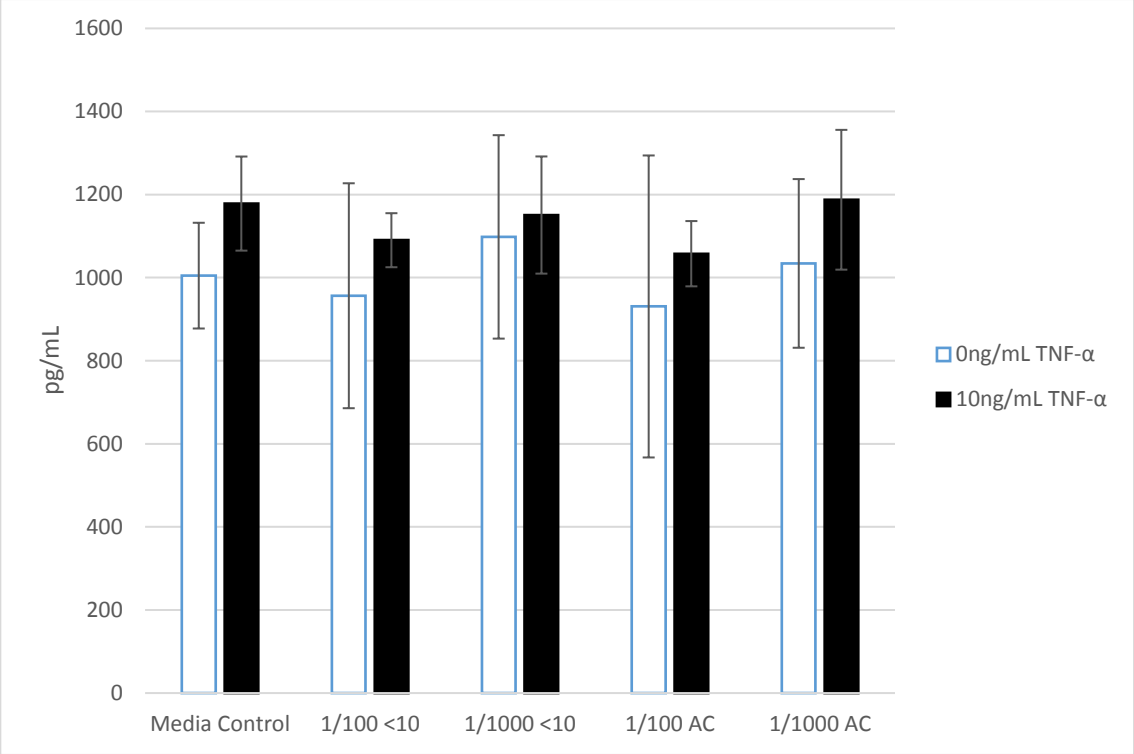



Fig 4: ELISA analysis of the immunomdulatory effects of Bacterial Secretome Co-incubation on production of MCP-1 by HUVEC. For all treatments, including media controls, HUVEC cells were seeded at a density of 5000 cells/cm$^2$ into collagen coated, tissue culture treated 6 well plates (Falcon) and allowed to grow to a confluent monolayer over 8 days. Confluent wells containing HUVEC cells were incubated for 48 hours using either 3mL of plain supplemented MCDB 131 media (0ng/mL), 3 mL of MCDB 131 media containing 10ng/mL TNF-α (10ng/mL), 1/100 or 1/1000 dilutions of bacterial secretome (<10) or acidified lactic acid control (AC) diluted into 3 mL of MCDB131 media or 1/100 or 1/1000 dilutions of bacterial secretome (<10) or acidified lactic acid diluted into 3mL supplemented MCDB131 media containing 10ng/mL TNF-α. Experiments were repeated in triplicate and tested for significance using the SigmaPlot statistical analysis program. No significant differences were seen.

## 3.3 – Conditioned Macrophage Supernatant

The effects of conditioned macrophage supernatant on TNF-α activated HUVECs were assayed using both the ELISA assay and the GUAVA immuno-fluorescent cytometry assay. Cells were assayed for the cytokines, chemokines and surface markers macrophage colony stimulating factor (MCSF), monocyte chemotactic protein 1 (MCP-1), soluble intercellular adhesion molecule 1 (sICAM-1), and intercellular adhesion molecule 2 (ICAM-2). In the pre-incubation and post-incubation experiments, conditioned HUVEC media was removed every 24 hours to assess the individual effects of TNF-α and supernatant on the cells. In the co-incubation experiments media was removed after 48 hours in order to assess the combined effects of simultaneous TNF-α and macrophage supernatant exposure on the HUVEC. All experiments, save for the ICAM-2 cytometric analysis, were performed in triplicate and the results were analyzed through one way ANOVA through the Sigma Plot statistical analysis program.

Fig. 5 represents the effects of a 24 hour filtered macrophage supernatant and TNF-α co-incubation on the expression of ICAM-2 by HUVEC. The effects of the co-incubation were analyzed using the GUAVA flow-cytometry assay mentioned above. This assay was done in an attempt to identify a surface marker that may have responded better to stimulation with the macrophage supernatant. The 1/100 dilution of the supernatant, but not the acidified control appeared to increase the expression of ICAM-2 on the surface, but the presence of TNF-α greatly reduced this expression. The macrophage supernatant appeared unable to dampen the effects of TNF-α activation and no further investigation into ICAM-2 expression was carried out.

Figures 6-8 represent the effect of a 48 hour incubation of filtered macrophage supernatant on MCSF, MCP-1 and sICAM-1 expression on TNF-α activated HUVEC. Following the 48 hour incubation, media was removed and frozen for at least 24 hours before analysis using the respective R&D ELISA kit. No significant change in cytokine production by α activated HUVECs was detected.

Figure 9 represents the effects of a 72 hour whole macrophage secretome pre-incubation on HUVEC followed by a 24 hour TNF-α activation, in order to asses if pre-treatment might block the effects of activation. As above, following each incubation period media was removed and stored at -80$^{o}$C for at least 24 hours in order to assess the individual effects of TNF-α and macrophage supernatant on HUVEC. Here, sICAM-1 levels were analyzed using the R&D CD54/ICAM-1 ELISA kit and results were completed in triplicate in order to test for statistical significance. No significant change in cytokine production by TNF- α activated HUVECs was seen.

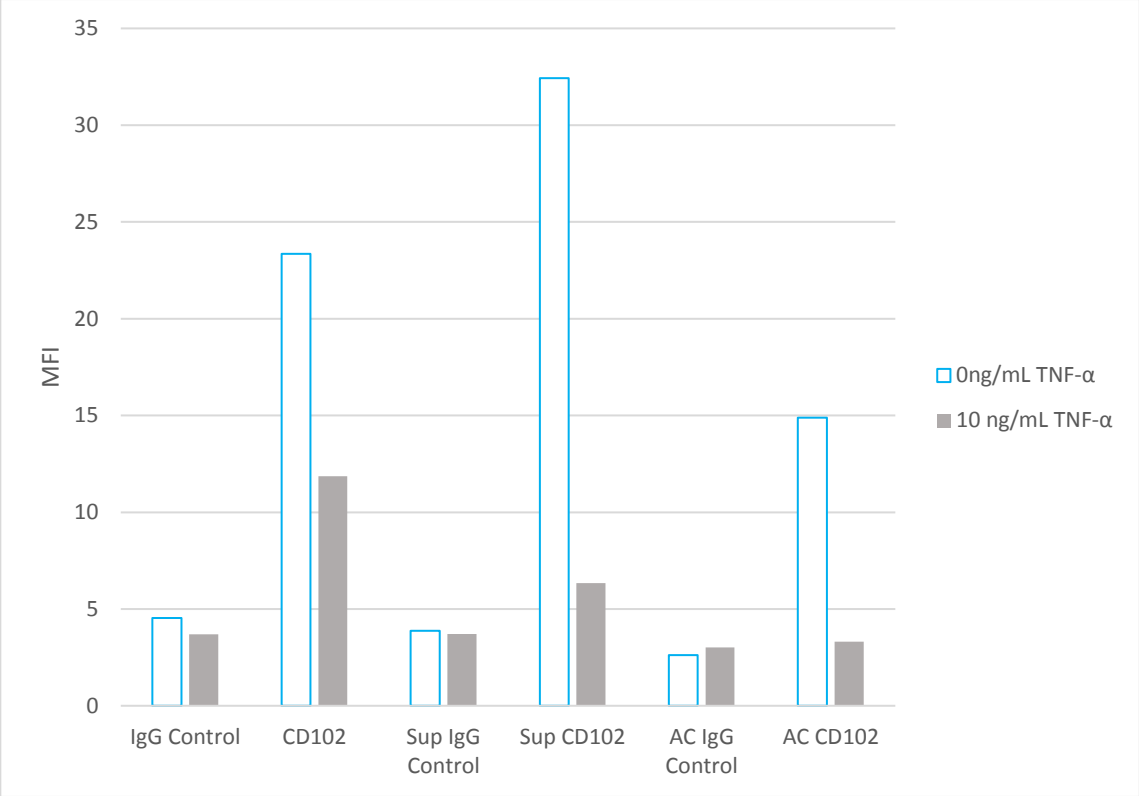



Fig 5: Flow cytometric analysis of the immunomodulatory effects of Conditioned Macrophage Supernatant on Endothelial cell expression of Intracellular Adhesion Molecule 2 (ICAM-2/CD102). For all treatments HUVEC cells were seeded at a density of 5000 cells/cm$^2$ into vented, collagen coated 25 cm$^2$ tissue culture flasks and allowed to form into a confluent monolayer over the course of 8 days. 25 cm$^2$ flasks have a workable culturing area of 25cm$^2$ which allowed for a population density of HUVEC amounting to approximately approximately 1-1.2 million cells per flask, on average. Confluent flasks containing HUVEC cells were incubated with 3mL of either: supplemented MCDB131 media (0ng), MCDB 131 supplemented with 1/100 dilutions of supernatant (Sup) or acidified control (AC), MCDB131 media supplemented with 10ng/mL of TNF-α (10ng) or MCDB 131 media supplemented with 10ng/mL TNF-α and a 1/100 dilution of supernatant (Sup) or acidified control (AC). Following the 24 hour co-incubation, media was removed and stored for later analysis. The HUVEC cells remaining in the flask were then detached from the flask following the trypsin protocol above, and then fixed and read following the flow cytometry protocol mentioned above. Cell suspensions were read on the GUAVA flow cytometer. Cells were analyzed for levels of fluorescence. The greater the fluorescent intensity, the greater the amount of ICAM-2 expression. Results were obtained once, and therefore statistical analysis was not possible.

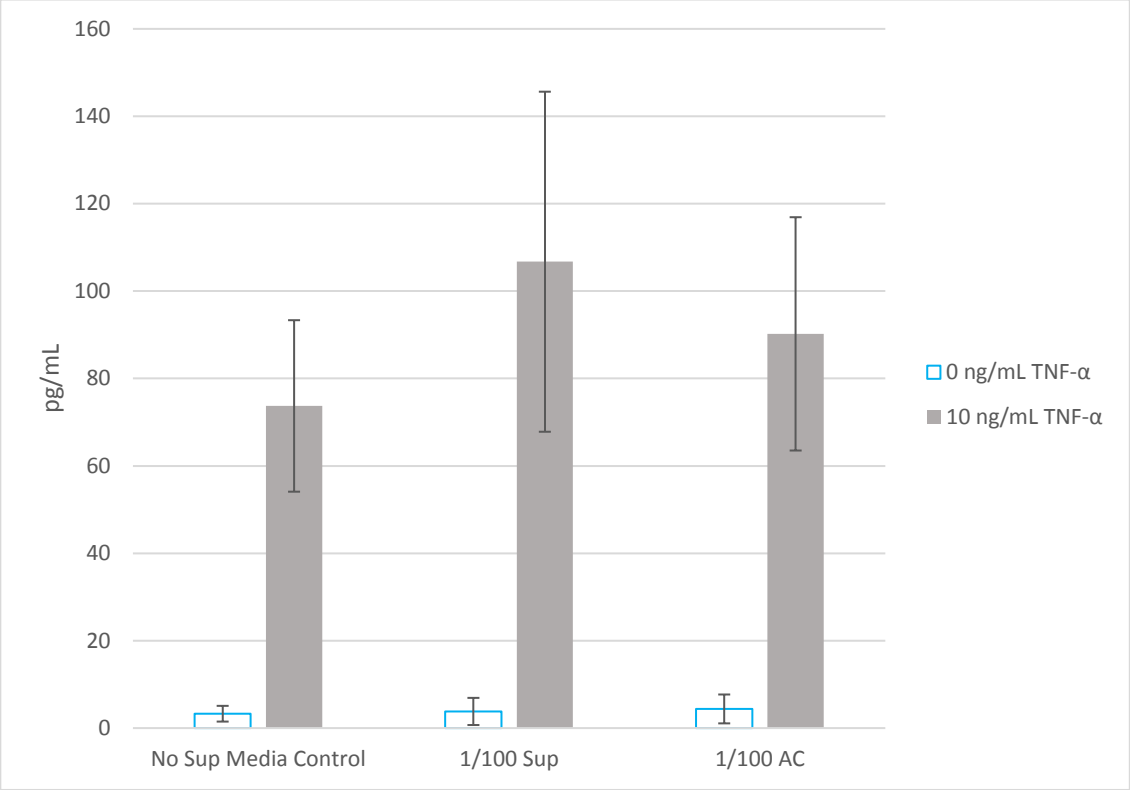



Fig 6: ELISA analysis of the immunomodulatory effects of supernatant from macrophages conditioned in filtered L. *rhamnosus* R0011 fermented milk with TNF-α co-incubation on production of MCSF by HUVEC. For all treatments, including media control, HUVECs were seeded at a density of 5000 cells/cm$^2$ into collagen coated, tissue culture treated 6 well plates (Falcon) and allowed to grow to a confluent monolayer over 8 days. 6-well plates have 9.5 cm$^2$ of available growth area which allowed for a population density of HUVEC amounting to approximately 456,000 to 500,000 cells on average per well. Confluent wells containing HUVEC cells were incubated for 48 hours using either MCDB 131 media alone (0ng/mL), MCDB 131 media containing 10 ng/mL of TNF-α (10 ng/mL), MCDB 131 media containing a 1/100 dilution of either the macrophage supernatant (sup) or the acidified lactic acid control (LA). After the 48 hour co-incubation, media was removed from the coated collagen plate and stored in a -80$^o$C freezer for at least 24 hours prior to use in the R&D MCSF ELISA assay. Experiments were repeated in triplicate and tested for significance using the SigmaPlot statistical analysis program.

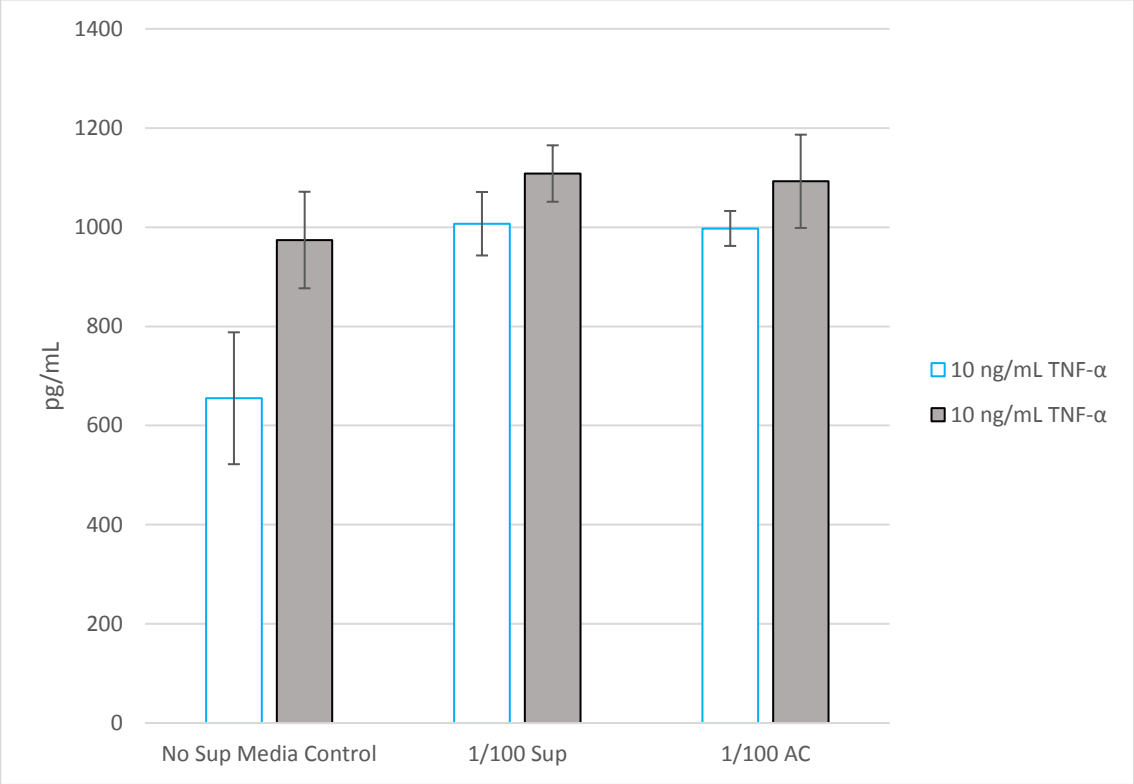



Fig 7: ELISA analysis of the immunomodulatory effects of supernatant from macrophages conditioned in filtered L. *rhamnosus* R0011 fermented milk and TNF-α Co-incubation on production of MCP-1 by HUVEC. For all treatments, including media control, HUVEC cells were seeded at a density of 5000 cells/cm$^2$ into collagen coated, tissue culture treated 6 well plates (Falcon) and allowed to grow to a confluent monolayer over 8 days. 6-well plates have 9.5 cm$^2$ of available growth area which allowed for a population density of HUVEC amounting to approximately 456,000 to 500,000 cells on average per well. Confluent wells containing HUVEC cells were incubated for 48 hours using either MCDB 131 media alone (0ng/mL), or MCDB 131 media containing 10 ng/mL of TNF-α (10ng/mL) plus either a 1/100 dilution of the macrophage supernatant (Sup) or the acidified lactic acid control (AC). After the 48 hour co-incubation, media was removed from the coated collagen plate and stored in a -80$^o$C freezer for at least 24 hours prior to use. Experiments were repeated in triplicate and tested for significance using the SigmaPlot statistical analysis program. No significant differences were seen.

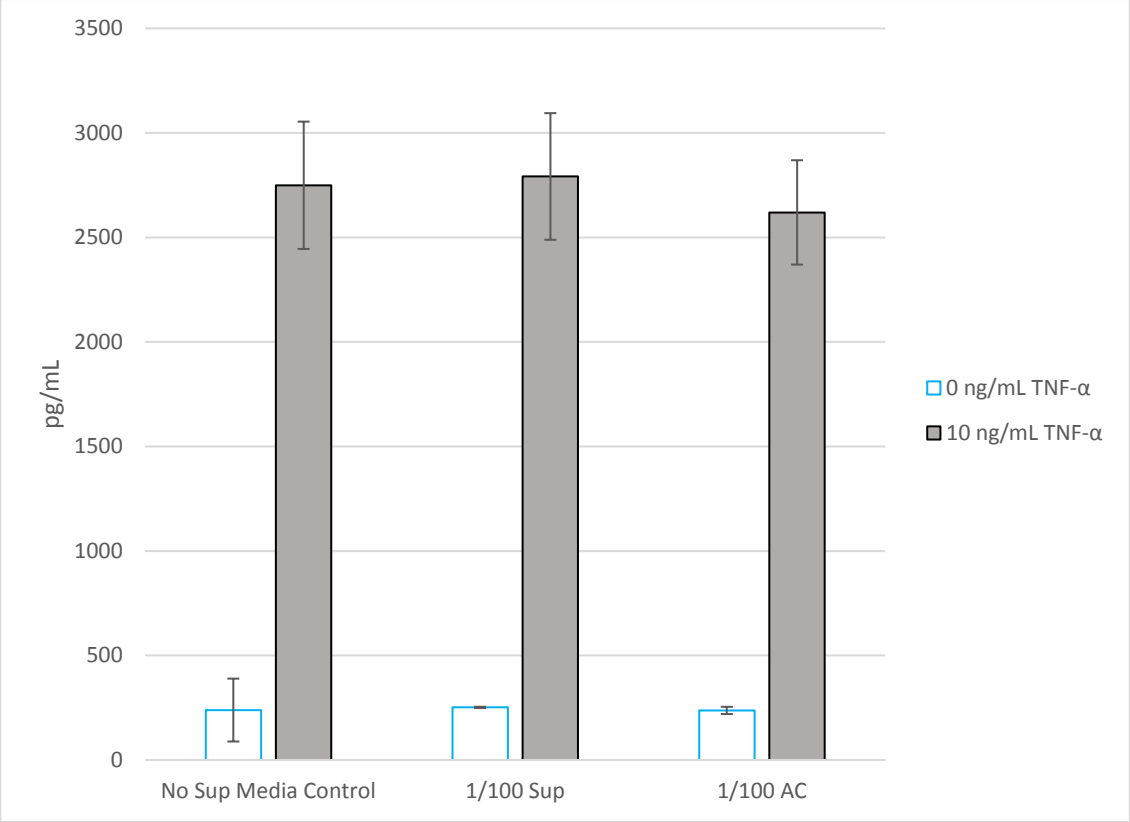



Fig 8: ELISA analysis of the immunomodulatory effects of supernatant from macrophages conditioned in filtered L. *rhamnosus* R0011 fermented milk and TNF-α Co-incubation on production of sICAM-1 by HUVEC. For all treatments, including media controls, HUVEC cells were seeded at a density of 5000 cells/cm$^2$ into collagen coated, tissue culture treated 6 well plates (Falcon) and allowed to grow to a confluent monolayer over 8 days. Confluent wells containing HUVEC cells were incubated for 48 hours using either MCDB 131 media alone (0ng/mL), or MCDB 131 media containing 10ng/mL of TNF-α (10ng/mL) plus a 1/100 dilution of either the macrophage supernatant (sup) or the acidified lactic acid control (LA). After the 48 hour co-incubation, media was removed from the coated collagen plate and stored in a -80$^o$C freezer for at least 24 hours prior to use in the R&D MCSF Enzyme Linked immunosorbent assay (ELISA). Experiments were repeated in triplicate and tested for significance using the SigmaPlot statistical analysis program. No significant differences were seen.

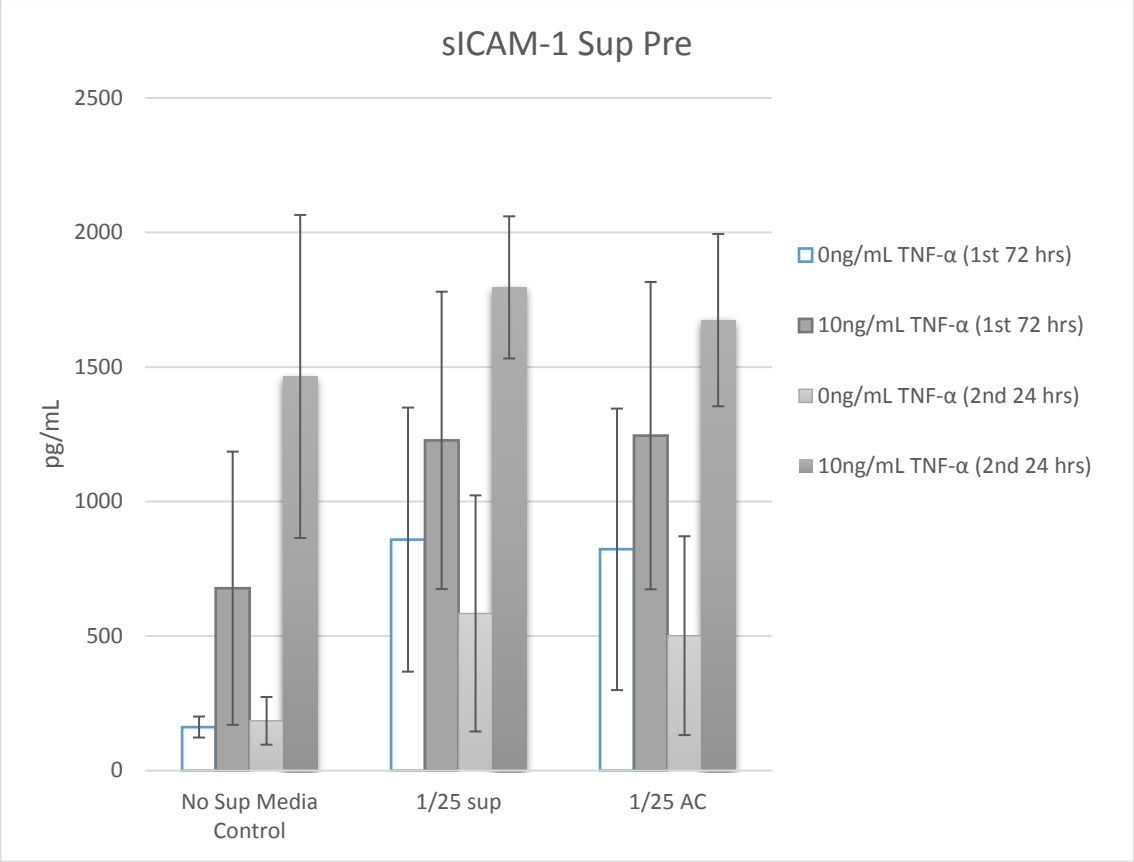



Fig. 9 ELISA analysis of the immunomodulatory effects of 72 hour pre-incubation of supernatant from macrophages conditioned in L. Rhamnosus R0011 whole milk followed by a 24 hour incubation with 10ng/mL TNF-α on production of sICAM-1 by HUVEC. For all treatments, including media controls, HUVEC cells were seeded at a density of 5000 cells/cm$^2$ into vented, collagen coated t-25 tissue culture flasks and allowed to form into a confluent monolayer over the course of 8 days. Confluent flasks containing HUVEC cells were incubated with 10mL of either: supplemented MCDB131 media (0ng, 10ng TNF-α), MCDB 131 supplemented with 1/25 dilutions of supernatant (Sup) or acidified control (AC) for 72 hours. Following this 72 hour pre-incubation flasks were then exposed to either supplemented MCDB 131 media (0ng/mL or 0ng/mL TNF-α plus a 1/25 dilution of supernatant) or supplemented MCDB 131 media containing 10ng/mL TNF-α (10ng/mL or 10ng/mL plus a 1/25 dilution of supernatant). Media was then removed from the tissue culture flasks and stored for at least 24 hours at -80$^o$C before use in the R&D ELISA protocol described above, specified for sICAM-1. Experiments were repeated in triplicate and tested for significance using the SigmaPlot statistical analysis program. No significant differences were seen.

## 3.4 – Nitric Oxide Production

Effects of synthesized milk ferment proteins (bioactive peptides) on production of the vasodilator NO by endothelial cells were assessed using the Cayman Nitrate/Nitrite assay kit, which measures the stable metabolites of NO production. Nitrate and nitrite are markers of NO production and measured due to the fact that NO only exists for approximately 30 seconds, making it impossible to measure with a colorimetric assay. Measurements of the total nitrate and nitrite levels give an estimate of the total NO produced by the HUVECs, as the main anti-inflammatory mediator released by endothelial cells is nitric oxide. The experiment was carried out in two phases. The first was to assess the NO production by HUVECs during exposure to <10 kDa fraction of L. *helveticus*, L. *rhamnosus*, and their associated non acidified (NAC) and acidified controls (AC). The fractions were placed into the media at dilutions of 0.1 μg/mL, 1 μg/mL, 3 μg/mL and 10 μg/mL for 24 hours. Following that 24 hours, the 96 well plates containing the HUVEC and HUVEC conditioned media were shaken to mix the nitrate and nitrite into the media. Figure 10 shows the increase in NO production by HUVEC exposed to 10 μg/mL of <10 kDa fractions of *L*. *helveticus* and *L*. *rhamnosus* fermented milk as well as their acidified (AC) and non-acidified (NAC) controls.

The second phase involved 12 synthesized peptides derived from the sequence of β-casein, obtained from Bio Basic, and diluted into MCDB 131 supplemented media. Each peptide has been shown to have ACE inhibitory activity (Nakamura, 1995; Tellez, 2010; Yamamoto, 1999; Boutrou, 2013; Migliore-samour, 1989) and were identified from the <10 fraction milk ferment peptides. Also obtained from Bio Basic were the

lactotripeptides valyl-prolyl-proline (VPP) and isoleucyl-prolyl-proline (IPP), which are peptides liberated from β-casein by *L. helveticus* and *S. cerevisiae.* Both have previously been shown by Hirota et al (2011) to induce NO production by endothelial cells and were therefore used as positive controls. The HUVEC were incubated with the peptides at dilutions of 3 μg/mL and 10 μg/mL for 24 hours. The cells were not activated by TNF-α for this test as we were not assessing the HUVECs in an inflammatory state. As can be seen below (Fig. 11 & 12) peptides 4 and 9 showed the greatest effect on the amount of NO production by endothelial cells, over control and were similar in NO production levels to known peptides VPP & IPP. Figure 12 shows a direct comparison of peptides 4 and 9 (HQPHQPLPPT and PGPIPN respectively) to VPP and IPP. Statistical analysis shows each peptide elicits more NO production than control and that NO production by peptides HQPHQPLPPT and PGPIPN is not statistically different from known vasodilators VPP and IPP.

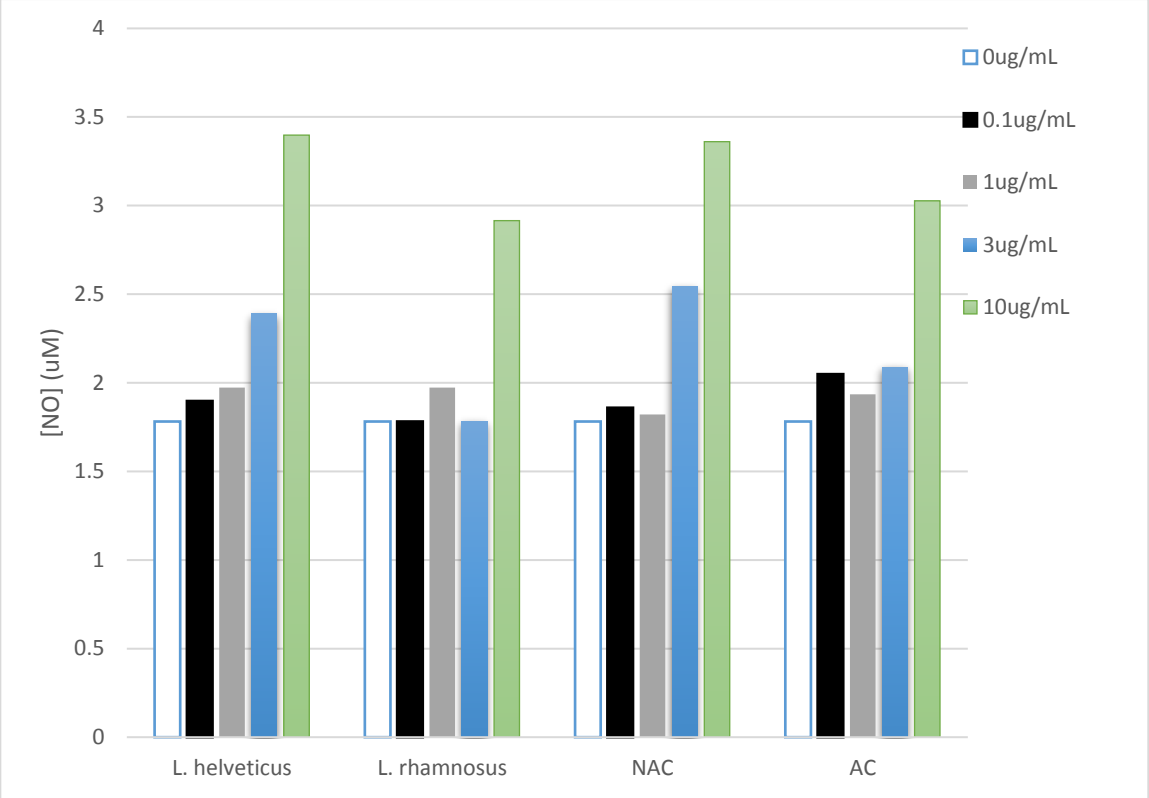



Fig 10. Effect of >10kDa fractions of milk fermented with L. *helveticus* and L. *rhamnosus* on untreated HUVECs. HUVEC cells were seeded at a density of 5000 cells/cm$^2$ into collagen coated, tissue culture treated 96 well plates (Falcon) and allowed to grow to a confluent monolayer over 8 days. Confluent wells containing HUVEC cells were incubated for 24 hours with either 100 μL of 0.1 μg/mL, 1 μg/mL, 3 μg/mL or 10 μg/mL of <10 kDa fraction or of L. *helveticus* or L. *rhamnosus*, non-acidified control (NAC) or acidified control (AC). Results were obtained once, and therefore statistical analysis was not possible.

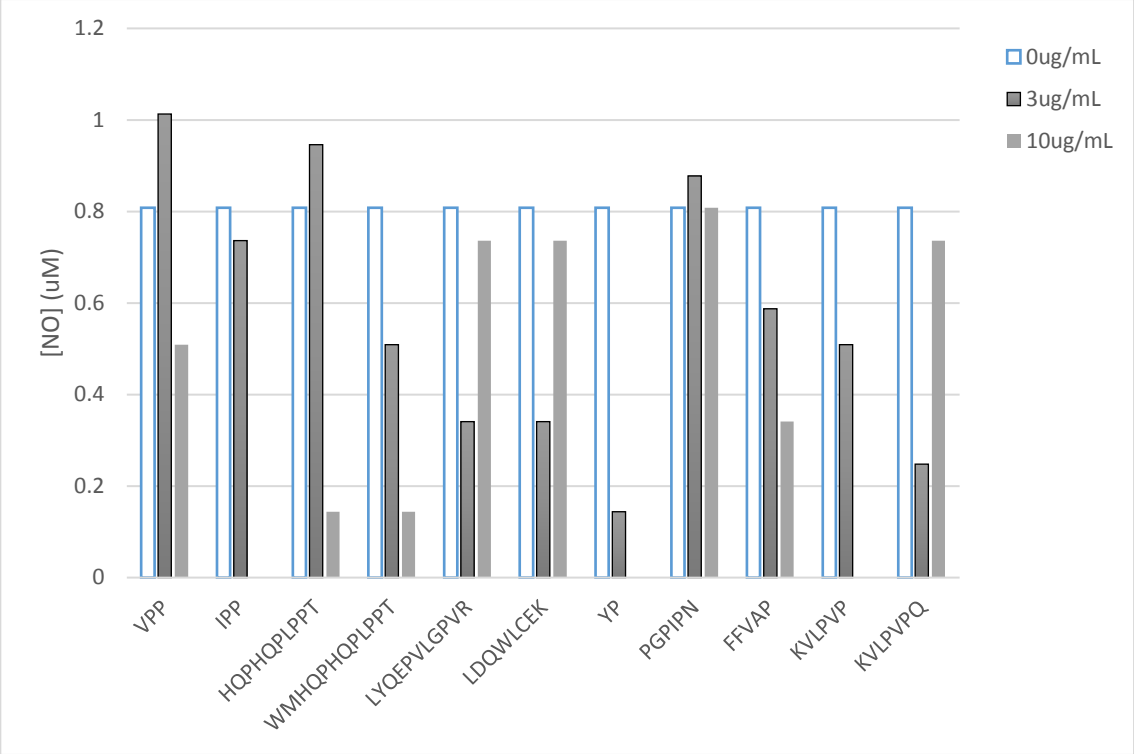



Fig.11: Effect of VPP, IPP and 9 isolated synthesized peptides on NO production in HUVECs. HUVEC cells were seeded at a density of 5000 cells/cm$^2$ into collagen coated, tissue culture treated 96 well plates (Falcon) and allowed to grow to a confluent monolayer over 8 days. Confluent wells containing HUVEC cells were incubated for 24 hours exposed to either 100 μL of 3 μg/mL or 10 μg/mL of 12 different synthesized peptides, using VPP & IPP as controls. Results were obtained once and therefore statistical analysis was not possible.

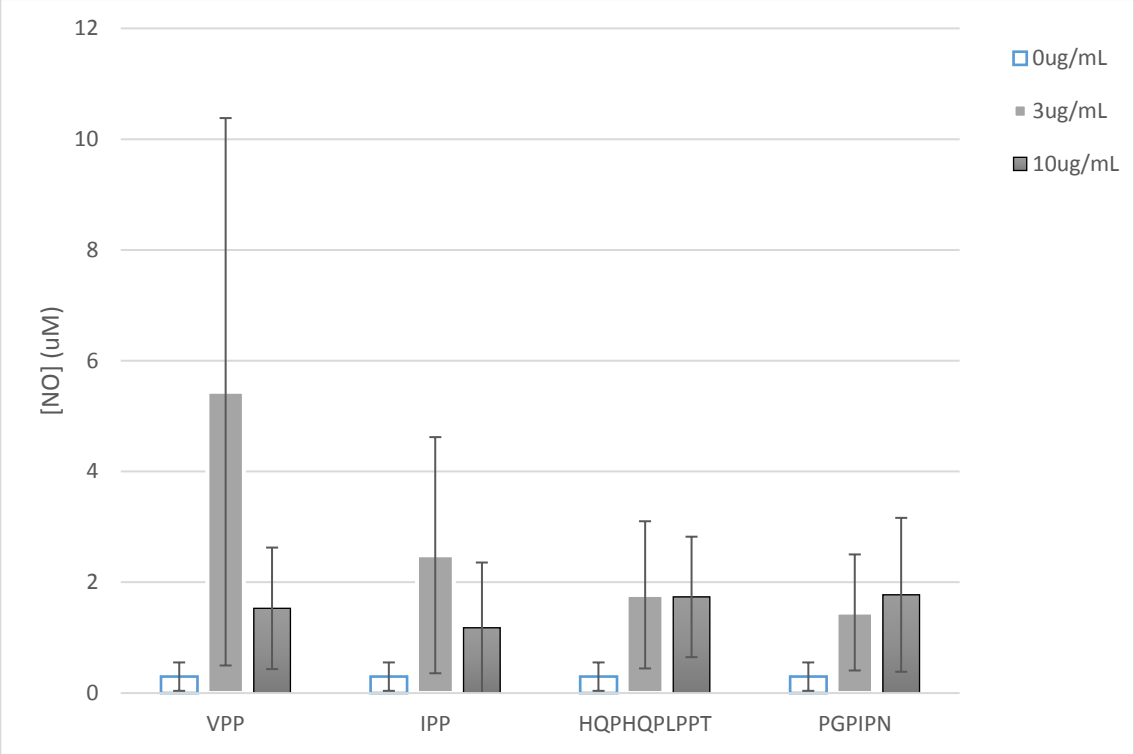



Fig. 12 Effect of VPP, IPP and 2 isolated synthesized peptides on NO production in HUVECs. HUVEC cells were seeded at a density of 5000 cells/cm$^2$ into collagen coated, tissue culture treated 96 well plates (Falcon) and allowed to grow to a confluent monolayer over 8 days. Confluent wells containing HUVEC cells were incubated for 24 hours exposed to either 100uL of 3ug/mL or 10ug/mL of either VPP (1) and IPP (2) controls, or one of 2 different synthesized peptides; HQPHQPLPPT (P4) and PGPIPN (P9). After the 24 hour incubation period the 96 well plates were shaken and 80uL of the conditioned media was transferred to a second 96 well plate and the nitric oxide assay was performed using Cayman's Nitrate/Nitrite Assay kit as described above. The nitric oxide assay was repeated in triplicate and tested for significance using the sigma plot statistical analysis program. No significant differences were seen.

# 4- Discussion

## ***4.1*** **– Exposure to Bacterial Secretome does not result in down regulation of inflammatory biomarker production by HUVECs**

Exposure of HUVECs to the bacterial secretome of *L. rhamnosus* was important as a control to test for the effects of proteins that bacteria would secrete normally. However, as can be seen in figures 1-4, the bacterial secretome did not seem to have any effect on the HUVEC at all, either when activated by TNF-α or not. These experiments tested whether proteins from the bacteria could have effects on the cell; further experiments are now focussed on the effects of proteins from milk ferments and peptides, not products from the bacteria themselves.

## ***4.2 – Exposure of HUVECs to Macrophage Supernatant does not result in down regulation of inflammatory biomarker production***

The macrophage supernatant tests were done using the secreted compounds from macrophages conditioned with either whole milk ferment, which contained *L. rhamnosus* cells, or filtered milk ferment, which had the *L. rhamnosus* cells filtered from it. Monocytes were first cultured in the laboratory of Dr. Julia Green-Johnson before being differentiated into M0 macrophages. Monocytes are cells that grow in suspension, but upon differentiation become adherent. These macrophages were then challenged in milk fermented with *L. rhamnosus* bacteria in one of two ways. Either the macrophages were incubated with exposure of whole ferment, which means that the fermented milk still contained the *L. rhamnosus* bacteria that fermented it, or they were incubated in a filtered fermented milk that had the bacteria filtered out of it. Using the whole and filtered ferments, we were able to investigate the effects of the macrophage

supernatant and thus investigate indirect effects on HUVEC cells. As shown in (Fig. 6- Fig. 9) the whole and filtered macrophage supernatant had no effect in preventing the endothelial cell secretion of sICAM-1, MCP-1 or MCSF as inflammatory molecules when co-incubated with 10 ng/mL TNF-α as challenge. The supernatant from cultured macrophages was a biological model that was tested in our study as a way to determine if products from these immune cells were altering HUVEC cell behaviour. Macrophages are found in the circulation during both healthy and inflamed states so it stands to reason that both the macrophages and their secreted products would be in close proximity to endothelial cells. We began this experiment optimistic that the macrophages would, upon exposure to milk ferments, secrete some protective signals that would exert an anti-inflammatory effect in an indirect manner on the HUVEC cells. In order to condition themselves and maintain immunity, macrophages will travel through the circulation to the GALT (gut associated lymphoid tissue) and extend processes out into the gut lumen. When we consume fermented milk products like yogurt or fermented milk drink, we are consuming not only the peptides from the milk itself but the healthy bacteria that cleaved milk proteins. This means that when the macrophages reach out they are being conditioned by the peptides and the bacteria. Then, as opposed to their secretions, the bacterium themselves or bioactive peptides making their way into the circulatory system, may each potentially mediate their effects by causing changes in the macrophages (Bermudez-Brito et al. 2014). Here we tested the effects of the supernatant of the macrophages. As can be seen with figure 6 to figure 9, the filtered macrophage supernatant did not seem to have any effect on the HUVECs

either positively or negatively once the HUVECs were activated by TNF-α. Once the HUVEC were exposed to the pro-inflammatory TNF-α, they seemed to stay activated in a pro-inflammatory state for at least 48 hours, which was the duration of the co-incubation test. Tellez et al (2010) showed that macrophages exposed to "cell free" L. *helveticus* fermented milk increased production of inflammatory biomarkers, suggesting that conditioned supernatants may contain further inflammatory signals. It may then be possible that macrophages increase production of cytokines in response to fermented milk, perhaps as a way to prime or modulate the immune system for more immediate action during infection. Work ongoing in our group has suggested that milk conditioned with L. *rhamnosus* appears to condition the macrophages into a regulatory phenotype, characterized by an increase in regulatory cytokines (M. Jeffrey, personal communication)

Next steps for this project would be to put the M0 macrophages in direct contact with the HUVECs after they have been conditioned with the L. *rhamnosus* fermented milk to investigate if direct contact is necessary. There is still a possibility that once conditioned, the macrophages prepared in Dr. Julia Green-Johnson's lab may be able to exert an anti-inflammatory effect on endothelial cells once they have a chance to adhere to them directly and this is proposed as a future extension of this project. Since TNF-α stimulation of HUVECs results in profound activation, another approach would be to co-culture with ferment exposed macrophages and investigate production of anti-inflammatory biomarkers or changes in gene expression. This would allow investigation of HUVECs expression of anti-inflammatory biomarkers through known gene regulatory

pathways as well as examining the decreased expression of pro-inflammatory cytokines. Marcone et al. (2015) describe an adhesion assay using THP-1 monocytes, as a method for testing the adhesive nature of activated macrophages and the effects that bioactive peptides have on those interactions. The methodology may be pursued in order to determine the effects that L. *rhamnosus* fermented milk fractions play on the interaction between activated HUVEC and monocytes/macrophages.

### ***4.3*** **– Exposure to Synthesized Peptide from β -*casein* Sequence shows NO production by cultured endothelial cells**

As was mentioned in the introduction, it has been shown that bioactive peptides can have a direct role to play in the prevention of atherosclerosis and cardiovascular disease (Donkor et al. 2007; Fekete et al. 2013; Papadimitriou et al. 2007; Hirota et al. 2011; Qian et al, 1995). Three suggested mechanisms for bioactive peptides' physiological role are: ACE-inhibition, NO release and interference in thrombus formation. (Phelan and Kerins, 2011). However, the ability of a bioactive peptide to exert any of these effects is dependent on which bacteria are responsible for its liberation during fermentation and also on the method of consumption. Specifically with the research discussed in this thesis, the bioactive peptides are proposed to be produced through the cleavage of β-casein by L. *helveticus* lactic acid bacteria (See Table 2). This is one of the many bacteria that have been shown to have potential angiotensin converting enzyme (ACE) inhibitory activity and thus it was important to test the effects of specific peptides in isolation. Of the 12 synthesized peptides obtained from the lab of Dr. Janice Strap, 2 peptides had an effect on NO production by endothelial cells greater than the media control (Fig 11&12). VPP and IPP, as they have been shown to induce NO

production by endothelial cells (Hirota et al. 2011) and have angiotensin converting enzyme (ACE) inhibitory activity *in vitro* (Nakamura, 1995), were used as positive controls. While not exceeding levels seen with VPP and IPP, HQPHQPLPPT (peptide 4) and PGPIPN (peptide 9) did show a moderate induction of [NO] when co-incubated with HUVECs. Why these peptides have an effect over others is still unknown; however these two sequences do show some similarities to VPP and IPP. Specifically, peptide 4 possesses two prolines at the C-terminal end and peptide 9 contains the sequence PIP, a rearrangement of IPP. Peptide 4 (HQPHQPLPPT) was initially identified by Tellez et al. (2010), and its effects were tested on a murine macrophage cell line. Peptide 4 was one of 5 peptides identified from an *L. helveticus* fermented milk fraction is reported to have significant effects on cytokine release by the murine macrophage cell line RAW 264-7 (Tellez et al. 2010). Along with IL-6 and TNF-α release, which are hypothesized to act to prime the immune system, NO was also released supporting our results seen in fig. 12 using a human endothelial cell line. Peptide 9 also shows the ability to induce NO production by human cells; since it possessed the amino acids PIP in its sequence, it is intriguing to speculate that the dipeptide IP may be in action, although the mechanism is not known. Further investigations into peptide 9 and its signaling effects on HUVECs should be pursued.

### ***4.4 – Human Umbilical Vein Endothelial Cells (HUVECs)***

Culturing Human Umbilical Vein Endothelial Cells (HUVEC) was a very challenging aspect of this research project. HUVECs are incredibly sensitive cells, requiring specific culture

methods to ensure the cells grow properly and at the appropriate rate. Ideally, HUVECs need a minimum of 8 days to grow to a confluent monolayer and be ready for use in experiments. Special attention to passaging was developed to mitigate lysing of cells upon each passage with trypsin. Cells received from Cedarlane were only viable for ~15 population doublings. This is different than 15 passages. The cells could only double their population 15 times and this doubling was only based on the number of cells available. When the majority of cells would lyse during passaging, the number of viable cells making it into the new culture flask was sparse. This lack of population density meant that in order for the HUVECs to populate a flask they had to divide more than normal, making the passage numbers misleading. Typically HUVEC cells are only viable for experimentation from passage 2 to passage 5, possibly 6 if the flasks have been seeded at a density higher than the recommended 5000 cells/$cm^2$. By having the cells lyse and decrease in population during passaging they exceeded their doubling limit well before the passage number indicated they should have. This issue also arose when we began using Cellstar tissue treated cell culture flask as, even though the flasks were treated specifically to allow most cell types to grow and thrive, there was still a problem with cells adhering to the plastic. This problem was solved by providing the flasks with a layer that works to imitate the basal lamina (found in the blood vessels) between the bottom of the flasks and the adherent cells. By coating the tissue culture flasks in collagen this not only provided a layer for the cells to adhere to so that they would not become stuck to the plastic, but also gave them access to proteins that allowed them to grow robustly at an appropriate rate. Combining the collagen coating protocol, with 30

minute incubation of supplemented MCDB131 prior to the addition of the HUVEC cells meant that when added, the HUVEC had everything they needed in order to adhere and propagate without sticking and lysing later. The addition of collagen also made passaging easier. When Accutase or more concentrated trypsin/EDTA were added, it dissolved the collagen by cleaving the peptide bonds that held it together. This released the HUVEC into suspension without ever having to manipulate the cells directly. Due to the extended propagation time of the HUVECs and the multiple cell culture problems; troubleshooting their growth took a significant amount of time. However, the final result was a very efficient and effective culture protocol that optimized later work. Lastly, ELISA and Flow Cytometry protocols were optimized for use with HUVEC. It was important to ensure that cell populations were large enough that the flow cytometry washing protocol wouldn't totally eliminate the cell population, while also being small enough that we could easily test multiple ferment components without using media at an irresponsible rate. Additionally, it was discovered that the HUVECs were too large to fit into the needle attached to the flow cytometer. In order to work around this we employed a fixation/dehydration protocol that allowed us to ensure any biomarkers stayed on the surface of the cell available for antibody adhesion, but dehydrated the cells to a point where they would easily fit through the GUAVA flow cytometer needle.

HUVEC are obtained directly from umbilical cords and immediately frozen down for culture later. This means that we are as close to donated human tissue as we could get for our research project. However it is worth noting that both human aortic

endothelial cells and human microvascular endothelial cells (HMEC) are available cell lines for research, as was seen previously in the literature.

### ***4.5*** **– Importance of Interventions for Cardiovascular Disease/Metabolic Syndrome/Diet/Diabetes**

Cardiovascular disease, atherosclerosis, obesity, the metabolic syndrome and the other so called diseases of civilization are seemingly here to stay. The abundance of processed, fatty, greasy and generally unhealthy food stuffs makes winning the war on unhealthy lifestyles seem insurmountable. The rates of obesity and incidence of cardiovascular disease have risen over the last 30 years (Flegal et al, 1998; Statistics Canada, 2011c), and continues to be a major health concern for the future. Additionally as third world countries continue to industrialize and become further exposed to more modern approaches to living, businesses have been making a push to provide their overly processed unhealthy foods to this emerging market (Curtis and McCklusky, 2007). If the populations of these countries begin to regularly consume Western type diets, rates of obesity and cardiovascular disease will increase and the "unhealthy lifestyle epidemic" will continue to worsen. It is, therefore, incredibly important to find easily executable interventions as quickly as possible in order to proactively prevent an unnecessary increase in deaths associated with atherosclerosis, obesity, cardiovascular disease and diabetes. Effort should first be placed on educating the public on the dangers of over-consumption, or consumption of diets too high in fatty foods, with available options for healthy alternatives and knowledge of nutritional benefits of foods such as dairy products. Along with this, exercise should also be recommended and heavily supported as a way to compliment and maximize the benefits of dietary changes.

Finding ways to supplement already popular foodstuffs is also an easy, and ideal, method to help prevent the insidious effects of an unhealthy diet. While it is possible to achieve weight loss through exercise, reversing more insidious consequences of poor dietary choices like the cardiovascular disease risk, fatty streak formation or atherosclerotic processes take more time. This time could be reduced or made more efficient by using components found in foods that have been shown to have beneficial effects on heart health. A systematic review by Fekete et al (2013) concluded that more robust, well designed randomized control trial are necessary to confirm the benefits of milk peptides on health as the existing evidence is limited and warrants further investigations

### ***4.6*** **– Investigating mechanisms of fermented milk products' effect is important for CVD interventions**

Fermented milk products have been shown to have beneficial effects on heart health (Sonestedt et al, 2011) but, so far, the mechanisms through which these products exert their effects have yet to be elucidated. Their importance, though, cannot be understated. A few of the most important examples of fermented products available immediately and to the widest population demographics are yogurt and fermented milk drinks, the latter of which are fast becoming part of North American diets, but have been more popular in Asian countries. Focusing on yogurt, though, we are presented with a very prevalent example of a fermented milk product that could be easily added into North American diets in order to assist with the lifestyle changes that are necessary for the prevention and treatment of cardiovascular disease, atherosclerosis and other diseases. Since yogurt is easily and readily accessible, it is a prime example of a potential

dietary staple or supplement that will assist in combatting cardiovascular disease. The discovery of what exactly makes yogurt so beneficial to heart health inform people of exactly why they should be incorporating it into their diet and, in turn, potentially alter consumption habits. Through the study of fermented dairy products like fermented milk, yogurt and various cheeses, we are working towards identifying novel peptides that seem to exert a cardio/athero-protective/preventative effect on the circulatory system. Identification of these peptides and a confirmation that they continue to exert their effects in isolation would be a benefit for its incorporation into functional foods. With isolation and characterization of components that make these products protective, supplementation of food with bioactive peptides and components could be proposed as an important health benefit. In this way, we make the idea of a healthy lifestyle change easier and less obtrusive to current lifestyles. Studying fermented milk products will, ideally, allow for us to come up with better preventative methods to promote heart health.

However, it seems as though that bioactivity *in vivo* cannot be established solely through *in vitro* experimentation (Saito et al, 2000; Abubakar et al, 1998). As was shown by Sonsetedt et al (2011) it is not just fermented milk that carries with it bioactive peptides and associated health benefits. Cheese, butter and cream, while not being at the level of fermented milk, still exert their own cardioprotective effects suggesting that the way someone consumes dairy products also plays a role in the overall health benefits of dairy products. Fekete et al (2013) reviewed several studies that showed bioactive peptides demonstrating potentially cardioprotective ACE-inhibitory activity *in*

*vitro*, but lose all effect when consumed and assessed *in vivo*. The ability of isolated bio-active peptides to survive digestion and which peptides are consumed play a large role in whether or not these peptides will be able to have cardio-protective effects for humans. Discovery of specific foods that best protect and carry isolated bio-active peptides, and allow them to display the full breadth of their health promoting effects will open up a new category of functional foods.

### ***4.7 –Future directions***

This project aimed to fill knowledge gaps in understanding how our endothelium responds to bioactive components of milk ferments, chemokines produced by macrophages, bacterial and isolated milk peptides through the measurement of modulation of cytokine release. Endothelial cells play an important role as the gatekeepers of the vascular system. They decide which cell types move in and out of circulation. Issues arise when determination of appropriate cell type and movement is compromised, a situation referred to as endothelial dysfunction. Investigating the endothelial cell's role in slowing macrophage accumulation and foam cell formation beneath the endothelial barrier of the blood vessels can be expanded to include other cell types and mechanisms. As our blood flow encounters branch points, the modulation in shear stress upon the vessel surface is enough to allow endothelial cells to become dysfunctional and allow the entrance of leukocytes. Our circulatory system has a plethora of branch points which are necessary in sending blood around our bodies; this makes endothelial dysfunction by shear stress a fact of life. Slowing the response of leukocyte invasion at these branch points may not be feasible. However, the second

component of the vascular wall that plays an incredibly important role in the pathogenesis of atherosclerosis is smooth muscle (Kumar et al, 2007). Smooth muscle would be an important cell type to look at to bolster the results of the peptide NO assays. NO is both a potent anti-inflammatory and vasodilator. Assuming that the release of NO by the endothelial cells through exposure to bioactive peptides, would enact a vasodilatory function on the smooth muscle, this would cause widening of the vessels at areas prone to atherogenesis and might relieve some of the effects of shear stress on the vessels. In fact, Hirota et al (2011) showed that VPP and IPP caused vasorelaxation in isolated rat aorta. If these effects could be duplicated using novel bioactive peptides either alone, or in reversal of induced vasoconstriction, it would comprise a preventative function of bioactive peptides on vascular smooth muscle. Smooth muscle would also be an ideal next candidate for consideration due to its role in the pathogenesis of atherosclerosis. Once the fatty streak has progressed and foam cells have started to accumulate underneath the endothelial barrier, smooth muscle cells react to the accumulation of lipids and foam cells by starting to propagate (Zettler et al, 2003) and release MCSF to call for more monocytes to change to macrophages (Shimada et al, 1992). An investigation into the effects on vascular smooth muscle might begin with a measurement of MCSF secretion levels and cell proliferation.

Further investigation into smooth muscle cell responses to bioactive peptides would yield important information about effects on smooth muscle cells role in thrombus formation. The true danger with atherosclerosis is the thromboembolism, the end stage of its progression. When the atherosclerotic plaque becomes necrotic and exposes

smooth muscle cells to the circulation, the clotting process/ coagulation cascade begins and a blood clot forms at the damaged site (Marmur et al, 1992). Eventually this clot, through the function of may be dislodged from the atherosclerotic site and make its way through the circulation until it arrives at a smaller vessel, becomes stuck, and begins impeding blood and therefore oxygen from arriving at that site. It is this blockage that causes the ischemic cardiovascular disease mentioned earlier and that could eventually proceed to cause a heart attack. Discovering if bioactive peptides have any effect on smooth muscle's activation and incorporation into the atherosclerotic plaque would be an interesting future direction.

Experiments may also include investigations into possible modification of macrophage scavenger receptors (so that they do not consume ox-LDL) or anti-oxidant properties of the peptides that would slow the formation of ox-LDL so the incidence of foam cell formation decreases. Testing the effects of bioactive peptides on LDL cholesterol would lead to more knowledge concerning either the direct anti-oxidant properties or how they may interfere with scavenger receptors of macrophages would also be a potential research path for the future (Hernández-Ledesma et al, 2005). It has been shown, previously, that isolated peptides from L. *helveticus* fermented β-casein fraction exert an anti-oxidant effect twice as potent as the vitamin E derivative, trolox (Stressler et al, 2013). If bioactive peptides exerted this anti-oxidant effect on LDL, they would interfere with the formation of ox-LDL, and the rate of macrophages changing to foam cells would be decreased. Alternatively, the bioactive peptides could act in such a

way that they prevent macrophages from recognizing and consuming ox-LDL, therefore preventing their transformation into foam cells.

Finally, it is very compelling that a recent report by Marcone et al (2015) showed inhibition of pro-inflammatory cytokine production in TNF-α activated cultured endothelial cell lines at much higher concentrations than described here. It is possible that in our incubations of 3-10 μg/mL of peptide was too dilute to exert an effect. It has been shown that pre-incubation of human aortic endothelial cells with 30-300ug/mL of <5kDa proteins from *Enterococcus* hydrolyzed milk results in downregulation of ICAM-1, VCAM-1 and e-selectin gene and cell surface protein expression (Marcone et al, 2015). The net amount of peptide present may be a key determinant to that fraction's effect. Leclerc et al (2002) showed that 1-2.5 g/kg of caseinate rich L. *helveticus* (R211 and R389) fermented milk showed significant reductions in mean arterial blood pressure over a 24 hour period in spontaneously hypertensive rats. Increasing the protein concentrations in our incubations would potentially produce different results, although the focus on biologically relevant concentrations may be lost.

## ***4.8 – Conclusion***

Fermented milk has been shown by Sonestedt et al (2011) to exert cardio-protective or anti-atherogenic effects. In our experiments, 2 bioactive peptides synthesized from the β-casein sequence showed moderate effects of NO production, a potent vasodilator and anti-inflammatory compound, release by HUVECs. In order to confirm and expand these results, experiments should look further into the preparation and effects of bioactive peptides. The effects of bioactive peptides have been shown to

change depending on the milk used, the bacteria used and the method of ingestion. In this project we focused specifically on bioactive peptides produced by L. *rhamnosus*, a lactic acid bacteria suggested to produce bioactive peptides that exert ACE-inhibitory effects. While their effects on inflamed endothelial cells at low concentrations were not robust, their anti-inflammatory activity will require further investigation to fully elucidate any mechanisms. It will be important to discover whether or not the effects of the bioactive peptides produced by L. *rhamnosus* change depending on the method of consumption, as *in vitro* and *in vivo* effects can differ (Sonestedt et al, 2011). As cardiovascular disease and atherosclerosis become more and more prevalent with a worldwide shift towards a modern diet, it is becoming increasingly more important to discover interventions that will combat the more insidious effects of high fat diets. Discovery of functional foods that will assist in protecting our cardiovascular system from the damaging effects of free radicals and atherosclerosis will be a step in the right direction for assisting individuals with necessary lifestyle changes.

**Literature Cited**

Abhijit, S., Bhaskaran, R., Narayanasamy, A., Chakroborty, A., Manickam, N., Dixit, M., Visawanathan, M., & Balasubramanyam, M. (2013). Hyperinsulinemia-induced vascular smooth muscle cell (VSMC) migration and proliferation is mediated by converging mechanisms of mitochondrial dysfunction and oxidative stress. *Molecular and Cellular Biochemistry*, *373*(1-2), 95-105.

Abubakar, A., Saito, T., Kitazawa, H., Kawai, Y., & Itoh, T. (1998). Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion. *Journal of Dairy Science*, *81*(12), 3131-3138.

Albers, G. W., Caplan, L. R., Easton, J. D., Fayad, P. B., Mohr, J. P., Saver, J. L., & Sherman, D. G. (2002). Transient ischemic attack—proposal for a new definition. *New England Journal of Medicine*, *347*(21), 1713-1716.

Alberti, K. G. M. M., Eckel, R. H., Grundy, S. M., Zimmet, P. Z., Cleeman, J. I., Donato, K. A., ... & Smith, S. C. (2009). Harmonizing the Metabolic Syndrome A Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity.*Circulation*, *120*(16), 1640-1645.

Aldrovandi, M., & O'Donnell, V. B. (2013). Oxidized PLs and vascular inflammation. *Current Atherosclerosis Reports*, *15*(5), 1-8.

Arcaro, G., Cretti, A., Balzano, S., Lechi, A., Muggeo, M., Bonora, E., & Bonadonna, R. C. (2002). Insulin causes endothelial dysfunction in humans sites and mechanisms. *Circulation*, *105*(5), 576-582.

Barreiro, O., Yáñez-Mó, M., Serrador, J. M., Montoya, M. C., Vicente-Manzanares, M., Tejedor, R., Furthmayr, H., & Sánchez-Madrid, F. (2002). Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes. *The Journal of Cell Biology*, *157*(7), 1233-1245.

Bermudez-Brito, M., Munoz-Quezada, S., Gomez-Llorente, C., Romero, F., & Gil, A. (2014). Lactobacillus rhamnosus and its cell-free culture supernatant differentially modulate inflammatory biomarkers in Escherichia coli-challenged human dendritic cells. *British Journal of Nutrition, 111*(10), 1727-1737.

Björntorp, P., Bergman, H., & Varnauskas, E. (1969). Plasma free fatty acid turnover rate in obesity. *Acta Medica Scandinavica*, *185*(1-6), 351-356.

Boden, G., Jadali, F., White, J., Liang, Y., Mozzoli, M., Chen, X., Coleman, E., & Smith, C. (1991). Effects of fat on insulin-stimulated carbohydrate metabolism in normal men. *Journal of Clinical Investigation*, *88*(3), 960.

Boden, G., & Shulman, G. I. (2002). Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and β-cell dysfunction. *European Journal of Clinical Investigation*, *32*(s3), 14-23.

Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A., Benamouzig, R., Tomé, D., & Leonil, J. (2013). Sequential release of milk protein–derived

bioactive peptides in the jejunum in healthy humans. *The American Journal of Clinical Nutrition*, *97*(6), 1314-1323.

Carrera-Bastos, P., Fontes-Villalba, M., O'Keefe, J. H., Lindeberg, S., & Cordain, L. (2011). The Western Diet and Lifestyle and Diseases of Civilization. *Research Reports in Clinical Cardiology*, *2*, 15-35.

Champagne, B., Tremblay, P., Cantin, A., & Pierre, Y. S. (1998). Proteolytic cleavage of ICAM-1 by human neutrophil elastase. *The Journal of Immunology*,*161*(11), 6398-6405.

Chouinard, J. A., Grenier, G., Khalil, A., & Vermette, P. (2008). Oxidized-LDL induce morphological changes and increase stiffness of endothelial cells. *Experimental Cell Research*, *314*(16), 3007-3016.

Conference Board of Canada. (2010, February). The Canadian Heart Health Strategy: Risk Factors and Future Cost Implications Report.

Cordain, L., Eaton, S. B., Sebastian, A., Mann, N., Lindeberg, S., Watkins, B. A., ... & Brand-Miller, J. (2005). Origins and evolution of the Western diet: health implications for the 21st century. *The American Journal of Clinical Nutrition*, *81*(2), 341-354.

Curtis, K. R., & McCluskey, J. J. (2007). Consumer preferences for western-style convenience foods in China. *China Economic Review*, *18*(1), 1-14.

Dai, S., Bancej, C., Bienek, A., Walsh, P., Stewart, P., & Wielgosz, A. (2009). Report summary tracking heart disease and stroke in canada 2009. *Chronic Diseases in*

*Canada, 29*(4), 192-193. Retrieved from Public Health Agency of Canada. (2010, July 23). *Six Types of Cardiovascular Disease.*

De Caterina, R., Libby, P., Peng, H. B., Thannickal, V. J., Rajavashisth, T. B., Gimbrone Jr, M. A., ... & Liao, J. K. (1995). Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *Journal of Clinical Investigation*, *96*(1), 60.

de la Fuente, M. A., Singh, H., & Hemar, Y. (2002). Recent advances in the characterisation of heat-induced aggregates and intermediates of whey proteins. *Trends in Food Science & Technology*, *13*(8), 262-274.

de Fougerolles, A. R., Stacker, S. A., Schwarting, R., & Springer, T. A. (1991). Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. *The Journal of Experimental Medicine*, *174*(1), 253-267.

Deshmane, S. L., Kremlev, S., Amini, S., & Sawaya, B. E. (2009). Monocyte chemoattractant protein-1 (MCP-1): an overview. *Journal of Interferon & Cytokine Research*, *29*(6), 313-326.

Donkor, O. N., Henriksson, A., Singh, T. K., Vasiljevic, T., & Shah, N. P. (2007). ACE-inhibitory activity of probiotic yoghurt. *International Dairy Journal*,*17*(11), 1321-1331.

Edmonds, M. E., & Foster, A. V. M. (2006). ABC of wound healing: Diabetic foot ulcers. *BMJ: British Medical Journal*, *332*(7538), 407.

Fang, Y. Z., Yang, S., & Wu, G. (2002). Free radicals, antioxidants, and nutrition. *Nutrition*, *18*(10), 872-879.

Fekete, A. A., Givens, D. I., & Lovegrove, J. A. (2013). The impact of milk proteins and peptides on blood pressure and vascular function: a review of evidence from human intervention studies. *Nutrition Research Reviews*, *26*(02), 177-190.

Festa, A., D'Agostino, R., Howard, G., Mykkänen, L., Tracy, R. P., & Haffner, S. M. (2000). Chronic subclinical inflammation as part of the insulin resistance syndrome the Insulin Resistance Atherosclerosis Study (IRAS). *Circulation*,*102*(1), 42-47.

Fixe, P., & Praloran, V. (1998). M-CSF: haematopoietic growth factor or inflammatory cytokine?. *Cytokine*, *10*(1), 32-37.

Flegal, K. M., Carroll, M. D., Kuczmarski, R. J., & Johnson, C. L. (1998). Overweight and obesity in the United States: prevalence and trends, 1960-1994. *International Journal of Obesity and Related Metabolic Disorders: Journal of the International Association for the Study of Obesity*, *22*(1), 39-47.

Flemming, K. D., Wijdicks, E. F., St Louis, E. K., & Li, H. (1999). Predicting deterioration in patients with lobar haemorrhages. *Journal of Neurology, Neurosurgery & Psychiatry*, *66*(5), 600-605.

Fulop, T., Tessier, D., & Carpentier, A. (2006). The metabolic syndrome. *Pathologie Biologie*, *54*(7), 375-386.

Gerrity, R. G. (1981). The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. *The American Journal of Pathology*, *103*(2), 181.

Grundy, S. M., Abate, N., & Chandalia, M. (2002). Diet composition and the metabolic syndrome: what is the optimal fat intake?. *The American Journal of Medicine*, *113*(9), 25-29.

Guyton, A. C., & Hall, J. E. (2006) *Textbook of Medical Physiology (11th edition)*. Philadelphia, PA: Elsevier Saunders.

Halai, K., Whiteford, J., Ma, B., Nourshargh, S., & Woodfin, A. (2014). ICAM-2 facilitates luminal interactions between neutrophils and endothelial cells in vivo. *Journal of Cell Science*, *127*(3), 620-629.

Haverstick, D. M., Cowan, J. F., Yamada, K. M., & Santoro, S. A. (1985). Inhibition of platelet adhesion to fibronectin, fibrinogen, and von. *Blood*, *66*(4), 946-952.

Hernández-Ledesma, B., Miralles, B., Amigo, L., Ramos, M., & Recio, I. (2005). Identification of antioxidant and ACE-inhibitory peptides in fermented milk. *Journal of the Science of Food and Agriculture*, *85*(6), 1041-1048.

Hirota, T., Nonaka, A., Matsushita, A., Uchida, N., Ohki, K., Asakura, M., & Kitakaze, M. (2011). Milk casein-derived tripeptides, VPP and IPP induced NO production in cultured endothelial cells and endothelium-dependent relaxation of isolated aortic rings. *Heart and Vessels*, *26*(5), 549-556.

Hubbard, A. K., & Rothlein, R. (2000). Intercellular adhesion molecule-1 (ICAM-1) expression and cell signaling cascades. *Free Radical Biology and Medicine*,*28*(9), 1379-1386.

Jamison, D. T., Breman, J. G., Measham, A. R., Alleyne, G., Claeson, M., Evans, D. B., Jha, P., Mills, A., & Musgrove, P. (Eds.). (2006). *Disease Control Priorities in Developing Countries*. World Bank Publications.

Jensen, M. D., Haymond, M. W., Rizza, R. A., Cryer, P. E., & Miles, J. (1989). Influence of body fat distribution on free fatty acid metabolism in obesity. *Journal of Clinical Investigation*, *83*(4), 1168.

Jun, C. D., Carman, C. V., Redick, S. D., Shimaoka, M., Erickson, H. P., & Springer, T. A. (2001). Ultrastructure and function of dimeric, soluble intercellular adhesion molecule-1 (ICAM-1). *Journal of Biological Chemistry*,*276*(31), 29019-29027.

Kim, J. A., Montagnani, M., Koh, K. K., & Quon, M. J. (2006). Reciprocal relationships between insulin resistance and endothelial dysfunction molecular and pathophysiological mechanisms. *Circulation*, *113*(15), 1888-1904.

Kumar, V., Abbas, A., Fausto, N., & Mitchell, R. N. (2007). *Robbins Basic Pathology* (8th edition). Pennsylvania, United States of America: Saunders Elsevier.

Lawson, C., & Wolf, S. (2009). ICAM-1 signaling in endothelial cells. *Pharmacological Reports*, *61*(1), 22-32.

Leclerc, P. L., Gauthier, S. F., Bachelard, H., Santure, M., & Roy, D. (2002). Antihypertensive activity of casein-enriched milk fermented by Lactobacillus helveticus. *International Dairy Journal*, *12*(12), 995-1004.

Leeuwenberg, J. F., Smeets, E. F., Neefjes, J. J., Shaffer, M. A., Cinek, T., Jeunhomme, T. M., & Buurman, W. A. (1992). E-selectin and intercellular adhesion molecule-1 are released by activated human endothelial cells in vitro. *Immunology*, *77*(4), 543.

Lemieux, I., Pascot, A., Couillard, C., Lamarche, B., Tchernof, A., Alméras, N., ... & Després, J. P. (2000). Hypertriglyceridemic Waist A Marker of the Atherogenic Metabolic Triad (Hyperinsulinemia; Hyperapolipoprotein B; Small, Dense LDL) in Men. *Circulation*, *102*(2), 179-184

Lewis, G. F., Uffelman, K. D., Szeto, L. W., Weller, B., & Steiner, G. (1995). Interaction between free fatty acids and insulin in the acute control of very low density lipoprotein production in humans. *Journal of Clinical Investigation*,*95*(1), 158.

Libby, P. (2006). Inflammation and cardiovascular disease mechanisms. *The American Journal of Clinical Nutrition*, *83*(2), 456S-460S.

Libby, P., Ridker, P. M., & Maseri, A. (2002). Inflammation and atherosclerosis. *Circulation*, *105*(9), 1135-1143.

Ling, P. R., Mueller, C., Smith, R. J., & Bistrian, B. R. (2003). Hyperglycemia induced by glucose infusion causes hepatic oxidative stress and systemic inflammation, but not STAT3 or MAP kinase activation in liver in rats. *Metabolism*, *52*(7), 868-874.

Macciocchi, S. N., Diamond, P. T., Alves, W. M., & Mertz, T. (1998). Ischemic stroke: relation of age, lesion location, and initial neurologic deficit to functional outcome. *Archives of Physical Medicine and Rehabilitation*, *79*(10), 1255-1257.

Madonna, R., Pandolfi, A., Massaro, M., Consoli, A., & De Caterina, R. (2004). Insulin enhances vascular cell adhesion molecule-1 expression in human cultured endothelial cells through a pro-atherogenic pathway mediated by p38 mitogen-activated protein-kinase. *Diabetologia*, *47*(3), 532-536.

Malek, A. M., Alper, S. L., & Izumo, S. (1999). Hemodynamic shear stress and its role in atherosclerosis. *The Journal of the American Medical Association*, *282*(21), 2035-2042.

Marcone, S., Haughton, K., Simpson, P. J., Belton, O., & Fitzgerald, D. J. (2015). Milk-derived bioactive peptides inhibit human endothelial-monocyte interactions via PPAR-γ dependent regulation of NF-κB. *Journal of Inflammation*, *12*(1), 1.

Marmur, J. D., Rossikhina, M., Guha, A., Fyfe, B., Friedrich, V., Mendlowitz, M., ... & Taubman, M. B. (1993). Tissue factor is rapidly induced in arterial smooth muscle after balloon injury. *Journal of Clinical Investigation*, *91*(5), 2253.

Matthys, K. E., & Bult, H. (1997). Nitric oxide function in atherosclerosis. *Mediators of Inflammation*, *6*(1), 3-21.

McGill, H. C., McMahan, C. A., Herderick, E. E., Zieske, A. W., Malcom, G. T., Tracy, R. E., & Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group. (2002). Obesity accelerates the progression of coronary atherosclerosis in young men. *Circulation*, *105*(23), 2712-2718.

Meyer, D. M., Dustin, M. L., & Carron, C. P. (1995). Characterization of intercellular adhesion molecule-1 ectodomain (sICAM-1) as an inhibitor of lymphocyte function-associated molecule-1 interaction with ICAM-1. *The Journal of Immunology*, *155*(7), 3578-3584.

Migliore-Samour, D., Floc'h, F., & Jolles, P. (1989). Biologically active casein peptides implicated in immunomodulation. *Journal of Dairy Research*, *56*(03), 357-362.

Muller, W. A. (2014). How endothelial cells regulate transmigration of leukocytes in the inflammatory response. *The American Journal of Pathology*,*184*(4), 886-896.

Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S., & Takano, T. (1995). Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *Journal of Dairy Science*, *78*(4), 777-783.

O'Keefe, J. H., & Cordain, L. (2004). Cardiovascular disease resulting from a diet and lifestyle at odds with our Paleolithic genome: how to become a 21st-century hunter-gatherer. In *Mayo Clinic Proceedings* (Vol. 79, No. 1, pp. 101-108). Elsevier.

Palmer, R. M., Ferrige, A. G., & Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature,* 327, 524-526.

Palucka, K. A., Taquet, N., Sanchez-Chapuis, F., & Gluckman, J. C. (1998). Dendritic cells as the terminal stage of monocyte differentiation. *The Journal of Immunology*, *160*(9), 4587-4595.

Panagiotakos, D. B., Pitsavos, C. H., Zampelas, A. D., Chrysohoou, C. A., & Stefanadis, C. I. (2010). Dairy products consumption is associated with decreased levels of inflammatory markers related to cardiovascular disease in apparently healthy adults: the ATTICA study. *Journal of the American College of Nutrition*, *29*(4), 357-364.

Papadimitriou, C. G., Vafopoulou-Mastrojiannaki, A., Silva, S. V., Gomes, A. M., Malcata, F. X., & Alichanidis, E. (2007). Identification of peptides in traditional and probiotic sheep milk yoghurt with angiotensin I-converting enzyme (ACE)-inhibitory activity. *Food Chemistry*, *105*(2), 647-656.

Park, Y. W. (Ed.). (2009). *Bioactive components in milk and dairy products*. John Wiley & Sons.

Phelan, M., & Kerins, D. (2011). The potential role of milk-derived peptides in cardiovascular disease. *Food & Function*, *2*(3-4), 153-167.

Pihlanto, A., Virtanen, T., & Korhonen, H. (2010). Angiotensin I converting enzyme (ACE) inhibitory activity and antihypertensive effect of fermented milk.*International Dairy Journal*, *20*(1), 3-10

Pirro, M., Mauriège, P., Tchernof, A., Cantin, B., Dagenais, G. R., Després, J. P., & Lamarche, B. (2002). Plasma free fatty acid levels and the risk of ischemic heart disease in men: prospective results from the Quebec Cardiovascular Study. *Atherosclerosis*, *160*(2), 377-384.

Pober, J. S., & Sessa, W. C. (2007). Evolving functions of endothelial cells in inflammation. *Nature Reviews Immunology*, *7*(10), 803-815.

Public Health Agency of Canada (2015, June 1). *Cardiovascular Disease*. Retrieved from: www.phac-aspc.gc.ca/cd-mc/cvd-mcv/index-eng.php

Qian, Z. Y., Jollès, P., Migliore-Samour, D., Schoentgen, F., & Fiat, A. M. (1995). Sheep κ-casein peptides inhibit platelet aggregation. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1244*(2), 411-417.

Ridker, P. M., Hennekens, C. H., Roitman-Johnson, B., Stampfer, M. J., & Allen, J. (1998). Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. *The Lancet*, *351*(9096), 88-92.

Roberts KC, Shields M, de Groh M, Aziz A, Gilbert J. Overweight and obesity in children and adolescents: Results from the 2009 to 2011 Canadian Health Measures Survey. Health Reports 2012;23(3):3-7

Rollins, B. J., Yoshimura, T., Leonard, E. J., & Pober, J. S. (1990). Cytokine-activated human endothelial cells synthesize and secrete a monocyte chemoattractant, MCP-1/JE. *The American Journal of Pathology*, *136*(6), 1229

Ross, R. (1999). Atherosclerosis—an inflammatory disease. *New England Journal of Medicine*, *340*(2), 115-126.

Saito, T., Nakamura, T., Kitazawa, H., Kawai, Y., & Itoh, T. (2000). Isolation and structural analysis of antihypertensive peptides that exist naturally in Gouda cheese. *Journal of Dairy Science*, *83*(7), 1434-1440.

Schächinger, V., Britten, M. B., & Zeiher, A. M. (2000). Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation*, *101*(16), 1899-1906.

Shimada, M., Inaba, T., Shimano, H., Gotoda, T., Watanabe, Y., Yamamoto, K., ... & Yamada, N. (1992). Platelet-derived growth factor BB-dimer suppresses the expression of macrophage colony-stimulating factor in human vascular smooth muscle cells. *Journal of Biological Chemistry*, *267*(22), 15455-15458.

Sidossis, L. S., Mittendorfer, B., Chinkes, D., Walser, E., & Wolfe, R. R. (1999). Effect of hyperglycemia-hyperinsulinemia on whole body and regional fatty acid metabolism. *American Journal of Physiology-Endocrinology and Metabolism*, *276*(3), E427-E434.

Sima, A. V., Stancu, C. S., & Simionescu, M. (2009). Vascular endothelium in atherosclerosis. *Cell and tissue research*, *335*(1), 191-203.

Siso, M. G. (1996). The biotechnological utilization of cheese whey: a review. *Bioresource Technology*, *57*(1), 1-11.

Stancu, C. S., Toma, L., & Sima, A. V. (2012). Dual role of lipoproteins in endothelial cell dysfunction in atherosclerosis. *Cell and Tissue Research*, *349*(2), 433-446.

Statistics Canada. (2006, July 6). Canadian Community Health Survey: Overview of Canadians' eating habits. Retrieved from: http://www.statcan.gc.ca/daily-quotidien/060706/dq060706b-eng.htm

Statistics Canada. (2011c, October). Mortality, summary list of causes 2008.

Stressler, T., Eisele, T., & Fischer, L. (2013). Simultaneous monitoring of twelve angiotensin I converting enzyme inhibitory peptides during enzymatic β-casein hydrolysis using Lactobacillus peptidases. *International Dairy Journal*,*30*(2), 96-102.

Sonestedt, E., Wirfält, E., Wallström, P., Gullberg, B., Orho-Melander, M., & Hedblad, B. (2011). Dairy products and its association with incidence of cardiovascular disease: the Malmö diet and cancer cohort. *European Journal of Epidemiology*, *26*(8), 609-618.

Tellez, A., Corredig, M., Brovko, L. Y., & Griffiths, M. W. (2010f. *Journal of Dairy Research*, *77*(02), 129-136.

Valente, A. J., Irimpen, A. M., Siebenlist, U., & Chandrasekar, B. (2014). OxLDL induces endothelial dysfunction and death via TRAF3IP2: inhibition by HDL3 and AMPK activators. *Free Radical Biology and Medicine*, *70*, 117-128.

Wagar, L. E., Champagne, C. P., Buckley, N. D., Raymond, Y., & Green-Johnson, J. M. (2009). Immunomodulatory properties of fermented soy and dairy milks prepared with lactic acid bacteria. *Journal of Food Science*, *74*(8), M423-M430.

Wang, J. M., Colella, S., Allavena, P., & Mantovani, A. (1987). Chemotactic activity of human recombinant granulocyte-macrophage colony-stimulating factor. *Immunology*, *60*(3), 439.

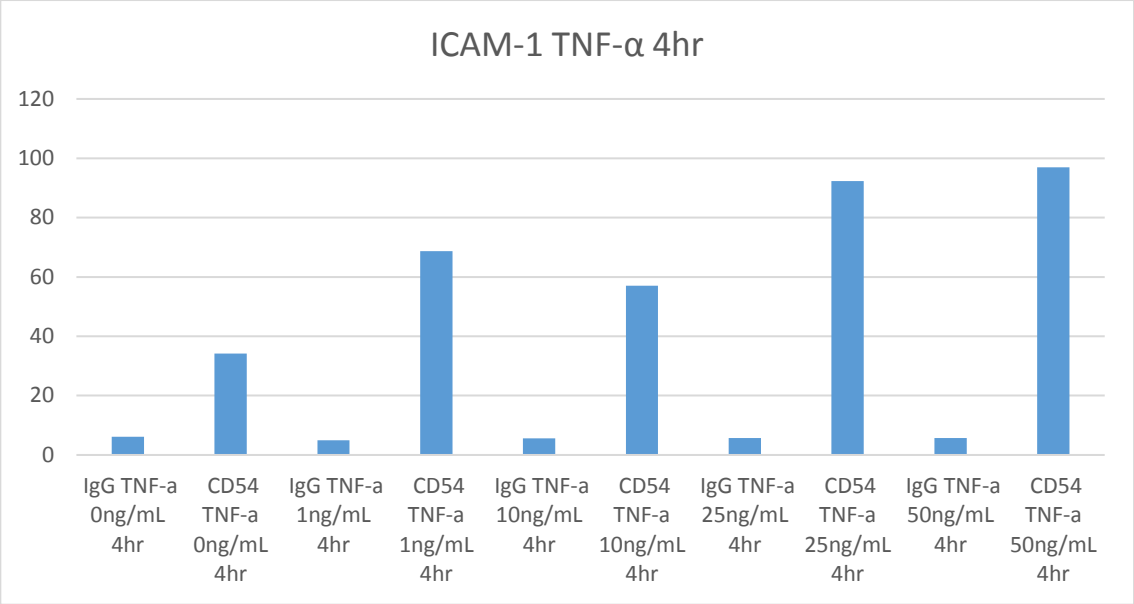
Wuerzner, G., Peyrard, S., Blanchard, A., Lalanne, F., & Azizi, M. (2009). The lactotripeptides isoleucine-proline-proline and valine-proline-proline do not inhibit the N-terminal or C-terminal angiotensin converting enzyme active sites in humans. *Journal of Hypertension*, *27*(7), 1404-1409.

Yamamoto, N., Maeno, M., & Takano, T. (1999). Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by Lactobacillus helveticus CPN4. *Journal of Dairy Science*, *82*(7), 1388-1393.

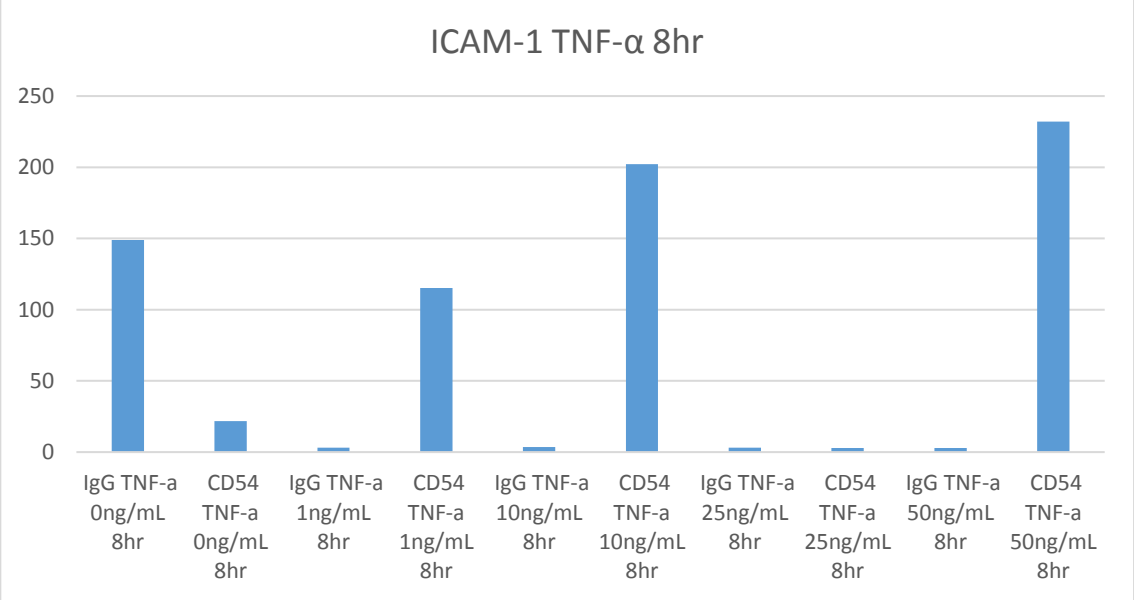
Yuan, S.Y., & Rigor, R.R. (2012). *Regulation of Endothelial Barrier Function.* San Rafael, CA: Morgan & Claypool.

Zettler, M. E., Prociuk, M. A., Austria, J. A., Massaeli, H., Zhong, G., & Pierce, G. N. (2003). OxLDL stimulates cell proliferation through a general induction of cell cycle proteins. *American Journal of Physiology-Heart and Circulatory Physiology*, *284*(2), H644-H653.

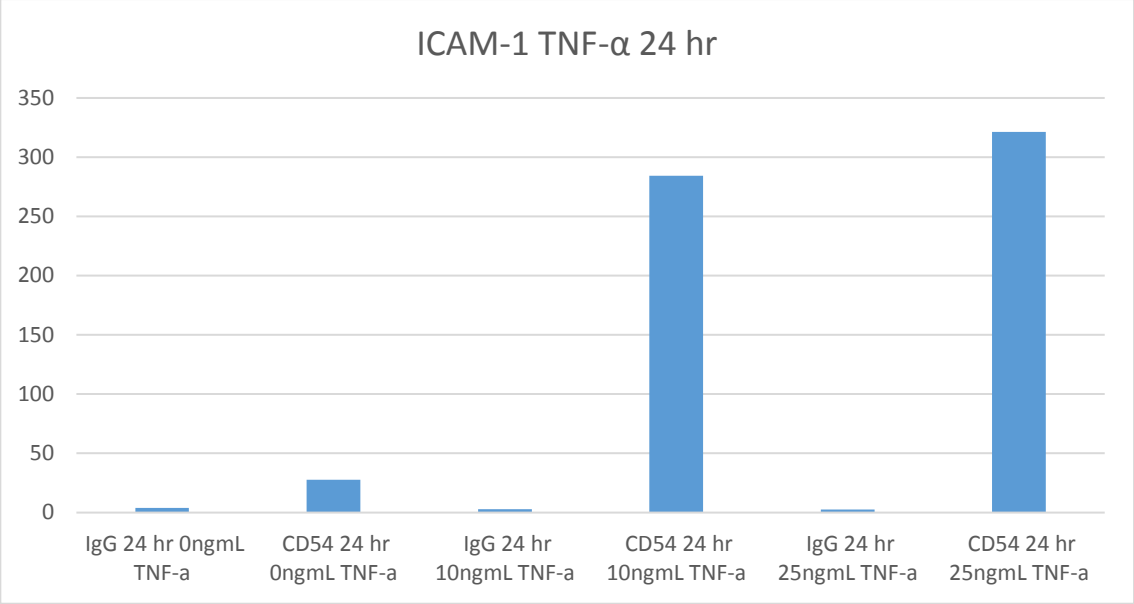
Appendix 1



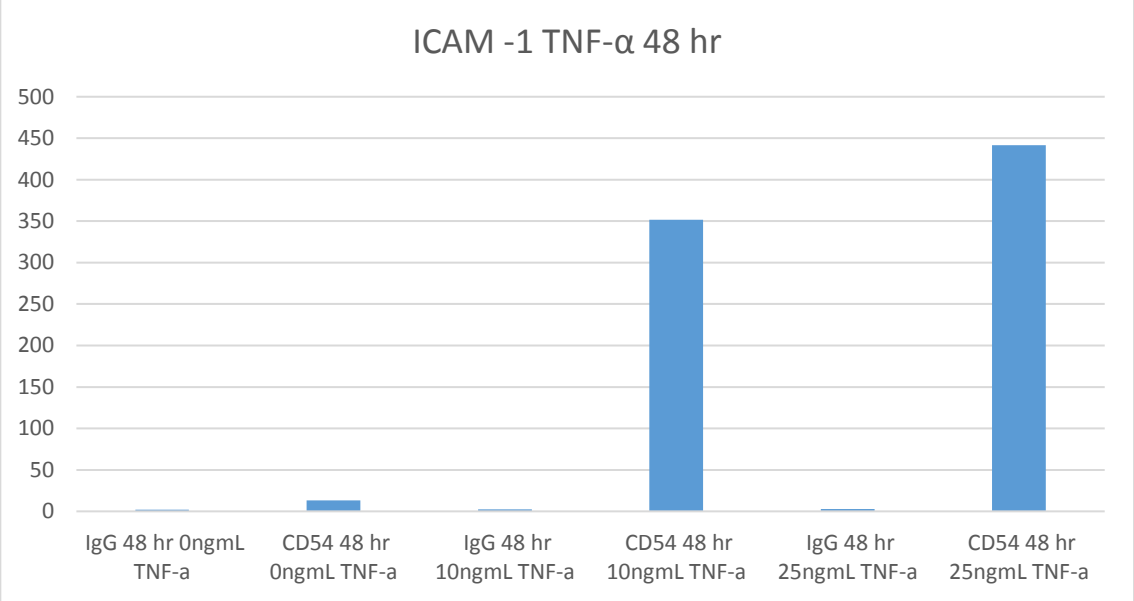

Supplementary Figure 1



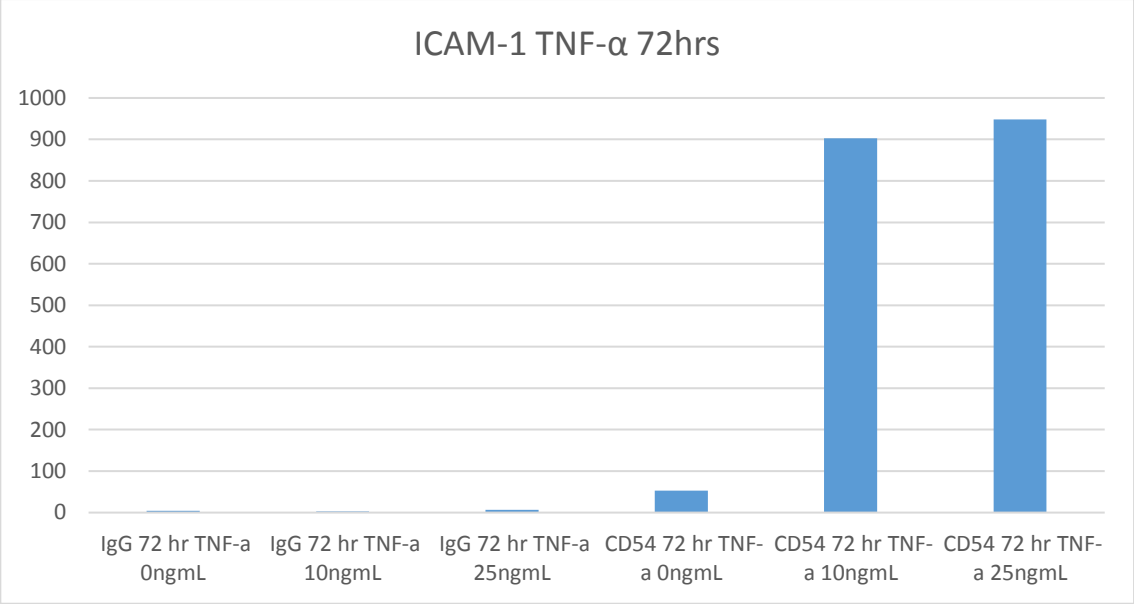

Supplementary figure 2




Supplementary Figure 3




Supplementary figure 5




Supplementary Figure 6