

**Characterizing immunomodulatory activity of lactic acid
bacteria on cell types involved in innate immunity: Analysis of
the effects of *Lactobacillus rhamnosus* R0011-derived milk
ferments on macrophage polarization and of secretome effects
on intestinal epithelial cell cytokine production**

By

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Abstract

Probiotic lactic acid bacteria have been associated with a wide array of host-immune modulatory effects including down-regulation of pro-inflammatory gene transcription and expression following pro-inflammatory challenge, through direct and indirect contact with host immune cells. However, it is still unknown how many of these microorganisms convey these beneficial immune effects to the host. *Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0389 were examined for their immunomodulatory activity on challenged human HT-29 and rat IEC-6 intestinal epithelial cells (IEC). Secreted products from both strains demonstrated the ability to down-regulate IL-8 production from challenged HT-29 with differential effects on CINC-1 production from challenged IEC-6 cells. Further, *L. rhamnosus* R0011-derived milk ferments were tested for their ability to modulate pro-atherogenic cytokine production from challenged human THP-1 and U937 monocytes and macrophages. Culturing THP-1 and U937 macrophages with milk ferments resulted in the up-regulation of regulatory cytokine production in response to challenge with lipopolysaccharide indicating possible polarization into the regulatory M2b macrophage phenotype. Taken together, *L. rhamnosus* R0011 has been shown to have immunomodulatory activity on a variety of cells involved in innate immunity.

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List of Abbreviations

AHR	Aryl hydrocarbon receptor
ATTC	American Type Culture Collection
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
cAMP	Cyclic adenosine monophosphate
CD40	Cluster of differentiation 40
CD54/ICAM-1	Intercellular adhesion molecule-1
CLA	Conjugated linoleic acid
CVD	Cardiovascular disease
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
eATP	Extracellular ATP
ELISA	Enzyme-linked immunosorbant assay
FBS	Fetal bovine serum
fMLF	N-formyl methionyl peptide
GABA	Gamma-aminobutyric acid
GALT	Gut-associated lymphoid tissue
IEC	Intestinal epithelial cell
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-18	Interleukin-18
IL-1Ra	Interleukin-1 receptor antagonist
IL-1 β	Interleukin-1 β
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8

IP-10/CXCL10	Interferon gamma-induced protein 10
LDL	Low-density lipoprotein
LFA-1	Lymphocyte function associated antigen-1
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAMP	Microbe associated molecular pattern
MCP-1	Macrophage colony-stimulating factor
MRS	de Man, Rogosa and Sharpe medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation primary response gene
NF- κ B	Nuclear factor- κ B
NOD	Nucleotide-binding oligomerization domain receptor
oxLDL	Oxidized low-density lipoprotein
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PMA	Phorbol-12-myristate-13-acetate
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
qPCR	Real-time polymerase chain reaction
RIPK-1	Receptor-interacting serine/threonine-protein kinase 1
RIPK-2	Receptor-interacting serine/threonine-protein kinase 2
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute-1640

sCD54	Soluble CD54/ICAM-1
SCFA	Short chain fatty acid
SEM	Standard error of the mean
TCR	T-cell receptor
TGF- β	Transforming growth factor beta
TLR	Toll-like receptor
TLR-2	Toll-like receptor 2
TLR-4	Toll-like receptor 4
TLR-5	Toll-like receptor 5
TNFAIP3	Tumour Necrosis Factor α -Induced Protein 3
TNF-R	Tumor necrosis factor-receptor
TNF- α	Tumor necrosis factor alpha
TRAF-6	Tumour necrosis factor receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon- β
VCAM-1	Vascular cell adhesion molecule-1
XTT	2,3-Bis(2-methoxy-4-nitro-5-sulfohenyl)-2H-tetrazolium-5-carboxanilide

Introduction

The Human Gut Microbiome

The human gut microbiome encompasses every living microorganism within our gastrointestinal tract. The gut is a transient microenvironment influenced by diet, sex, and genetics (Mueller *et al.*, 2006; Kovacs *et al.*, 2011). With the advent of next-generation sequencing, it has been determined that each individual contains approximately 600,000 unique microbial genes within the gastrointestinal tract (Guarner, 2015). Under homeostatic conditions, the relationship between the gut microbiome and the host is mutualistic. These gut microbial symbionts aid in the digestion of many complex carbohydrates and fibres. The breakdown of these food stuffs leads to the production of many immunologically active metabolites such as short chain fatty acids (SCFAs). However, dysbiosis, or perturbations in the normal gut microbiota, has recently been associated with obesity, cardiovascular disease, and inflammatory bowel diseases (Carding *et al.*, 2015).

Members of the human gut microbiome have been identified as probiotics. Probiotics are defined as living organisms that when consumed in adequate amounts elicit certain health benefits to the host (FAO/WHO, 2001). These health benefits include gut pathogen antagonism, modulation of host immune responses, and enhancement of the structural integrity of the intestinal epithelial barrier within the host (Lebeer *et al.*, 2010). In some cases, these microorganisms are already part of a healthy individual's gut microflora. Although most of the health benefits elicited by probiotics are localized in the gut, there is mounting evidence suggesting that these organisms play a much more

significant role in the systemic immune system (Smelt *et al.*, 2012; Borchers *et al.*, 2009; McCarthy *et al.*, 2003; Moro-Garcia *et al.*, 2012).

The microorganisms within the gastrointestinal tract are in constant contact with the cells of the mucosal immune system and have been shown to beneficially modulate host immune responses by promoting physiological homeostasis through induction of regulatory cytokine production (Thomas and Versalovic, 2010). Further, through direct interactions with resident DCs, macrophages, and T cells within the gastrointestinal tract, these bacteria have been shown to have a role in the maturation of the naïve mucosal immune system (Hooper *et al.*, 2012).

The Mucosal Immune System

Cells lining the mucosal surfaces of the gastrointestinal tract are in continual contact with both pathogenic and non-pathogenic bacteria. These intestinal epithelial cells (IECs) provide both a mechanical and chemical barrier that prevents these bacteria from entering systemic circulation (Artis, 2008). IECs are connected to one another through tight junctions providing a physical barrier which the normal gut microbiota and pathogenic bacteria cannot readily cross. Directly above these cells, there is a thick layer of glycosylated proteins known as the mucin layer. This mucin layer is produced by goblet cells along the gastrointestinal tract and provides additional protection from invading pathogens and commensal microorganisms (Sansonetti, 2004). IECs also express a myriad of pattern recognition receptors (PRRs) which are used to recognise bacteria and other microorganisms. These include numerous toll-like receptors (TLR), inflammasomes, and the cytosolic nucleotide-binding and oligomerisation domain (NOD)

proteins. If the mucin layer is breached, these PRRs become activated inducing IECs to produce pro-inflammatory cytokines and chemokines, such as interleukin-8 (IL-8), which act as potent chemoattractant proteins for neutrophils and other members of the immune system in order to maintain the structural integrity of the IEC layer (Pitman and Blumberg, 2000).

Directly beneath the IEC layer there is a layer of connective tissue called the lamina propria containing numerous tissue macrophages, dendritic cells, and other members of the adaptive immune system such as T cells and B cells. Within the small intestine, there are also organised lymphoid structures which have been collectively called the gut-associated lymphoid tissues (GALT). The GALT is comprised of the Peyer's patches, which are specialised lymphoid tissues containing high numbers of B cells, T cells, and antigen-presenting dendritic cells (DC). Directly above the Peyer's patches are specialised epithelial cells called microfold cells (M cells). M cells allow for the direct transport of dietary and microbial-associated antigens across the IEC layer into the underlying lymphoid tissues. DCs present within these tissues process antigens and present them to the surrounding T cells, optimising antigen-presenting cell, T cell and B cell interactions. Interestingly, these activated T and B cells can leave the Peyer's patches via the mesenteric lymph node and provide protection at other systemic mucosal locations (Mowat and Agace, 2014).

Depending on the antigen sampled, macrophages and DCs beneath M cells mount an appropriate immunological response. When this antigen is associated with a pathogen, there is a massive increase in pro-inflammatory cytokine, chemokine, and antibody production by surrounding immune cells, usually resulting in the eventual clearance of

the invading pathogen. However, once the invading pathogen or danger signal is cleared, the inflammatory signal is terminated and there is an increase in the transcription of regulatory cytokines in an attempt to return to physiological homeostasis (Barton, 2008). Although inflammation plays an important role in host defence, continuous expression of these inflammatory biomarkers is associated with the pathogenesis of several chronic inflammatory diseases (Manabe, 2011).

The Innate Immune System

The innate immune system is the first line of defense against invading microorganisms. Unlike the adaptive immune system, innate immunity is characterised by a general, non-specific inflammatory response to invading microorganisms with no immunological memory (Janeway and Medzhitov, 2002). Cells of the innate immune system distinguish between self and non-self by recognizing microbe-associated molecular patterns (MAMPs) and pathogen-associated molecule patterns (PAMPs) on the surface of invading microorganisms through PRRs (Kumar *et al.*, 2011; Takeda and Akira, 2015). Once recognized, these cells invoke an inflammatory response through the production of cytokines and chemokines. Cytokines are small, soluble proteins secreted by activated cells which elicit an effect on nearby cells (and sometimes have autocrine or long range effects). Chemokines are chemoattractant proteins with a major role in controlling homing and migration of immune cells. Once secreted, cytokines and chemokines attract other members of both the innate and adaptive immune systems in an attempt to clear the invading pathogen and restore physiological homeostasis.

PRRs include the TLRs and the NOD proteins. To date, there have been 13 TLRs identified in mammals, each recognizing a different, unique MAMP (Pandey *et al.*, 2015). These PRRs are present on both the cell surface and within the intracellular environment of innate immune cells. For example, toll-like receptor-4 (TLR-4), present on the surface of many members of the innate immune system, recognises lipopolysaccharide (LPS), the major constituent of the outer membrane of Gram-negative bacteria. Toll-like receptor-5 (TLR-5), also present on the surface of many cells, recognises bacterial flagellin, while toll-like receptor-3 (TLR-3), an intracellular TLR, recognises double-stranded RNA (Takeda and Akira, 2015). Once bound, these TLRs induce a signalling cascade through the recruitment of several adaptor molecules including myeloid differentiation factor 88 (MyD88), TIR domain-containing adaptor-inducing interferon- β (TRIF), and TRIF-related adaptor molecule (TRAM). Interestingly, these TLRs use different combinations of these adaptor molecules in order to elicit different cellular responses. For example, TLR-4 utilizes both MyD88 and the MyD88-independent TRIF/TRAM pathways to induce the activation of different transcription factors (Pandey *et al.*, 2015). These intracellular signal cascades terminate with the activation of the Nuclear Factor- κ B (NF- κ B) transcription factor, the interferon regulatory factors (IRF), the activator protein (AP-1) family of proteins, or the mitogen-activated protein kinases (MAPKs) (Pandey *et al.*, 2015). Activated transcription factors induce the transcription of numerous pro-inflammatory cytokines and chemokines. Unlike the TLRs, NODs are solely intracellular PRRs which recognize peptidoglycan motifs present on both Gram-positive and Gram-negative bacteria. Once activated, these PRRs induce a similar intracellular signalling pathway as the TLRs terminating with the

activation of the NF- κ B transcription factor and the increased transcription of pro-inflammatory cytokines and chemokines. Inflammasomes contain intracellular PRRs which respond to PAMPs and damage-associated molecular patterns (DAMPs) such as extracellular adenosine triphosphate (ATP). These inflammasomes often contain a member of the NOD family of proteins and activation of these PRRs results in the induction of caspase-1, an enzyme responsible for the cleaving of pro-interleukin-1 β (IL-1 β) to the biologically active, pro-inflammatory IL-1 β .

Within the innate immune system, there are a number of cells each with their own unique repertoire of PRRs to recognize specific MAMPs and PAMPs. The majority of these cells are derived from the bone marrow. These pluripotent hematopoietic stem cells give rise to a common myeloid progenitor which can further differentiate into granulocytes and monocytes. Granulocytes are characterised by granules in their cytoplasm and give rise to monocytes, neutrophils, eosinophils, and the basophils (Janeway and Medzhitov, 2002). Monocytes are circulating sentinels of the innate immune system which differentiate into tissue macrophages upon stimulation with specific cytokines and chemokines or pathogens. Once differentiated, these macrophages are tasked with scavenging, pathogen recognition and elimination, and antigen presentation to the adaptive arm of the immune system (Janeway and Medzhitov, 2002).

Atherosclerosis and the Innate Immune System

Cardiovascular disease (CVD) has become the leading cause of death globally, accounting for approximately 30% of all deaths worldwide and 48% of all deaths which are not associated with communicable diseases (WHO, 2011). Current risk factors

contributing to CVD are hypertension, diabetes, smoking, poor diet, underlying autoimmune disease, and previous infections with pathogens (Rosenfield and Campbell, 2011; WHO, 2011). The most frequent underlying cause of CVD is atherosclerosis (WHO, 2011). Atherosclerosis is a disease which affects both large and mid-sized arteries, resulting in the formation of plaques (lesions). Although this disease progresses silently for years, it can ultimately result in myocardial infarction, ischemic stroke, ischemic damage to kidneys and intestines, and other life-threatening events due to plaque rupture (Libby *et al.*, 2013; Lusis, 2000). Traditionally, it was believed that atherosclerosis resulted solely from the buildup of cholesterol in the arterial wall. Understanding of this process has changed as the underlying mechanisms behind the development of plaques have become better understood and a role for inflammation has been recognised. More recently, both the innate and adaptive arms of the immune system have been implicated in the pathogenesis of this disease (Libby *et al.*, 2011).

Atherosclerotic plaques are initiated through the accumulation of apolipoprotein B (apoB)-containing low density lipoproteins (LDL) within the endothelial cell layer of arteries (the barrier between blood and tissues) in areas of low shear stress (Tabas *et al.*, 2007; Skalen *et al.*, 2002; Zand *et al.*, 1999). It is thought that these LDLs bind to proteoglycans present within the subendothelial space via an ionic interaction between the apo-B100 moiety on the surface of LDLs and the negatively charged sulphate groups present on the proteoglycans (Boren *et al.*, 1998). Once bound, these LDLs undergo a variety of oxidative modifications (oxLDL), primarily due to the activity of lipoxygenase enzymes and reactive oxygen species (Cyrus *et al.*, 1999). These oxLDL molecules activate endothelial cells resulting in the increased transcription and expression of

leukocyte adhesion molecules such as P-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (CD54/ICAM-1) (Cominacini *et al.*, 1996; Khan *et al.*, 1994). Adhesion molecules are expressed on the surface of the endothelial cells and facilitate the binding of migrating leukocytes. Further, these activated endothelial cells secrete chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and chemokine (C-C motif) ligand 5 (CCL5/RANTES) (Gautier *et al.*, 2009). Travelling monocytes are directed towards the activated endothelial cell layer where they bind to P-selectin on the surface of the endothelial cells and ultimately adhere via binding of leukocyte function-associated molecule-1 (LFA-1) and very late antigen-4 (VLA-4) (expressed on the travelling monocytes) to CD54/ICAM-1 and VCAM-1, respectively (Legein *et al.*, 2013; Ley *et al.*, 2007; Mestas and Ley, 2008). Monocytes eventually traverse the endothelial cell layer into the intima (the area between the endothelial cell layer and smooth muscle cells) through diapedesis. Once inside the intima, monocytes secrete lipoprotein-binding proteoglycans, further perpetuating the accumulation of LDL and monocyte accumulation at the activated endothelium (Legein *et al.*, 2013).

Monocytes which have migrated across the endothelial cell layer into the intima eventually become differentiated into macrophages through the activity of macrophage colony-stimulating factor (M-CSF) (Moore and Tabas, 2011). These differentiated macrophages exist as a heterogeneous population consisting of M1 and M2 cell populations in atherosclerotic plaques (Legein *et al.*, 2013). M1 macrophages, also known as classical macrophages, are activated by TLR recognition of conserved PAMPs such as LPS and peptidoglycan. Once activated, M1 macrophages produce inflammatory cytokines such as tumour necrosis factor- α (TNF- α), interleukin-6 (IL-6), IL-1 β , and

interleukin-12 (IL-12) (Moore and Tabas, 2011). Prolonged M1 macrophage activation can potentially lead to tissue damage and impair wound healing (Legein *et al.*, 2013). In contrast, M2 macrophages are activated by parasite antigens and cytokines secreted by T-helper 2 (Th2) cells such as interleukin-4 (IL-4) and interleukin-13 (IL-13) (Moore and Tabas, 2011). Unlike M1 macrophages, M2 macrophages have been shown to produce regulatory cytokines such as interleukin-10 (IL-10), interleukin-1 receptor antagonist (IL-1Ra), and transforming growth factor- β (TGF- β) and mediate tissue healing through the secretion of collagen (Legein *et al.*, 2013; Moore *et al.*, 2013). In human atherosclerotic plaques, both M1 and M2 macrophages are present (Stoger *et al.*, 2012; Johnson *et al.*, 2009). However, macrophages displaying the M1 phenotype are the dominant cell phenotype in progressing atherosclerotic plaques (specifically areas within the plaque that are prone to rupture) (Khallou-Laschet *et al.*, 2010). In experimental models which reverse the progression of the atherosclerotic plaque, M1 macrophages undergo a phenotype switch to M2 macrophages indicating a significant role for M1 macrophages in the inflammation associated with atherosclerosis (Feig *et al.*, 2011).

Once differentiated, both M1 and M2 macrophages begin to take up oxLDL through the scavenger receptors SRA (scavenger receptor type A) and CD36 (Legein *et al.*, 2013). Once inside the intracellular environment, the oxLDL release cholesterol. These cells can also take up unmodified LDL through micropinocytosis (Libby *et al.*, 2013). This massive increase in oxidized lipid influx and subsequent cholesterol release overwhelms the cell's ability to remove these molecules resulting in the formation of macrophage foam cells (Legein *et al.*, 2013). Eventually, these cells die and lead to the formation of necrotic bodies within the atherosclerotic plaque (Libby *et al.*, 2013).

Accumulating necrotic bodies, coupled with the inability to efficiently remove the necrotic debris, are thought to contribute to plaque size and promote plaque progression (Moore and Tabas, 2011).

Table 1. Macrophage phenotypes present within atherosclerotic plaques

Macrophage Phenotype ¹	Role in Atherosclerosis	Cytokine Profile	Reference
M1	<ul style="list-style-type: none"> • Pro-inflammatory • Promotes plaque progression 	IL-12, IL-6, IL-23, TNF- α	Colin <i>et al.</i> , 2014
M2a	<ul style="list-style-type: none"> • Involved in wound healing • Atheroprotective 	IL-10, TGF- β , IL-1Ra	Colin <i>et al.</i> , 2014
M2b	<ul style="list-style-type: none"> • Immunoregulation • Atheroprotective 	IL-1 β , IL-6, IL-10	Mantovani <i>et al.</i> , 2004
M2c	<ul style="list-style-type: none"> • Tissue-remodelling • Immunoregulation • Atheroprotective 	IL-10, TGF- β	Mantovani <i>et al.</i> , 2004

¹This is a non-comprehensive list of macrophage phenotypes present within atherosclerotic plaques

Other cells of the immune system implicated in atherosclerosis pathology are dendritic cells (DCs) (Bobryshev, 2005), which are professional antigen-presenting cells responsible for activating and facilitating the cross-talk between the innate and adaptive arms of the immune system. As is seen with macrophages, there is a heterogeneous cell population of different dendritic cells in atherosclerotic plaques (Legein *et al.*, 2013). Dendritic cells are also involved in the uptake of oxLDLs which increases antigen presenting efficacy to T cells (Alderman *et al.*, 2002). Further, DCs produce cytokines which actively recruit T cells to the atherosclerotic plaque. T cells, or those lymphocytes with the characteristic T-cell receptor (TCR) on their surface, migrate to the growing atherosclerotic plaque in a similar fashion to monocytes (Legein *et al.*, 2013). It is thought that the antigen which activates these T cells is the ApoB100 moiety found on

the surface of LDLs (as mentioned above); however this is still under investigation (Legein *et al.*, 2013). Once activated, these cells elicit a wide range of responses, including the production of pro-inflammatory cytokines further perpetuating the development of the atherosclerotic plaque.

Recently, TLRs have been implicated in the pathogenesis of atherosclerosis (Libby *et al.*, 2013; Cole *et al.*, 2013). For example, TLR-4 has been implicated in increasing macrophage differentiation into foam cells by increasing oxLDL uptake in the presence of LPS (Howell *et al.*, 2011). In fact, the cell-surface expression of TLR-4 on macrophages and endothelial cells is significantly higher in human atherosclerotic plaques than in normal arteries suggesting that this receptor may play an important role in the development of atherosclerosis (Edfeldt *et al.*, 2002). As such, it has become increasingly evident that these innate pattern recognition receptors may play an integral role in the development of atherosclerosis.

Probiotic-Derived Fermented Dairy Products and their Effect on the Human Immune System and CVDs

Dairy products, including milk, yogurt, and cheese, are major constituents of the North American and European diet. Historically, consumption of milk and other dairy products was closely associated with an increase in both serum cholesterol levels and incidence of stroke in epidemiological studies (Appleby *et al.*, 1999; Larsson *et al.*, 2009). However, these claims have been refuted by multiple studies suggesting consumption of dairy products protects against cardiovascular disease and may modulate host immune responses (Soedamah-Muthu *et al.*, 2011; Chrysant and Chrysant, 2013).

These effects on host immune responses are largely attributable to bioactive proteins present within milk such as lactoferrin, immunoglobulins, and growth factors (Chatterton *et al.*, 2013). For example, β -lactoglobulin and α -lactalbumin, two major proteins found within bovine whey, up-regulate the production of IL-1Ra in human neutrophils through a NF- κ B-dependent mechanism (Rusu *et al.*, 2009).

Many of the organisms defined as “probiotic” belong to the Gram-positive lactic acid-producing family of bacteria (Naidu *et al.*, 1999) and have been used in dairy starter cultures for milk fermentation since ancient times. It was hypothesized in 1907 by Élie Metchnikoff that dairy products fermented with lactic acid bacteria confer health benefits to the host (Metchnikoff, 1907). Since then, there has been extensive research examining Metchnikoff’s health claims (Shiby *et al.*, 2013). These health-related effects include the down-regulation of pro-inflammatory cytokines in IEC, antioxidant activity, alleviation of food allergies and sensitivities (including improved lactose tolerance), alleviation of antibiotic-induced diarrhea, and antagonism of gut-associated pathogens (Parvez *et al.*, 2006; Wagar *et al.*, 2009; Virtanen *et al.*, 2006). Recent evidence has also suggested that the consumption of fermented milk products can change host metabolism of certain food stuffs and even reduce social anxiety in humans (McNulty *et al.*, 2011; Hilimire *et al.*, 2015).

Lactic acid bacteria primarily convert lactose into lactic acid during milk fermentation. Some of these bacteria concurrently secrete proteases which release bioactive peptides from milk proteins (casein and whey) and some synthesize vitamins such as A and E (Beermann and Hartung, 2013). The bioactive peptides released during the proteolytic degradation of milk proteins have also been shown to have

antihypertensive (through the inhibition of the angiotensin-converting-enzyme) and antioxidant properties (Beermann and Hartung, 2013). Other fermentation products have also been associated with anti-hypertensive attributes. For example, the consumption of a dairy product high in gamma-aminobutyric acid (GABA), a product of bacterial fermentation, was shown to significantly lower blood pressure in patients suffering from mild hypertension (Inoue *et al.*, 2003). It has also been shown in a double-blind randomised clinical trial that a yogurt formulation with *L. reuteri* NCIMB 30242 significantly reduced serum cholesterol levels in hypercholesterolemic adults (Jones *et al.*, 2012). Further, there was a 15% reduction in cardiovascular disease risk seen in subjects consuming fermented dairy products when compared to those subjects consumed non-fermented milk (Sonestedt *et al.*, 2011). Collectively, these observations suggest that fermentation of milk with select lactic acid bacteria increases the nutritional value of the milk prior to consumption and may improve cardiovascular health.

Probiotic-Derived Secreted Bioactive Molecules

Traditionally, it was believed that in order to confer their immunomodulatory benefits, probiotic microorganisms had to interact directly with IECs and other parts of the immune system. Recently, there has been a growing body of scientific literature suggesting that these bacteria also secrete an array of bioactive molecules having immunomodulatory activity. Bioactive molecules include short-chain fatty acids, lipoteichoic acid, adenosine triphosphate, tryptophan metabolites, and lactocepins and other secreted proteins. These secreted molecules retain their biologically active properties following intestinal transport through the IEC layer in the GI tract and may have an effect on systemic tissues (Menard *et al.*, 2004; Wikoff *et al.*, 2009).

Short-Chain Fatty Acids

As mentioned previously, one of the major metabolites of bacterial fermentation of dietary fibres in the gut are SCFAs including lactic acid, acetic acid, propionic acid, and butyric acid. However, acetic acid, propionic acid, and butyric acid are the predominant bacterially-derived fermentation products within the gut. These SCFAs have been associated with many immunomodulatory activities including decreased pro-inflammatory cytokine and chemokine transcription and decreased leukocyte recruitment to inflamed tissues through the modulation of cellular adhesion molecules (Vinolo *et al.*, 2011). SCFAs bind to the G-protein coupled receptors, GPR41 and GPR43, which are expressed by IECs, adipose tissue, and cells of the immune system (Kimura *et al.*, 2014).

Butyric acid, the most widely studied of the SCFAs, is the predominant energy source for colonocytes. It has been shown to have numerous immunomodulatory activities which are largely attributed to its ability to act as a histone deacetylase inhibitor. This leads to the decreased transcription of many pro-inflammatory cytokines and chemokines (Sanderson, 2004).

Lactic acid is the major metabolite produced during the fermentation of milk by lactic acid bacteria. It exists in two optical isomers, L(+)- and D(-)-lactic acid. The ratio between the two isomers within cultures depends on the microorganism used for fermentation. Lactic acid has immunomodulatory activity by suppressing the production of pro-inflammatory cytokine production, proliferation and cytotoxic activity of cytotoxic T-cells (Fischer *et al.*, 2007).

Lipoteichoic Acid

Lipoteichoic acid (LTA) is a major constituent of the cell walls of Gram-positive bacteria and regulates the activity of cell wall hydrolases involved in bacterial cell replication. LTAs typically contain a glycolipid anchor with a hydrophilic tail containing 1,2-phosphodiester-linked polymers of glycerol-phosphates. These glycerol-phosphates can be substituted with D-alanyl or sugar residues (Neuhaus and Baddiley, 2003). LTA can be liberated and secreted by certain Gram-positive bacteria during replication or bacteriolysis caused by β -lactam antibiotics (Ginsburg, 2002). Once liberated, it is recognised by members of the innate immune system through toll-like receptor-2 (TLR-2) with CD14 and CD36 acting as co-receptors. The traditional view has been that once LTA binds to TLR-2, there is an increase in the transcription of the pro-inflammatory cytokines IL-1, IL-5, IL-6, and IL-8 (Ginsburg, 2002). However, recent research has indicated that the immunogenic properties of LTA are species-dependant, and vary depending on certain structural features of LTA (Lebeer *et al.*, 2012). For example, LTA isolated from *Staphylococcus aureus* is highly immunostimulatory, resulting in the increased transcription of many pro-inflammatory cytokines including TNF- α (Schröder *et al.*, 2003). In contrast, LTA isolated from *Lactobacillus plantarum* down-regulates TNF- α induced pro-inflammatory cytokine transcription *in vitro*. This inhibition was mediated through a TLR-2 dependant mechanism and resulted in the inactivation of the NF- κ B signalling pathway (Kim *et al.*, 2012). Further, LTA isolated from *L. plantarum* was attributed with the ability to down-regulate LPS-induced inflammation *in vitro* and atherosclerotic plaque formation *in vivo* in ApoE^{-/-} mice by modulating the expression of pro-inflammatory cytokines and cell surface receptors (Kim *et al.*, 2013). These

differences in immunostimulatory capacities of different LTA molecules are often the result of sugar substitutions or replacement of D-alanyl moieties with L-alanyl within the hydrophilic tail (Lebeer *et al.*, 2012).

Adenosine Triphosphate

Adenosine triphosphate, a nucleoside triphosphate involved in energy transfer within the cell, has recently been identified as an important mediator of the immune response (Trautmann, 2009). Extracellular ATP (eATP) has now been identified as a DAMP which is released by cells that are under physiological stress. Once released, eATP often triggers inflammatory immune responses acting through the P2Y purinergic receptors expressed by immune cells. This inflammatory immune response involves the increased transcription of pro-inflammatory cytokines and cell-adhesion molecules. In fact, eATP can also act as a chemotactic molecule recruiting white blood cells towards sites of tissue stress (Di Virgilio and Vuerich, 2015).

eATP can be hydrolysed into adenosine through the activity of membrane-bound enzymes. CD39, or ecto-nucleoside triphosphate diphosphohydrolase 1, catalyses the conversion of ATP to adenosine monophosphate (AMP) and CD73, or ecto-5'-nucleotidase, dephosphorylates AMP into adenosine. These enzymes are constitutively expressed on the surface of many cells of the immune system and their activity is dictated by the local environment (Antonioli *et al.*, 2013).

Liberated adenosine can bind to immune cells through the A₂A adenosine receptor resulting in an increase in the intracellular concentration of cyclic-AMP (cAMP). This increase in intracellular cAMP leads to the phosphorylation of cAMP

response element binding protein (CREB) by protein kinase A (PKA). This ultimately results in the decreased activity of NF- κ B and subsequent transcription of pro-inflammatory mediators (Gerlo *et al.*, 2011). In inflammatory conditions or when the levels of eATP are high, CD39 and CD73 rapidly convert the pro-inflammatory ATP into the immunoregulatory adenosine. In fact, decreased expression of these enzymes is associated with the pathogenesis of many chronic inflammatory conditions including atherosclerosis (Antonioli *et al.*, 2013).

Recent evidence has suggested that some species of gut-associated bacteria can secrete ATP into the extracellular environment when grown in glucose-rich medium (Hironaka *et al.*, 2013). Although it is still unclear if these bacteria secrete ATP in order to facilitate cross-talk within the host, commensal-derived ATP drives the differentiation of Th17 cells within the lamina propria (Atarashi *et al.*, 2008).

Tryptophan Metabolites

Dietary tryptophan can be metabolized in the gut lumen by Lactobacilli which convert tryptophan into indole. Indoles have recently garnered attention as they have been implicated in modulating host immune responses. Bacterially-derived indoles bind to aryl hydrocarbon receptors (AHR) present in immune cells within the GALT (Esser *et al.*, 2009). AHRs are intracellular receptors predominantly found within cells lining the skin, lungs, and the gastrointestinal tract. They are responsible for recognizing and binding small molecular weight endogenous and exogenous molecules, such as serotonin and tryptophan metabolites (Noakes, 2015). Once bound, the AHR translocates to the nucleus where it acts as a transcription factor inducing the transcription of genes involved

in xenobiotic metabolism or interacting with other signalling pathways such as the NF- κ B signalling pathway suggesting a role of the AHRs in inflammation (Vogel *et al.*, 2014). Indoles produced by Lactobacilli induce the production of interleukin-22 (IL-22) in innate lymphoid cells (Zelante *et al.*, 2013). IL-22 belongs to the IL-10 superfamily and is involved in the initiation of the immune response against invading pathogens (Dumoutier *et al.*, 2000). The induction of IL-22 was mediated by the binding of indole-3-amine to the aryl hydrocarbon receptor (Zelante *et al.*, 2013)

Endogenous tryptophan metabolites can also activate the AHRs leading to modulation of inflammatory processes within the cell. Tryptophan is primarily metabolised through the kynurenine pathway via the indoleamine 2,3-dioxygenase (IDO) enzyme (Noakes, 2013). This enzyme has also been implicated in having a role in the pathogenesis of atherosclerosis (Polyzos *et al.*, 2015). Interestingly, some Lactobacilli modulate the expression of IDO and subsequent tryptophan metabolism *in vitro*. H₂O₂ produced by *Lactobacillus johnsonii* significantly inhibited the activity of IDO in HT-29 IECs *in vitro* (Valladares *et al.*, 2013).

Bacteriocins

Bacteriocins are small (less than 10 kDa in size), antimicrobial peptides produced by many lactic acid bacteria. These antimicrobial peptides are heat-stable and active in acidic environments (pH less than 5) (Jack *et al.*, 1995) and inhibit the growth of several gut-associated pathogens *in vitro* and antagonise the adherence of gut pathogens to IECs *in vivo* (Hudault *et al.*, 1997; O'Shea *et al.*, 2012). It is believed that these molecules permeabilize bacterial cell membranes leading the efflux of intracellular components and

ultimately leading to bacterial cell death (Nes and Holo, 2000; Diep *et al.*, 2007). For example, a bacteriocin produced by *Lactobacillus plantarum* KLDS1.0391 was shown to disrupt the membrane potential of *S. typhimurium* cells leading to the release of ATP, potassium ions, and other integral intracellular components (Gong *et al.*, 2010).

Lactocepins and other Secreted Molecules

Lactocepins are biologically active proteases that can be secreted or anchored to bacterial outer cell membranes. They were first identified in Lactococci species and have since been identified in Lactobacilli. These proteases have the ability to degrade casein to liberate free amino acids required for bacterial cell growth during milk fermentation. Recently, a novel role for some lactocepins has been identified. A lactocepin produced by *Lactobacillus paracasei* selectively degraded the pro-inflammatory IP-10 chemokine produced by IECs in a clinically induced mouse colitis model. This led to the abrogation of cecal tissue inflammation and to reduced infiltration of lymphocytes in the inflamed tissue (von Schillde *et al.*, 2012).

Other Lactobacilli secreted proteins have also garnered attention for their anti-inflammatory activity. Most notably, p75 and p40, secreted by *Lactobacillus rhamnosus* GG have been shown to reduce cytokine-induced epithelial cell damage in both human and mouse colonic epithelial cells. These proteins are cell wall hydrolases with endopeptidase activity which are responsible for normal separation of *L. rhamnosus* GG cells during replication (Claes *et al.*, 2012). It was determined that these proteins activated the Akt cellular pathways to prevent TNF- α induced cell death (Yan *et al.*, 2007).

N-formyl peptides (fMLF) are bacterially produced peptides which contain the *N*-formyl methionine amino acid. fMLFs are produced by both pathogenic and commensal bacteria and have been recently been found to bind to formyl peptide receptors (FPR) present on cells of the innate immune system. Once bound, these receptors induce the transcription of pro-inflammatory cytokine and chemokine production and reactive oxygen species (ROS) generation (Migeotte *et al.*, 2006). fMLFs produced by *L. rhamnosus* GG have recently been shown to up-regulate ROS production in IECs (Wentworth *et al.*, 2011). This increase in ROS production at the IEC level has been shown to down-regulate pro-inflammatory cytokine transcription by inhibiting NF- κ B activation and promote intestinal barrier function (Kumar *et al.*, 2007; Lin *et al.*, 2009; Alam *et al.*, 2014).

Potential Mechanisms of Action of Probiotics

The molecular mechanisms of action behind many of the observed beneficial effects of probiotics on the host immune system have remained elusive. However, there has been considerable attention spent examining gene alteration involved in the negative regulation of the NF- κ B signalling pathway in response to probiotic treatment including the up-regulation of the A20 and the peroxisome proliferator-activated receptors (PPAR) family of proteins.

A20 (TNFAIP3: Tumour Necrosis Factor α -Induced Protein 3)

The A20 protein is responsible for the termination of the pro-inflammatory NF- κ B signalling pathway and TNF- α induced apoptosis. Activation of the NF- κ B pathway results in the increased transcription of a wide array of pro-inflammatory genes. The A20

protein has recently emerged as a potential marker in the pathogenesis of several immune-related disorders as small loss-of-function mutations in the gene locus encoding this protein have been associated with inflammatory diseases such as Crohn's disease, rheumatic heart disease, and atherosclerosis (Vereecke *et al.*, 2009). In most cell types, it is not constitutively expressed as it is a NF- κ B target gene. Therefore, A20 expression is dependent upon stimulation via the TLR, IL-1R, NOD receptors, or TNF-receptor (TNF-R) pathways. Once expressed, A20 antagonizes a variety of molecular targets in the NF- κ B signalling pathway (dependent upon which receptor was activated) in an attempt to regulate the cell's response to pro-inflammatory stimuli and allow the cell to return to homeostatic conditions. These molecular targets include: TNF receptor associated factor-6 (TRAF-6) in response to TLR and IL-1R stimulus, receptor-interacting serine/threonine kinase -2 (RIPK-2) in response to NOD stimulus, and receptor-interacting serine/threonine kinase-1 (RIPK-1) in response to TNF-R stimulus (Ma and Malynn, 2012). Once bound, the A20 protein effectively terminates any subsequent signalling induced by the activation of the aforementioned receptors blocking the activation of NF- κ B.

As mentioned previously, inflammation has been implicated in the pathogenesis of atherosclerosis. As such, much attention has been focused on examining the potential role of the negative regulators of the immune system, such as the A20 protein, during the development of atherosclerosis. In an experiment conducted using atherosclerosis-sensitive mice (ApoE^{-/-}) which were also haploinsufficient in A20, the A20^{-/-} phenotype led to premature death due to severe inflammation in several organs (Lee *et al.*, 2000). Mice lacking the A20 protein had increased surface expression of VCAM-1,

CD54/ICAM-1, and increased levels of NF- κ B induced pro-inflammatory cytokines in their serum (Wolfrum *et al.*, 2007). In a separate experiment, it was found that A20 exerted beneficial effects at the endothelial level by suppressing CD40 activation and apoptosis in human coronary artery endothelial cells (Longo *et al.*, 2003). CD40 is expressed on the surface of endothelial cells and CD40 ligand (CD40L) binding has been implicated in the pathogenesis of atherosclerosis. Diabetic, hyperglycemic mice were increasingly susceptible to atherosclerosis due to the glucose-triggered post-translational modification of the A20 protein which ultimately led to proteasome degradation of the protein (Shrikhande *et al.*, 2010). Taken together, these studies suggest that the A20 protein has a profound influence in the pathogenesis and severity of atherosclerosis.

Some evidence suggests that probiotic organisms may down-regulate pro-inflammatory biomarkers via increased expression of the A20 protein (O'Callaghan *et al.*, 2012; O'Flaherty and Klaenhammer, 2012; Villena *et al.*, 2012; Tomosada *et al.*, 2013). This increase in A20 expression effectively down-regulates any subsequent expression of pro-inflammatory biomarkers at the transcriptome level by terminating the transcriptional activity of NF- κ B.

Peroxisome Proliferator-Activated Receptors (PPARs)

The PPARs are a set of nuclear hormone receptors involved in the regulation of lipid metabolism, glucose homeostasis, and attenuation of the inflammatory response (Duval *et al.*, 2002). The PPAR receptors (α , δ , γ) are activated by naturally derived fatty acid molecules such as conjugated linoleic acid, prostaglandins, and 9-hydroxyoctadecadienoic acid (9-HODE). Recently, these PPARs have been shown to

have anti-atherogenic properties and have been found in both human and mouse atherosclerotic plaques (Duval *et al.*, 2002). Both PPAR α and PPAR γ down-regulate cytokine induced VCAM-1 expression in endothelial cells via inhibition of the NF- κ B pathway (Marx *et al.*, 2000; Pasceri *et al.*, 2000). PPAR γ activation has also been attributed with the ability to prime human peripheral blood monocytes into regulatory M2 macrophages (Bouhlef *et al.*, 2007). Further, it has been shown that genetic knock-outs of the PPAR γ gene in macrophage-derived foam cells resulted in decreased cholesterol efflux suggesting another possible anti-atherogenic role of this receptor (Akiyama *et al.*, 2002; Chinetti *et al.*, 2001).

Some probiotics modulate the expression of pro-inflammatory cytokines through the increased transcription/activation of the PPARs. It is thought that some probiotic organisms activate the PPARs, specifically PPAR γ , through the local production of conjugated linoleic acid (CLA). In fact, CLA produced by probiotic organisms decreased dextran sodium sulfate-induced colitis in a mouse model by activating PPAR γ in macrophages (Bassaganya-Riera *et al.*, 2012). Orally administered probiotics have also been shown to protect against sepsis-induced liver and colonic damage in mice through the activation of PPAR γ (Ewaschuk *et al.*, 2007). Further, mice which were supplied with capsules containing fermented milk products showed increased expression of PPAR α mRNA in their liver (Sari *et al.*, 2013). Collectively, these studies suggest that these probiotic organisms can alter systemic expression and activation of PPAR receptors and that their actions are not isolated to the gastrointestinal tract.

Objectives

Chapter 1:

Determination of the Immunomodulatory Activity of the *L. rhamnosus* R0011 and *L. helveticus* R0389 Secretomes on Challenged Intestinal Epithelial Cells

Aim of Study:

Lactobacillus rhamnosus R0011 has been shown to have immunomodulatory activity when cultured with IECs (Wagar *et al.*, 2009; Foster *et al.*, 2011; McCarville, 2012, M. Sc. Thesis, UOIT). However, the immunomodulatory activity of *Lactobacillus helveticus* R0389 has been less characterised. Further, it is unclear whether these bacteria secrete bioactive molecules retaining immunomodulatory activity. The aim of this study was to determine the immunomodulatory activity of *L. rhamnosus* R0011 and *L. helveticus* R0389 secretomes on both human HT-29 and rat IEC-6 IECs stimulated with a wide array of pro-inflammatory stimuli. The secretome encompasses both proteins and other bioactive molecules secreted or shed by the cell. The transformed human HT-29 IECs have been used as a model *in vitro* cell system to study the effects of bacterial interactions with IECs and secrete IL-8 in response to challenge from innate immune stimulants (Kelly *et al.*, 1994; Abreu-Martin *et al.*, 1995). Similarly, the primary, non-transformed rat IEC-6 IEC cell line has been used as a model cell system to study IEC function *in vitro* (Quaroni *et al.*, 1979). Initial characterisation of the bioactive constituents of both secretomes was also conducted in an attempt to elucidate potential mechanisms of action. Further, the ability of the *L. rhamnosus* R0011 secretome to inhibit the growth of *Salmonella typhimurium*, a known gut pathogen, was determined.

1) Determination of the effects of *L. rhamnosus* R0011 and *L. helveticus* R0389 on constitutive levels of IL-8 production by human HT-29 IEC

Previous analysis has indicated that *L. rhamnosus* R0011 down-regulates constitutively produced IL-8 from human HT-29 IECs (Wallace *et al.*, 2003). However, the effects of the secreted products from these Lactobacilli strains on basal IL-8 production have not been elucidated. For this reason, HT-29 IECs were cultured with the *L. rhamnosus* R0011 and the *L. helveticus* R0389 secretomes to determine whether it is a secreted bioactive molecule from these Lactobacilli which confers the immunomodulatory activity.

2) Determination of the effects of *L. rhamnosus* R0011 and *L. helveticus* R0389 on levels of IL-8 production by human HT-29 IEC challenged with a wide array of pro-inflammatory stimuli:

The ability of many strains of Lactobacilli to down-regulate pro-inflammatory cytokine production by human IECs challenged with innate immune stimulants has been characterized extensively (Thomas and Versalovic, 2010; Lebeer *et al.*, 2008; Lebeer *et al.*, 2010). However, it is unclear whether these bacteria secrete bioactive molecules which have anti-inflammatory activity into the extracellular environment. For this reason, HT-29 IECs were stimulated with a wide array of pro-inflammatory stimuli (TNF- α , IL-1 β , Poly (I:C), Flagellin, LPS). Effects of concurrent administration of either the *L. rhamnosus* R0011 or the *L. helveticus* R0389 secretomes on the response induced by

these stimuli were examined to determine whether these Lactobacilli secrete bioactive molecules with the ability to down-regulate pro-inflammatory cytokine production.

3) Determination of the effects of *L. rhamnosus* R0011 and *L. helveticus* R0389 on constitutive levels of CINC-1 production by rat IEC-6 intestinal epithelial cells:

Immunomodulatory activities of the *L. rhamnosus* R0011 and *L. helveticus* R0389 secretomes were also tested on rat IEC-6 IECs as described previously in Objective (1). These cells are non-transformed, primary IECs. Changes in constitutive levels of the pro-inflammatory chemokine, CINC-1, were measured in response to incubation with the secretomes.

4) Determination of the effects of both *Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0389 on levels of CINC-1 production by rat IEC-6 intestinal epithelial cells challenged with a wide array of pro-inflammatory stimuli:

Immunomodulatory activities of the *L. rhamnosus* R0011 and *L. helveticus* R0389 secretomes were also tested on rat IEC-6 IECs as described previously in Objective (2). Changes in levels of pro-inflammatory chemokine, CINC-1, were measured in response to concurrent incubation with the secretomes and either poly (I:C), flagellin, or LPS.

5) Characterisation of the bioactive constituents of the *L. rhamnosus* R0011 and the *L. helveticus* R0389 secretomes:

Some probiotic microorganisms secrete small bioactive molecules retaining anti-inflammatory activity (Ganguli *et al.*, 2013). For this reason, size-fractionation of the two secretomes using a filter with a MW cutoff of 10 kDa was conducted in order to determine whether the secreted bioactive molecule is less than 10 kDa in size. Further characterization was done by neutralizing the pH of the secretome to physiological pH with sodium hydroxide, determining the isomer configuration of the lactic acid produced by both strains of Lactobacilli, and the ability of each strain to produce extracellular ATP.

6) Determination of the ability of the *L. rhamnosus* R0011 secretome to inhibit the growth of *Salmonella typhimurium*

Previous analysis has shown that Lactobacilli can antagonize the attachment of *S. typhimurium* to human IECs (Lehto and Salimen, 1997). We hypothesized that *L. rhamnosus* R0011 secretes antimicrobial compounds capable of antagonizing the growth of *S. typhimurium*. To test this hypothesis, overnight cultures of *S. typhimurium* were cultured with the *L. rhamnosus* R0011 secretome and growth was monitored over a 6-hour period.

Chapter 2:

Analysis of the effects of *L. rhamnosus* R0011-derived fermented milk on the expression of pro-atherogenic biomarkers using an *in vitro* cell system

Aim of Study:

The aim of this study was to examine the effects of *L. rhamnosus* R0011 fermented dairy milk on the expression of pro-atherogenic biomarkers using *in vitro* cell models.

- 1) Determination of the effects of *L. rhamnosus* R0011 fermented milk on cytokine expression by human THP-1 and U937 monocyte cells challenged with LPS:

In an attempt to elucidate the range of bioactivity of milk fermented with *L. rhamnosus* R0011, milk ferments were tested directly on human monocyte cells using standard cell line models. Since there have been reports describing differences in cytokine profiles between cell types following challenge, milk ferments were tested on both human THP-1 and U937 cells. The THP-1 cell line is a well-established model for studying the roles of both monocytes and macrophages in atherosclerosis (Qin, 2012; Chanput *et al.*, 2014). Stimulation of THP-1 and U937 cells with LPS has been shown to elicit a similar cytokine expression profile as those observed in monocytes and macrophages in atherosclerotic lesions (Sharif *et al.*, 2007; Huang *et al.*, 2012). Therefore, monocytes were exposed to LPS and cultured with *L. rhamnosus* R0011 milk ferments or acidified and unfermented milk controls. Further, cells were pre-treated with *L. rhamnosus* R0011 milk ferments prior to LPS challenge to determine whether timing

plays an integral role on any observed immunomodulatory activity of the milk ferments. Changes in both pro-inflammatory and regulatory cytokines were measured.

2) Determination of the effects of *Lactobacillus rhamnosus* R0011 fermented milk on cytokine and cell surface molecule expression by THP-1 and U937 derived resting macrophages challenged with lipopolysaccharide:

Since macrophages play an integral role in the pathogenesis of atherosclerosis, milk ferments prepared with *L. rhamnosus* R0011 were tested on both U937- and THP-1-derived macrophages. Changes in cytokine profiles in response to challenge with LPS and *L. rhamnosus* R0011 ferments were measured. As macrophages exist in a heterogeneous population in atherosclerotic plaques and produce specific cytokine profiles, the ferments were tested for the ability to transform M0 resting macrophages into the regulatory/anti-inflammatory M2 macrophage. Further, the ability of the milk ferments to down-regulate surface expression of the CD54/ICAM-1 adhesion molecule was examined.

Materials and Methods

Cell Culture

The HT-29 human colorectal adenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC) (ATCC #HTB-38) and was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 0.05 mg/mL gentamicin (Sigma-Aldrich, MO, USA) and were grown in 75cm² tissue culture flasks (Greiner-Bio-One, NC, USA) at 37°C, 5% CO₂ in a humidified incubator (Thermo Fisher, MA, USA). Cells were passaged every three days or when cells reached approximately 90% confluency. To passage, HT-29 cell culture medium was aspirated and replaced with 4 mL of 20% trypsin-EDTA (Sigma-Aldrich, MO, USA); flasks were then incubated at 37°C for 5 minutes to detach cells from the tissue culture flask. Trypsin was neutralized by the addition of 8 mL of HT-29 cell culture medium (containing FBS) to the flask. Cells were then collected by centrifugation at 300 x g for 10 minutes. Following centrifugation, the supernatant was removed and the HT-29 IEC were resuspended in 4 mL of HT-29 cell culture medium. A 1 in 10 dilution was performed of the resuspended cell culture with HT-29 cell culture medium into the tissue culture flask.

The non-transformed rat IEC-6 (ATCC #CRL-1592) small intestinal cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, MO, USA) supplemented with 10% FBS and 0.05mg/mL gentamicin, and cell cultures were maintained in a similar manner to HT-29 cells.

The THP-1 human peripheral blood monocyte cell line (ATCC #TIB-202) was obtained from the ATCC. Cells were maintained in RPMI-1640 medium supplemented

with 0.05 mM β -mercaptoethanol, 10% FBS and 0.05 mg/mL gentamicin in a humidified incubator at 37°C and 5% CO₂. THP-1 cells were sub-cultured every 4-5 days by centrifuging cells at 300 x g for 10 minutes. Supernatants were aspirated and cells were resuspended with 4 mL of THP-1 cell culture medium and a 1 in 10 dilution of the resuspended cell culture was performed with THP-1 cell culture medium into the tissue culture flask.

The U937 human myeloid monocyte cell line (ATCC #CRL-1593.2) was obtained from the ATCC. Cells were maintained in RPMI-1640 supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamate and 0.05 mg/mL of gentamicin. Cells were maintained similarly to the THP-1 cells.

Preparation of the *L. rhamnosus* R0011 and *L. helveticus* R0389 secretomes

Lactobacillus rhamnosus R0011 was obtained from Lallemand Health Solutions Inc. (Montreal, Quebec, Canada). Starting from a lyophilized stock, bacteria were weighed aseptically and washed 3X in sterile phosphate-buffered saline (PBS). Between each wash step, bacteria were centrifuged at 3000 x g for 20 minutes at 4°C. Following washes, the bacteria were diluted 1 in 50 in deMan, Rogosa and Sharpe (MRS) medium (Difco, Canada) and placed in a shaking incubator at 37°C and 200-220 rpm for 17 hours or until an OD₆₂₀ = 1.64 was reached. This had previously been determined to indicate stationary phase for these bacteria. The bacterial culture was then diluted 1 in 5 in either non-supplemented RPMI-1640 or DMEM medium and allowed to further propagate for an additional 23 hours under the same conditions. A control consisting of MRS only, or MRS diluted in either RPMI or DMEM, was incubated concurrently with the bacterial

culture. Following incubation, the pH of the *L. rhamnosus* R0011 culture was measured and the pH of the control was adjusted to that of the bacterial culture using concentrated HCl. Both the bacterial culture and the control were then centrifuged at 3000 x g for 20 minutes and filtered through a 0.22 µm filter (Progene, Canada) to remove any bacteria and stored at -80°C. For some experiments, the filtered secretome samples were also subjected to size fractionation using a <10 kDa Amicon Ultra – 15 centrifugal filter (EMD Millipore, MA, USA). The less than 10 kDa fraction of the secretome was also stored at -80°C. A portion of the filtered secretome was plated on MRS agar to ensure that all bacteria had been removed.

Lactobacillus helveticus R0389 was obtained from Lallemand Health Solutions Inc. (Montreal, Quebec). Starting from a frozen stock, bacteria were diluted 1 in 50 in fresh MRS medium and subjected to the same incubation periods as those described for production of the *L. rhamnosus* R0011 secretome. Following incubation, cultures were also filtered through a 0.22 µm filter and a portion of the secretome was also subjected to centrifugal filtration through a <10 kDa filter. Aliquots of the secretome and the less than 10 kDa fraction of the R0389 secretome were stored at -80°C.

IEC challenge with innate immune stimulants and *L. rhamnosus* R0011 and *L. helveticus* R0389 secretomes

For cell challenges, HT-29 and IEC-6 IEC were enumerated and viability determined using Trypan Blue following sub-culturing. Cells were then resuspended in complete culture medium and seeded at a concentration of 5×10^5 cells/mL in 96-well tissue culture treated plates. Plates were then placed in the CO₂ incubator for 48 hours or until cells formed a complete monolayer. Cells were then exposed to varied innate immune stimulants and dilutions of the *L. rhamnosus* R0011 or the *L. helveticus* R0389 secretome or acidified controls concurrently for 6 hours at 37°C and 5% CO₂ in a humidified incubator. Innate immune stimulants included human IL-1 β (Cedarlane, ON, Canada), human TNF- α (PeproTech, QC, Canada), poly (I:C) (Sigma-Aldrich, MO, USA), flagellin isolated from *Salmonella typhimurium* (Enzo Life Sciences, ON, Canada), and LPS isolated from *Escherichia coli* K12 (InvivoGen). For some cell challenges, cells were cultured with an A₂A receptor antagonist (Sigma-Aldrich, MO, USA), a CD73 blocker (Adenosine 5'-(α,β -methylene)diphosphate) (Sigma-Aldrich, MO, USA), a PPAR γ antagonist (GW9662) (Sigma-Aldrich, MO, USA), or pH neutralised (pH=7.4) *L. rhamnosus* secretome. Following IEC challenge, supernatants were collected and stored at -80°C for cytokine quantification and IEC viabilities were determined. All cell challenges were done using FBS-free RPMI-1640 medium for HT-29 IECs or non-supplemented DMEM medium for IEC-6 IECs (with the exception of LPS challenge, where FBS was included, to provide LPS binding protein (LBP)).

Table 2. Innate immune stimulants used to incite pro-inflammatory cytokine production by HT-29 and IEC-6 IECs

Innate Immune Stimulant	Concentration	Receptor
Lipopolysaccharide (LPS)	20 ng/mL	TLR-4
Poly (I:C)	40 µg/mL	TLR-3
Human Tumor Necrosis Factor – α (TNF-α)	50 ng/mL	TNF-Receptor
Flagellin	100 ng/mL	TLR-5
Human Interleukin-1β (IL-1β)	30 ng/mL	IL-1 Receptor

Salmonella typhimurium growth inhibition assay

Overnight cultures of *Salmonella typhimurium* were grown in tryptone soya broth (TSB) (Oxoid) prior to use. *S. typhimurium* was incubated with 40% v/v of the *L. rhamnosus* R0011 secretome and either an HCl acidified control or L (+) lactic acid control to account for any effect of pH or lactic acid on the growth of *S. typhimurium*, for 6 hours at 37°C under agitation. The OD₆₀₀ of the cultures were determined every hour for a total of 6 reads. Serial dilutions of *S. typhimurium* were plated on TSB agar after each read to enumerate the bacteria to determine the viability of the bacteria following the different treatments.

Preparation of the *L. rhamnosus* R0011 milk ferment

3.25% milk was inoculated with 1x10⁸ CFU/mL of *L. rhamnosus* R0011, placed in an incubator at 37°C with agitation, and allowed to ferment until a pH of 4.7 ± 0.1 was

obtained. A non-fermented milk control and a lactic acid acidified milk control were also subjected to the same incubation period under the same conditions. A portion of the *L. rhamnosus* R0011 ferment was subjected to filtration through 5.0 µm, 1.2 µm, and 0.2 µm to remove the bacteria to determine whether or not any observed immunomodulatory activity could be attributed to fermentation products of the bacteria. The lactic acid acidified milk control was prepared by determining the concentration of lactic acid in the *L. rhamnosus* R0011 ferment and adding the determined amount of lactic acid to the acidified control. If further reduction in pH was necessary following the addition of the correct concentration of lactic acid, HCl was added to reduce the pH of the acidified control to that of the *L. rhamnosus* R0011 ferment. *L. rhamnosus* R0011 was enumerated following fermentation by plating serial dilutions on MRS agar plates. Ferments, filtered ferments, non-fermented and acidified milk controls were immediately frozen in 1mL aliquots at -80°C.

Determination of the concentration of lactic acid in the *L. rhamnosus* R0011 ferment and secretome

The lactic acid concentration in the *L. rhamnosus* R0011 milk ferment and secretome was determined by using the commercially available Lactic Acid Determination Kit from Megazyme (Wicklow, Ireland). This kit also allows for the determination of the isoform configuration of the lactic acid present within the sample. Briefly, D-LDH and L-LDH were added to the fermented milk/secretome samples in the presence of NAD⁺ and D-glutamate-pyruvate transaminase (D-GPT). The amount of NADH produced following the addition of D/L-LDH to the sample is stoichiometric with

the amount of D(-)/L(+)-lactic acid present. Following incubation, the absorbance (nm) of the samples was measured and compared to the lactic acid standard.

Determination of ATP concentration in the *L. rhamnosus* R0011 ferment and secretome

ATP concentrations in the *L. rhamnosus* R0011 milk ferment and the *L. rhamnosus* R0011 and *L. helveticus* R0389 secretomes were determined using the commercially available ATP Determination Kit from Molecular Probes (Invitrogen, ON, Canada). D-luciferin, firefly luciferase, and dithiothreitol were added to samples in black 96-well plates (Greiner Bio-One, NC, USA). Luminescence was measured using a Synergy HTTR microplate reader (Bio-Tek, VT, USA). Total ATP concentration within the samples was determined by developing a standard curve and using the equation of the line of best fit.

Determination of indole production by *L. rhamnosus* R0011 and *L. helveticus* R0389

The indole spot-test was used to determine whether *L. rhamnosus* R0011 or *L. helveticus* R0389 produce indoles in culture (Vracko and Sherris, 1963). Briefly, a few drops of the indole spot-test reagent (Pro-Lab Diagnostics, ON, Canada) were placed onto Whatman filter paper. Pure cultures of each *Lactobacillus* species were smeared onto the spots and allowed to incubate for three minutes. If indoles are present, the 4-(Dimethylamino) cinnamaldehyde within the indole spot-test reagent will turn blue. Results were confirmed using the Kovac's reagent (Sigma-Aldrich, MO, USA).

Differentiation of THP-1 human peripheral blood monocyte cells into M0 macrophages

Differentiation of THP-1 monocyte cells into resting M0 macrophages was conducted as previously performed by Chanput *et al.* (2014). THP-1 cells were seeded in 12-well tissue culture treated plates (Greiner Bio-One, NC, USA) at a concentration of 1×10^6 cells/mL. These cells were treated with 100 ng/mL of phorbol-12-myristate-13-acetate (PMA) for 48 hours at 37°C, 5% CO₂ in a humidified incubator. Following this incubation, the culture medium containing PMA was removed and cells were washed 2X with complete THP-1 medium and allowed to rest in complete medium for an additional 24 hours prior to challenge with a pro-inflammatory stimulus. Differentiation was confirmed by immunophenotyping for increased expression of the macrophage cell surface marker CD11b.

Differentiation of U937 human myeloid monocyte cells into M0 macrophages

U937 cells were seeded at a concentration of 1×10^6 cells/mL in 12-well tissue culture treated plates in non-supplemented RPMI-1640 medium. These cells were stimulated with 1 μM all-trans retinoic acid (ATRA) for 24 hours at 37°C, 5% CO₂ in a humidified incubator, as this has previously been shown to induce U937 differentiation to the monocyte stage (Masotti, M. Sc. Thesis, UOIT, 2010; Zhang *et al.*, 2008). Medium containing ATRA was aspirated following incubation prior to cell challenge. Differentiation was confirmed by immunophenotyping for increased expression of the macrophage cell surface marker CD11b.

THP-1 and U937 cell challenge with lipopolysaccharide and *Lactobacillus rhamnosus* R0011 milk ferments

U937 and THP-1 monocyte cells were seeded at a concentration of 1×10^6 cells/mL in a 96-well tissue culture treated plate and challenged at a final concentration of 5×10^5 cells/mL in RPMI-1640 medium supplemented with 10% FBS. Cells were either pre-incubated with the *L. rhamnosus* R0011 milk ferments (either whole or filtered) or lactic acid acidified controls (either whole or filtered) or an unfermented milk control for 18 hours prior to challenge with 125 ng/mL of LPS for 6 hours or cultured with *L. rhamnosus* R0011 ferments or controls with LPS concurrently for 24 hours. Following challenge, cells were collected or removed by centrifugation at $300 \times g$ for 10 minutes and supernatants were collected and stored at -80°C for cytokine analysis. Initial characterisation of the effects of the *L. rhamnosus* R0011 milk ferment/controls was conducted by testing a series of dilutions (1/10, 1/50, 1/100, 1/1000) of the ferments and acidified or non-acidified controls on the cells. All subsequent challenges only examined the effects of the 1/50 ferment dilution (and pH-matched controls) as this concentration was shown to modulate the immune response without having a negative impact on cell viability.

Differentiated U937 and THP-1 M0 macrophages were challenged in a similar manner as the U937 and THP-1 monocyte cells. M0 macrophages were either pre-incubated with *L. rhamnosus* R0011 ferments or controls for 18 hours prior to challenge with 125 ng/mL LPS or cultured with *L. rhamnosus* R0011 ferments or controls concurrently for 24 hours. Following challenge, cultures were centrifuged at $300 \times g$ for

10 minutes prior to supernatant collection, and supernatants were immediately frozen at -80°C until analyzed for cytokine concentrations.

Cytokine Quantification

Human IL-1 β (R&D Systems, Catalogue #DY201), IL-6 (R&D Systems, Catalogue #DY206), IL-1Ra (R&D Systems, Catalogue #DY280), IL-8 (R&D Systems, Catalogue #DY208), TNF- α (R&D Systems, Catalogue #DY210), TGF- β (R&D Systems, Catalogue #DY208), and rat CINC-1 (R&D Systems, Catalogue #DY515) were quantified from cell culture supernatants using enzyme-linked immunosorbant assays (ELISA) following manufacturer's protocols (R&D Systems, MN, USA). IL-10 (BioLegend, Catalogue #430603) and IL-12(p70) (BioLegend, Catalogue #431702) were also quantified from cell culture supernatants following manufacturer's protocols (BioLegend, CA, USA). All ELISAs were done using 96-well high-binding Microlon 600 ELISA plates (Greiner Bio-One, NC, USA). Plates were coated with capture antibody (at the concentration indicated by the manufacturer) and incubated at 4°C overnight. Following sample loading and the addition of biotinylated detection antibody, horseradish peroxidase-conjugated streptavidin and its substrate, 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma-Aldrich, MO, USA), were added. The reaction was stopped with 1.8 N H₂SO₄ and plates were read at a wavelength of 450nm using a Synergy HTTR microplate reader (Bio-Tek Instrumentation, VT, USA).

Determination of cell surface molecule expression

Cell surface molecule expression was quantified using the Millipore Guava Personal Cell Analysis (PCA) System (EMD Millipore, MA, USA). Following cell

challenge, HT-29 IEC or differentiated U937 and THP-1 macrophages were dislodged from the tissue culture plates using a cell scraper, and collected by centrifugation at 300 x g for 10 minutes. Cells were counted (and viability determined) using the Trypan Blue exclusion assay and resuspended at a concentration of 1×10^6 cells/mL in 100 μ L of Double Protein Staining Buffer (5% FBS, 5% BSA in PBS, pH 7.4). Cells were stained with phycoerythrin (PE) conjugated antibodies and their corresponding isotype controls for 30 minutes on ice in the dark. Antibodies used were human anti-CD54 (Clone HCD54) (BioLegend, CA, USA), human anti-CD11b (Clone ICRF44) (BioLegend, CA, USA), human anti-CD73 (Clone (BioLegend, CA, USA), and isotype controls were IgG1, κ (Clone MOPC-21) (BioLegend, CA, USA) and IgG2a, κ (Clone MOPC-173) (BioLegend, CA, USA). Following incubation, cells were washed two times in 1mL of Double Protein Staining Buffer. Between each wash step, cells were centrifuged at 300 x g for 5 minutes at 4°C and supernatants were aspirated. After the final wash, cells were resuspended in 500 μ L of sterile, ice-cold PBS and incubated on ice, in the dark, for 5 minutes. Data were collected using the Guava Express Software and exported to Microsoft Excel. All data were reported as mean fluorescence intensity (MFI). FCS Express Flow Cytometry Software was used for generation of histograms and the Overton subtraction to determine percentage positive CD11b and CD73 expression.

Determination of cell viability following cell challenges

Cell viability was measured to ensure that any change in cytokine production was not due to cell death. Both the XTT cell viability assay (Roehm *et al.*, 1991) and Trypan Blue exclusion assay were used to determine cell viability of both U937 and THP-1 cells following cell challenge. Briefly, XTT reagent (0.2 mg/mL) was added to all wells which

did not receive the whole ferment as bacteria also yield a positive result when incubated with the XTT reagent. If cells were alive, a soluble formazan product would be produced. Following incubation for 2 hours at 37°C, the absorbance was measured at 450 nm and a background wavelength of 650 nm was subtracted. The Trypan Blue exclusion assay was used to determine the viability of those cells exposed to the whole ferment. All treatments were compared to the negative control (those cells which only received cell culture medium) and the % viability, relative to the negative control, was determined.

For determination of IEC viability following challenge, the MTT cell viability assay was used (Mosman, 1983). Briefly, the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) reagent (Sigma-Aldrich, MO, USA) was diluted to 0.5 mg/mL in either non-supplemented RPMI-1640 or DMEM medium. Cell culture supernatants were removed and replaced with the diluted MTT reagent. If cells were actively respiring, cells would produce an insoluble formazan product. Following incubation for 3 hours at 37°C, formazan crystals were dissolved using dimethyl sulfoxide and the absorbance was then measured at 600 nm. All treatments were compared to the negative control (those cells which only received cell culture medium) and the % viability, relative to the negative control, was determined.

Statistical Analysis

Statistical analysis was done using GraphPad Prism's one-way analysis of variance (ANOVA) and further analysis was done using Tukey's multiple comparison test when the ANOVA indicated significant differences were present. For some analyses, the student's paired T-test was used when only two groups were compared. All data are

shown as the mean \pm standard error of the mean (SEM) where one biological replicate (n=1) is representative of three technical replicates; for most conditions, n = 3 or greater.

Results

Effects of the *L. rhamnosus* R0011 and *L. helveticus* R0389 Secretomes on Challenged IEC and Characterisation of the Bioactive Constituents of the Secretomes

The *L. rhamnosus* R0011 and *L. helveticus* R0389 secretomes do not down-regulate constitutive IL-8 production from human HT-29 IEC

In order to elucidate whether the *L. rhamnosus* R0011 or the *L. helveticus* R0389 secretomes could down-regulate constitutive IL-8 production from HT-29 cells, cells were cultured with either secretome for six hours and levels of IL-8 were measured. Neither the *L. rhamnosus* R0011 nor the *L. helveticus* R0389 secretomes down-regulated constitutive IL-8 production from HT-29 IEC (Figures 1 and 2).

The *L. rhamnosus* R0011 and *L. helveticus* R0389 secretomes down-regulate IL-8 production from challenged human HT-29 IEC

To determine whether or not the *L. rhamnosus* R0011 or the *L. helveticus* R0389 secretomes could down-regulate IL-8 production from challenged HT-29 IEC, cells were cultured with either the *L. rhamnosus* R0011 or the *L. helveticus* secretome and an innate immune stimulant for six hours and changes in IL-8 production were measured. Both secretomes significantly down-regulated IL-1 β -induced IL-8 production (Figures 3 and 4), poly (I:C)-induced IL-8 production (Figures 5 and 6), LPS-induced IL-8 production (Figures 8 and 9), and TNF- α -induced IL-8 production (Figures 10 and 11) from HT-29 IECs. Further, the *L. rhamnosus* R0011 secretome was able to down-regulate flagellin-induced IL-8 production from HT-29 IEC (Figure 7). The less than 10 kDa fraction of

both the *L. rhamnosus* R0011 and the *L. helveticus* R0389 secretomes significantly down-regulated IL-8 production from activated HT-29 IEC as well (Figures 3-11).

The *L. rhamnosus* R0011 and *L. helveticus* R0389 secretomes do not down-regulate constitutive CINC-1 production from rat IEC-6 intestinal epithelial cells

Rat IEC-6 cells were cultured with either the *L. rhamnosus* R0011 or the *L. helveticus* R0389 secretomes for six hours and changes in production of the pro-inflammatory chemokine CINC-1 were measured. It was determined that neither secretomes significantly down-regulated constitutive CINC-1 production from IEC-6 cells (Figures 12 and 13).

The *L. rhamnosus* R0011 and *L. helveticus* R0389 secretomes have differential effects on CINC-1 production from challenged rat IEC-6 cells

To determine whether the two secretomes could down-regulate pro-inflammatory cytokines secreted by challenged IEC-6 cells, cells were cultured with different innate immune stimulants and either the *L. rhamnosus* R0011 or the *L. helveticus* R0389 secretomes for six hours and changes in CINC-1 were measured. The *L. rhamnosus* R0011 secretome did not down-regulate poly (I:C)-induced CINC-1 production from IEC-6 cells (Figure 14), indicating a difference from the observed effects on poly(I:C)-induced IL-8 production by HT-29 IEC. In contrast, the *L. rhamnosus* R0011 secretome did down-regulate flagellin-induced CINC-1 production and LPS-induced CINC-1 production from IEC-6 cells. This down-regulation was also observed in IEC-6 cells cultured with the less than 10 kDa fraction of the secretome (Figures 16 and 18).

However, the *L. helveticus* R0389 secretome did not down-regulate poly (I:C)-induced

CINC-1 production, flagellin-induced CINC-1 production, or LPS-induced CINC-1 production by IEC-6 cells. The same trend was also observed when cells were cultured with the less than 10 kDa fraction of the R0389 secretome (Figures 15, 17, and 19).

Characterisation of the bioactive constituents of the *L. rhamnosus* R0011 and *L. helveticus* R0389 secretomes

Initial characterisations of secreted products within the *L. rhamnosus* R0011 and the *L. helveticus* R0389 secretomes were conducted in an attempt to identify any bioactive constituents. It was found that *L. rhamnosus* R0011 predominately secretes L(+)-lactic acid while the *L. helveticus* R0389 predominately secretes D(-)-lactic acid (Figure 20). Further, it was determined that both the *L. rhamnosus* R0011 and the *L. helveticus* R0389 secretomes contain ATP, with some degree of difference in the ATP levels between strains (Figure 21). Finally, the ability of these two strains of Lactobacilli to produce indoles was determined. Both strains tested negative for indole production (Table 4).

The *L. rhamnosus* R0011 secretome did not modulate the expression of the AMP converting enzyme CD73 (Ecto-5'-Nucleotidase) by HT-29 IEC

HT-29 IEC express CD73 on the cell surface (Figures 22 and 23). To determine whether or not the *L. rhamnosus* R0011 secretome could modulate the expression of this adenosine generating enzyme, HT-29 IECs were cultured with the less than 10 kDa fraction of the secretome. Effects on CD73 expression were also measured following addition of TNF- α (50ng/mL). There was no significant difference in the expression of CD73 on HT-29 IEC cultured with the less than 10 kDa fraction of the secretome or any

of the other treatment groups, and TNF- α did not induce increased CD73 expression (Figure 24). Further, blocking of the CD73 enzyme with Adenosine 5'-(α,β -methylene) diphosphate (50 μ M; Schenck *et al.*, 2013) did not appear to reverse the down-regulation of IL-8 production by HT-29 IEC cultured with TNF- α and the *L. rhamnosus* R0011 secretome (Figure 25).

The *L. rhamnosus* R0011 Secretome did not act through the A₂A Adenosine Receptor

To determine whether or not the *L. rhamnosus* R0011 secretome immunomodulatory activity is mediated by the A₂A adenosine receptor, HT-29 IECs were cultured with the selective A₂A adenosine receptor antagonist 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385; 500nM) (Schenck *et al.*, 2013), the less than 10 kDa fraction of the secretome, and changes in TNF- α -induced IL-8 production were measured. The addition of the A₂A adenosine receptor antagonist did not appear to reverse the down-regulation of TNF- α -induced IL-8 production by HT-29 IEC cultured with the *L. rhamnosus* R0011 secretome (Figure 26).

Down-regulation of IL-8 production by the *L. rhamnosus* R0011 Secretome was not mediated through PPAR γ

HT-29 IECs were cultured with the *L. rhamnosus* R0011 secretome, TNF- α , and GW9662, a selective PPAR γ antagonist (5 μ M; Lea *et al.*, 2004; Leesnitzer *et al.*, 2002) and changes in TNF- α -induced IL-8 production were measured. The PPAR γ antagonist was unable to reverse the ability of the R0011 secretome to down-regulate TNF- α -induced IL-8 production (Figure 27).

Neutralising the pH of the *L. rhamnosus* R0011 secretome reverses the effect on IL-8 production

The pH of the *L. rhamnosus* R0011 secretome was neutralised to physiological pH (pH = 7.4) by the addition of sodium hydroxide, in order to determine whether the bioactivity of the secretome was pH-dependant. Neutralised *L. rhamnosus* R0011 or the less than 10 kDa fraction of the R0011 secretome did not significantly reduce TNF- α -induced IL-8 production from HT-29 IEC ($P > 0.05$) (Figure 28).

The *L. rhamnosus* R0011 secretome inhibits the growth of *S. typhimurium*

Overnight cultures of *S. typhimurium* were cultured with the *L. rhamnosus* R0011 secretome, the less than 10 kDa fraction of the R0011 secretome, or acidified controls, over a six hour period. *L. rhamnosus* R0011 and its less than 10 kDa secretome fraction significantly inhibited the growth of *S. typhimurium*. This growth inhibitory effect was not seen with the HCl or the L(+)-lactic acid controls (Figure 29).

Effects of *L. rhamnosus* R0011-derived Fermented Milk on the Expression of Pro-atherogenic Biomarkers by U937 and THP-1 cells

Characterisation of the *L. rhamnosus* R0011 milk ferments

Fermentation kinetics of *L. rhamnosus* R0011 grown in 3.25% milk were determined using different inoculum sizes. Growth was monitored over time and changes in pH were measured. It was determined that a starting inoculum of 1×10^8 CFU/mL yielded optimal fermentation kinetics (Figure 30). Further it was determined that *L. rhamnosus* R0011 primarily produces L(+)-lactic acid (Figure 31) and secretes ATP into the fermented milk product (Figure 32), although ATP levels were lower than those observed in the R0011 secretome (Figure 21).

Pre-incubating U937 or THP-1 human monocyte cells with *L. rhamnosus* R0011-derived fermented milk prior to LPS challenge has differential effects on cytokine production

U937 human monocyte cells were pre-incubated with *L. rhamnosus* R0011-derived milk ferments followed by LPS challenge and changes in production of IL-8, sCD54, and IL-1Ra were measured. Levels of IL-8 production increased in those cells pre-incubated with the filtered *L. rhamnosus* R0011 milk ferments and the unfermented milk control (Figure 33). In contrast, there were no changes in sCD54 (Figure 34) or IL-1Ra production (Figure 35) between LPS-challenged cells and those cells pre-incubated with the *L. rhamnosus* R0011 milk ferments or milk controls.

In contrast, pre-incubating THP-1 human monocyte cells with *L. rhamnosus* R0011-derived milk ferments followed by LPS challenge had no effect on IL-8 (Figure 39), sCD54 (Figure 40) or IL-1Ra production (Figure 41).

Culturing U937 or THP-1 human monocyte cells with *L. rhamnosus* R0011-derived fermented milk and LPS has differential effects on cytokine production

U937 human monocyte cells were cultured with the *L. rhamnosus* R0011-derived milk ferments and modulation of IL-8, sCD54, and IL-1Ra was determined. There was no change in IL-1Ra (Figure 38) or IL-8 (Figure 36) production between LPS-challenged cells and those cells cultured with LPS and the *L. rhamnosus* R0011-derived milk ferments or milk controls. However, there was a significant down-regulation of sCD54 in those cells cultured with the whole *L. rhamnosus* R0011 milk ferment. This down-regulation was also observed for U937 monocyte cells cultured with the lactic acid and non-fermented milk controls (Figure 37), suggesting an effect of a component of milk not associated with fermentation or acidification.

In contrast, THP-1 human monocyte cells which were cultured in media containing the *L. rhamnosus* R0011-derived milk ferments or milk controls showed no changes in LPS-induced IL-8 (Figure 42), sCD54 (Figure 43), or IL-1Ra production (Figure 44).

Culturing U937 or THP-1-derived macrophages with *L. rhamnosus* R0011-derived fermented milk and LPS has differential effects on cytokine production

U937 human monocytes were differentiated into macrophages using ATRA and differentiation was confirmed by immunophenotyping for increased expression of CD11b

(Figure 45 and 46). U937-derived resting macrophages were cultured with the *L. rhamnosus* R0011-derived milk ferments and LPS and changes in IL-10, IL-1Ra, sCD54, total TGF- β , IL-8, IL-6, and IL-1 β were measured. There was a significant increase in LPS-induced IL-10 production by those cells cultured with the *L. rhamnosus* R0011 whole milk ferment (Figure 47) relative to LPS-challenged cells cultured with the whole lactic acid control or the unfermented milk control. There was also a significant increase in LPS-induced IL-1Ra production by cells cultured with both the whole and filtered *L. rhamnosus* R0011 milk ferments relative to the negative control (Figure 48). Finally, there was a significant increase in LPS-induced IL-6 production by cells cultured with the whole *L. rhamnosus* R0011 ferment. This increase in LPS-induced IL-6 production was not observed for cells incubated with the lactic acid or non-fermented milk controls (Figure 52). In contrast, there was no modulation of sCD54 production (Figure 49), total TGF- β production (Figure 50), IL-8 production (Figure 51), IL-1 β production (Figure 53), IL-12p70 production or TNF- α production (Table 5) between cells challenged with LPS or those cultured with *L. rhamnosus* R0011-derived milk ferments or controls and LPS.

THP-1 human monocytes were differentiated into macrophages using PMA and differentiation was confirmed by immunophenotyping for increased expression of CD11b (Figure 55 and 56). THP-1 resting macrophages were cultured with LPS and the *L. rhamnosus* R0011-derived milk ferments or milk controls and changes in IL-10, IL-1Ra, sCD54, total TGF- β , IL-8, IL-6, and IL-1 β were measured. IL-10 production was significantly increased in those cells cultured with LPS and the *L. rhamnosus* R0011 whole ferment. This increase was not seen in the acidified or non-fermented milk controls

(Figure 57). There was also a significant increase in IL-1 β production from cells cultured with LPS and the *L. rhamnosus* R0011 whole milk ferment. This increase was not observed in cells cultured with the acidified or non-fermented milk controls (Figure 63). In contrast, there was no change in LPS-induced IL-1Ra production (Figure 59), IL-8 production (Figure 60), IL-6 production (Figure 62), total TGF- β production (Figure 64), IL-12p70 (Table 5) production or TNF- α production (Figure 61).

***L. rhamnosus* R0011 milk ferments do not decrease LPS-induced CD54 expression on U937 and THP-1 resting macrophages**

Both U937 and THP-1 resting macrophages were cultured with LPS and *L. rhamnosus* R0011-derived milk ferments or milk controls and changes in the surface expression of CD54/ICAM-1 was measured. LPS significantly increased the expression of CD54/ICAM-1 in both U937 and THP-1 macrophages (Figures 54 and 65). However, neither the *L. rhamnosus* R0011-derived milk ferments nor the milk controls were found to modulate the expression of LPS-induced CD54/ICAM-1 expression on U937 (Figure 54) and THP-1 macrophages (Figure 65).

Table 3. Cytokines and chemokines involved in the pathogenesis of atherosclerosis.

Cytokine¹	Cellular Source	Cellular Target	Function and Role in Atherogenesis	Reference
IL-1 β	Macrophages, Lymphocytes, Endothelial cells	Myriad of cells including smooth muscle cells and endothelial cells	<ul style="list-style-type: none"> • Pro-atherogenic • Pro-inflammatory 	Tedgui and Mallat, 2006; Girn <i>et al.</i> , 2007
IL-6	Macrophages, T cells, Endothelial cells	T cells, B cells, Endothelial cells, Smooth muscle cells	<ul style="list-style-type: none"> • Pro-atherogenic • Differentiation agent of monocytes • Increases CD54/ICAM-1 expression 	Tedgui and Mallat, 2006
IL-10	Monocytes, Macrophages (M2),	Macrophages, T cells, B cells	<ul style="list-style-type: none"> • Anti-atherogenic • Inhibits the production of proinflammatory cytokines 	Girn <i>et al.</i> , 2007; Tedgui and Mallat, 2006
TGF- β 1	Macrophages, Monocytes, T cells	Myriad of cell types	<ul style="list-style-type: none"> • Anti-atherogenic 	Tedgui and Mallat, 2006
TNF- α	Monocytes, Macrophages, ECs, T cells	Cells including smooth muscle cells and endothelial cells	<ul style="list-style-type: none"> • Pro-inflammatory • Pro-atherogenic • Activates endothelial cells 	Tedgui and Mallat, 2006
IL-1Ra	Monocytes, Macrophages	Macrophages and Endothelial cells	<ul style="list-style-type: none"> • Anti-atherogenic • Antagonizes the effect of IL-1β 	Girn <i>et al.</i> , 2007
Chemokines				
IL-8 (CXCL8)	Monocytes, Macrophages, Endothelial cells, Epithelial cells	Neutrophils and T cells	<ul style="list-style-type: none"> • Pro-atherogenic • Potent chemoattractant of neutrophils and T cells 	Tedgui and Mallat, 2006

¹This is a non-comprehensive list of cytokines and chemokines involved in the pathogenesis of atherosclerosis

Table 4. Preliminary characterisation of the bioactive constituents found within the *L. rhamnosus* R0011 and the *L. helveticus* R0389 secretomes

<i>L. rhamnosus</i> R0111	<i>L. helveticus</i> R0389
L-lactic acid	D-lactic acid
High levels of ATP	Low levels of ATP
Indole negative	Indole negative
Bioactive molecule is <10 kDa in size	Bioactive molecule is <10 kDa in size

Table 5. Immunomodulatory activity of the *L. rhamnosus* R0011 milk ferments on LPS-challenged U937 and THP-1-derived macrophages.

Cytokine/Chemokine	Modulation by <i>L. rhamnosus</i> R0011 Milk Ferment on LPS-challenged THP-1 Macrophages	Modulation by <i>L. rhamnosus</i> R0011 Milk Ferment on LPS-challenged U937 Macrophages
IL-1Ra	No Change ¹	Increase
IL-10	Increase	Increase
IL-1 β	Increase	No Change
TNF- α	No Change ²	No Change
IL-12p70	No Change ²	No Change ²
IL-6	No Change	Increase
TGF- β	No Change	No Change
IL-8	No Change	No Change
sCD54	No Change	No Change

¹There were no statistically significant differences between LPS-challenged cells and those cells cultured with the *L. rhamnosus* R0011-derived milk ferments and LPS

²Cytokine quantification were below levels of detection for all treatments

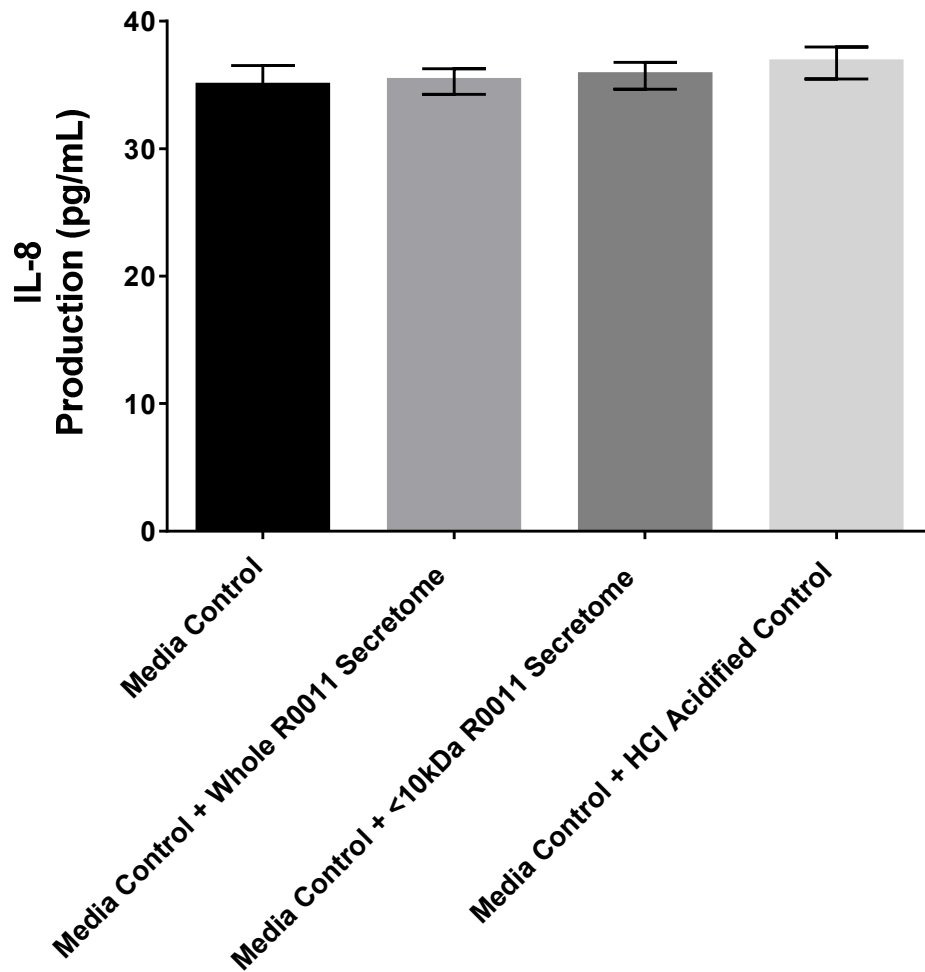


Figure 1. HT-29 IEC cultured with 40% v/v R0011 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=6). Data shown are the mean IL-8 production \pm SEM. Statistical analysis was performed using one-way ANOVA ($P > 0.05$).

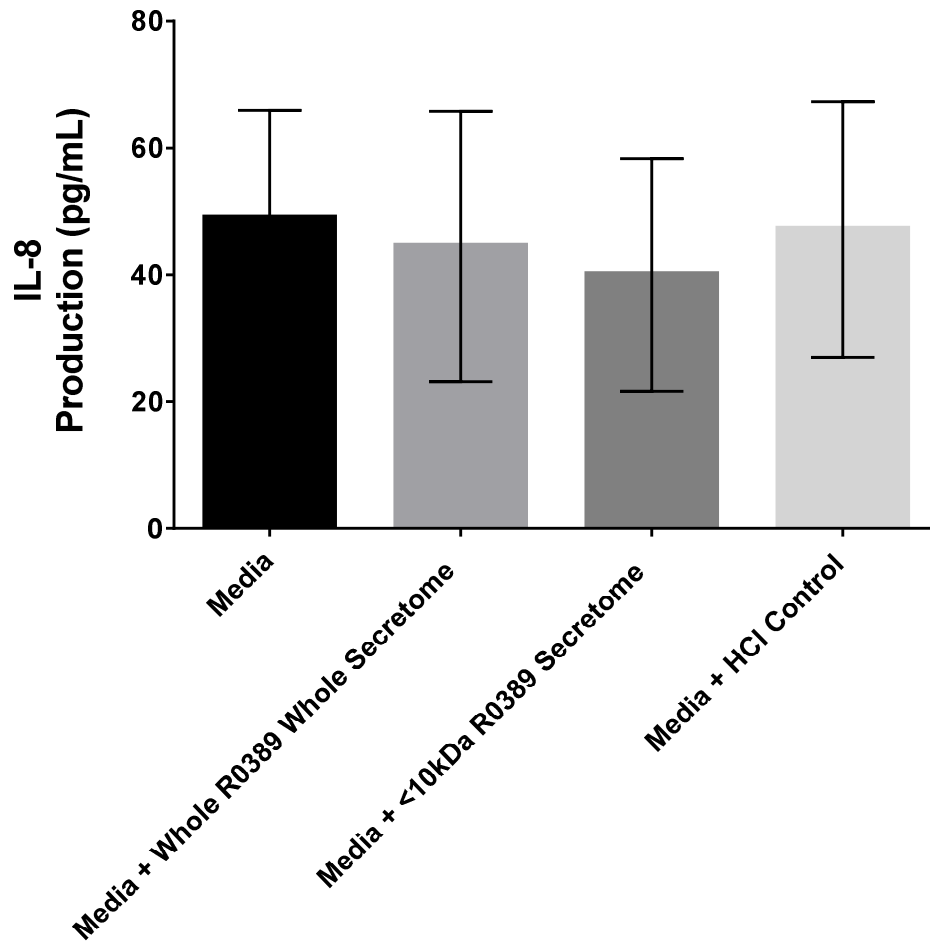


Figure 2. HT-29 IEC cultured with 40% v/v R0389 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=4). Data shown are the mean IL-8 production \pm SEM. Statistical analysis was performed using one-way ANOVA ($P>0.05$). Cell viabilities were greater than 75% for all treatments tested.

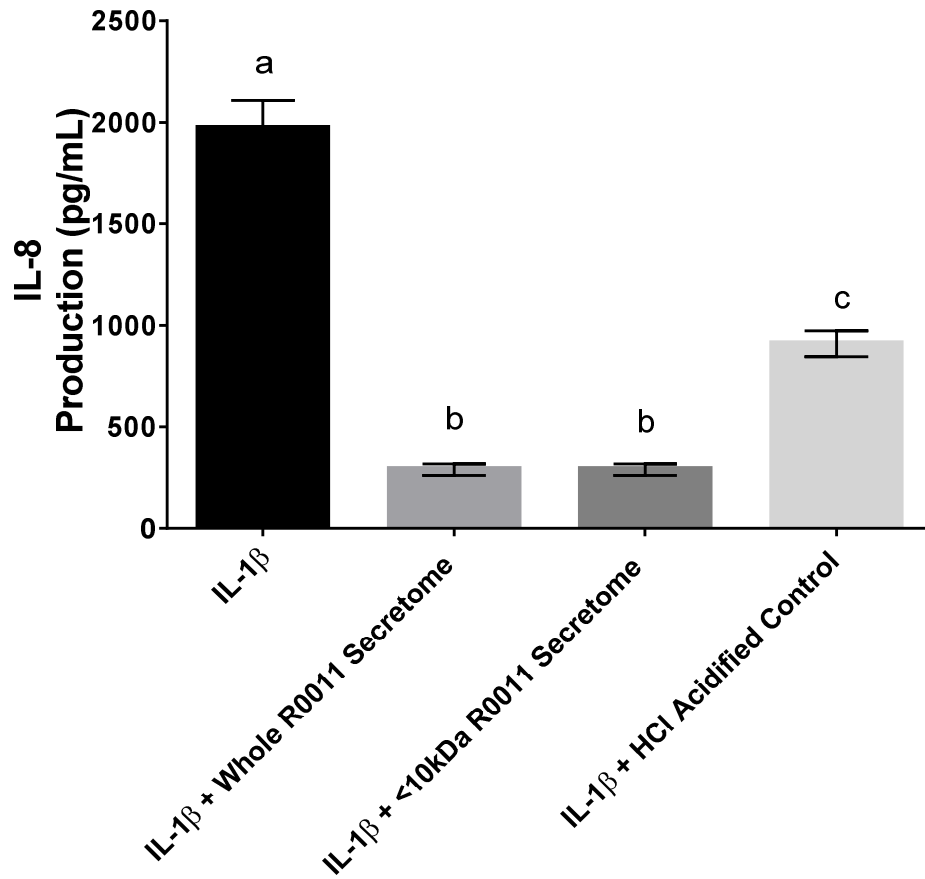


Figure 3. HT-29 IEC stimulated with IL-1 β and cultured with 40% v/v R0011 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=6). Data shown are the mean IL-8 production \pm SEM. Statistical analysis was performed using one-way ANOVA (P<0.001). Different letters denote significant differences between treatment groups. Cell viabilities were greater than 85% for all treatments tested.

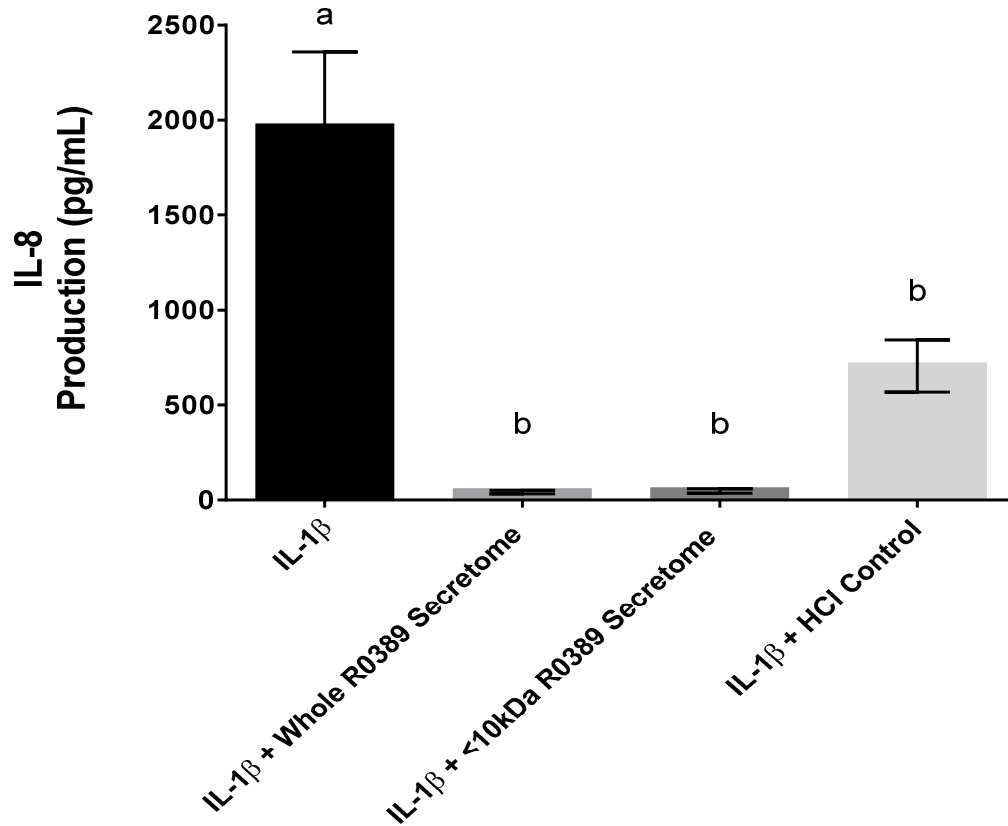


Figure 4. HT-29 IEC stimulated with IL-1 β and cultured with 40% v/v R0389 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=4). Data shown are the mean IL-8 production \pm SEM. Statistical analysis was performed using one-way ANOVA (P<0.001). Different letters denote significant differences between treatment groups. Cell viabilities were greater than 75% for all treatments tested.

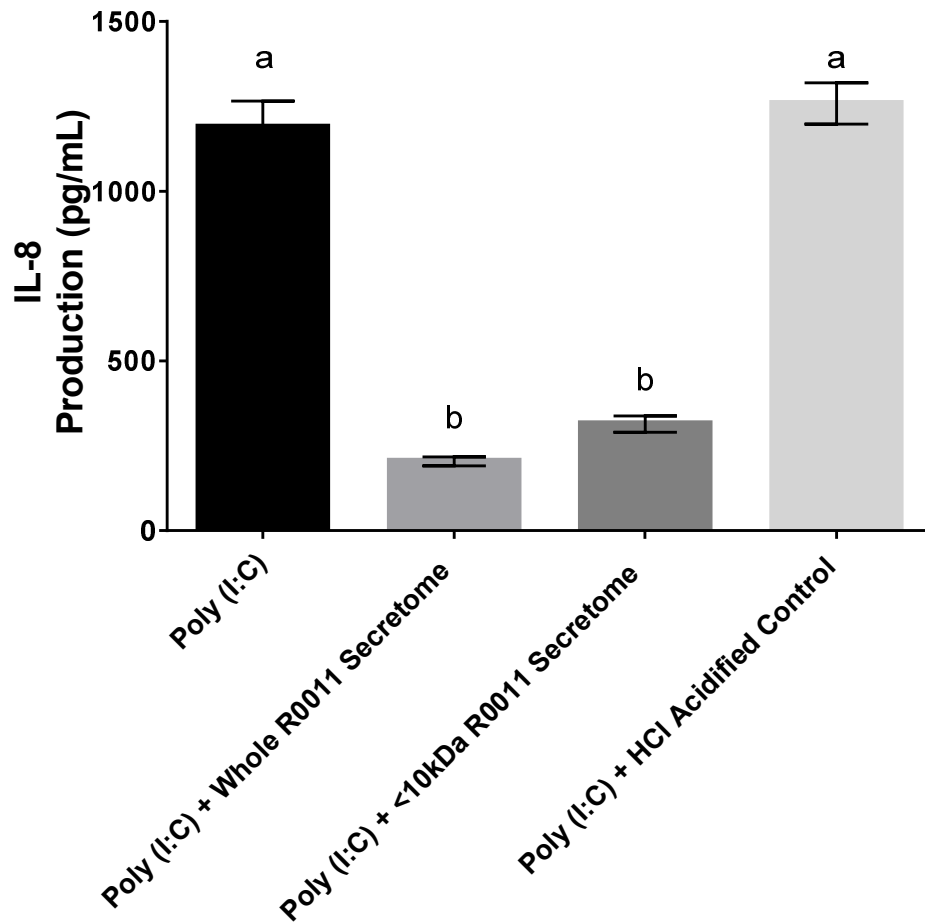


Figure 5. HT-29 IEC stimulated with Poly(I:C) and cultured with 40% v/v R0011 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=6). Data shown are the mean IL-8 production \pm SEM. Statistical analysis was performed using one-way ANOVA ($P < 0.001$). Different letters denote significant differences between treatment groups. Cell viabilities were greater than 80% for all treatments tested.

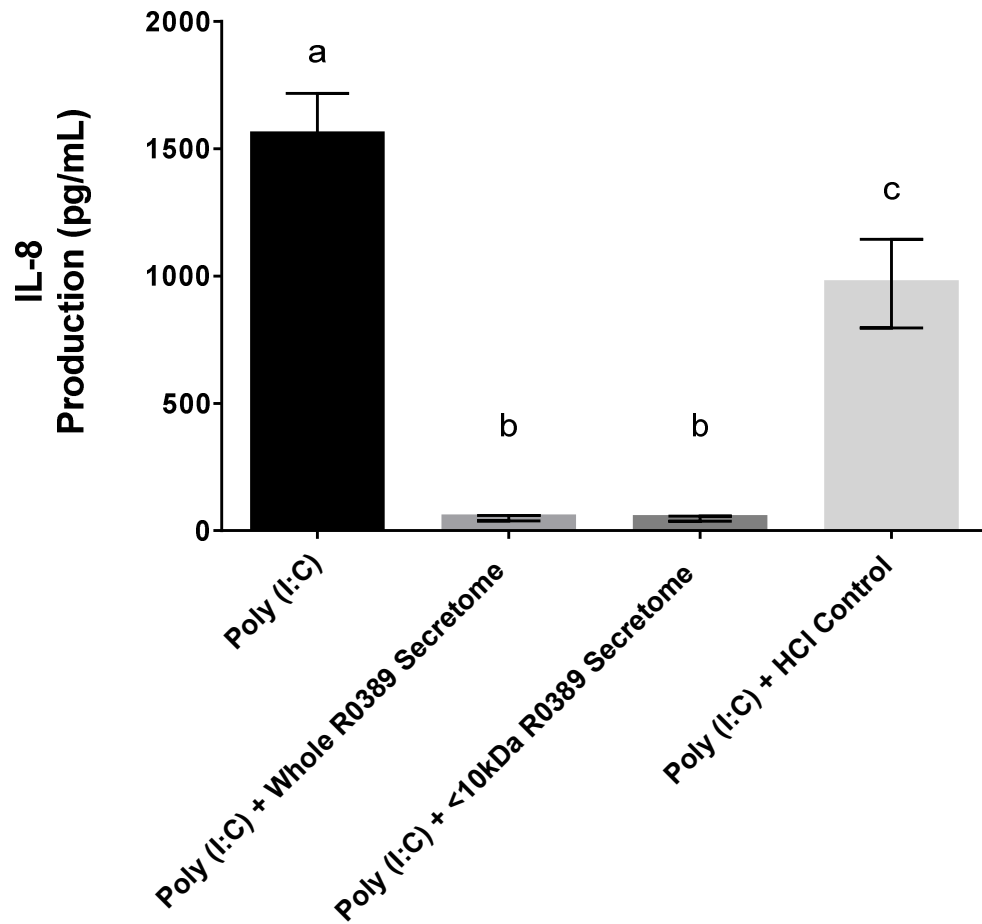


Figure 6. HT-29 IEC stimulated with Poly(I:C) and cultured with 40% v/v R0389 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=4). Data shown are the mean IL-8 production \pm SEM. Statistical analysis was performed using one-way ANOVA ($P < 0.001$). Different letters denote significant differences between treatment groups. Cell viabilities were greater than 70% for all treatments tested.

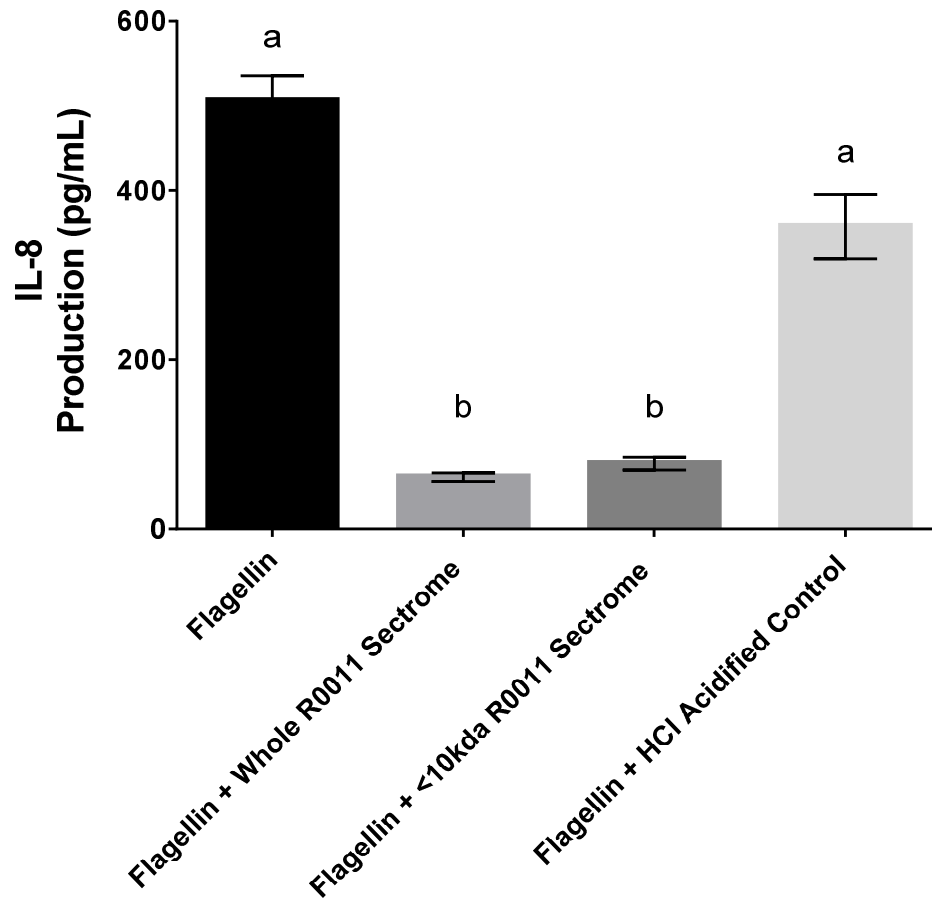


Figure 7. HT-29 IEC stimulated with Flagellin and cultured with 40% v/v R0011 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=6). Data shown are the mean IL-8 production \pm SEM. Statistical analysis was performed using one-way ANOVA ($P < 0.001$). Different letters denote significant differences between treatment groups. Cell viabilities were greater than 80% for all treatments tested.

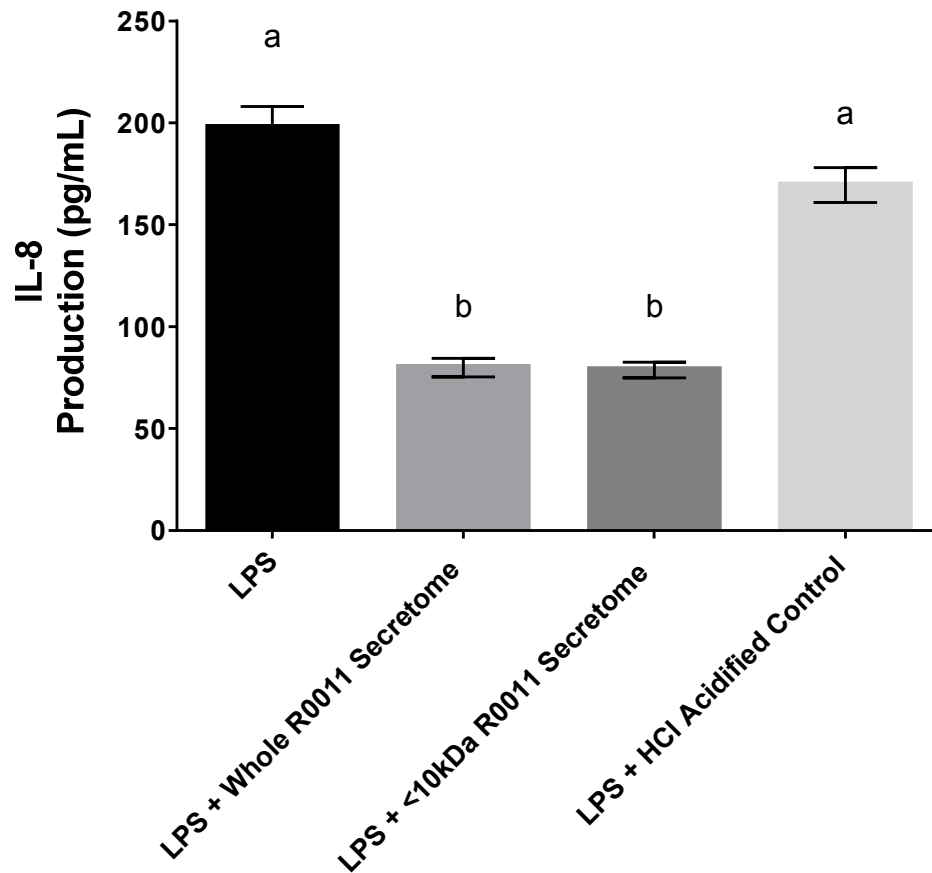


Figure 8. HT-29 IEC stimulated with LPS and cultured with 40% v/v R0011 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=6). Data shown are the mean IL-8 production \pm SEM. Statistical analysis was performed using one-way ANOVA ($P < 0.001$). Different letters denote significant differences between treatment groups. Cell viabilities were greater than 75% for all treatments tested.

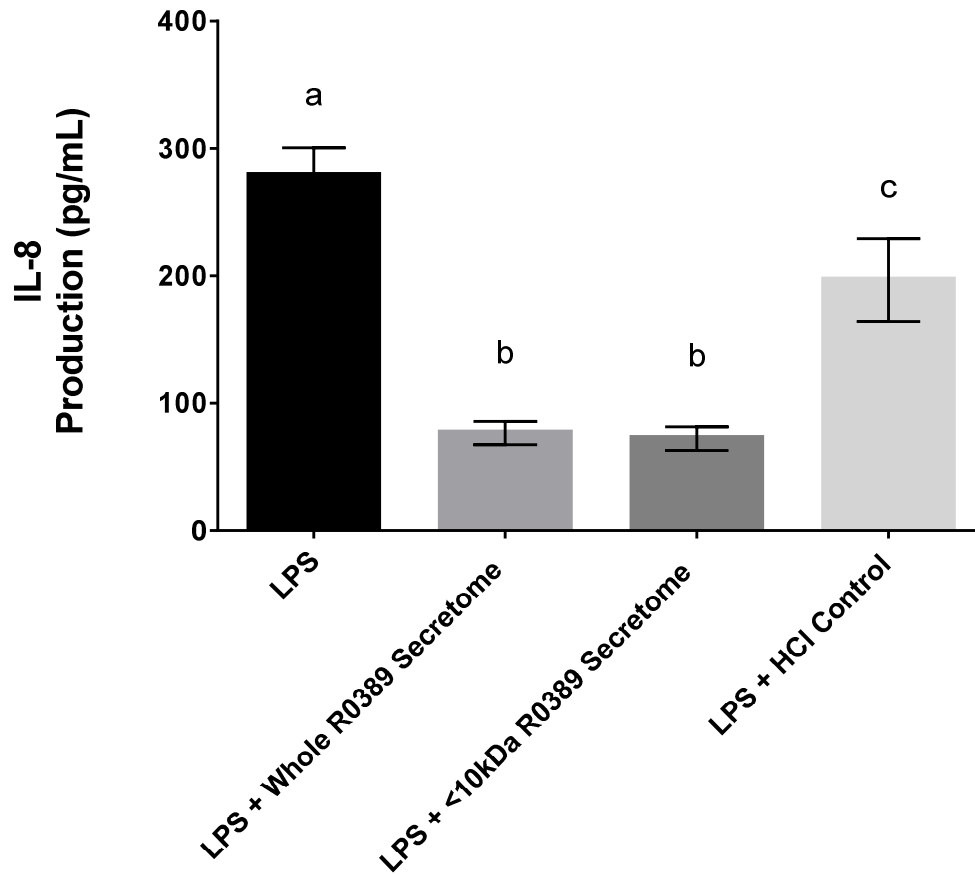


Figure 9. HT-29 IEC stimulated with LPS and cultured with 40% v/v R0389 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=4). Data shown are the mean IL-8 production \pm SEM. Statistical analysis was performed using one-way ANOVA ($P < 0.001$). Different letters denote significant differences between treatment groups. Cell viabilities were greater than 70% for all treatments tested.

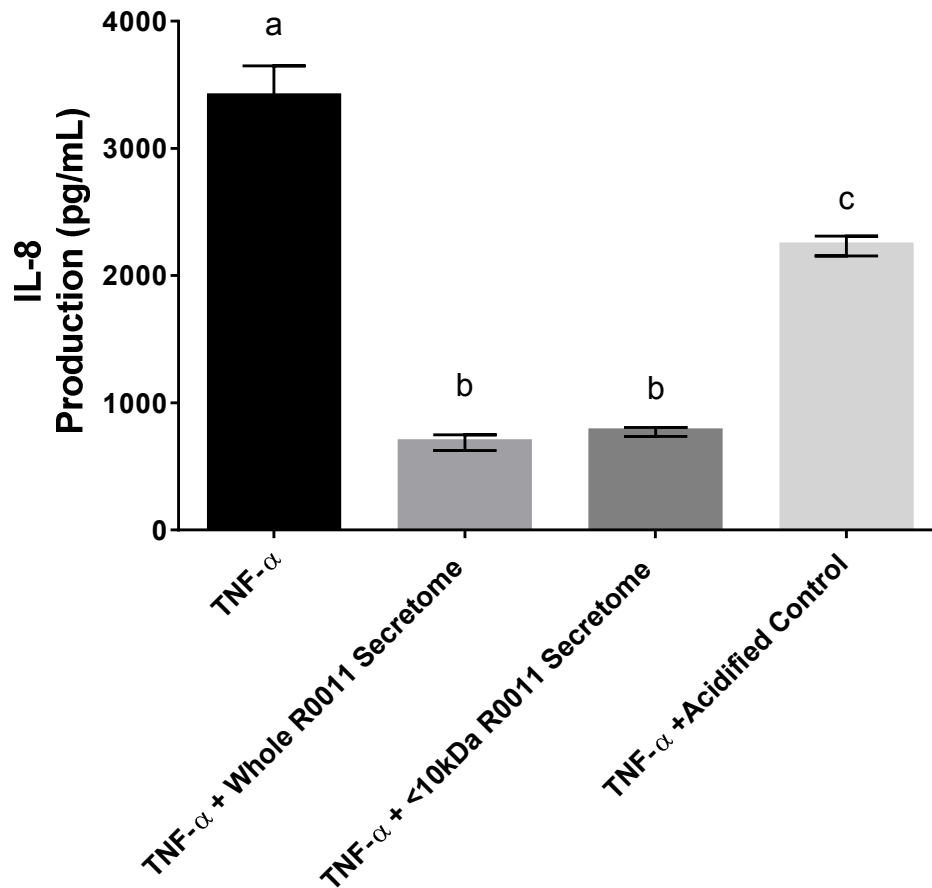


Figure 10. HT-29 IEC stimulated TNF- α and cultured with 40% v/v R0011 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=6). Data shown are the mean IL-8 production \pm SEM. Statistical analysis was performed using one-way ANOVA (P<0.001). Different letters denote significant differences between treatment groups. Cell viabilities were greater than 80% for all treatments tested.

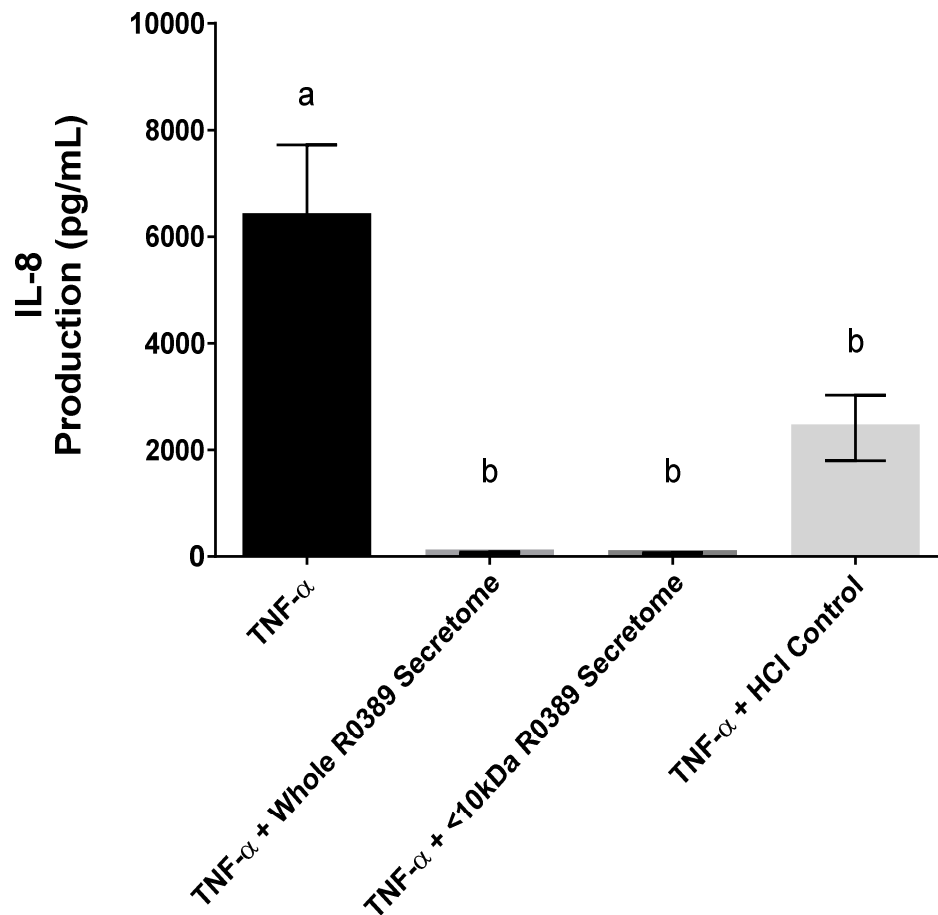


Figure 11. HT-29 IEC stimulated TNF- α and cultured with 40% v/v R0389 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=4). Data shown are the mean IL-8 production \pm SEM. Statistical analysis was performed using one-way ANOVA (P<0.001). Different letters denote significant differences between treatment groups. Cell viabilities were greater than 60% for all treatments tested.

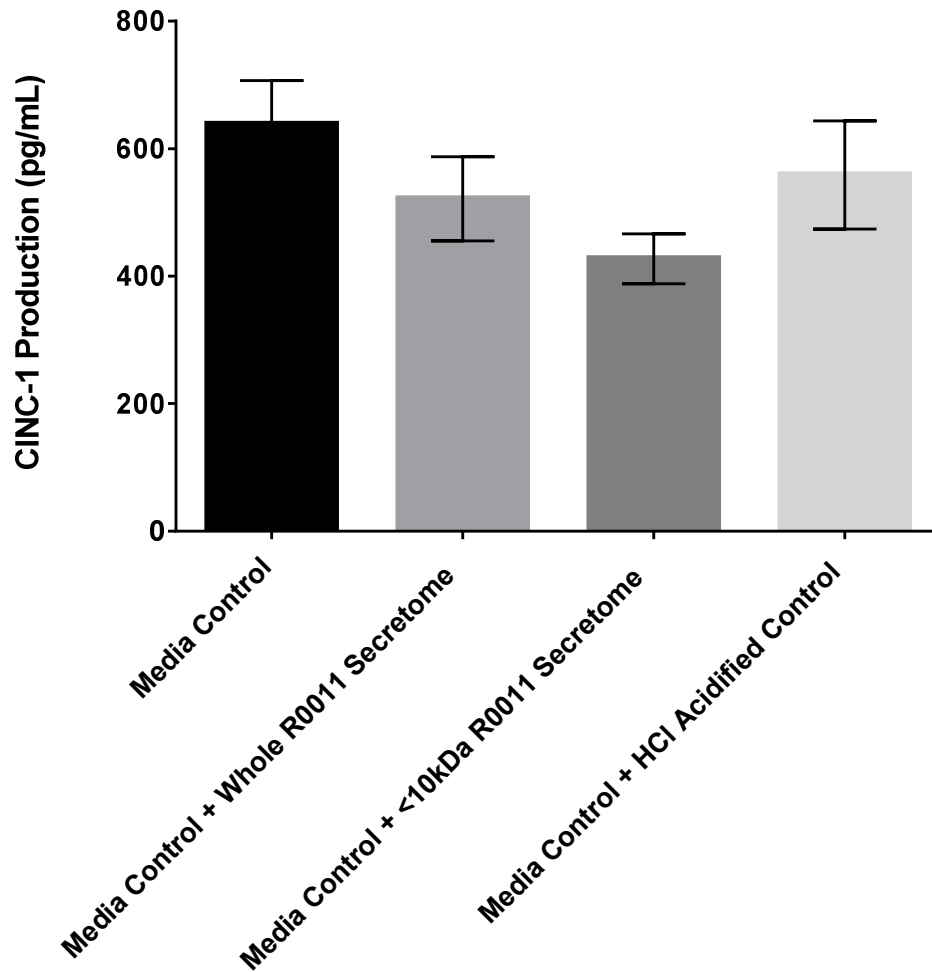


Figure 12. IEC-6 IEC cultured with 40% v/v R0011 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=6). Data shown are the mean CINC-1 production \pm SEM. Statistical analysis was performed using one-way ANOVA. There were no statistically significant differences between treatment groups. Cell viabilities were greater than 85% for all treatments tested.

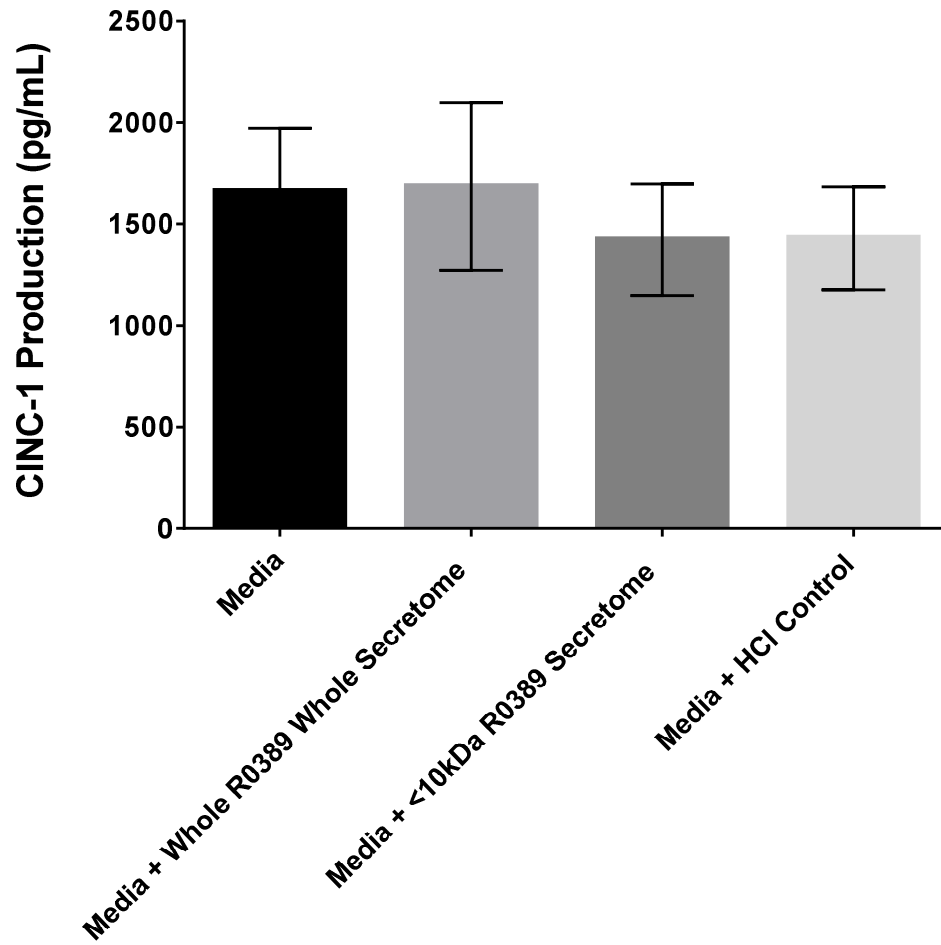


Figure 13. IEC-6 IEC cultured with 40% v/v R0389 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=3). Data shown are the mean CINC-1 production \pm SEM. Statistical analysis was performed using one-way ANOVA. There were no statistically significant differences between treatment groups. Cell viabilities were greater than 82% for all treatments tested.

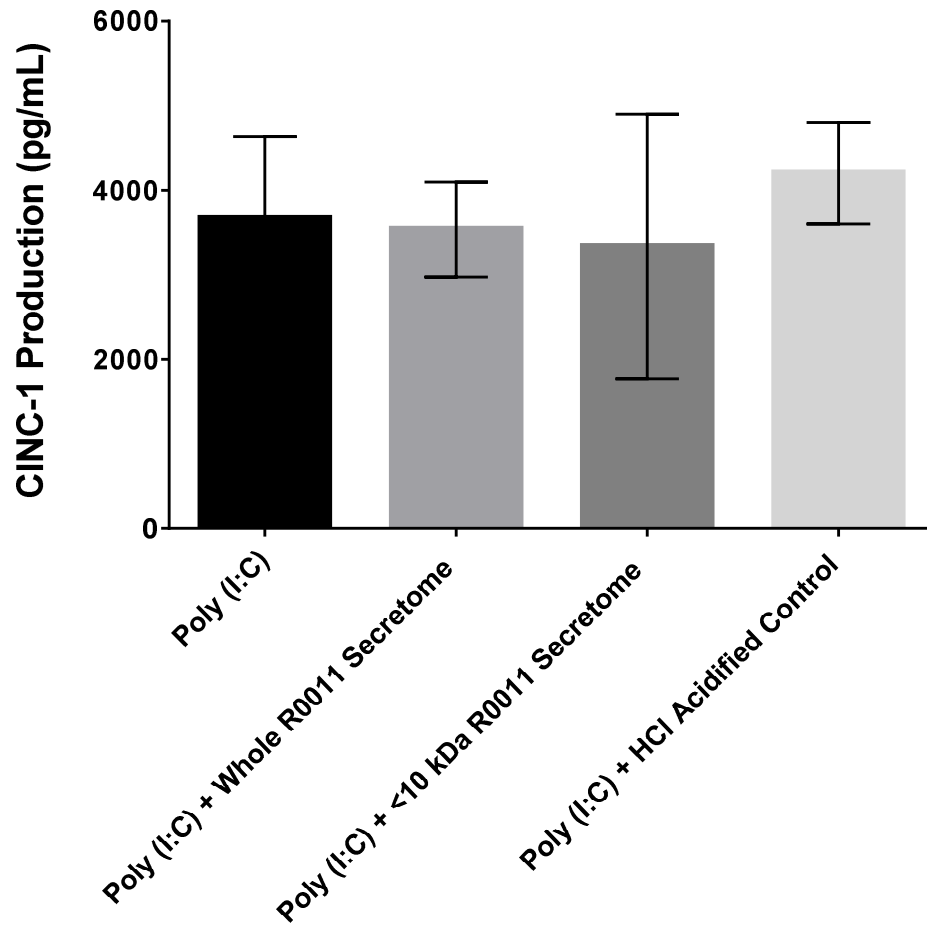


Figure 14. IEC-6 IEC stimulated with Poly(I:C) and cultured with 40% v/v R0011 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=6). Data shown are the mean CINC-1 production \pm SEM. Statistical analysis was performed using one-way ANOVA. There were no statistically significant differences between treatment groups. Cell viabilities were greater than 84% for all treatments tested.

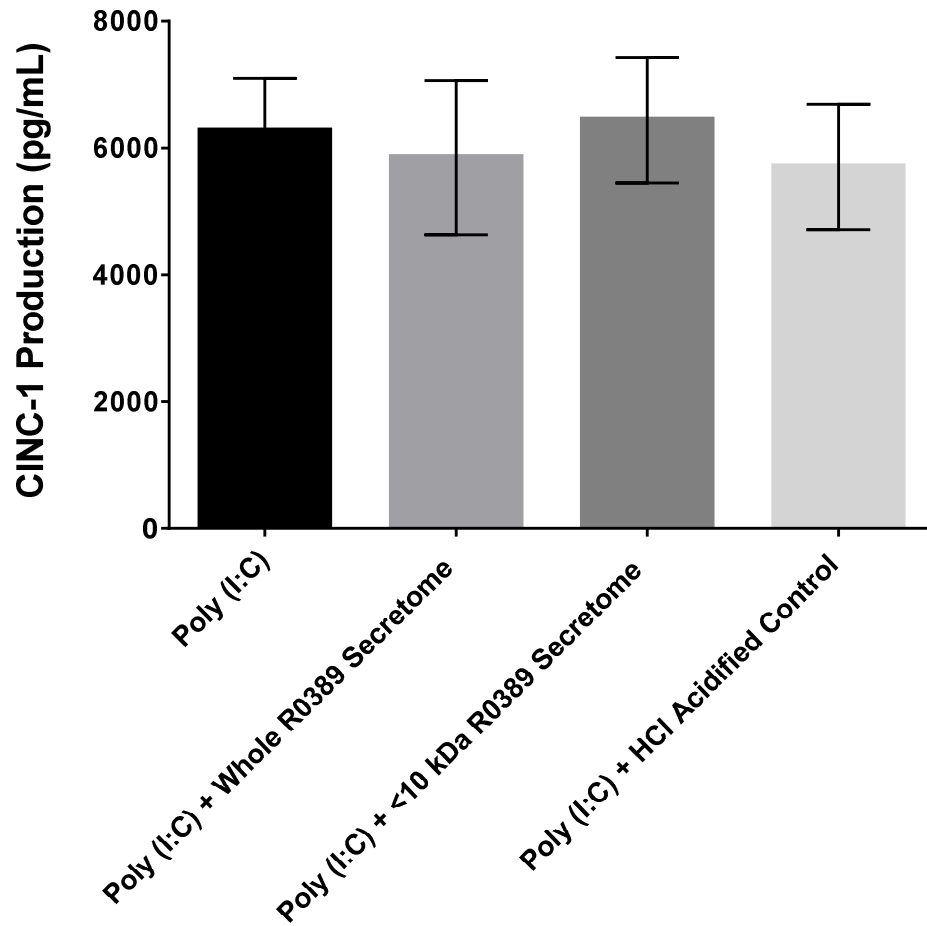


Figure 15. IEC-6 IEC stimulated with Poly(I:C) and cultured with 40% v/v R0389 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=3). Data shown are the mean CINC-1 production \pm SEM. Statistical analysis was performed using one-way ANOVA. There were no statistically significant differences between treatment groups. Cell viabilities were greater than 80% for all treatments tested.

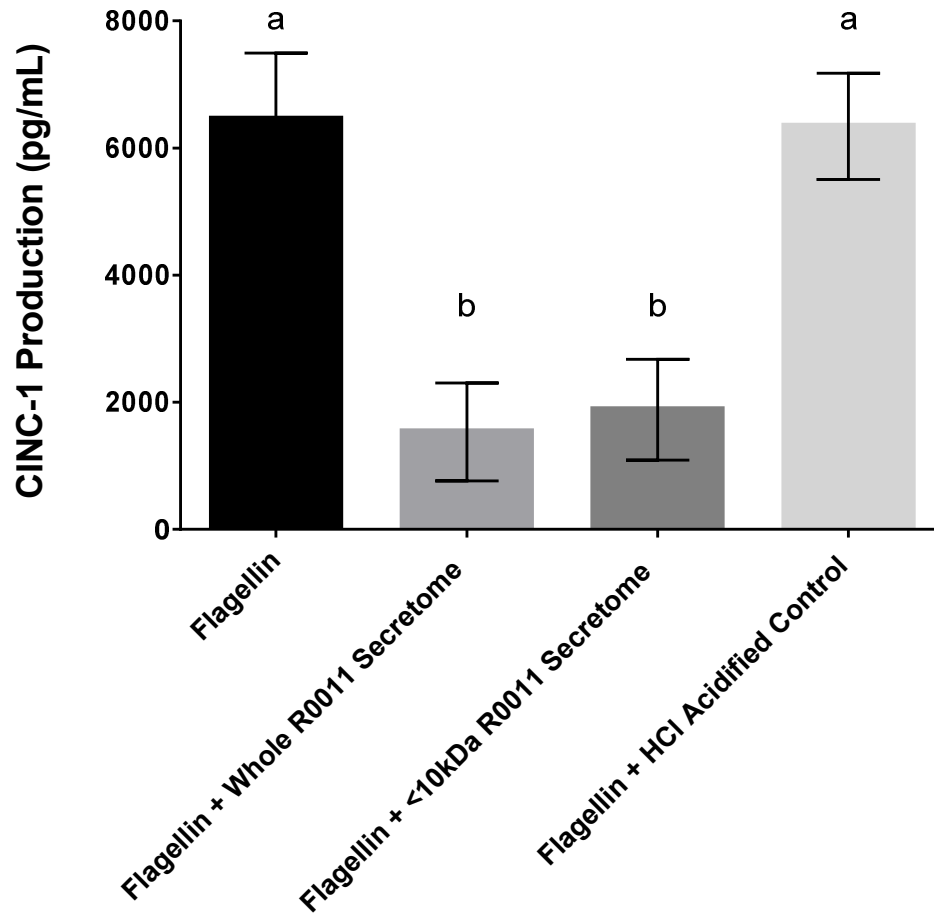


Figure 16. IEC-6 IEC stimulated with Flagellin and cultured with 40% v/v R0011 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=6). Data shown are the mean CINC-1 production \pm SEM. Statistical analysis was performed using one-way ANOVA ($P < 0.001$). Different letters denote significant differences between treatment groups. Cell viabilities were greater than 79% for all treatments tested.

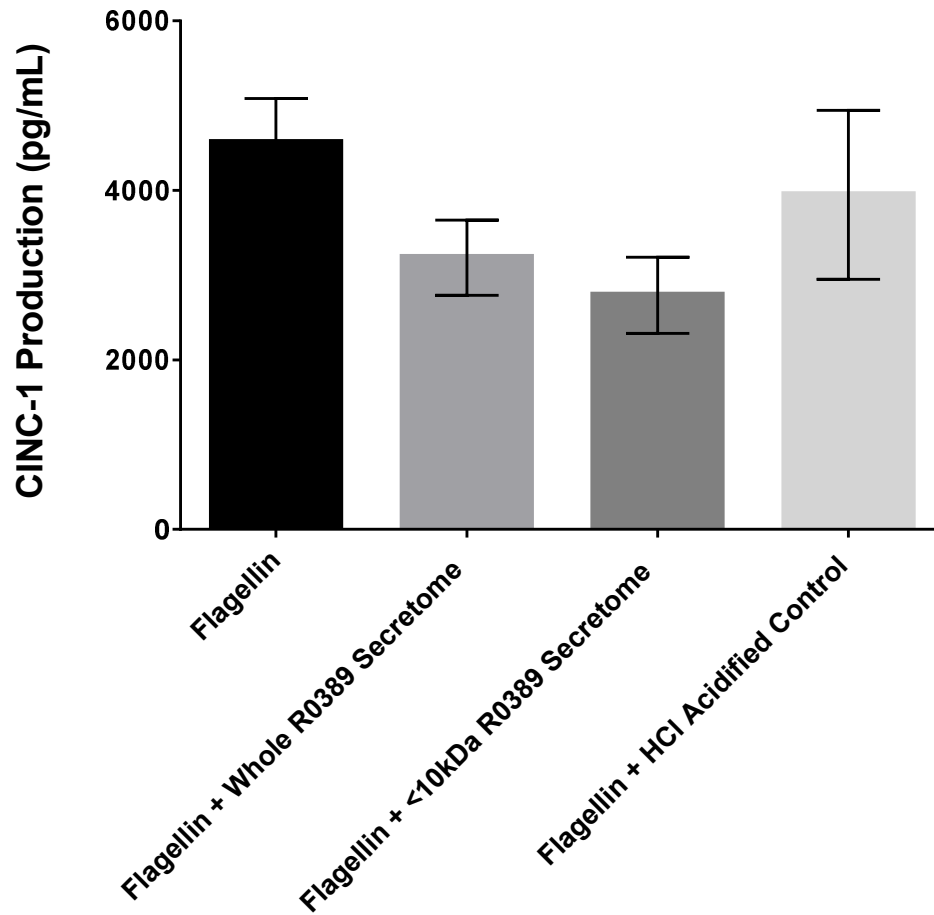


Figure 17. IEC-6 IEC stimulated with Flagellin and cultured with 40% v/v R0389 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=3). Data shown are the mean CINC-1 production \pm SEM. Statistical analysis was performed using one-way ANOVA. There were no statistically significant differences between treatment groups. Cell viabilities were greater than 82% for all treatments tested.

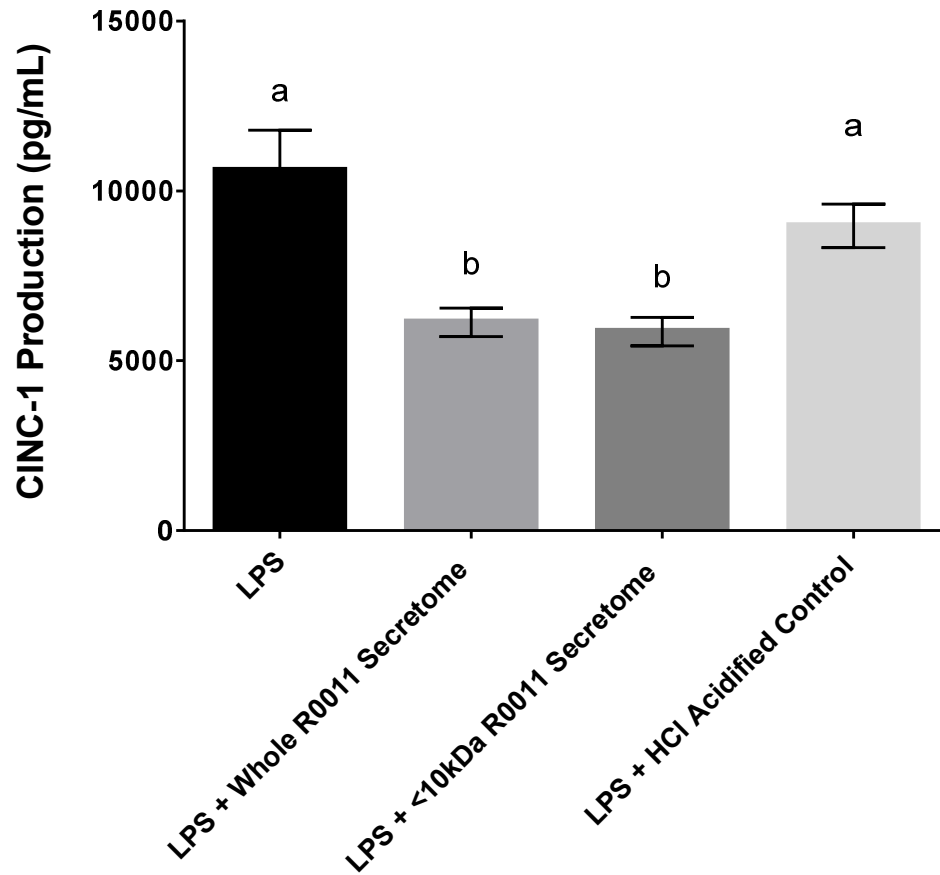


Figure 18. IEC-6 IEC stimulated with LPS and cultured with 40% v/v R0011 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=3). Data shown are the mean CINC-1 production \pm SEM. Statistical analysis was performed using one-way ANOVA (P<0.05). Different letters denote significant differences between treatment groups. Cell viabilities were greater than 76% for all treatments tested.

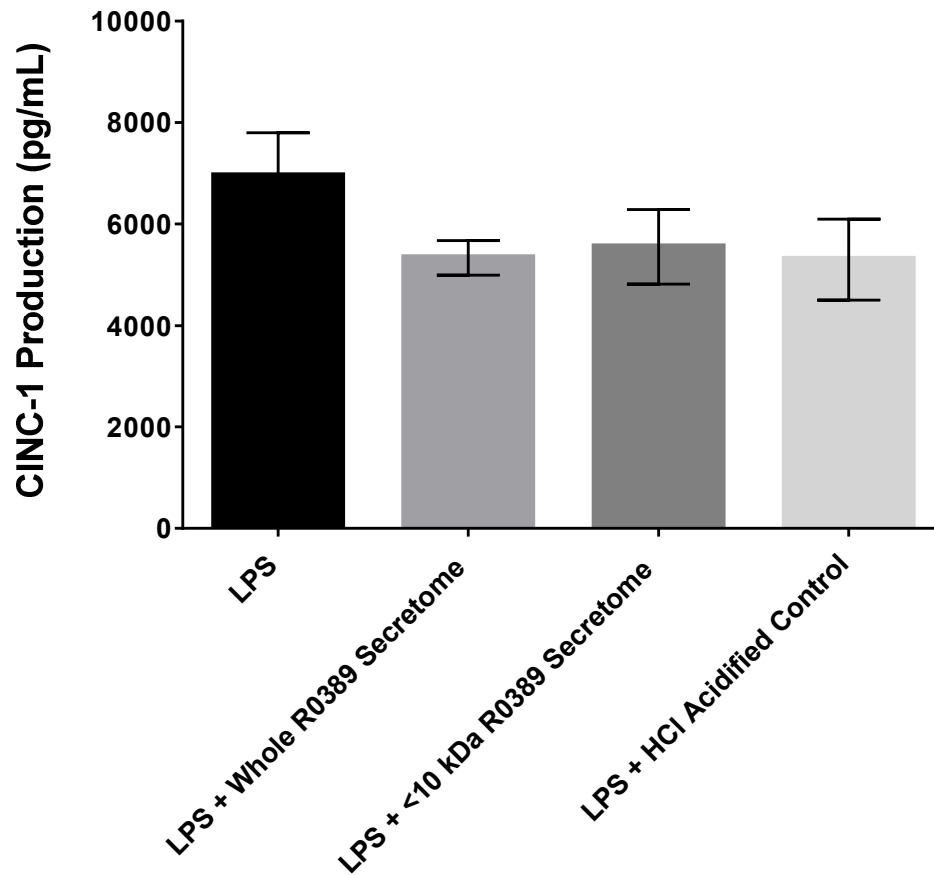


Figure 19. IEC-6 IEC stimulated with LPS and cultured with 40% v/v R0389 secretome (whole or <10 kDa fraction) or an HCl acidified control concurrently for 6 hours (n=3). Data shown are the mean CINC-1 production \pm SEM. Statistical analysis was performed using one-way ANOVA. There were no statistically significant differences between treatment groups. Cell viabilities were greater than 77% for all treatments tested.

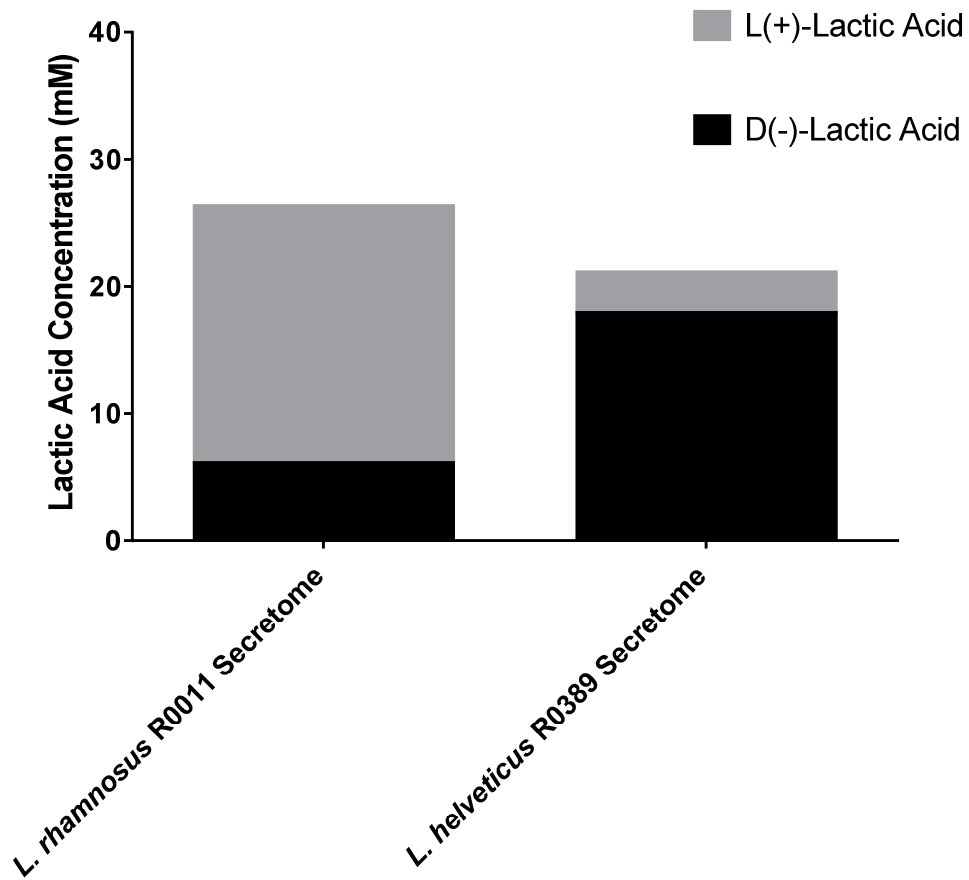


Figure 20. Lactic acid determination of both the *Lactobacillus helveticus* R0389 and the *Lactobacillus rhamnosus* R0011 secretomes. Data shown are the mean lactic acid concentration in mM (n=3).

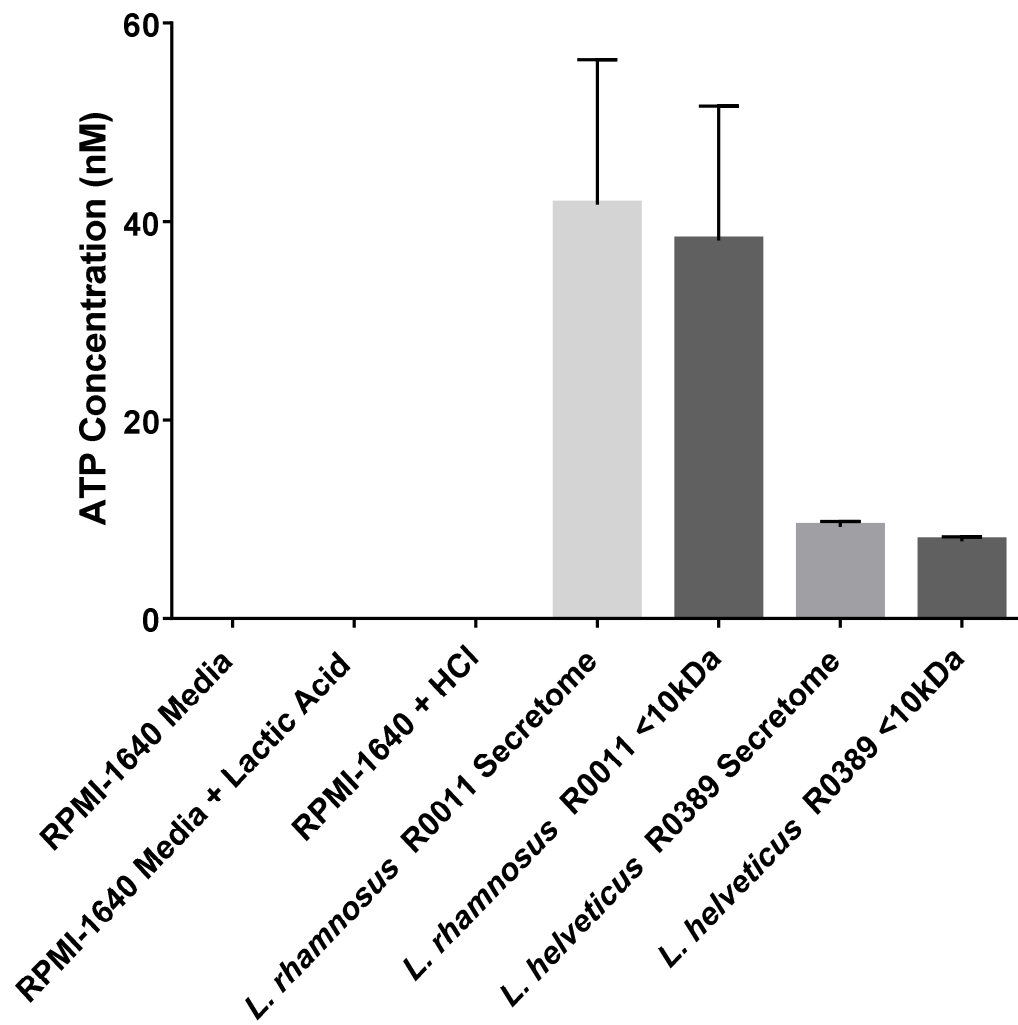


Figure 21. ATP determination within both the *Lactobacillus helveticus* R0389 and the *Lactobacillus rhamnosus* R0011 secretomes. Data shown are the mean ATP concentration in nM \pm SEM (n=3).

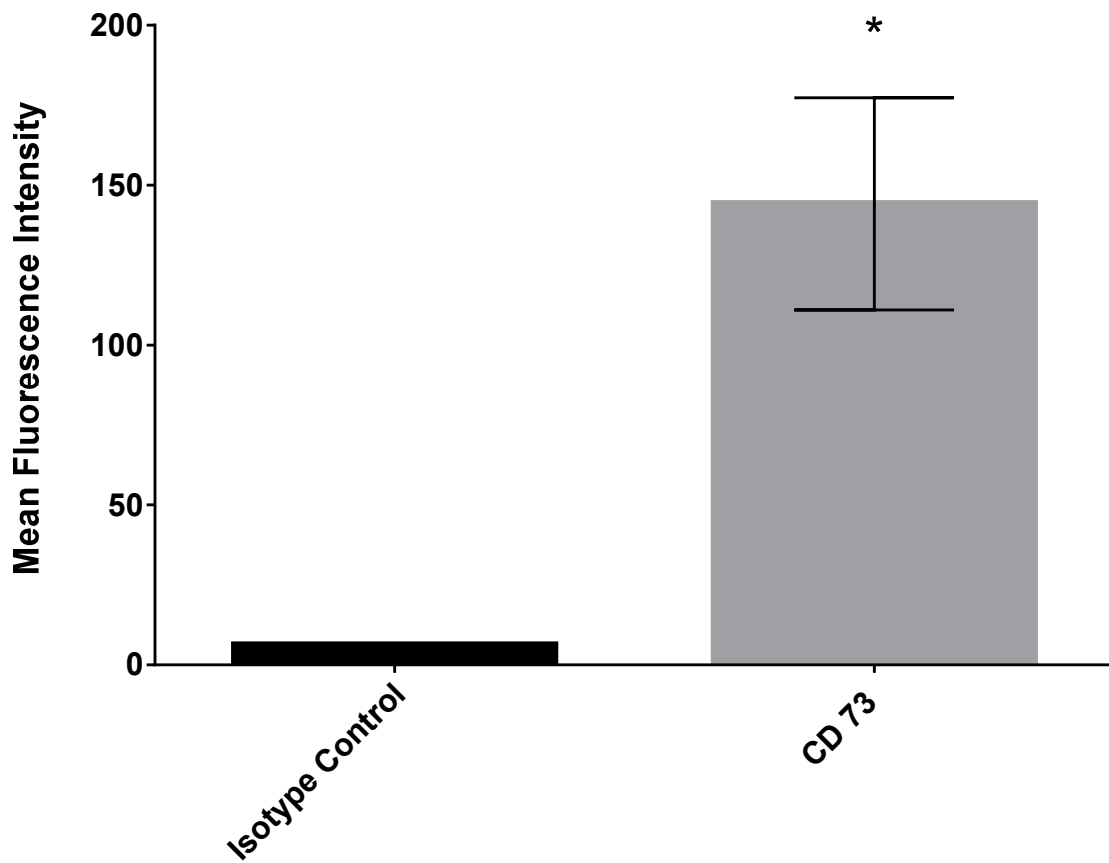


Figure 22. Constitutive CD73 expression on HT-29s. Data shown are the mean of the mean fluorescence intensity (MFI) \pm SEM (n=3). Statistical analysis was performed using Graphpad Student's t-test ($P < 0.05$). * indicates significance between groups. Cell viabilities were greater than 85%.

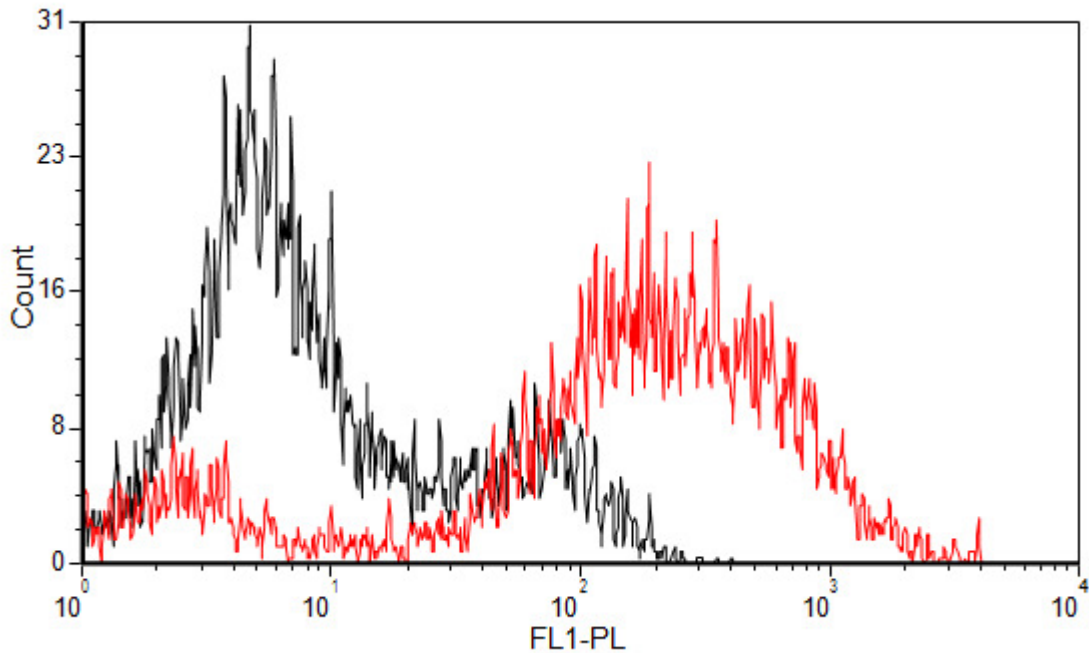


Figure 23. Histogram analysis of constitutive CD73 expression on HT-29 IECs. CD73 expression on HT-29 IEC (shown in red) is overlaid against the isotype control (shown in black). This is representative of four separate, independent experiments. The percent CD73+ population was determined to be s 57.14% using the Overton subtraction.

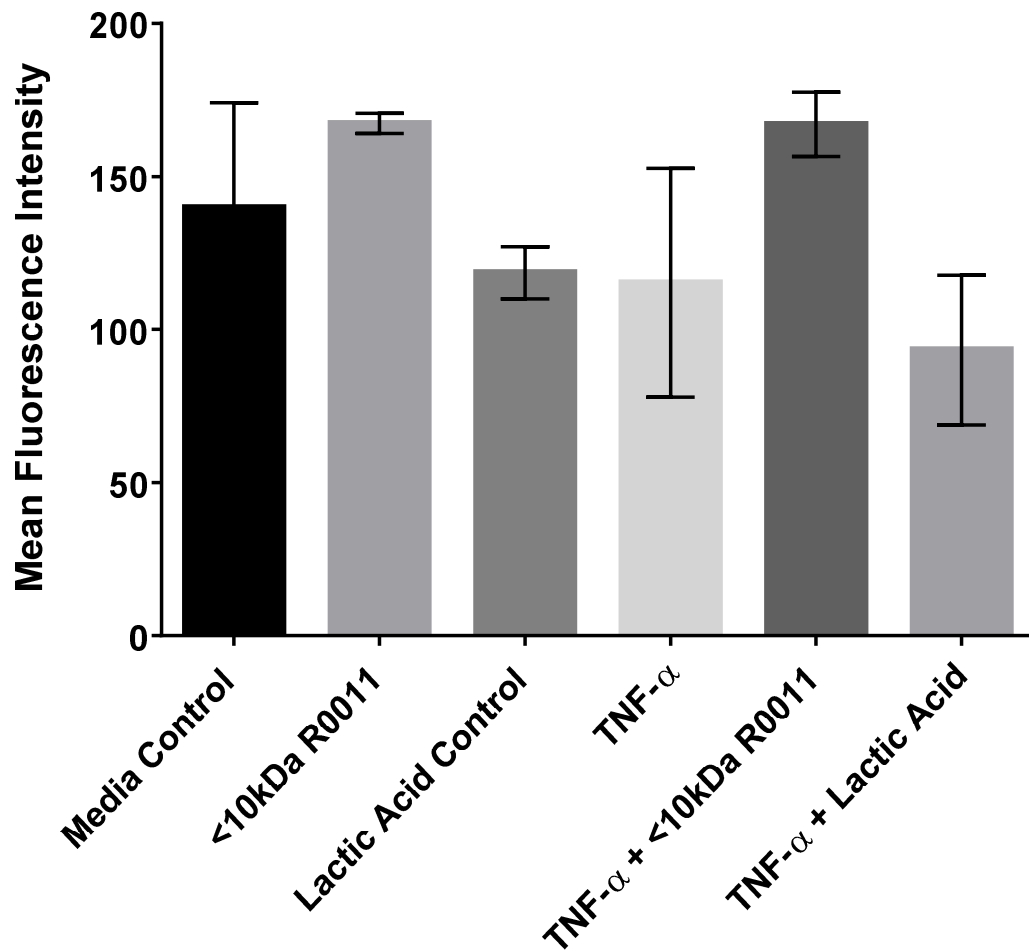


Figure 24. CD73 expression on HT-29s challenged with TNF- α \pm <10 kDa fraction of the *L. rhamnosus* R0011 secretome or a lactic acid control. Data shown are the mean of the mean fluorescence intensity (MFI) \pm SEM (n=3). Statistical analysis was performed using Graphpad Prism's one-way ANOVA ($P > 0.05$). There was no significant difference between any of the treatment groups. Cell viabilities were greater than 85%.

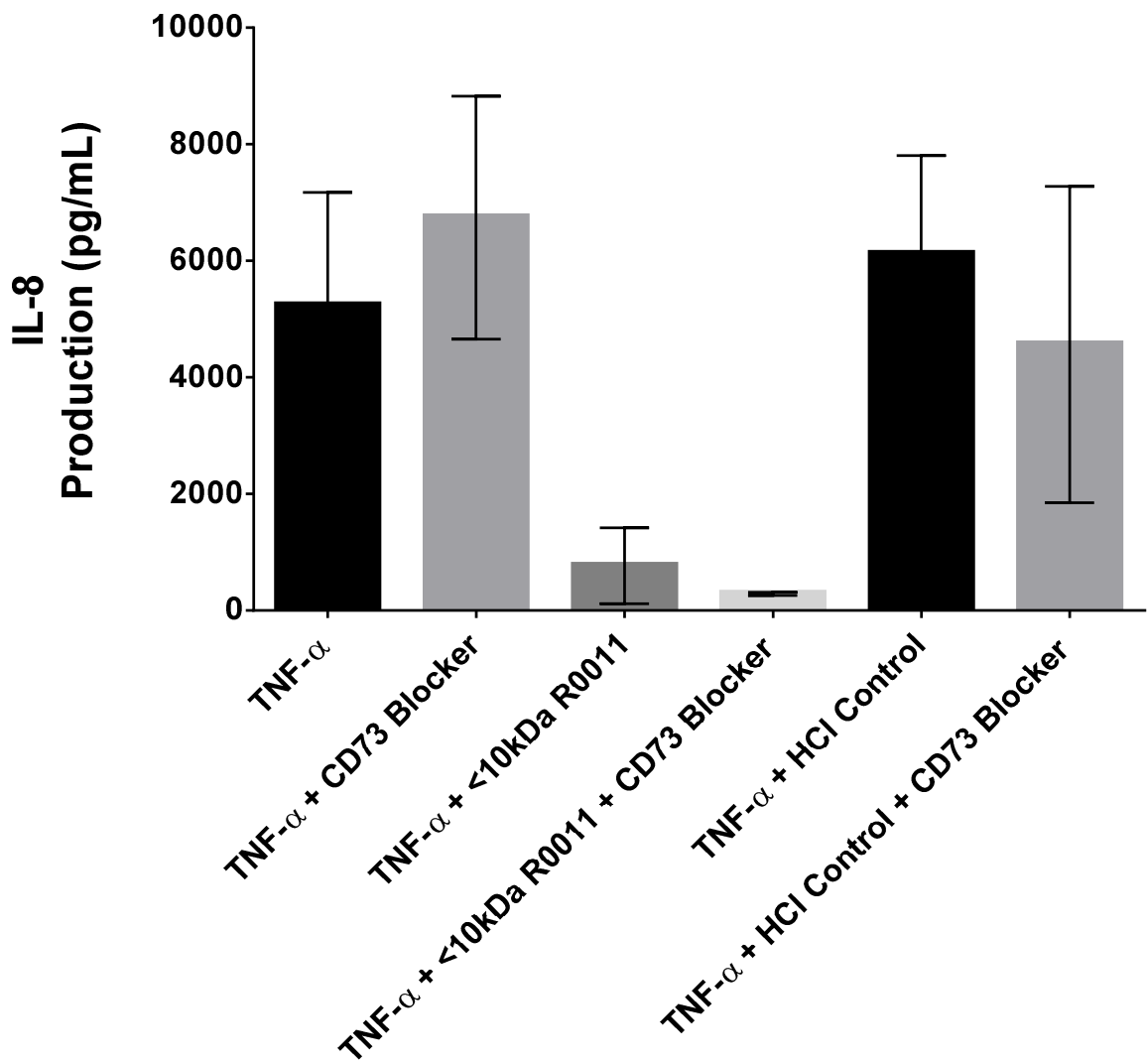


Figure 25. HT-29 IEC challenged with TNF- α \pm <10 kDa fraction of the *L. rhamnosus* R0011 secretome or a lactic acid control concurrently with a selective CD73 blocker (Adenosine 5'-(α,β -methylene)diphosphate; 50 μ M) for 4 hours. Data shown are the mean IL-8 production \pm SEM (n=2).

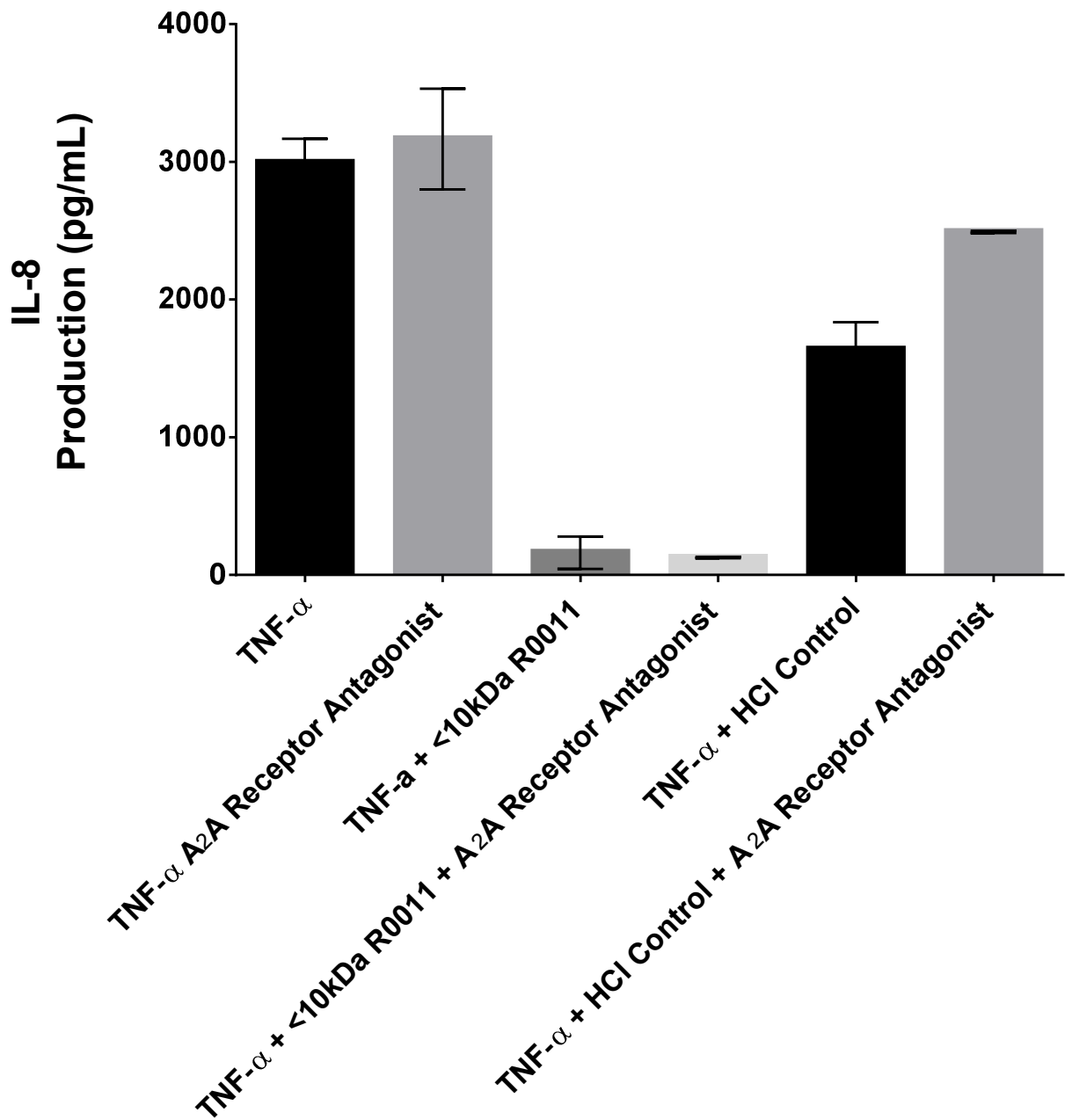


Figure 26. HT-29 IEC challenged with TNF- α \pm <10 kDa fraction of the *L. rhamnosus* R0011 secretome or an HCl acidified control concurrently with an A₂A receptor antagonist (ZM241385; 500 nM) for 6 hours. Data shown are the mean IL-8 production \pm SEM (n=2).

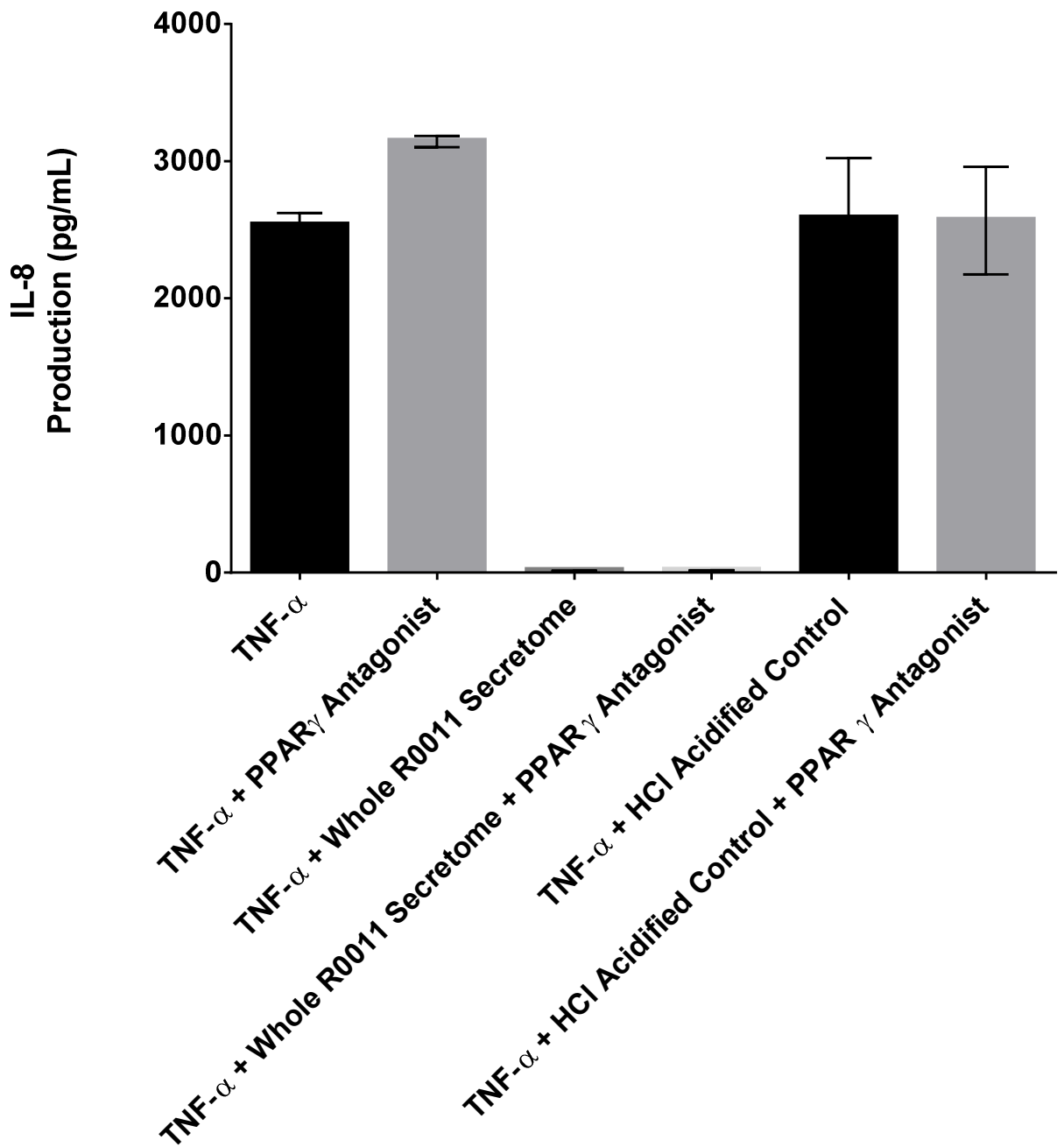


Figure 27. HT-29 IEC challenged with TNF- α \pm <10 kDa fraction of the *L. rhamnosus* R0011 secretome or an HCl acidified control concurrently with a PPAR γ receptor antagonist (GW9662; 5 μ M) for 6 hours. Data shown are the mean IL-8 production \pm SEM (n=2).

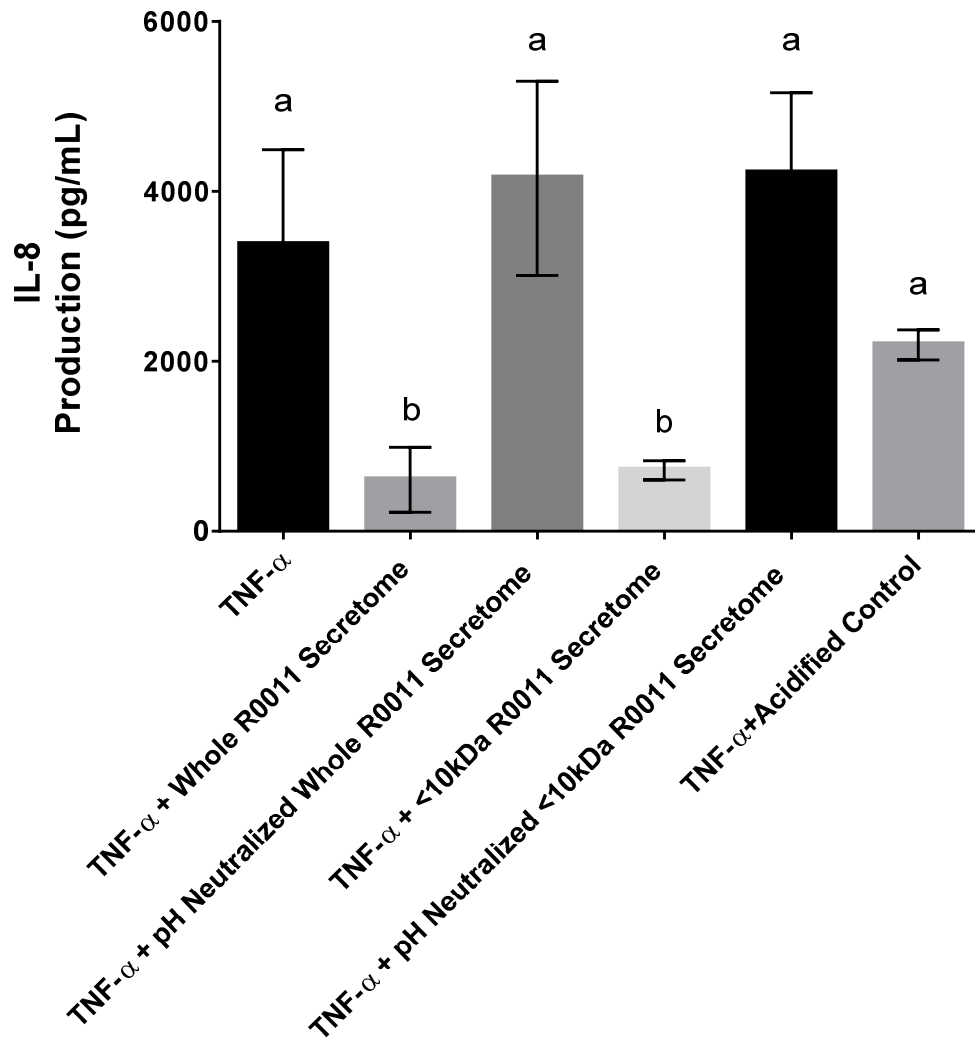


Figure 28. HT-29 IEC stimulated TNF- α and cultured with 40% v/v R0011 secretome (whole or <10 kDa fraction), pH neutralized R0011 secretome, or an HCl acidified control concurrently for 6 hours (n=3). Data shown are the mean IL-8 production \pm SEM. Statistical analysis was performed using one-way ANOVA (P=0.0004). Different letters denote significant differences between treatment groups. Cell viabilities were greater than 60% for all treatments tested

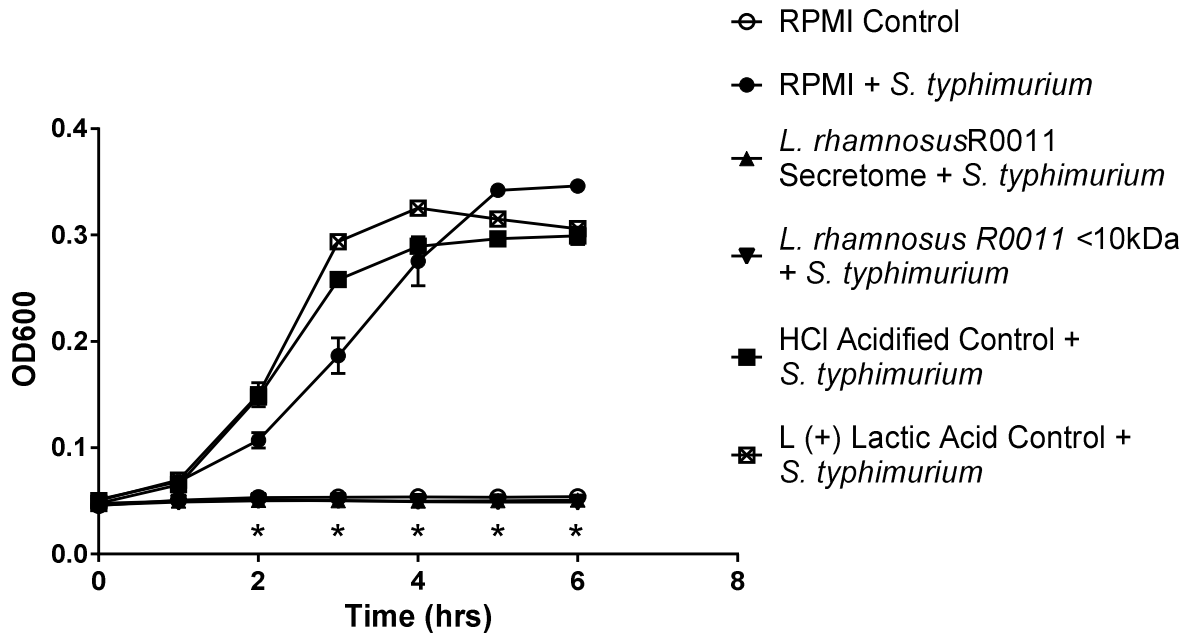


Figure 29. Growth of *Salmonella typhimurium* when cultured with the *L. rhamnosus* R0011 secretome over a 6 hour period. Data shown are the mean OD600 reading \pm SEM (n=3). * denotes statistical differences between RPMI-grown *S. typhimurium* and the different treatment groups as determined using ANOVA (P = 0.001) and Dunnett's multiple comparison test.

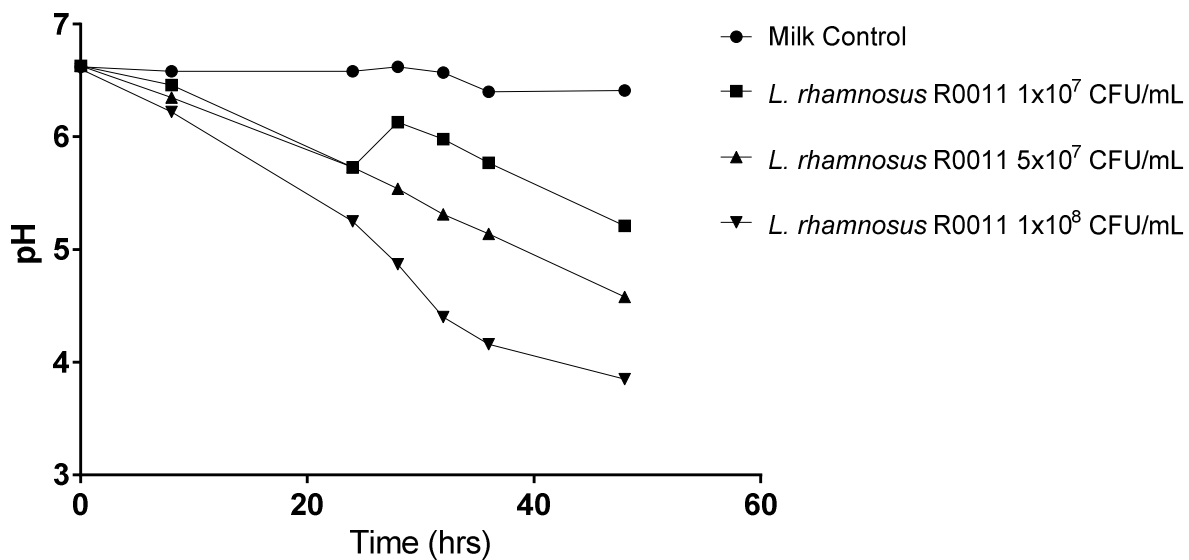


Figure 30. Fermentation kinetics of differing starting inoculum sizes of *L. rhamnosus* R0011 in 3.25% Milk Fat milk. Data shown are the change in pH over time (n=1).

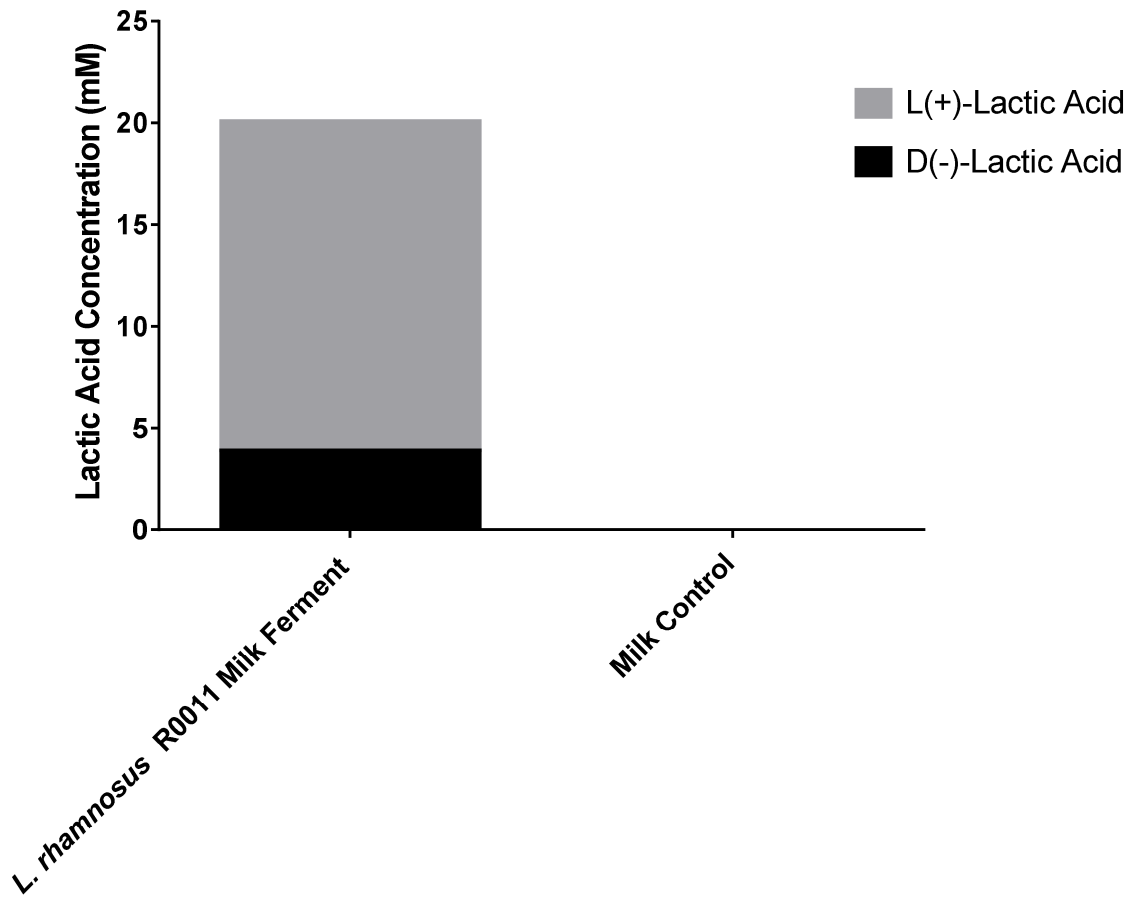


Figure 31. Lactic acid determination of the *L. rhamnosus* R0011 milk ferments. Data shown are the mean lactic acid concentration in mM (n=3).

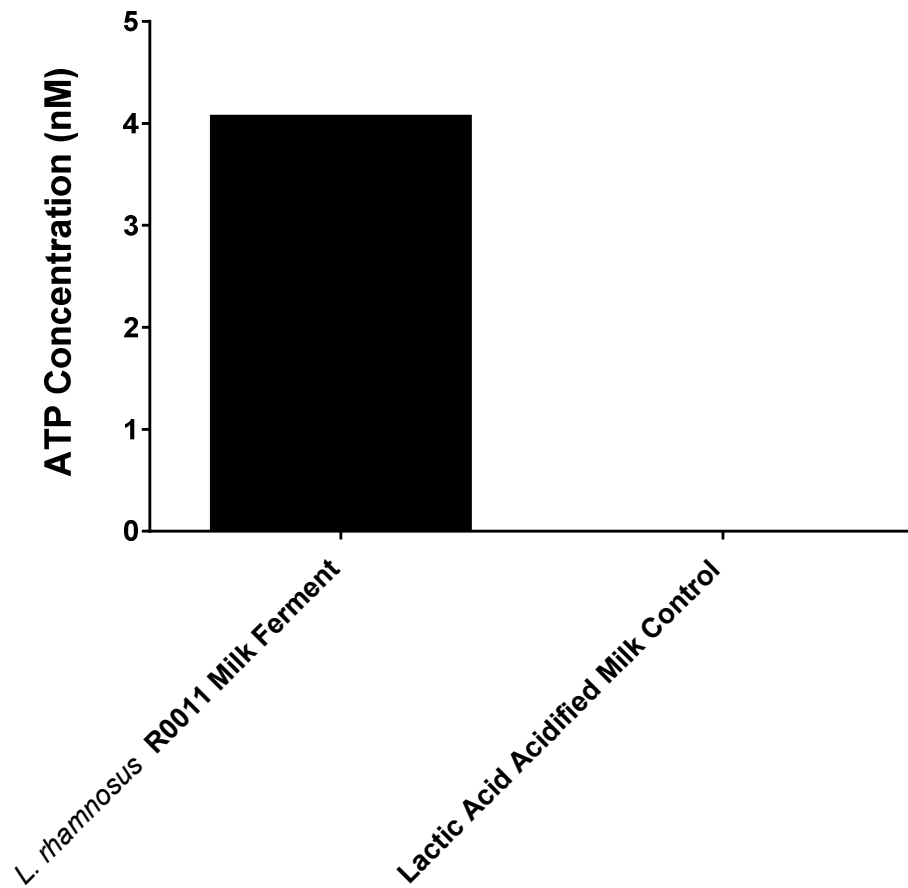


Figure 32. ATP determination within the *L. rhamnosus* R0011 milk ferment. Data shown are the mean ATP concentration in nM (n=2).

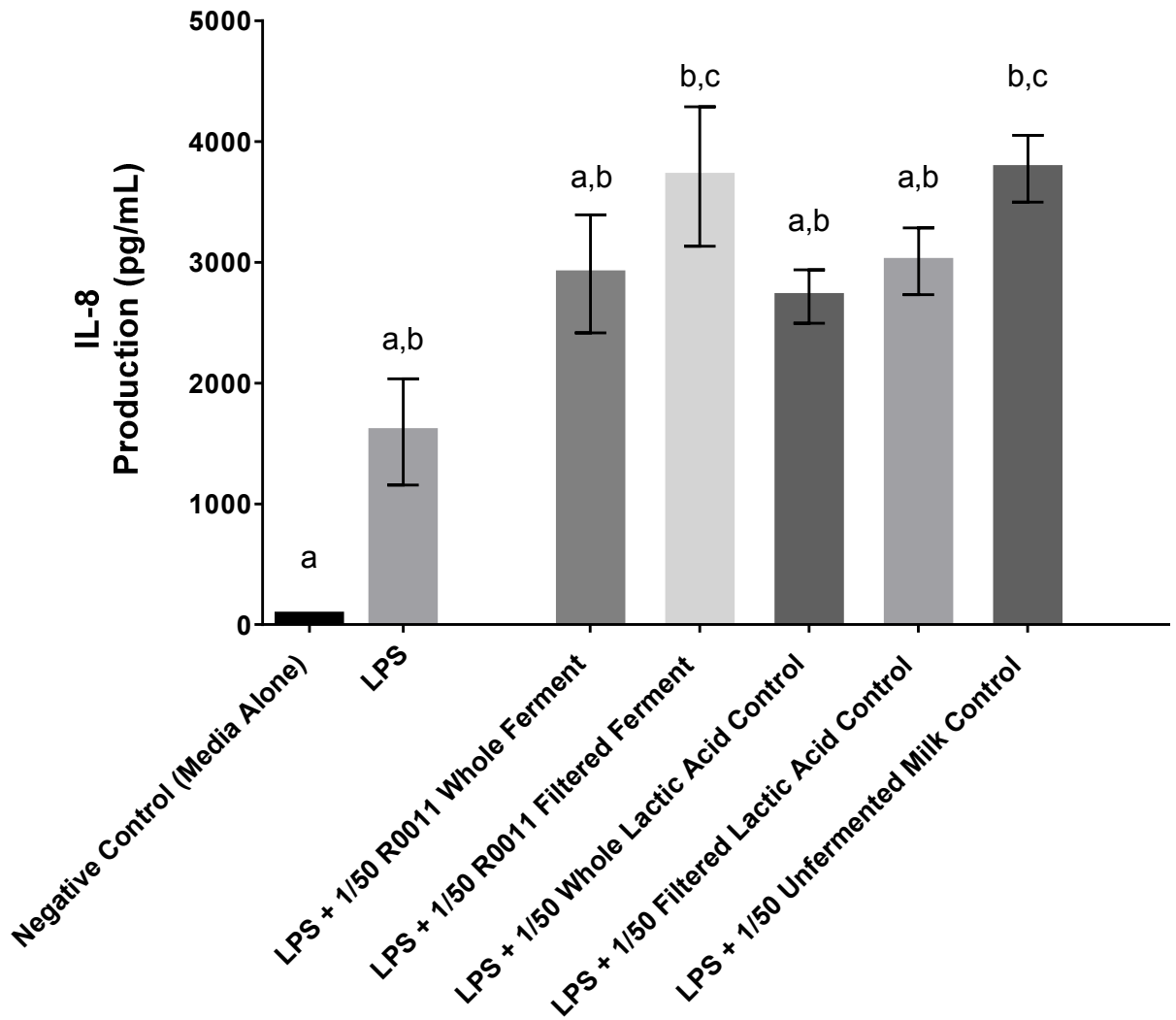


Figure 33. IL-8 production by U937 monocytes pre-treated with *L. rhamnosus* R0011 whole/filtered ferments or milk controls for 18 hours followed by stimulating with 125 ng/mL LPS for 6 hours (mean IL-8 production \pm SEM; n=3). Different letters denote statistically significant differences between treatment groups as determined using one-way ANOVA ($P < 0.05$) and Tukey's multiple comparison test. Cell viabilities were $> 80\%$ for all treatments as determined by XTT and Trypan Blue Exclusion Viability assay.

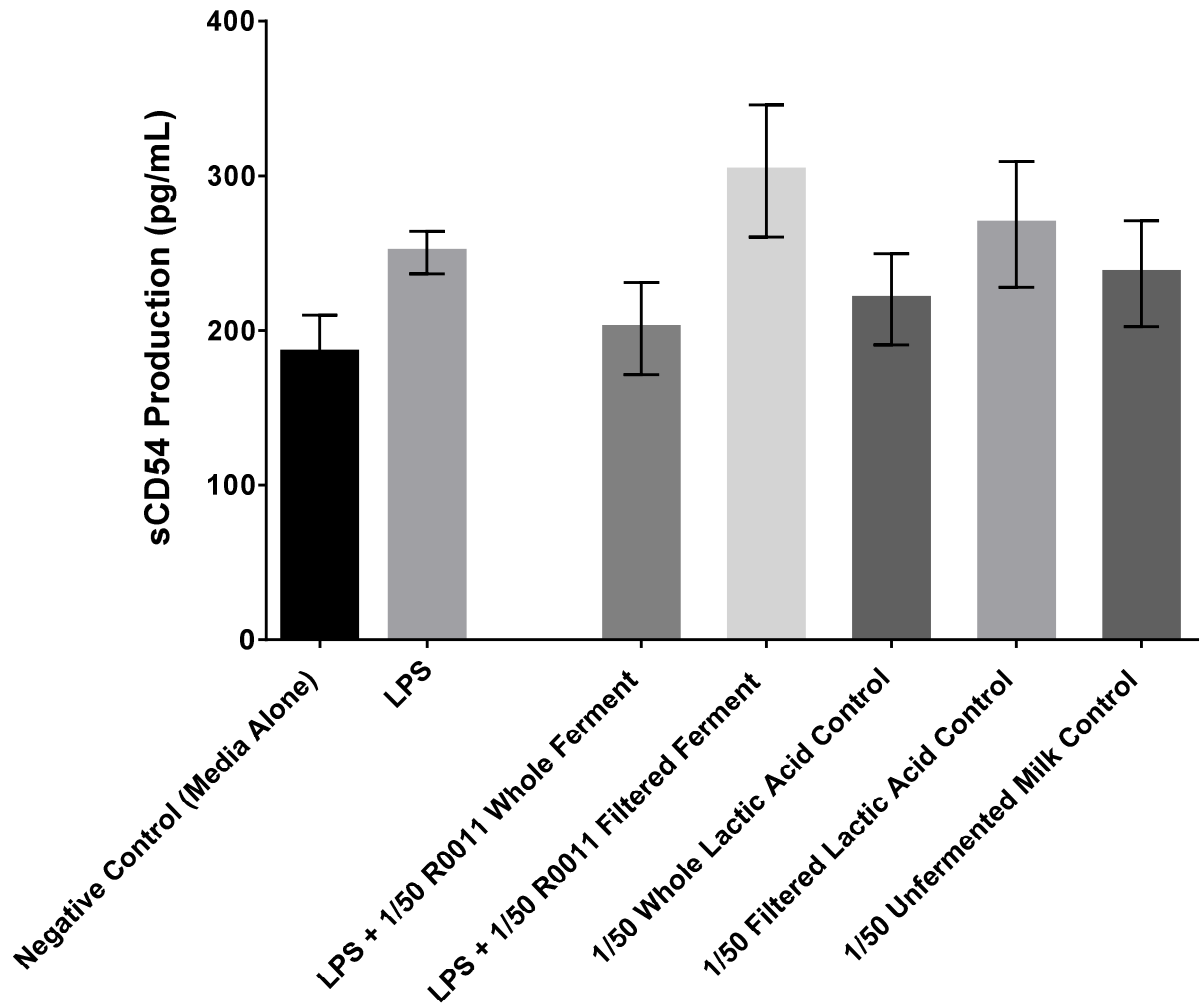


Figure 34. sCD54 production by U937 monocytes pre-treated with *L. rhamnosus* R0011 whole/filtered ferments or milk controls for 18 hours followed by stimulating with 125 ng/mL LPS for 6 hours (mean sCD54 production \pm SEM; n=3). There was no statistical significance between treatment groups as determined using one-way ANOVA ($P > 0.05$). Cell viabilities were $> 80\%$ for all treatments as determined by XTT and Trypan Blue Exclusion Viability assay.

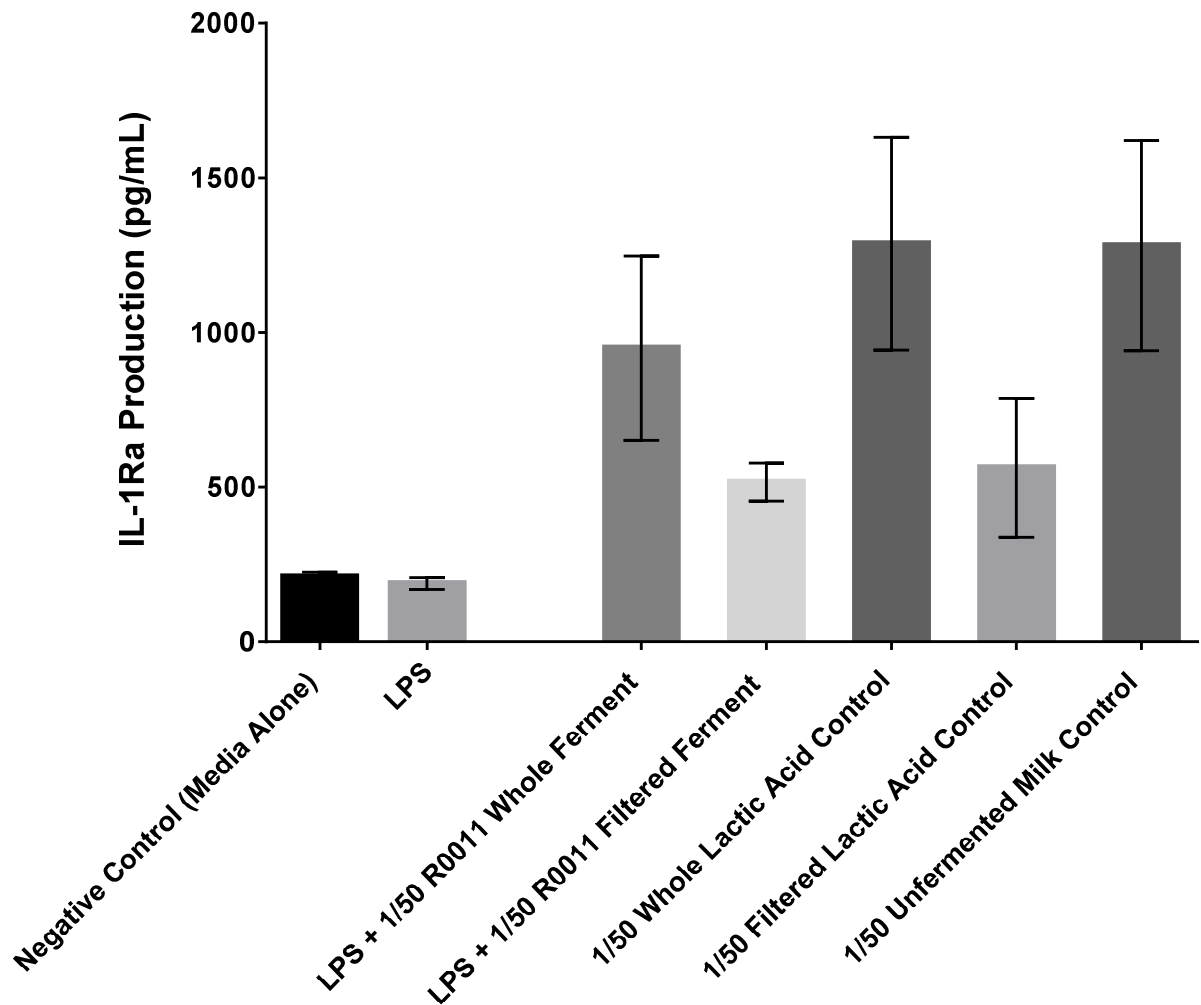


Figure 35. IL-1Ra production by U937 monocytes pre-treated with *L. rhamnosus* R0011 whole/filtered ferments or milk controls for 18 hours followed by stimulating with 125 ng/mL LPS for 6 hours (mean IL-1Ra production \pm SEM; n=3). There were no statistical significance between treatment groups as determined using one-way ANOVA ($P > 0.05$). Cell viabilities were $> 80\%$ for all treatments as determined by the XTT and Trypan Blue Exclusion Viability assay.

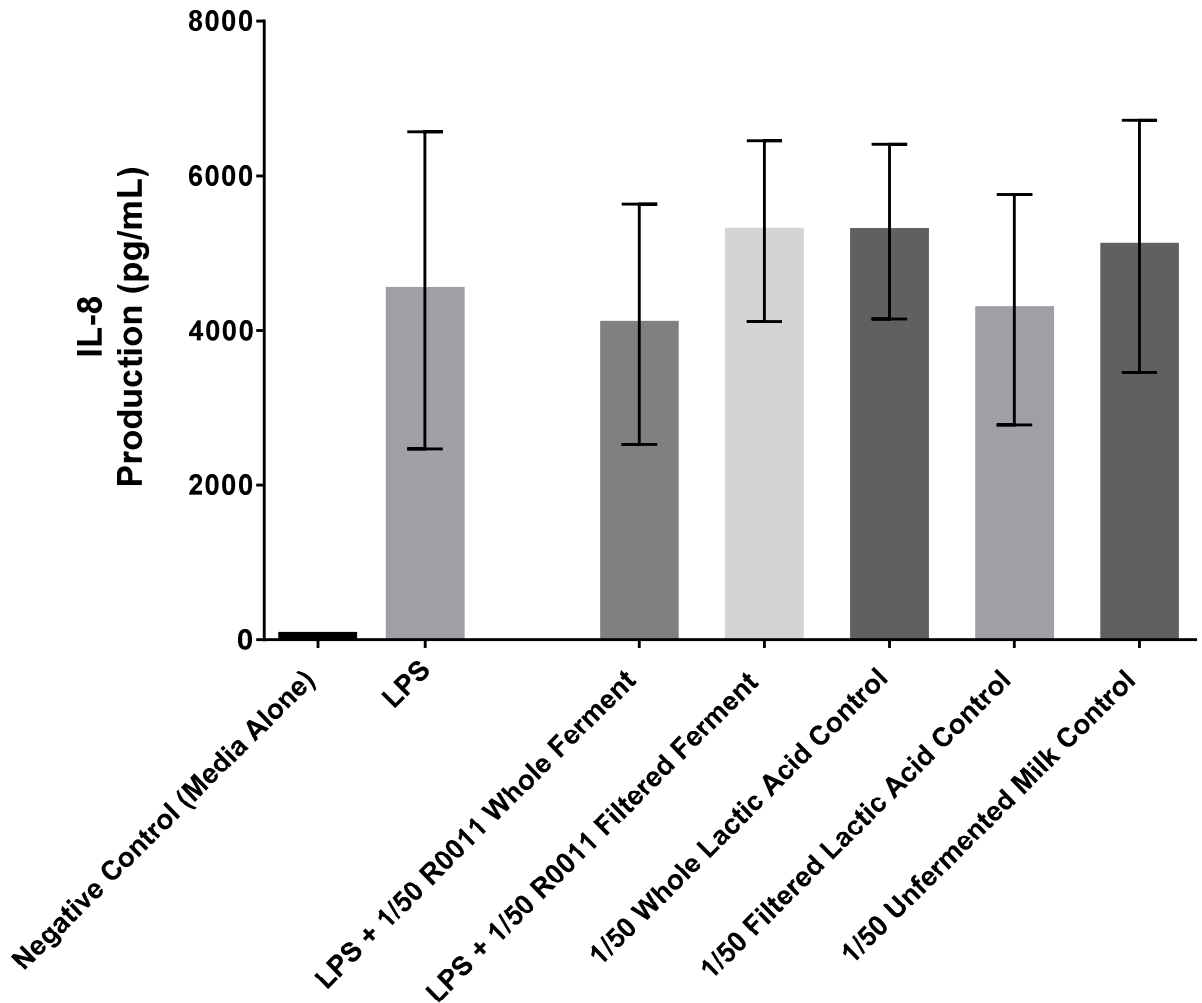


Figure 36. IL-8 production by U937 monocytes stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean IL-8 production \pm SEM; n=3). There were no significant differences between treatment groups as determined by one-way ANOVA ($P > 0.05$). Cell viabilities were $> 80\%$ for all treatments as determined by XTT and Trypan Blue Exclusion Viability assay.

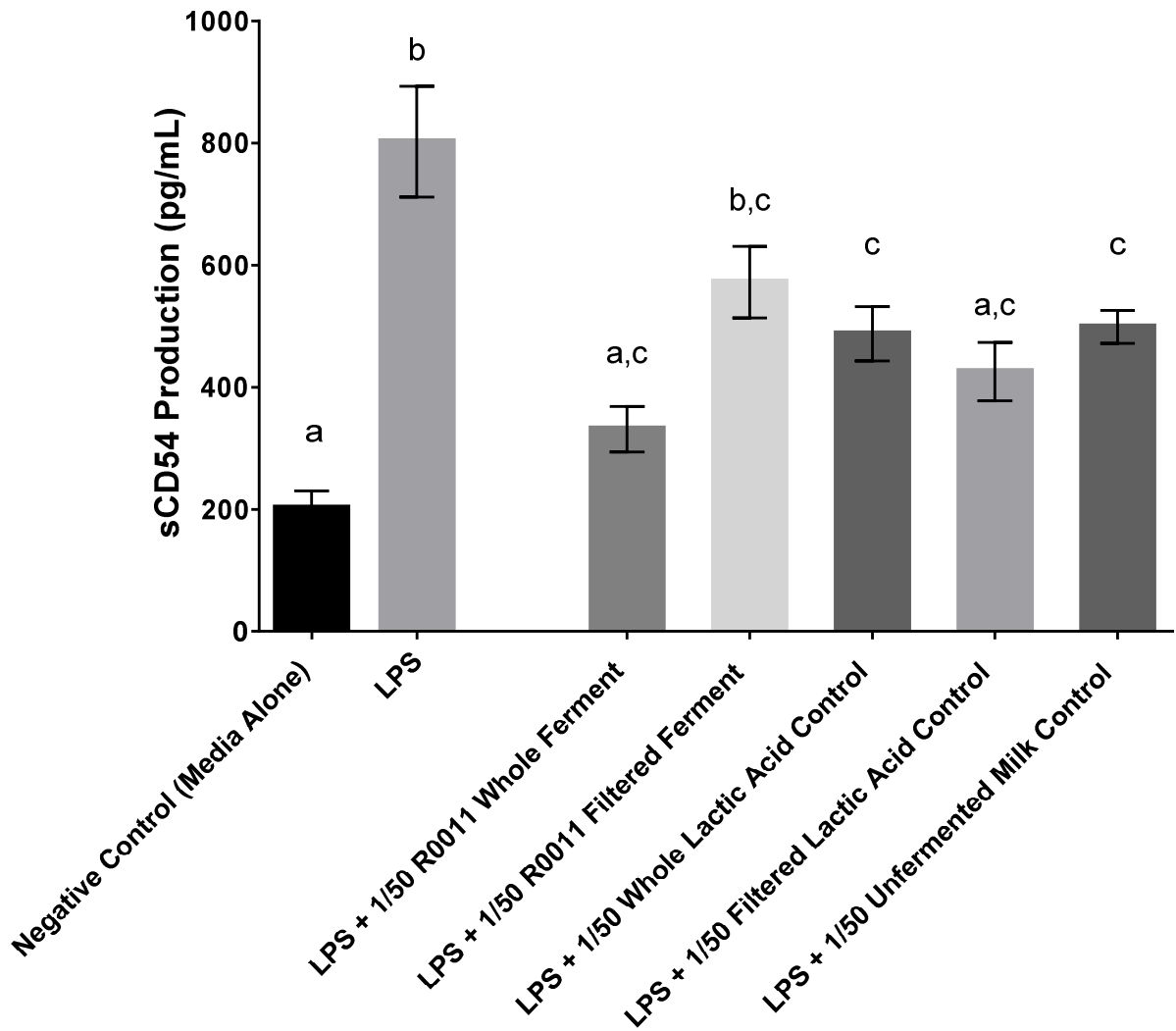


Figure 37. sCD54 production by U937 monocytes stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean sCD54 production \pm SEM; n=3). Different letters denote statistically significant differences between treatment groups as determined using one-way ANOVA ($P < 0.05$) and Tukey's multiple comparison test. Cell viabilities were $> 80\%$ for all treatments as determined by XTT and Trypan Blue Exclusion Viability assay.

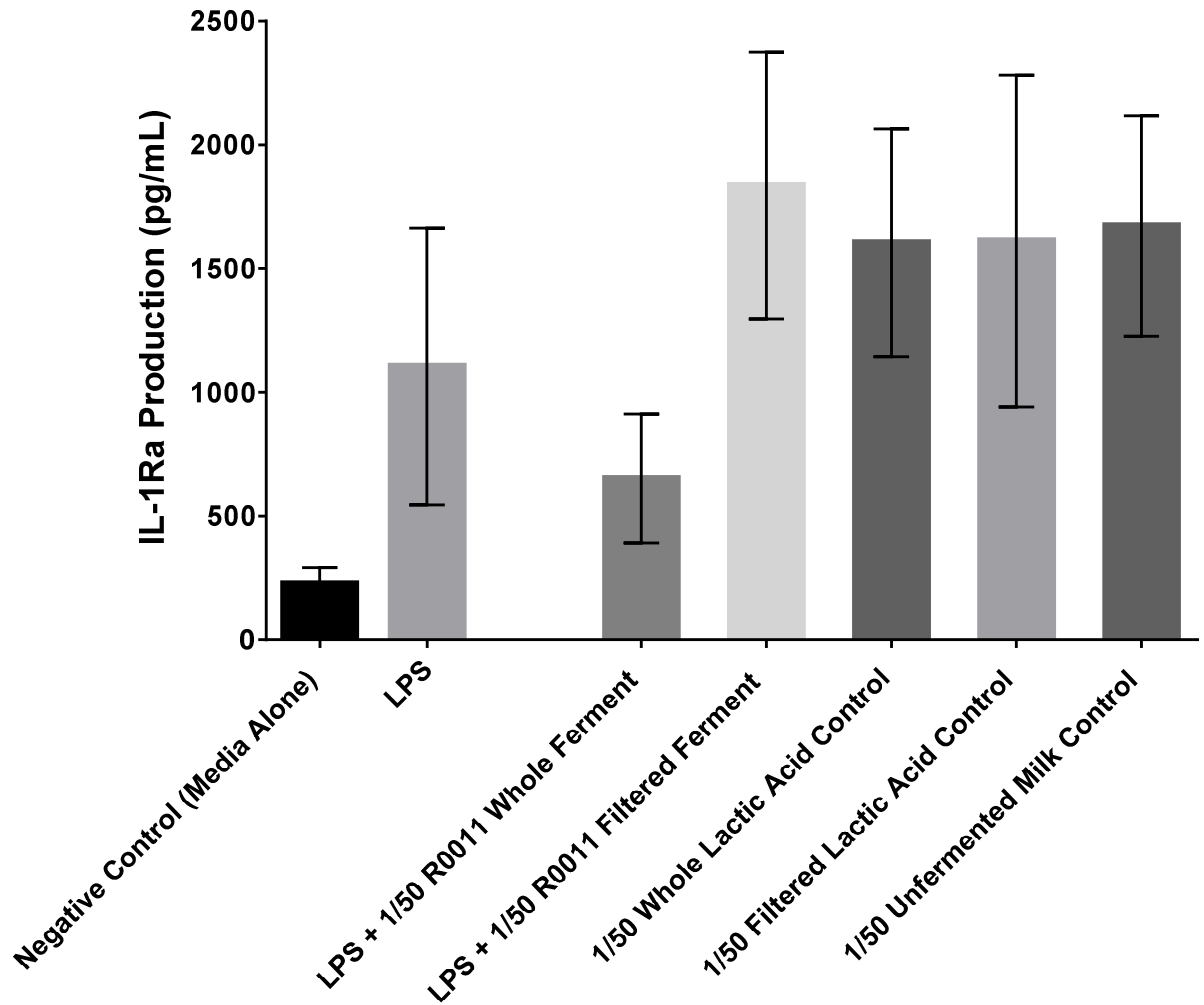


Figure 38. IL-1Ra production by U937 monocytes stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean IL-1Ra production \pm SEM; n=3). There was no significant differences between treatment groups as determined by one-way ANOVA ($P > 0.05$). Cell viabilities were $> 80\%$ for all treatments as determined by XTT and Trypan Blue Exclusion Viability assay.

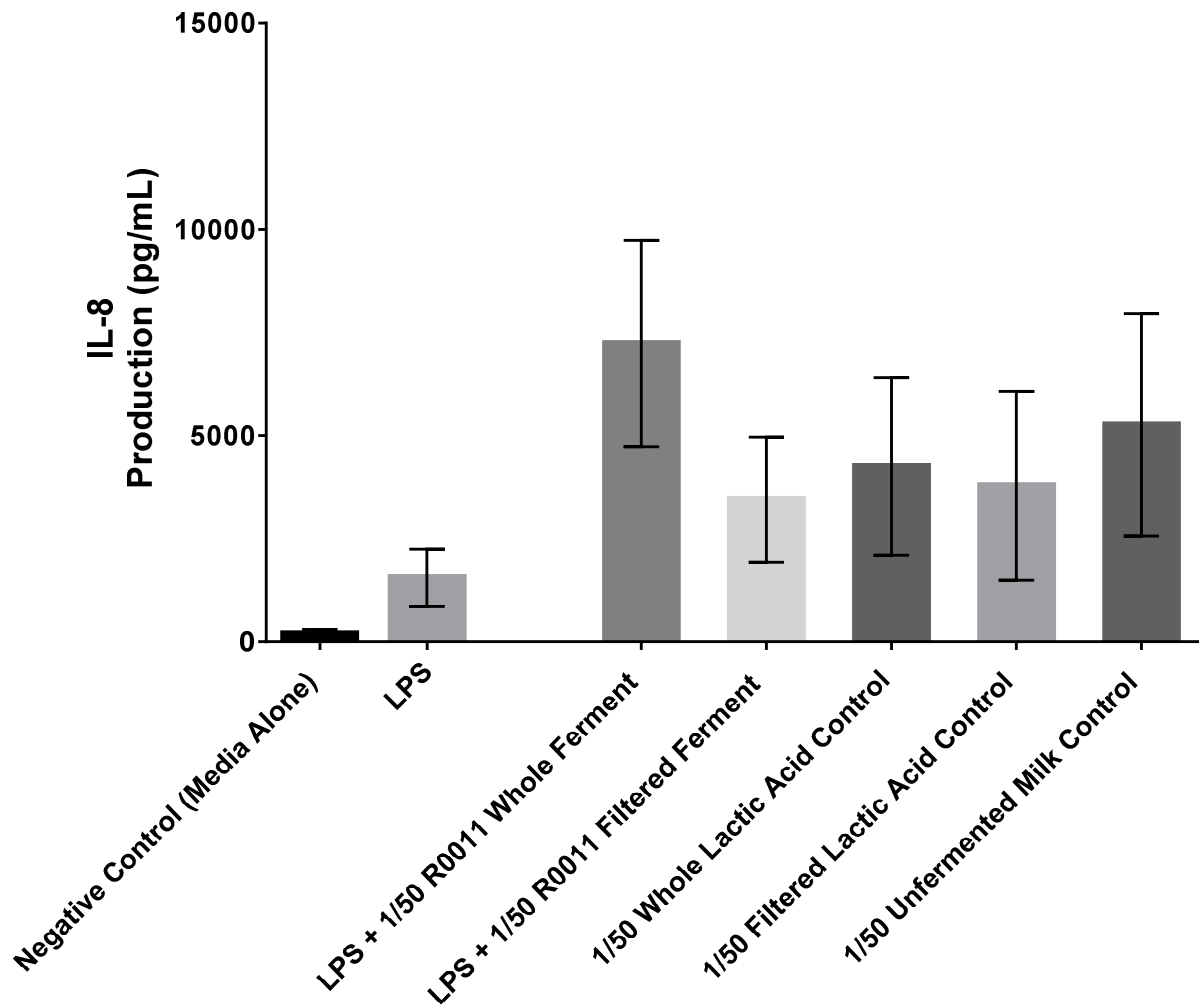


Figure 39. IL-8 production by THP-1 monocytes pre-treated with *L. rhamnosus* R0011 whole/filtered ferments or milk controls for 18 hours followed by stimulating with 125 ng/mL LPS for 6 hours (mean IL-8 production \pm SEM; n=3). There was no significant differences between treatment groups as determined by one-way ANOVA ($P > 0.05$). Cell viabilities were $> 80\%$ for all treatments as determined by XTT and Trypan Blue Exclusion Viability assay.

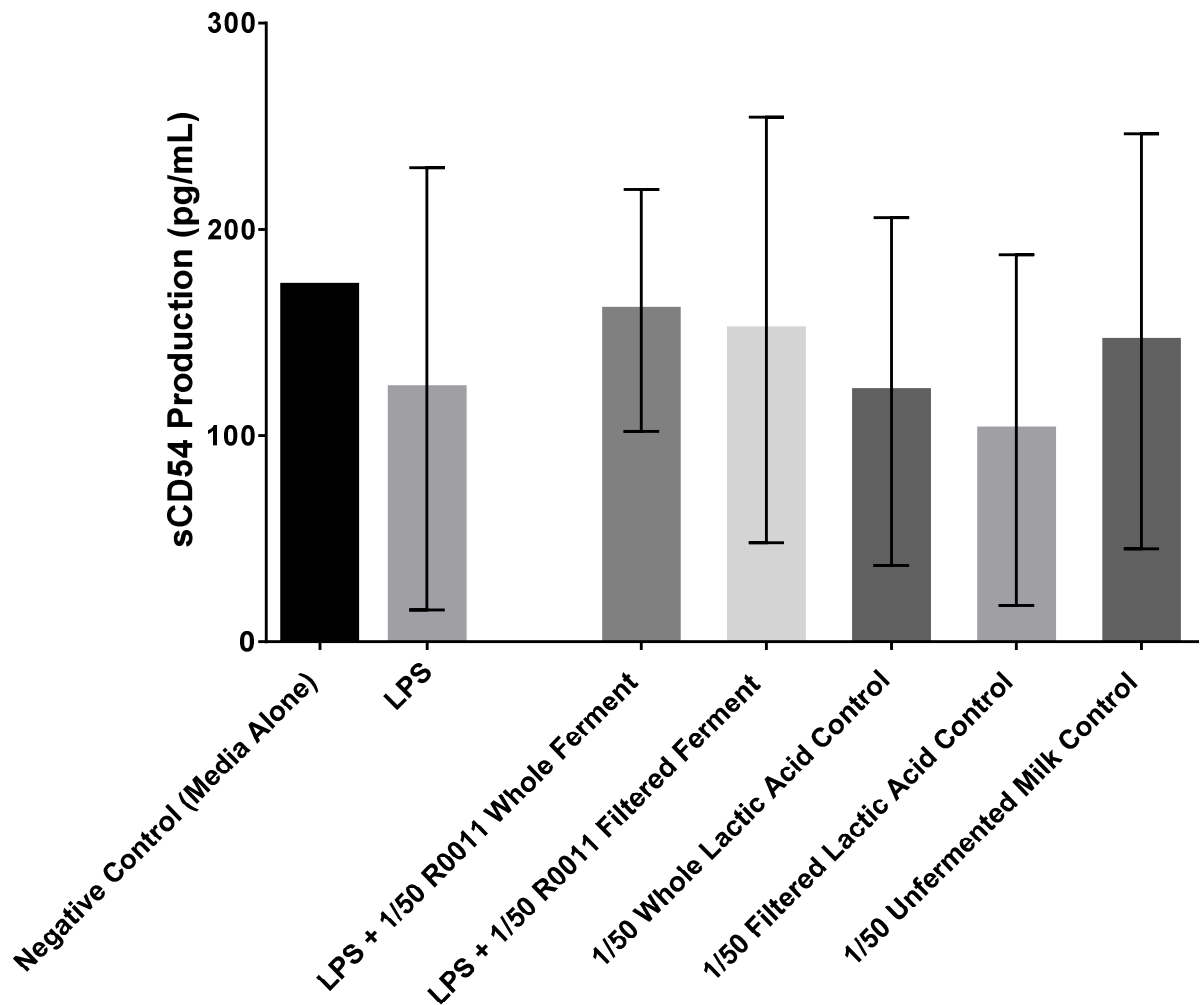


Figure 40. sCD54 production by THP-1 monocytes pre-treated with *L. rhamnosus* R0011 whole/filtered ferments or milk controls for 18 hours followed by stimulating with 125 ng/mL LPS for 6 hours (mean sCD54 production \pm SEM; n=3). There was no significant differences between treatment groups as determined by one-way ANOVA ($P > 0.05$). Cell viabilities were $> 80\%$ for all treatments as determined by XTT and Trypan Blue Exclusion Viability assay.

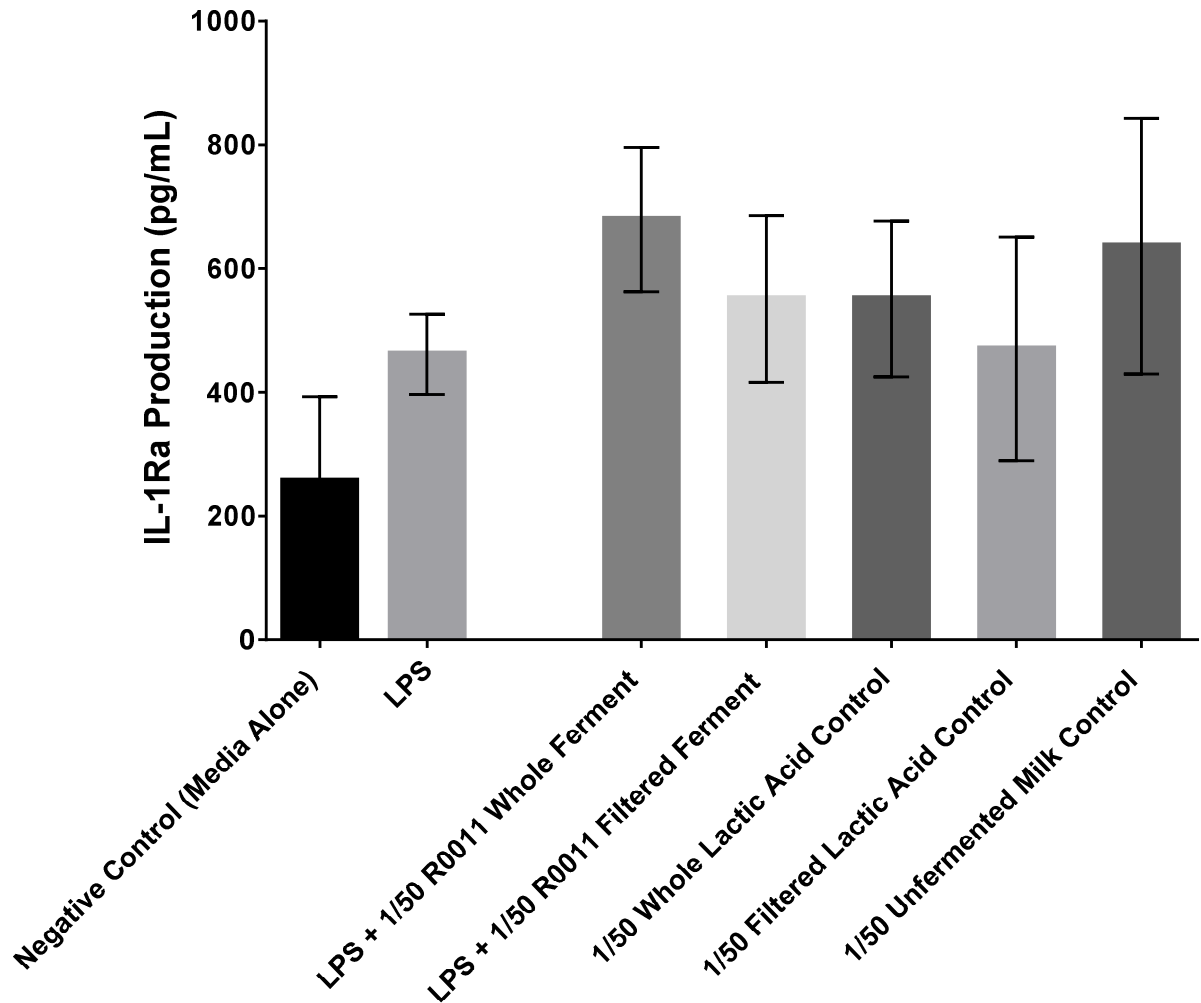


Figure 41. IL-1Ra production by THP-1 monocytes pre-treated with *L. rhamnosus* R0011 whole/filtered ferments or milk controls for 18 hours followed by stimulating with 125 ng/mL LPS for 6 hours (mean IL-1Ra production \pm SEM; n=3). There was no significant differences between treatment groups as determined by one-way ANOVA ($P > 0.05$). Cell viabilities were $> 80\%$ for all treatments as determined by XTT and Trypan Blue Exclusion Viability assay.

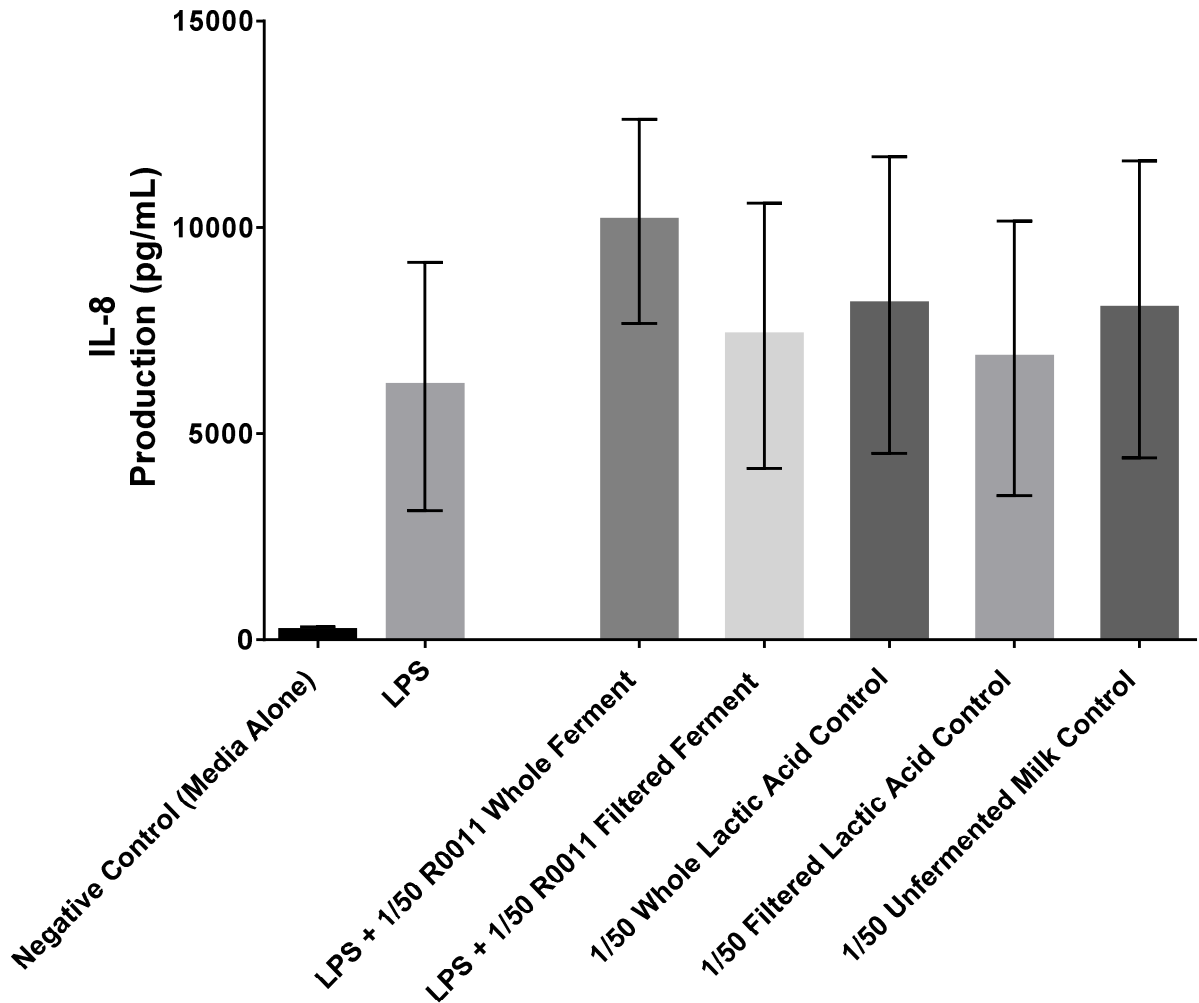


Figure 42. IL-8 production by THP-1 monocytes stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean IL-8 production \pm SEM; n=3). There was no significant differences between treatment groups as determined by one-way ANOVA ($P < 0.05$). Cell viabilities were $> 80\%$ for all treatments as determined by XTT and Trypan Blue exclusion viability assay.

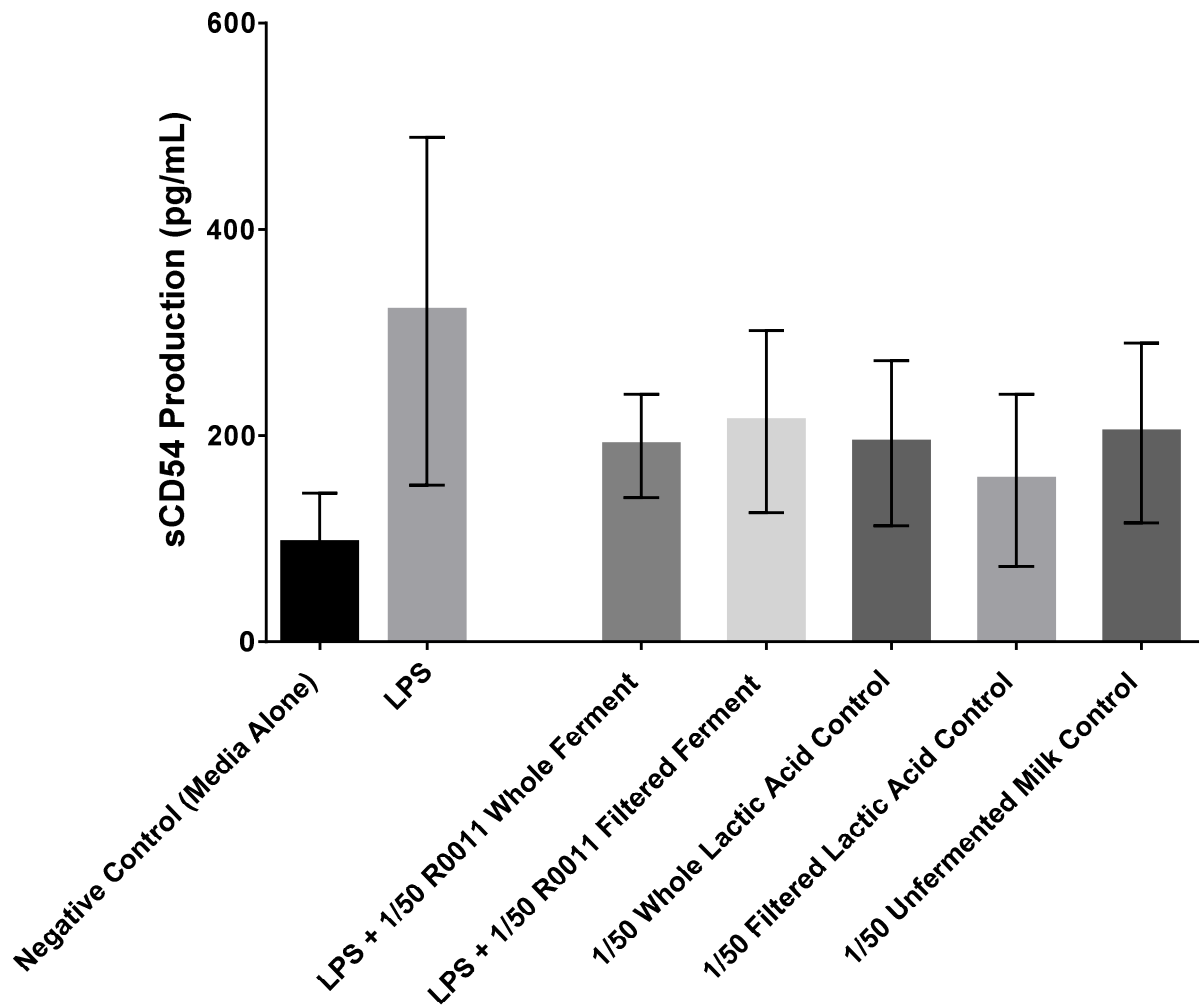


Figure 43. sCD54 production by THP-1 monocytes stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean sCD54 production \pm SEM; n=3). There was no significant differences between treatment groups as determined by one-way ANOVA ($P < 0.05$). Cell viabilities were $> 80\%$ for all treatments as determined by XTT and Trypan Blue Exclusion Viability assay.

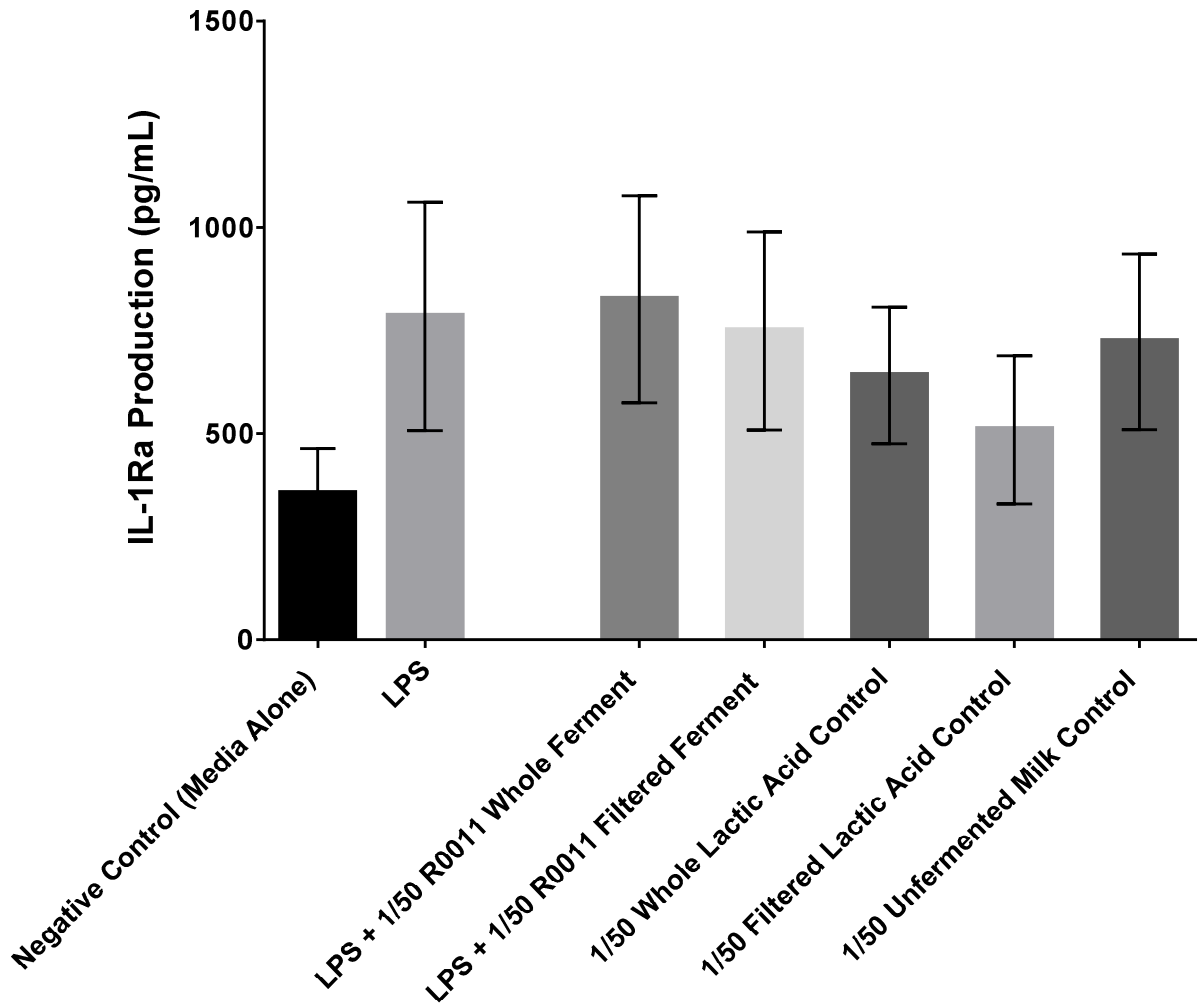


Figure 44. IL-1Ra production by THP-1 monocytes stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean IL-1Ra production \pm SEM; n=3). There was no significant differences between treatment groups as determined by one-way ANOVA ($P < 0.05$). Cell viabilities were $> 80\%$ for all treatments as determined by XTT and Trypan Blue Exclusion Viability assay.

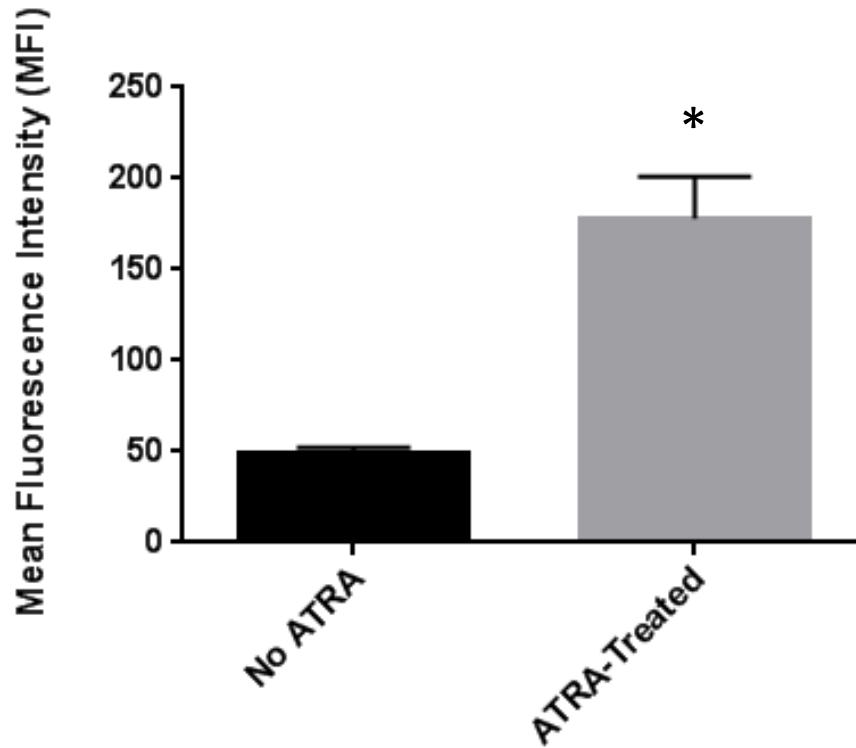


Figure 45. CD11b expression on ATRA-differentiated U937 macrophages. Data shown are CD11b expression (mean fluorescence intensity, n=6). * denotes statistical significance between treatment groups as determined using student's T-test (P = 0.0014). Cell viabilities were > 90% for all treatments as determined by Trypan Blue Exclusion Viability assay.

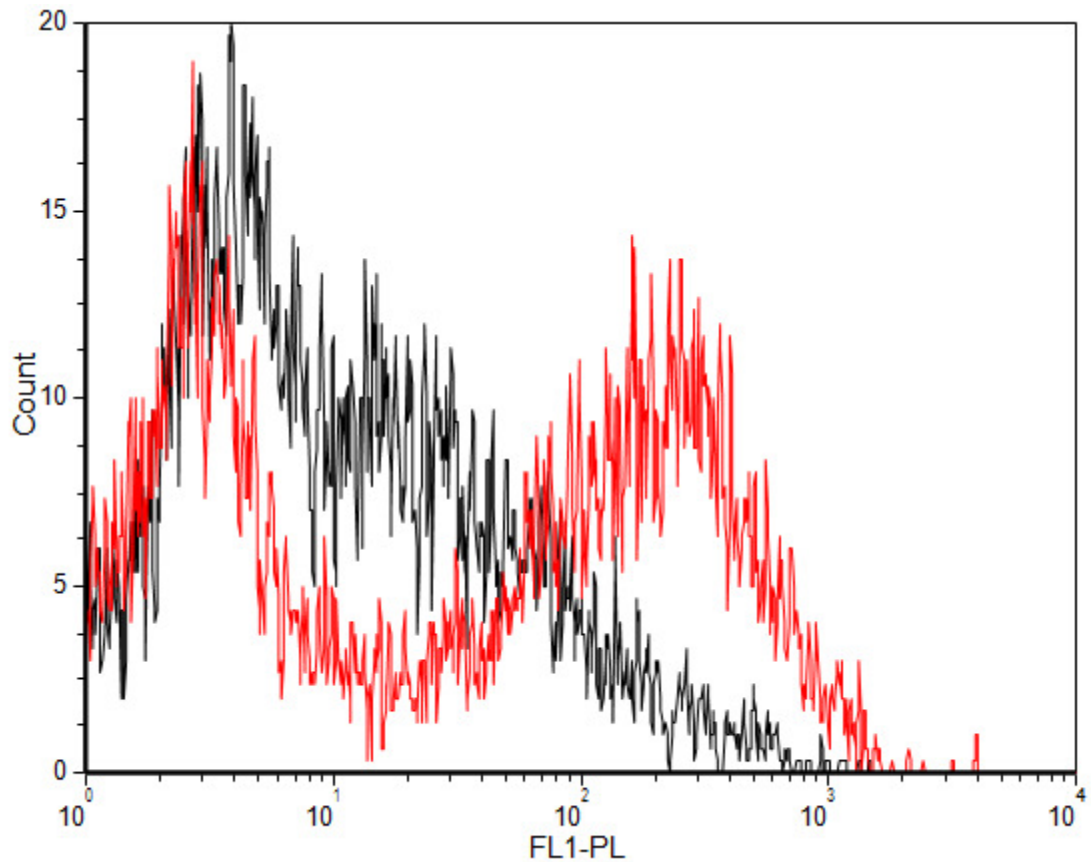


Figure 46. Histogram analysis of CD11b expression on U937 monocytes following differentiation with ATRA. CD11b expression by U937 cells treated with ATRA (shown in red) is overlaid with those cells which did not receive the ATRA treatment (shown in black). This is representative of six separate, independent experiments. The increase in the CD11b⁺ population following ATRA treatment was 45.9% as determined by the Overton subtraction.

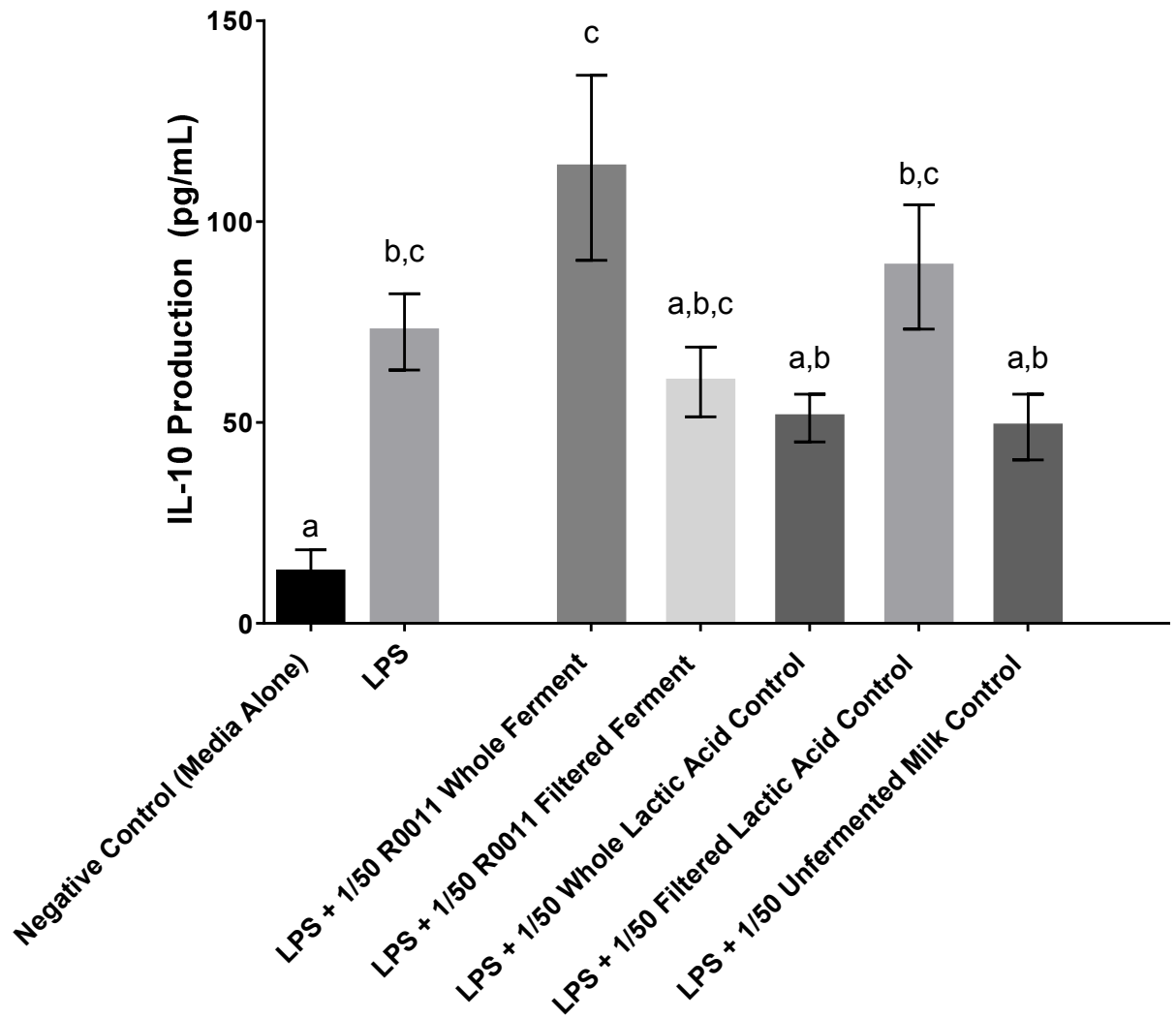


Figure 47. IL-10 production by ATRA-differentiated U937 macrophages stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean IL-10 production \pm SEM; n=5). Different letters denote statistically significant differences between treatment groups as determined using one-way ANOVA ($P < 0.05$) and Tukey's multiple comparison test. Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

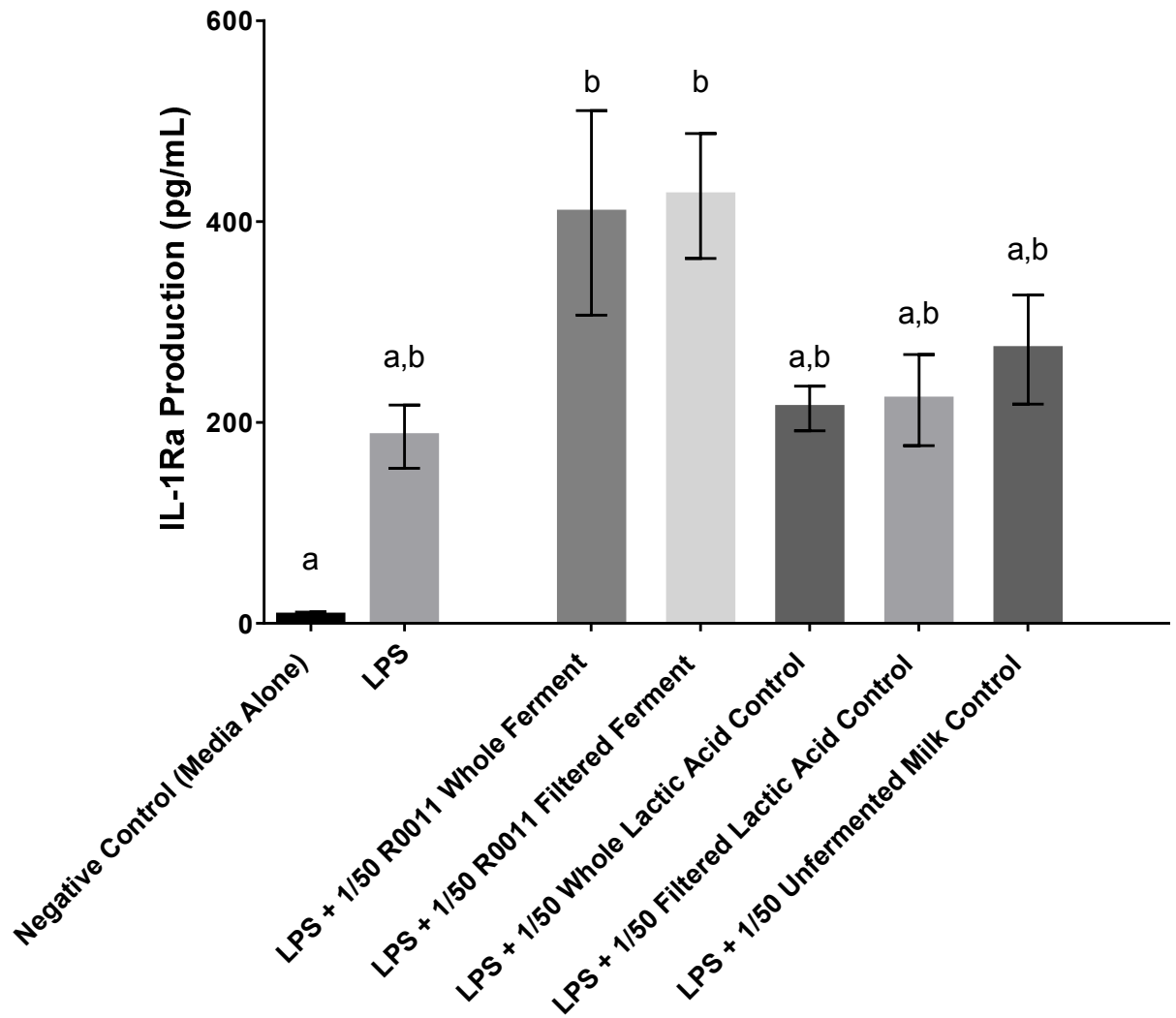


Figure 48. IL-1Ra production by ATRA-differentiated U937 macrophages stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean IL-1Ra production \pm SEM; n=4). Different letters denote statistically significant differences between treatment groups as determined using one-way ANOVA ($P < 0.05$) and Tukey's multiple comparison test. Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

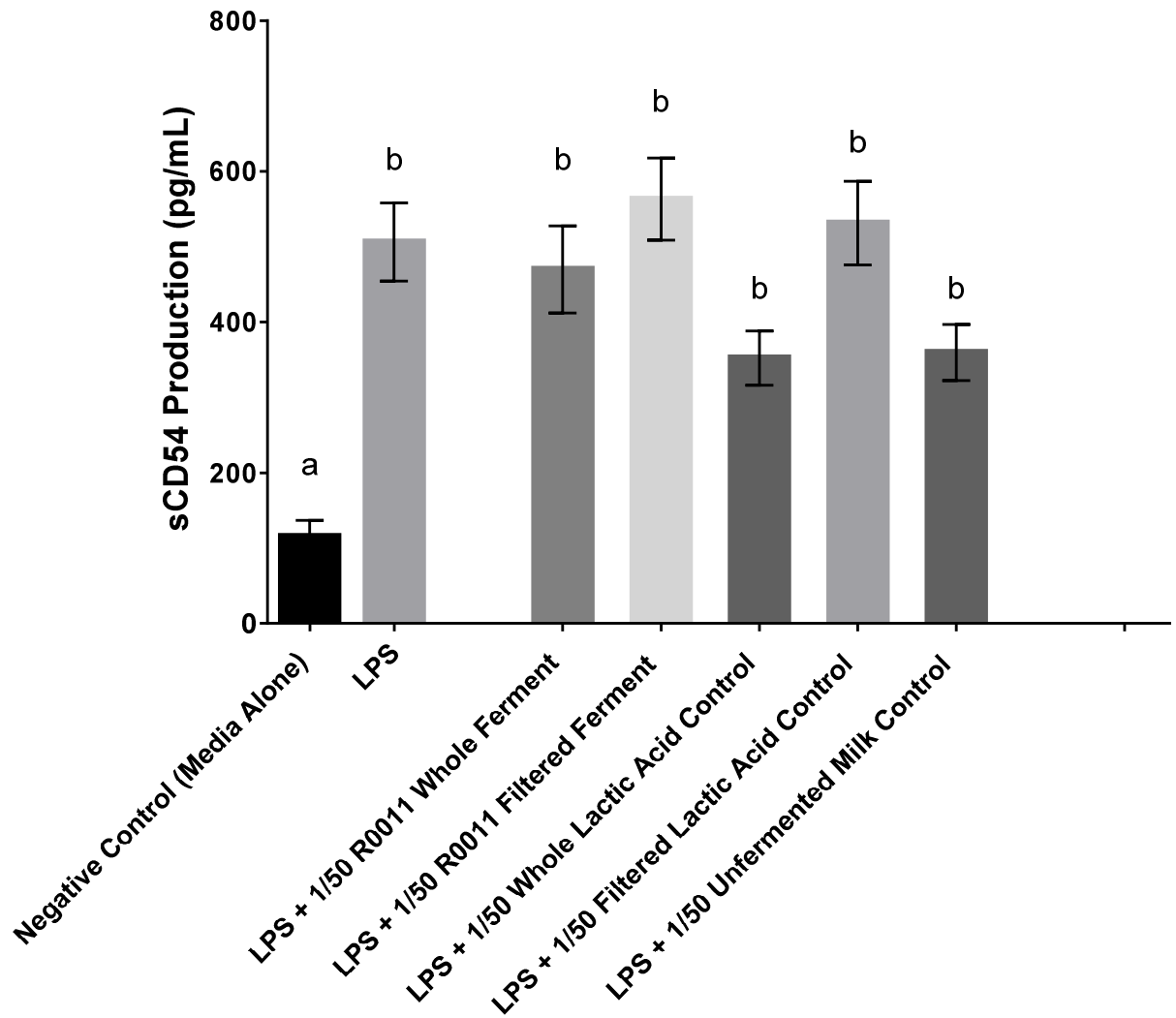


Figure 49. sCD54 production by ATRA-differentiated U937 macrophages stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean sCD54 production \pm SEM; n=3). Different letters denote statistically significant differences between treatment groups as determined using one-way ANOVA ($P < 0.05$) and Tukey's multiple comparison test. Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

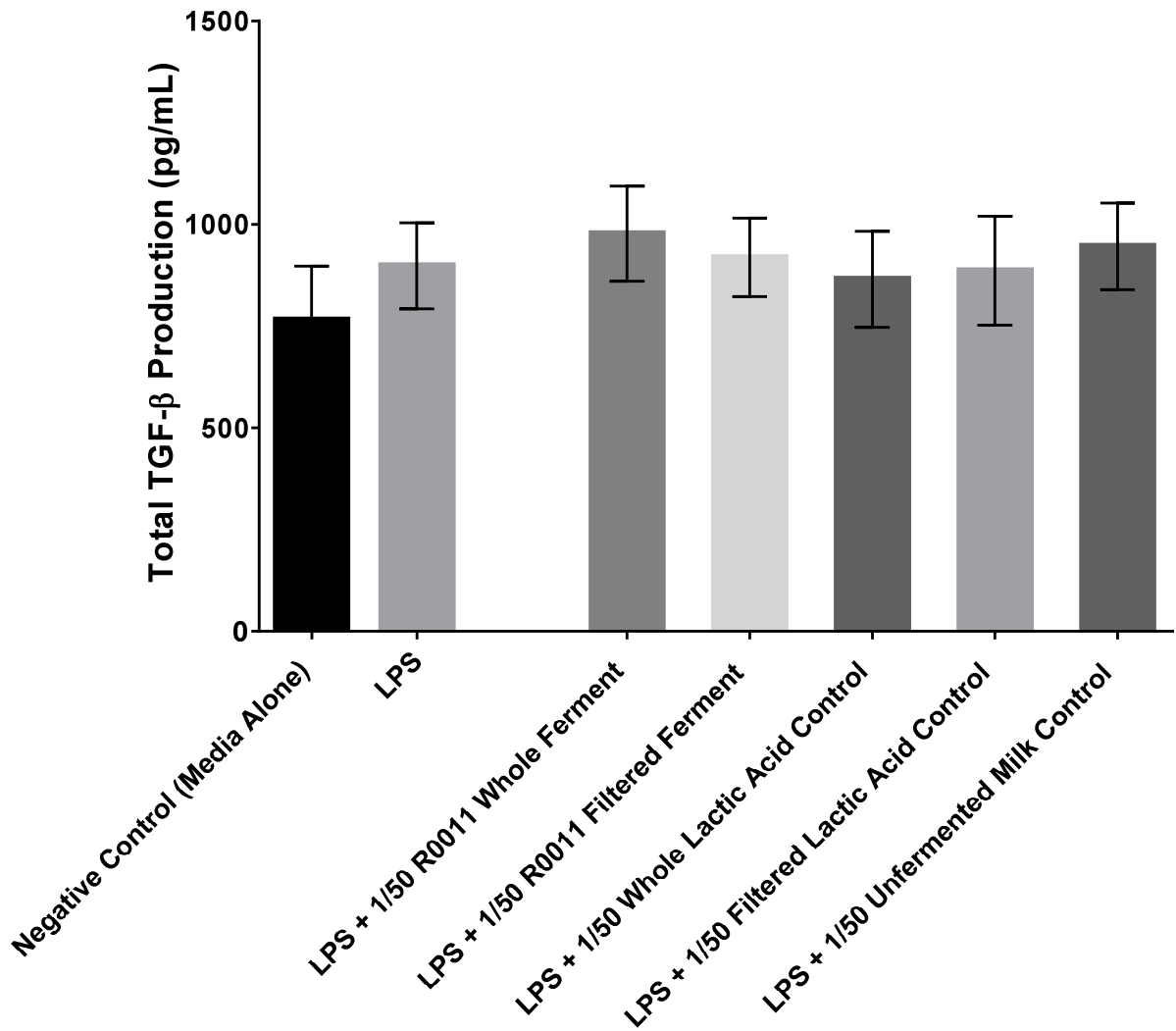


Figure 50. Total TGF- β production by ATRA-differentiated U937 macrophages stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean Total TGF- β production \pm SEM; n=3). There was no significant differences between treatment groups as determined by one-way ANOVA ($P < 0.05$). Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

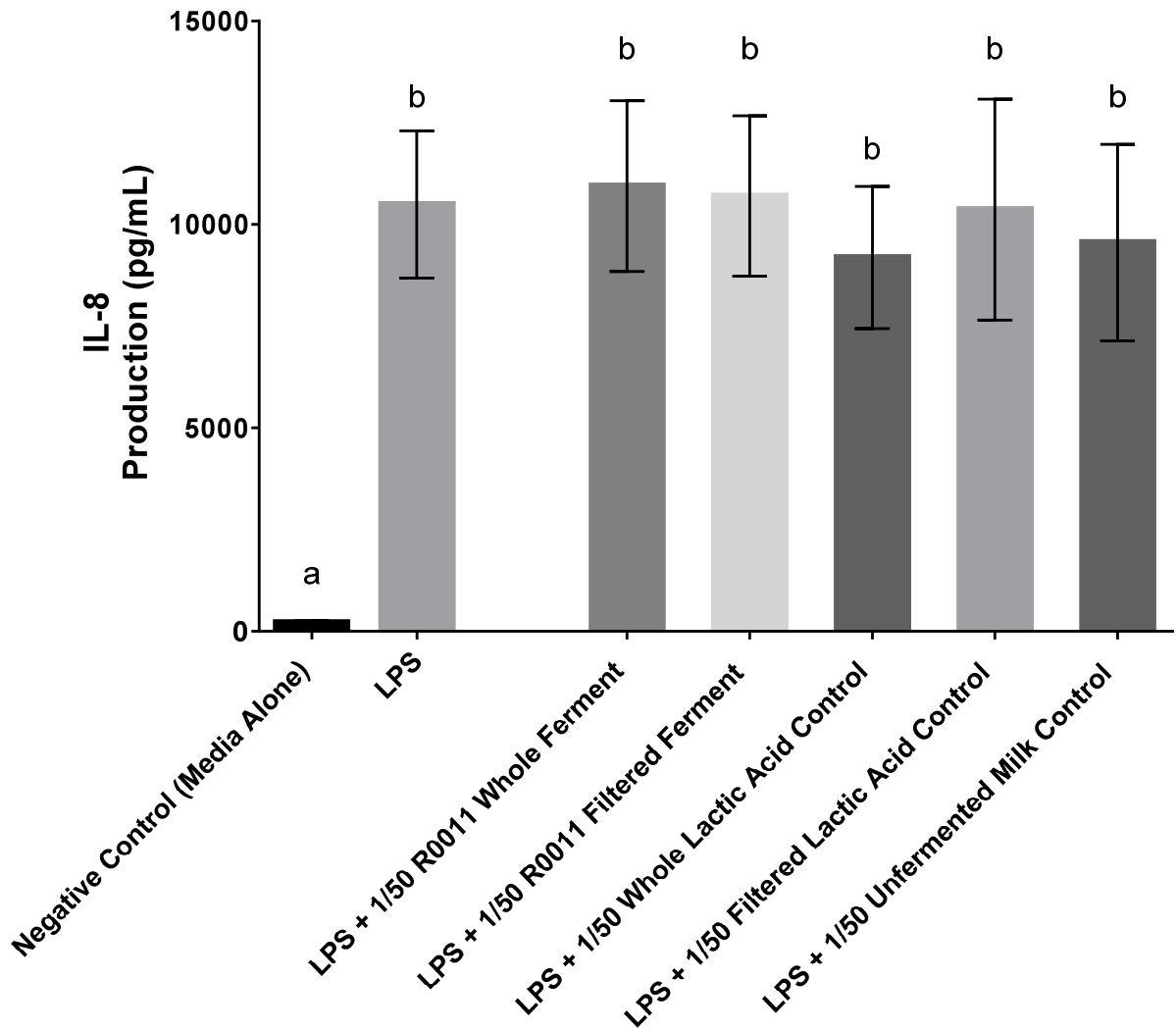


Figure 51. IL-8 production by ATRA-differentiated U937 macrophages stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean IL-8 production \pm SEM; n=3). Different letters denote statistically significant differences between treatment groups as determined using one-way ANOVA ($P < 0.05$) and Tukey's multiple comparison test. Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

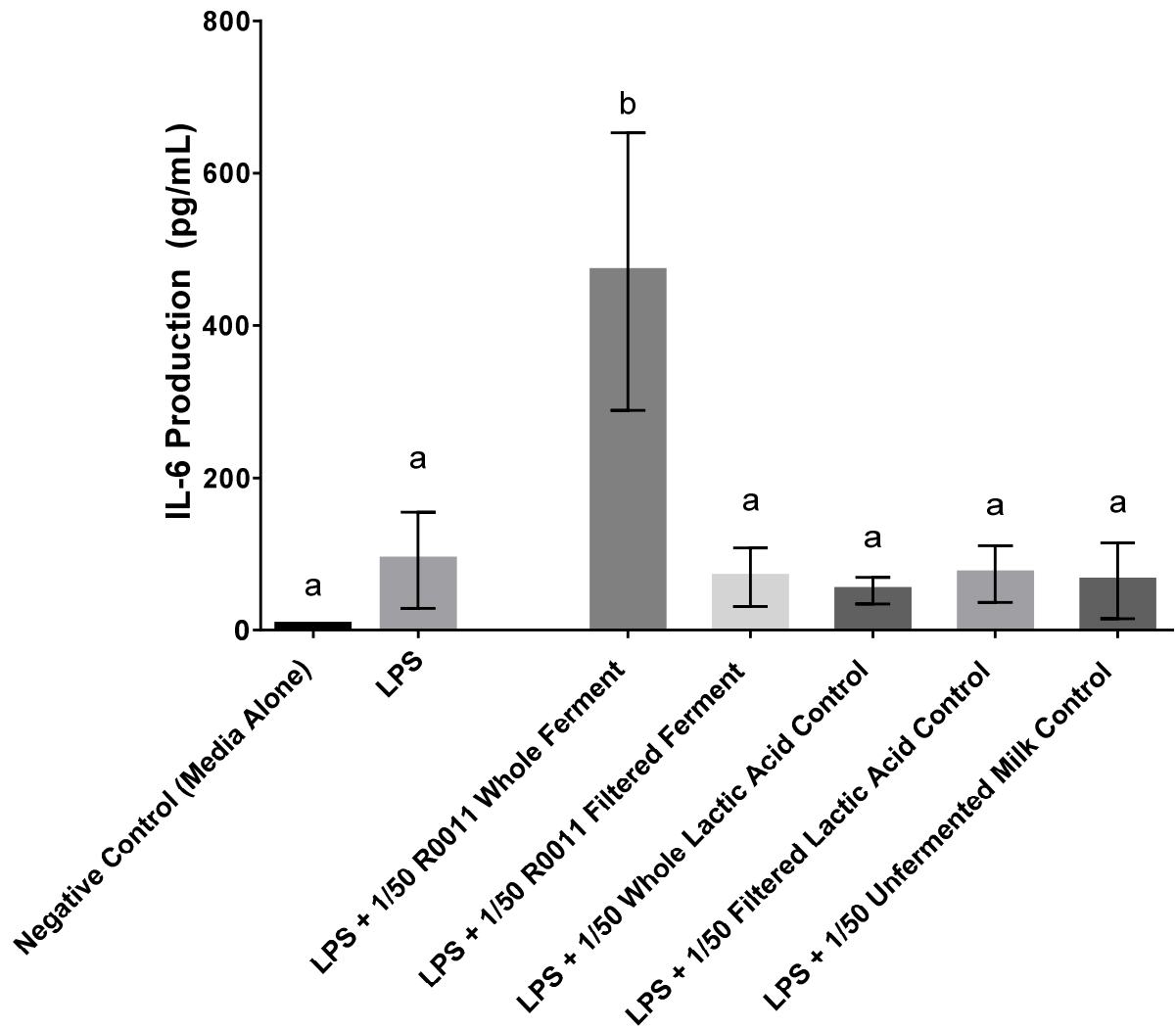


Figure 52. IL-6 production by ATRA-differentiated U937 macrophages stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean IL-6 production \pm SEM; n=3). Different letters denote statistically significant differences between treatment groups as determined using one-way ANOVA ($P < 0.05$) and Tukey's multiple comparison test. Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

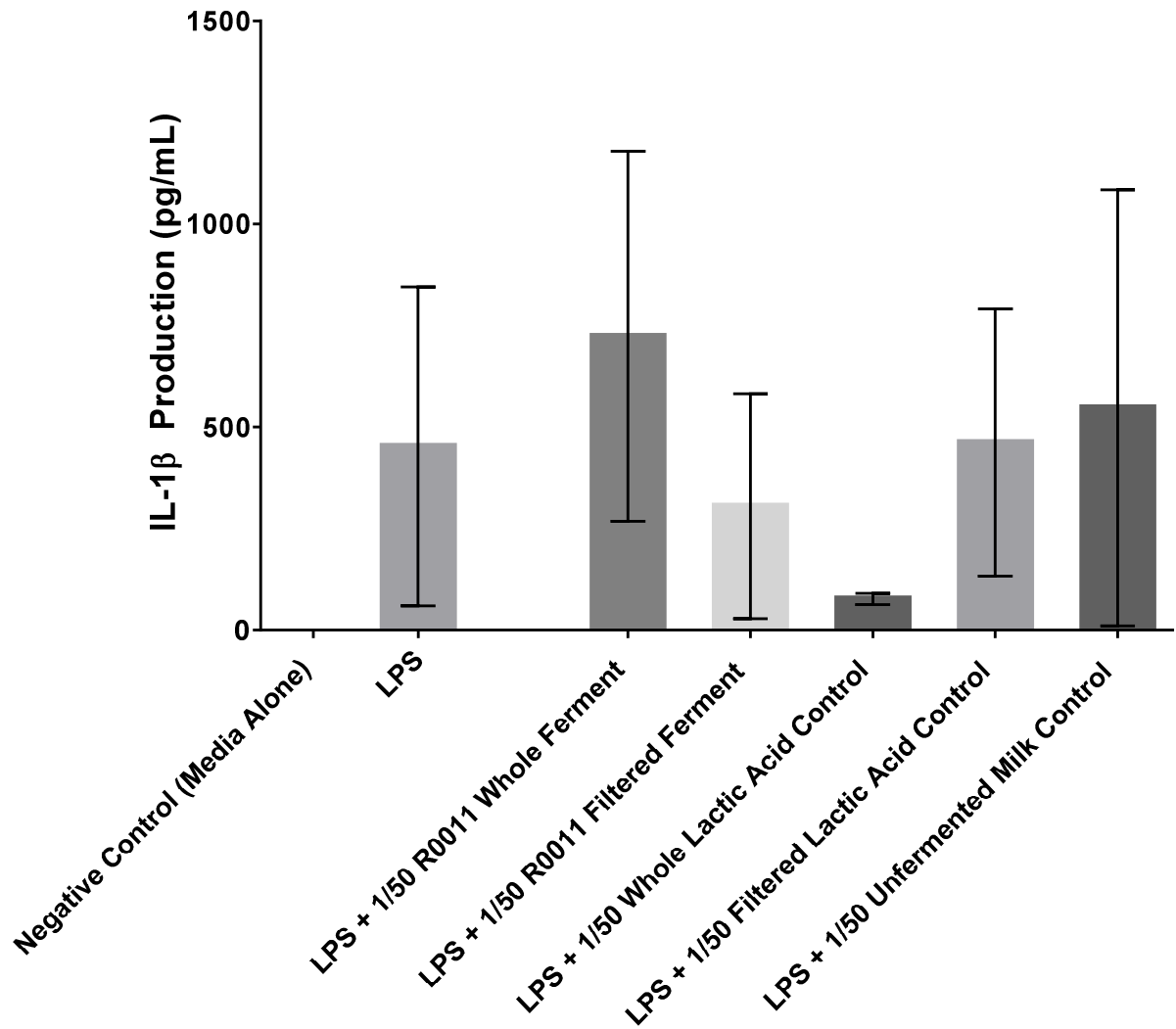


Figure 53. IL-1 β production by ATRA-differentiated U937 macrophages stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean IL-1 β production \pm SEM; n=3). Different letters denote statistically significant differences between treatment groups as determined using one-way ANOVA ($P < 0.05$) and Tukey's multiple comparison test. Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

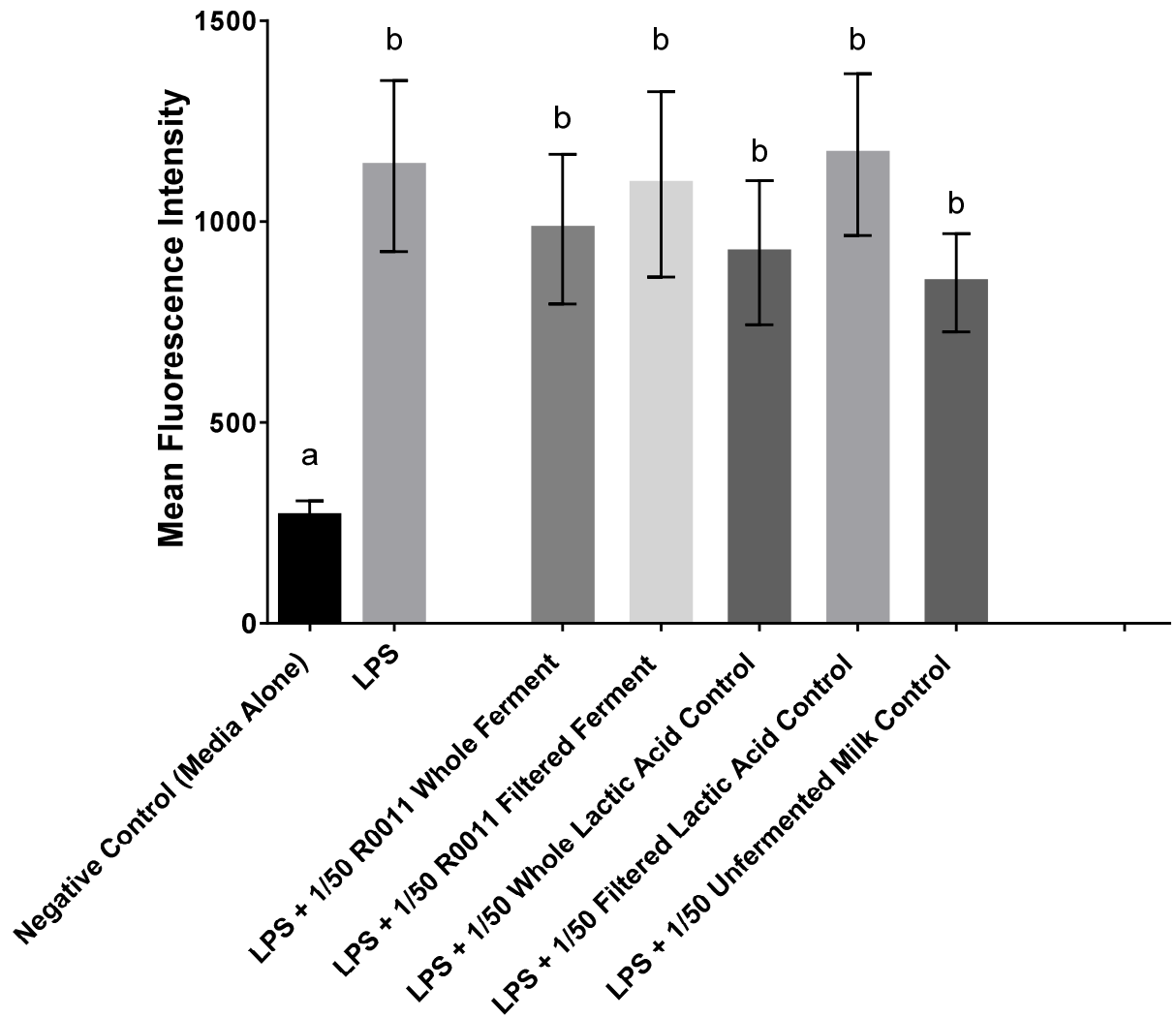


Figure 54. CD54 expression on ATRA-differentiated U937 macrophages stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean fluorescence intensity \pm SEM; n=3). Different letters denote statistically significant differences between treatment groups as determined using one-way ANOVA ($P < 0.05$) and Tukey's multiple comparison test. Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

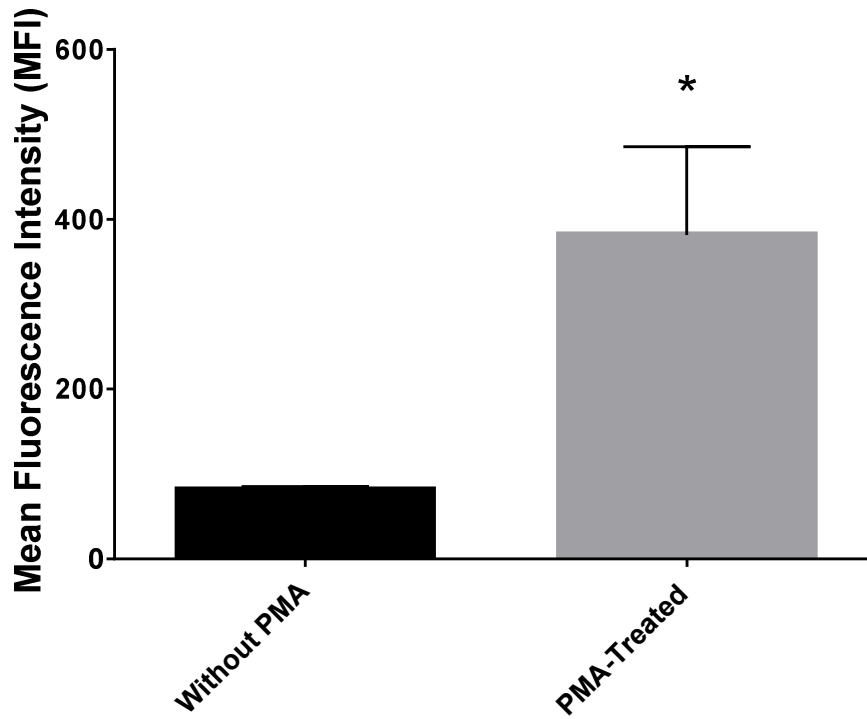


Figure 55. CD11b expression on PMA-differentiated THP-1 macrophages. Data shown are CD11b expression (mean fluorescence intensity, n=6). * denotes significant differences between treatment groups as determined using student's T-test ($P < 0.05$). Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

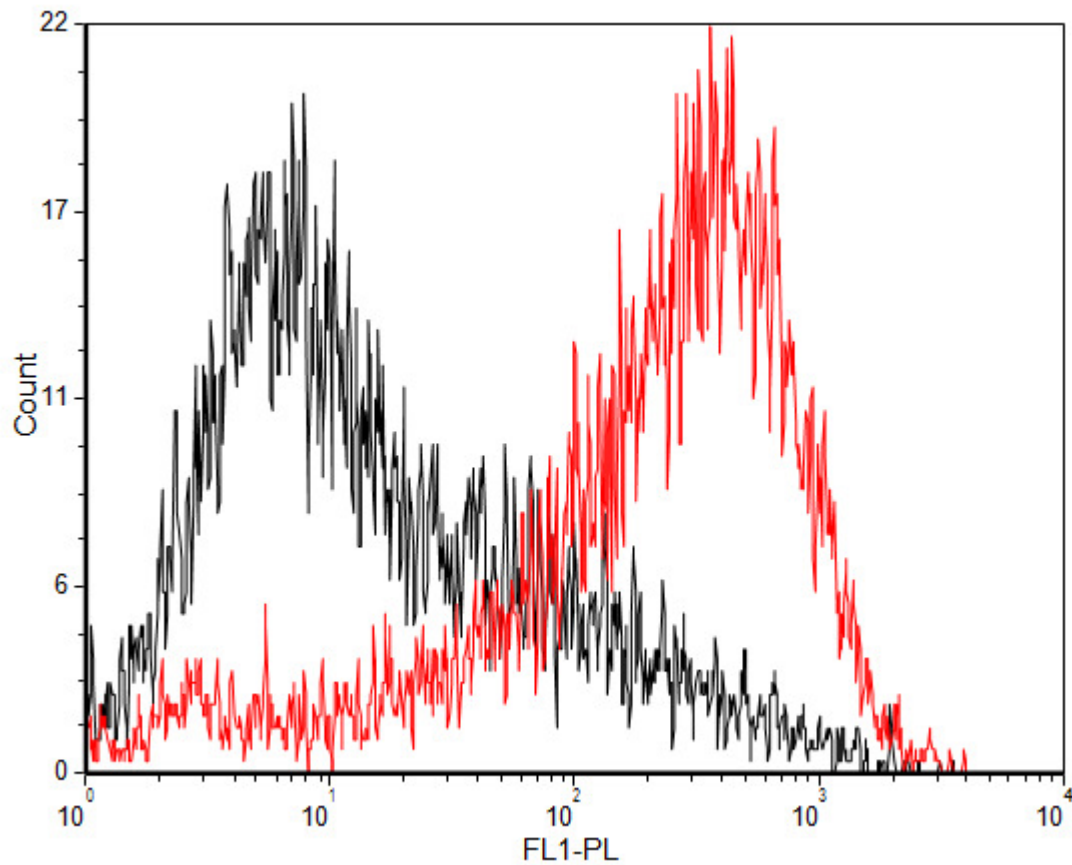


Figure 56. Histogram analysis of CD11b expression on THP-1 monocytes following differentiation with PMA. CD11b expression by PMA-treated THP-1 cells (shown in red) is overlaid with those cells which did not receive the PMA treatment (shown in black). This is representative of six separate, independent experiments. The increase in the CD11b⁺ population after PMA treatment was 59.9% as determined by the Overton subtraction.

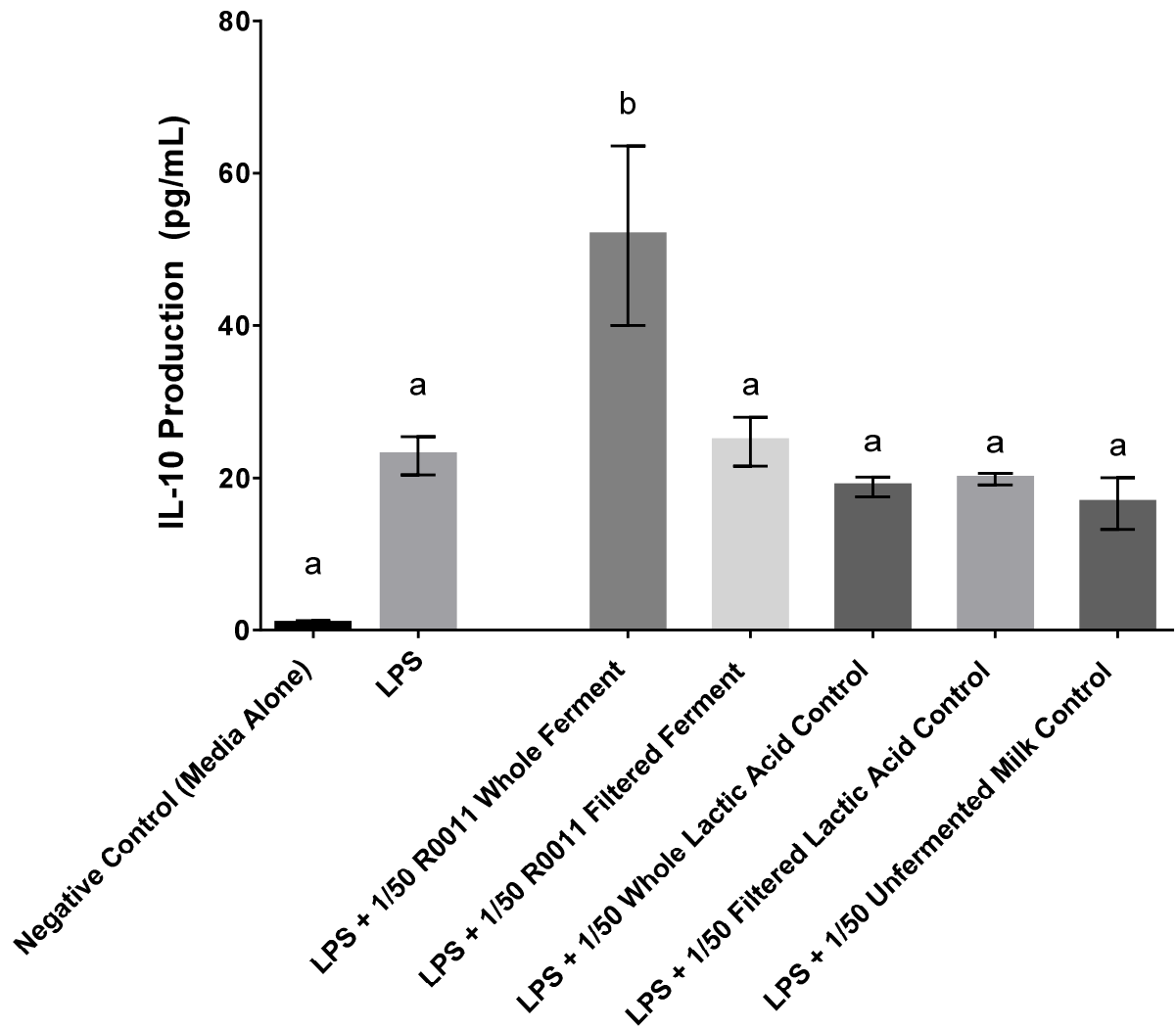


Figure 57. IL-10 production by PMA-differentiated THP-1 macrophages stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean IL-10 production \pm SEM; n=3). Different letters denote statistically significant differences between treatment groups as determined using one-way ANOVA ($P < 0.05$) and Tukey's multiple comparison test. Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

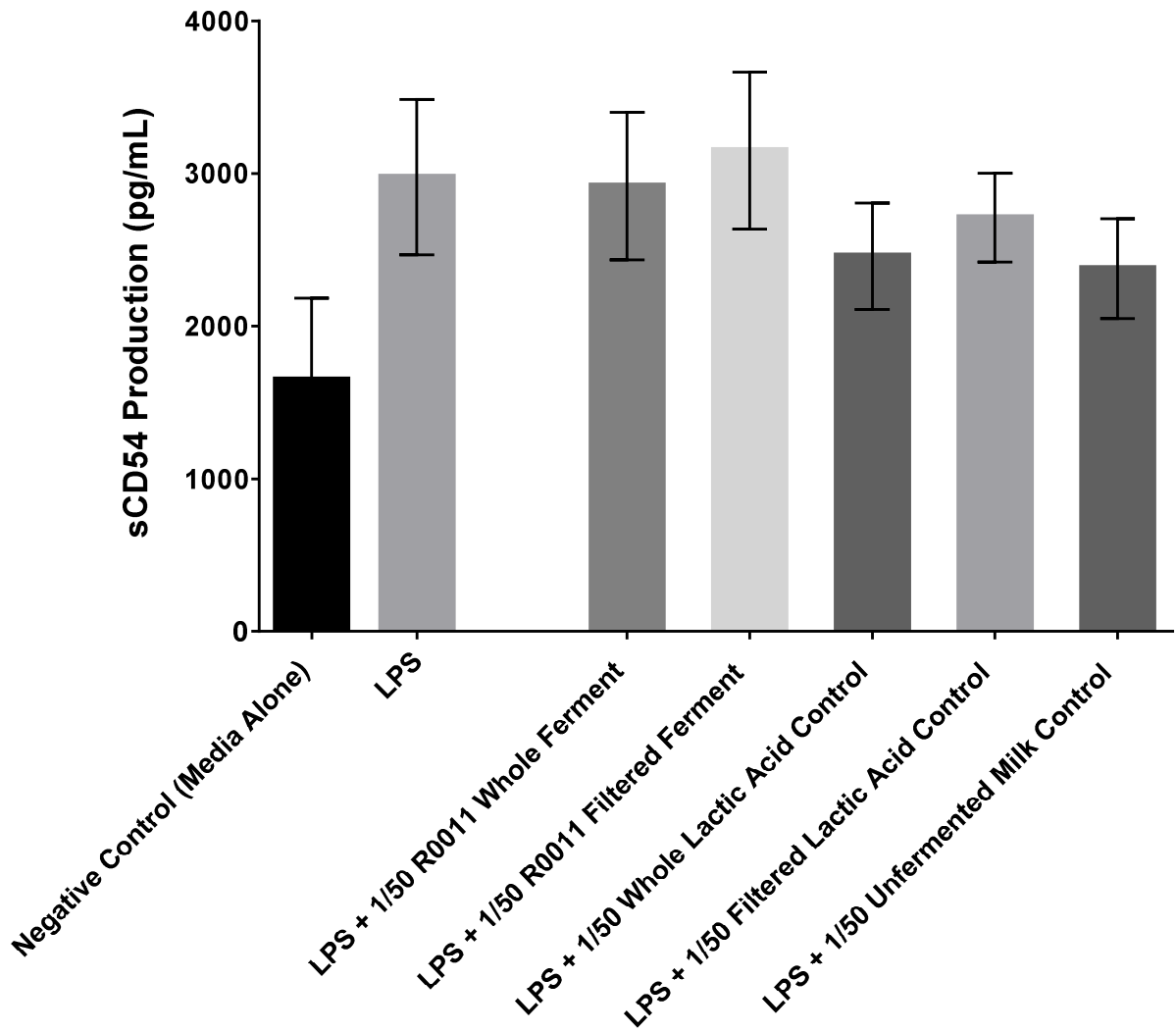


Figure 58. sCD54 production by PMA-differentiated THP-1 macrophages stimulated with 125 ng/mL LPS and *Lactobacillus rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean sCD54 production \pm SEM; n=3). There was no significant differences between treatment groups as determined by one-way ANOVA ($P > 0.05$). Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay

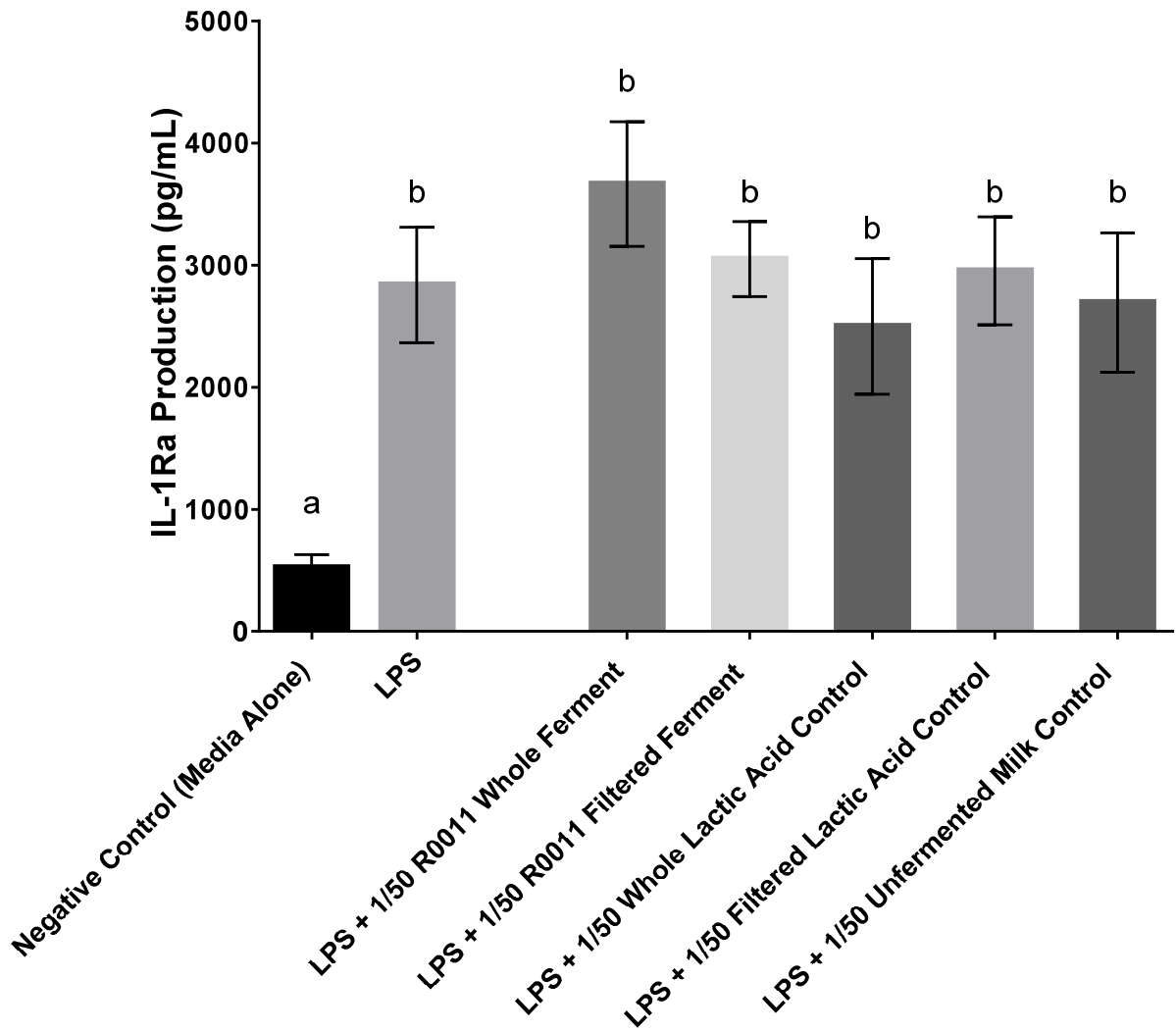


Figure 59. IL-1Ra production by PMA-differentiated THP-1 macrophages stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean IL-1Ra production \pm SEM; n=3). Different letters denote statistically significant differences between treatment groups as determined using one-way ANOVA ($P < 0.05$) and Tukey's multiple comparison test. Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

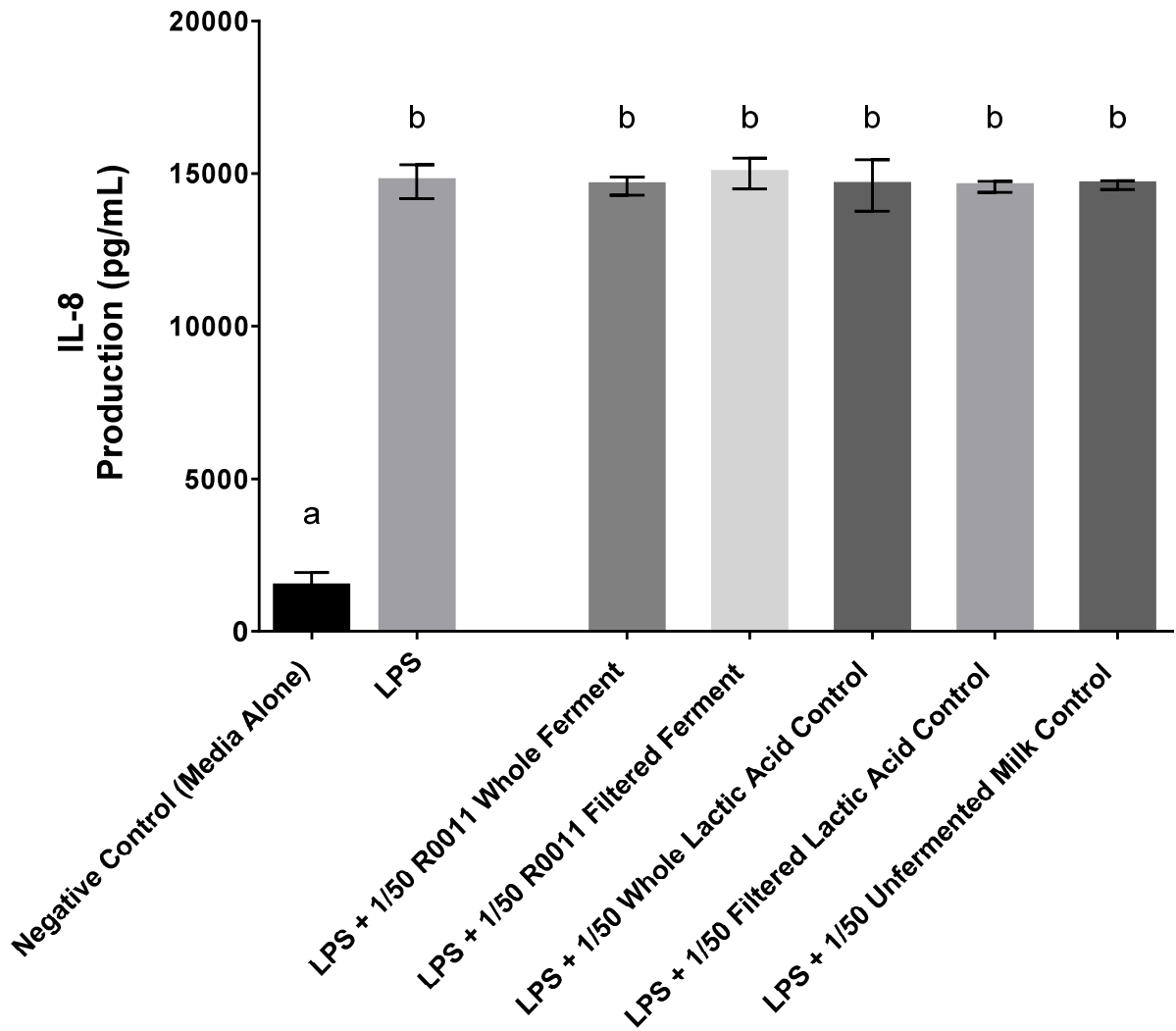


Figure 60. IL-8 production by PMA-differentiated THP-1 macrophages stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean IL-8 production \pm SEM; n=4). Different letters denote statistically significant differences between treatment groups as determined using one-way ANOVA ($P < 0.05$) and Tukey's multiple comparison test. Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

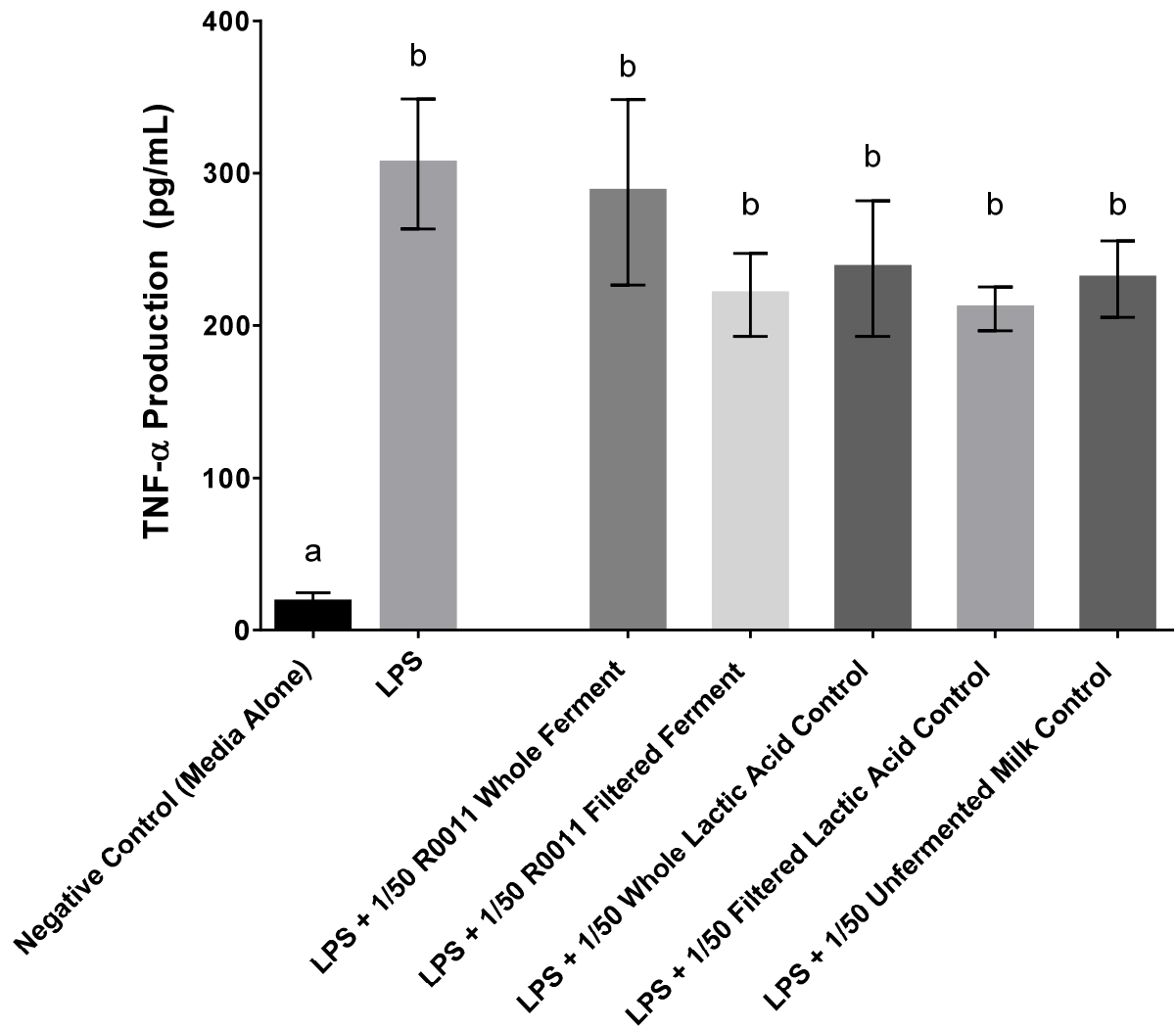


Figure 61. TNF- α production by PMA-differentiated THP-1 macrophages stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean TNF- α production \pm SEM; n=3). Different letters denote statistically significant differences between treatment groups as determined using one-way ANOVA ($P < 0.05$) and Tukey's multiple comparison test. Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

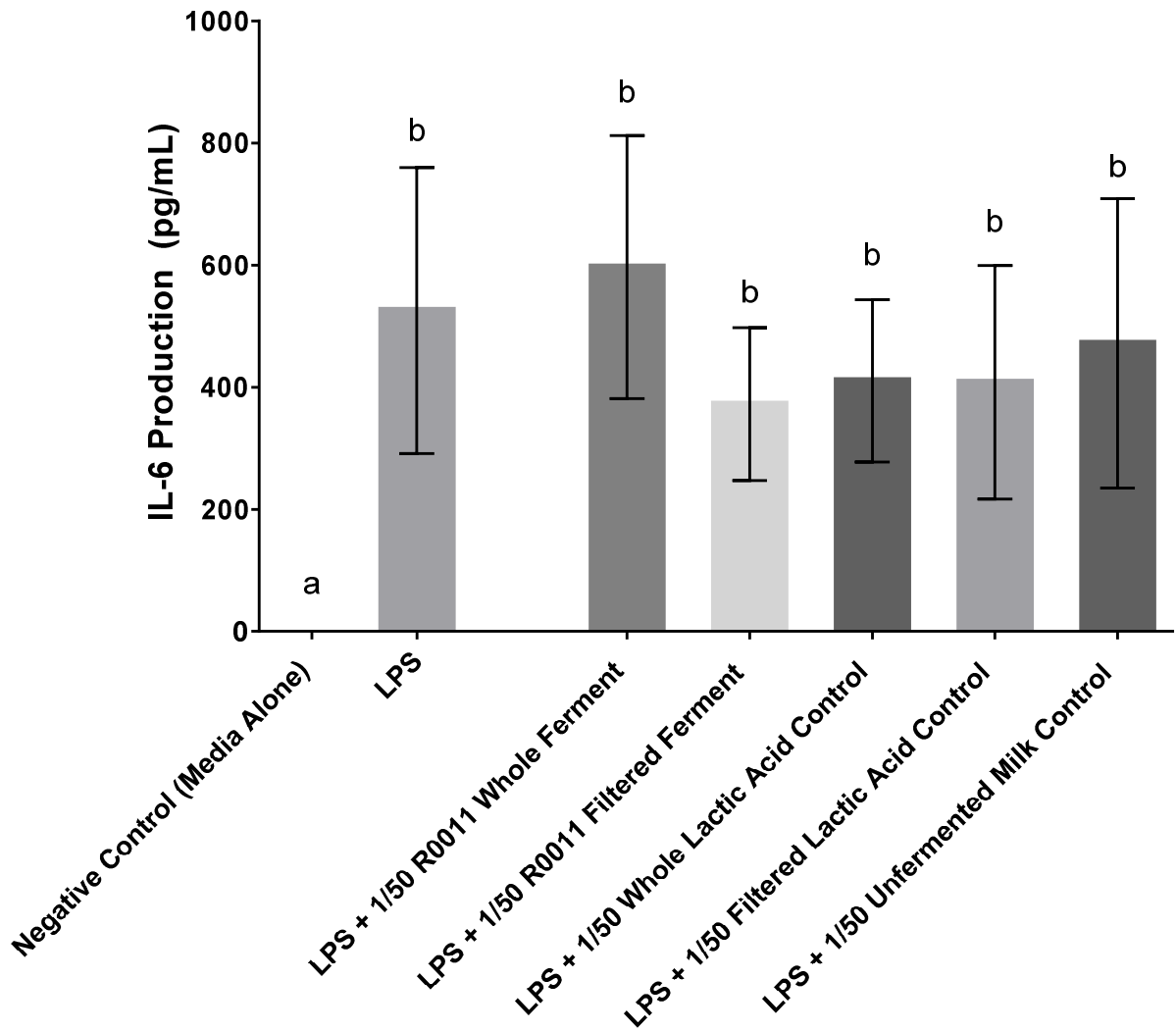


Figure 62. IL-6 production by PMA-differentiated THP-1 macrophages stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean IL-6 production \pm SEM; n=3). Different letters denote statistically significant between treatment groups as determined using one-way ANOVA ($P < 0.05$) and Tukey's multiple comparison test. Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

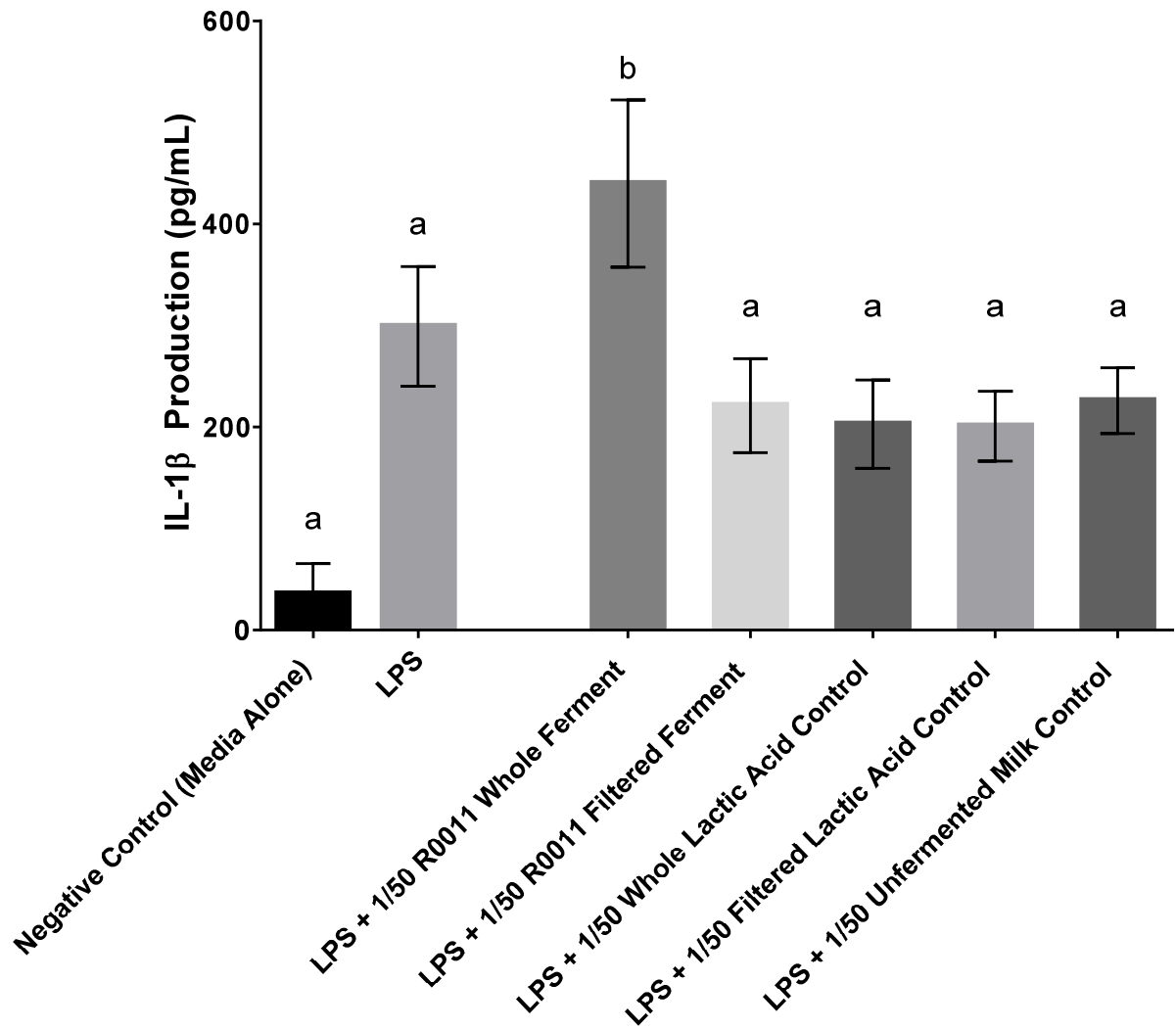


Figure 63. IL-1 β production by PMA-differentiated THP-1 macrophages stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean IL-1 β production \pm SEM; n=3). Different letters denote statistically significant differences between treatment groups as determined using one-way ANOVA ($P < 0.05$) and Tukey's multiple comparison test. Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

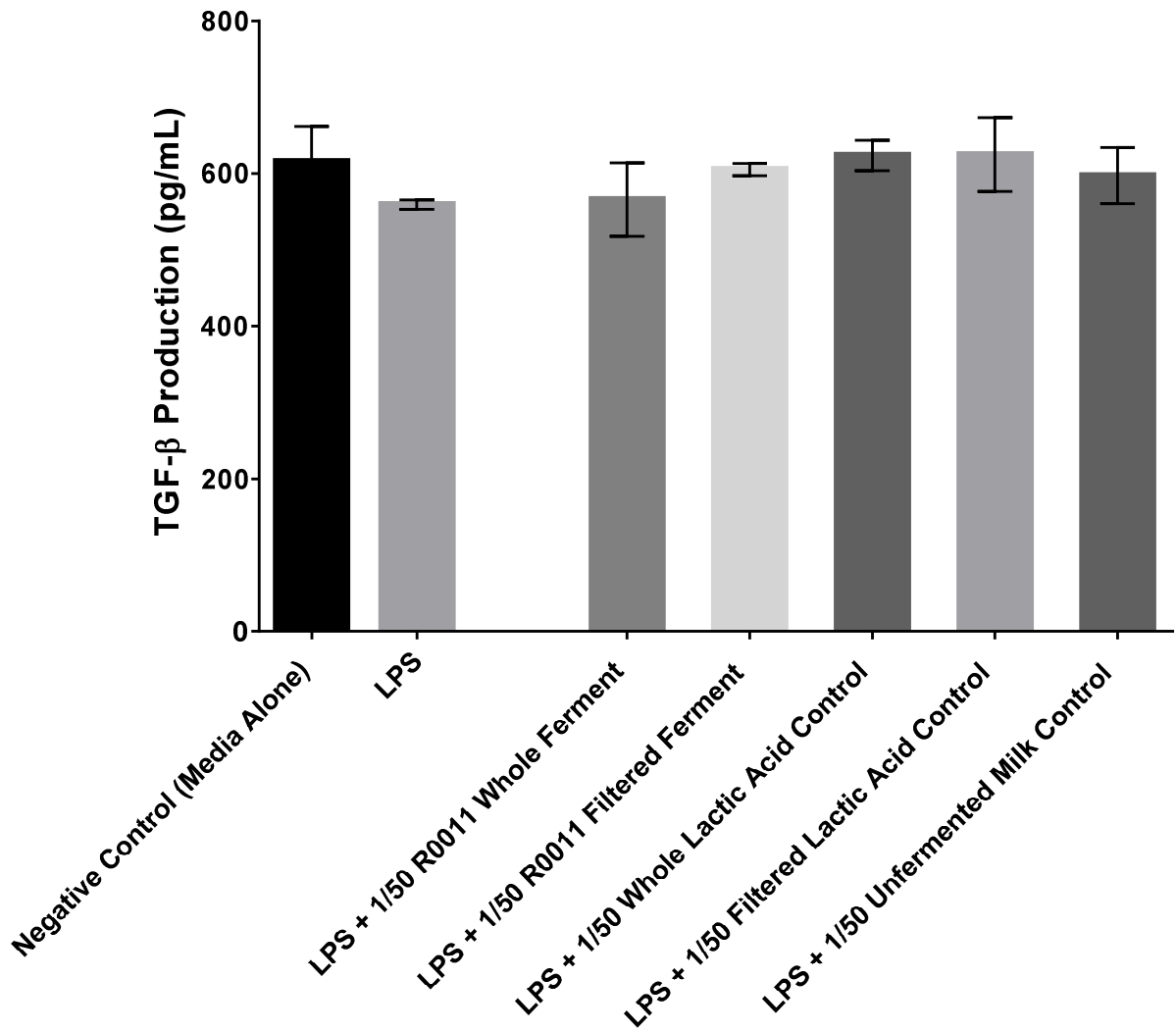


Figure 64. Total TGF- β production by PMA-differentiated THP-1 macrophages stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean Total TGF- β production \pm SEM; n=3). There was no significant differences between treatment groups as determined by one-way ANOVA ($P > 0.05$). Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

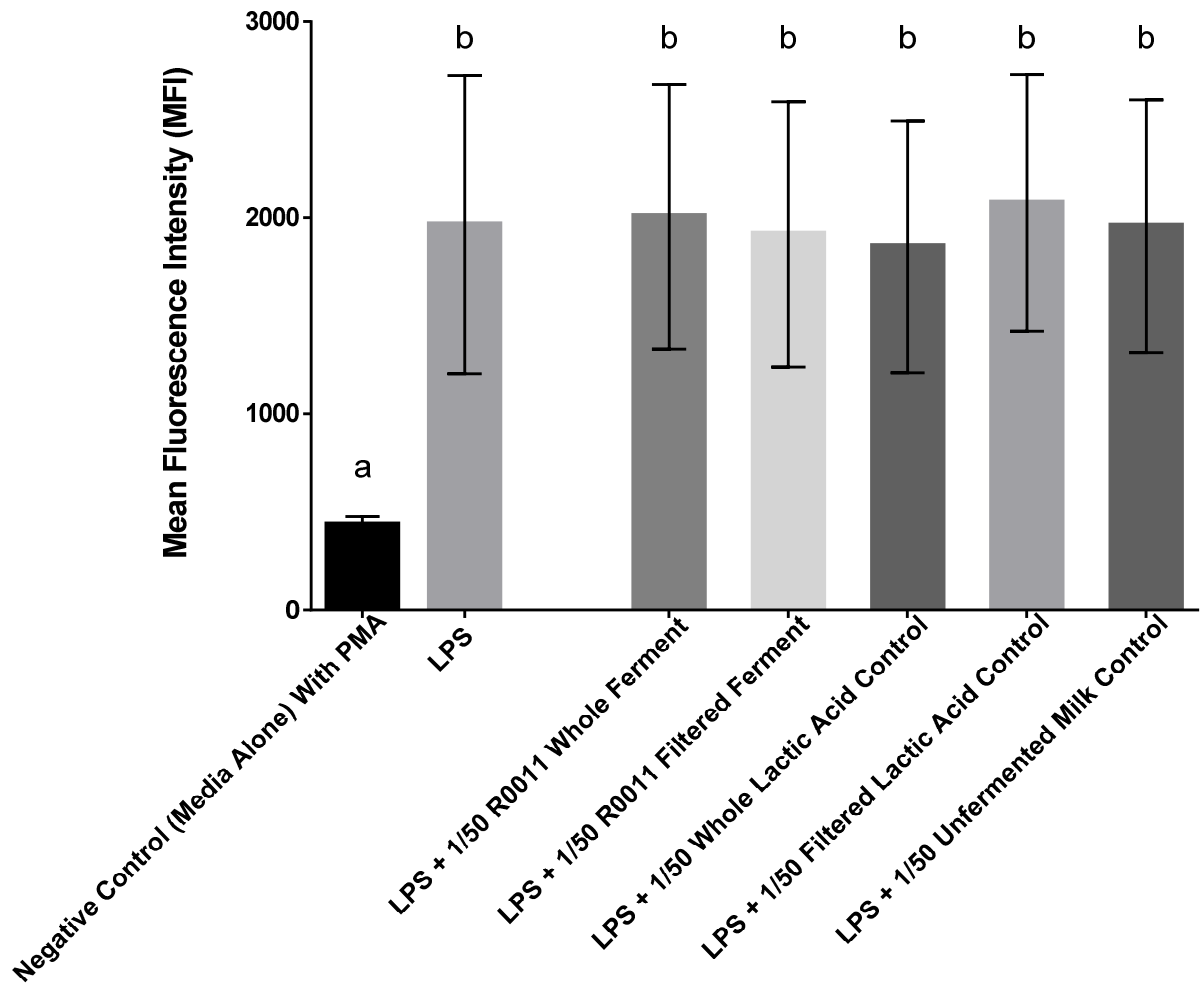


Figure 65. CD54 expression on PMA-differentiated THP-1 macrophages stimulated with 125 ng/mL LPS and *L. rhamnosus* R0011 whole/filtered ferments or milk controls concurrently for 24 hrs (mean fluorescence intensity \pm SEM; n=3). Different letters denote statistically significant differences between treatment groups as determined using one-way ANOVA ($P < 0.05$) and Tukey's multiple comparison test. Cell viabilities were $> 90\%$ for all treatments as determined by Trypan Blue Exclusion Viability assay.

Discussion

***L. rhamnosus* R0011 and *L. helveticus* R0389 differentially modulated pro-inflammatory chemokine production from human HT-29 and rat IEC-6 IECs**

Several species and strains of lactic acid bacteria have been characterised for their ability to modulate host immune responses through direct interactions with the mucosal immune system. Many of these bacteria have been classified as probiotic organisms defined as live microorganisms conferring health benefits to the host when consumed in adequate amounts (FAO/WHO, 2001). These microorganisms modulate host immune responses in response to pro-inflammatory challenges, antagonise the attachment of known-gut pathogens to IECs, improve gut epithelial barrier function, and improve dietary nutrient uptake (Bajaj *et al.*, 2015). However, the mechanisms used by many of these bacteria to exert these effects on the host remain unclear. Further, it is unknown whether these bacteria have to be in direct contact with host IECs or if they secrete bioactive molecules conferring immunological activity. As such, it is important to evaluate the range of bioactivity of these organisms and their products in order to elucidate potential mechanisms of action behind probiotic-induced effects on the host.

The current study indicates that *L. rhamnosus* R0011 and *L. helveticus* R0389 secrete a bioactive molecule (or molecules) of less than 10 kDa in size that attenuates pro-inflammatory chemokine production from challenged human HT-29 IECs. Further, the *L. rhamnosus* R0011 secretome and the less than 10 kDa fraction of the secretome decreased pro-inflammatory chemokine production from challenged rat IEC-6 IECs. These results suggest that these two Lactobacilli strains modulate IEC activity by

producing bioactive molecules that act to dampen signals delivered through key PRRs and cytokine receptors associated with pro-inflammatory activity. This is in agreement with other studies suggesting that some probiotic microorganisms secrete bioactive molecules retaining immunomodulatory activity. For example, cell-free supernatants from *L. kefir* IM002 have been shown to significantly inhibit IL-8 production from HT-29 IECs challenged with *S. typhimurium* (Carey and Kostrzynska, 2012). *Bifidobacterium infantis* and *Lactobacillus acidophilus* also secrete a bioactive molecule which down-regulated IL-8 and IL-6 production from IL-1 β and TNF- α challenged primary human enterocytes. Preliminary characterisation of the bioactive molecule found that it was less than 10 kDa in size and resistant to DNase, RNase, protease, heat, and acid treatment, although the precise nature of the bioactive molecule was not determined (Ganguli *et al.*, 2013).

Other bacteria have been characterised for their ability to secrete bioactive molecules in culture which have immunomodulatory activity on cells of the innate immune system. For example, *E. coli* strain Nissle 1917 has been shown to produce a bioactive molecule that suppresses TNF- α -induced IL-8 production from human IECs in an NF- κ B independent manner (Kamada *et al.*, 2008). The cell-free supernatant of *L. rhamnosus* CNCM I-4036 has also been shown to significantly down-regulate pro-inflammatory cytokine production in human DCs challenged with *E. coli*. Treatment with the cell-free supernatant resulted in an increase in TLR-1 and TLR-5 gene transcription in DCs, suggesting a possible role of these TLRs in the observed immunomodulatory activity (Bermudez-Brito *et al.*, 2014).

In contrast to the observed down-regulation of IL-8 production from challenged HT-29 IECs cultured with the *L. helveticus* R0389 secretome, there was no observed down-regulation of CINC-1 production from challenged IEC-6 IECs cultured with the secretome. Similarly, there was no observed down-regulation of poly (I:C)-induced CINC-1 production from IEC-6 IECs cultured with the *L. rhamnosus* R0011 secretome. This was the only instance in this study where the *L. rhamnosus* R0011 secretome did not down-regulate pro-inflammatory chemokine production from challenged IECs. These discrepancies between the observed immunomodulatory capacities of the secretomes between the two cell lines could be explained by differences between the species from which the cell lines were isolated or the location along the GI tract from which they originate. Another explanation may lie in the fact that the IEC-6 IEC cell line is a primary, non-transformed cell line while the HT-29 IECs were isolated from a colorectal adenocarcinoma. It has been noted that there are species- and tissue-specific responses to TLR stimulation (Rehli, 2002). For example, cells with myeloid lineage have been shown to respond in a NF- κ B-independent manner when challenged with poly (I:C) while non-myeloid cells do not (Lundberg *et al.*, 2007). This suggests that there are multiple different signalling pathways which TLR-3 can activate. It is also important to note that TLR-3 signalling is mediated through a MyD88-independent pathway while the other TLR stimuli used in this study are mediated through the MyD88-dependent pathway. The results of this study suggests that the immunomodulatory activity of the *L. rhamnosus* R0011 secretome is effective only for signals mediated through the MyD88-dependent pathway in IEC-6 cells, and that the *L. rhamnosus* R0011 secretome does not demonstrate the

ability to down-regulate CINC-1 production induced through the MyD88 independent pathway.

Preliminary characterisation of the constituents of both *L. rhamnosus* R0011 and *L. helveticus* R0389 secretomes was conducted in an attempt to elucidate the nature of the bioactive molecule(s) present. Since commensal-derived ATP can potentially alter host immune function (Atarashi *et al.*, 2008), the concentrations of ATP were measured in both secretomes. It was found that both the *L. rhamnosus* R0011 and *L. helveticus* R0389 secretomes contained ATP. It is unclear whether the ATP was secreted by the bacteria or released during bacterial cell lysis as cultures were harvested at stationary phase. Interestingly, the *L. rhamnosus* R0011 secretome contained an appreciably higher amount of ATP than that found in the *L. helveticus* R0389 secretome. Since ATP can be converted to the immunomodulatory molecule adenosine through the activity of CD73, CD73 expression was measured on HT-29 IEC and effects of incubation with the *L. rhamnosus* R0011 secretome were examined. Although CD73 expression was detected on HT-29 IEC, there were no significant changes in CD73 expression on HT-29 IEC following incubation with the R0011 secretome. Pharmacological blocking of CD73 did not appear to reverse the immunomodulatory activity of the secretome at the concentrations examined in this study. Further, pharmacological blocking of the A₂A adenosine receptor, the down-stream target of the liberated adenosine, did not appear to reverse the secretome's immunomodulatory activity. Stimulation of HT-29 IEC with *L. rhamnosus* R0011 (using heat-killed bacteria) induces an increase in intracellular cAMP production (Wood *et al.*, 2006). It appears that this observed up-regulation of intracellular

cAMP production and subsequent down-regulation of chemokine production from challenged IECs may not be mediated by ATP present within the secretome.

L. rhamnosus R0011 predominantly secretes L(+)-lactic acid while *L. helveticus* predominantly secretes D(-)-lactic acid. L(+)-lactic acid has been attributed with immunosuppressive properties characterised by the ability to inhibit pro-inflammatory cytokine and chemokine production from human monocytes and dendritic cells. Interestingly, D(-)-lactic acid does not appear to have the same immunosuppressive effects, as down-regulation of pro-inflammatory cytokine production was only seen in cells exposed to L(+)-lactic acid (Gottfried *et al.*, 2006; Goetze *et al.*, 2011). However, the effects of L(+)-lactic acid on IECs have not been extensively characterised. Preliminary analysis conducted at the time of writing has indicated that L(+)-lactic acid at a concentration similar to the concentration found in the *L. rhamnosus* R0011 secretome does not down-regulate TNF- α -induced IL-8 production from HT-29 IEC. Therefore, the observed down-regulation of IL-8 production in HT-29 IEC cultured with the *L. rhamnosus* R0011 or *L. helveticus* R0389 secretomes cannot be solely explained by the presence of lactic acid.

In an attempt to identify down-stream molecular targets of the secretome, HT-29 IEC were cultured with a PPAR γ antagonist to determine whether the bioactivity of the secretome is mediated through this nuclear receptor. However, the addition of this antagonist did not reverse the ability of the R0011 secretome to down-regulate TNF- α -induced IL-8 production from challenged HT-29 IEC. This suggests that the *L. rhamnosus* R0011 secretome may not activate the PPAR γ receptor, and that the mode of action differs from other members of the gut microbiota which have been shown to

down-regulate NF- κ B activation through modulation of the PPAR γ receptor (Kelly *et al.*, 2004; Eun *et al.*, 2007; Bassaganya-Riera *et al.*, 2012).

To determine whether or not the immunomodulatory activities of the two secretomes could be attributed to indole production within culture, both the *L. rhamnosus* R0011 and *L. helveticus* R0389 were tested for their ability to produce indoles. Both strains tested negative for indole production when grown in MRS as determined by the traditional indole spot test suggesting that the immunomodulatory activity of the two secretomes is not mediated through AHRs as described by others (Zelante *et al.*, 2013). However, the sensitivity of this test is low (Lombard and Dowell, 1983). High-performance liquid chromatography should be used to validate these results to determine if indoles are indeed present within the two secretomes.

Neutralisation of the pH of the *L. rhamnosus* R0011 secretome to physiological levels abrogated the observed down-regulation of IL-8 production from HT-29 cells challenged with TNF- α . This indicates that the bioactive molecule is pH sensitive and may provide a clue into its nature. However, it should be noted that sodium hydroxide was used to adjust the pH of the secretome. This is important as LTA has been shown to be sensitive to sodium hydroxide treatment (Ryu *et al.*, 2009). LTA isolated from *Enterococcus faecalis* treated with sodium hydroxide or calcium hydroxide lost the ability to induce pro-inflammatory cytokine and nitric oxide production from RAW 264.7 cells (Baik *et al.*, 2011). Interestingly, LTA isolated from some Gram positive bacteria is smaller than 10 kDa in size (Draing *et al.*, 2006). Although not characterised within this study, LTA from some lactic acid bacteria have been shown to modulate IL-8 production from HT-29 IECs in response to pro-inflammatory challenge. LTA isolated from

Lactobacillus johnsonii La1 completely inhibited IL-8 production from TNF- α and LPS challenged HT-29 IEC. Deacylation of this LTA via treatment with ammonium hydroxide reversed the anti-inflammatory effect (Vidal *et al.*, 2002).

The less than 10 kDa fraction of the *L. rhamnosus* R0011 secretome also inhibited the growth of *S. typhimurium*. This inhibition was not observed when *S. typhimurium* was cultured with a HCl pH-control or a L(+)-lactic acid control suggesting that the *L. rhamnosus* R0011 secretes a class II bacteriocin with bacteriostatic properties. Class II bacteriocins are characterised as less than 10 kDa in size with the ability to inhibit the growth of several gut-associated pathogens (Todorov, 2009). In fact, *L. johnsonii* LA1 and *L. plantarum* ACA-DC 287 significantly inhibit the growth of *S. typhimurium* by the production of an unknown inhibitory substance which is only active in the presence of lactic acid (Makras *et al.*, 2005). Further, Coconnier *et al.*, (1997) have shown that the cell-free supernatant of *L. acidophilus* strain LB reduces the viability of several Gram-positive and Gram-negative bacteria and reduces the infectivity of *S. typhimurium* in IECs *in vivo*, effects that the authors determined were not simply due to lactic acid (Coconnier *et al.*, 1997). Dimitrijevic *et al.* (2009) have also shown that *L. rhamnosus* strain 68 secretes a 6433.8 Da peptide that inhibits the growth of *Micrococcus lysodeikticus* ATCC 4698, but has no effect on the growth of *L. plantarum* 8014 or *L. plantarum* 39268 (Dimitrijevic *et al.*, 2009). Interestingly, Sil, a bacteriocin produced by *Streptococcus iniae*, a common pathogen in fish, has been shown to not only antagonise the growth of *Bacillus subtilis* but also modulate host immune function in fish. Orally administered recombinant Sil reduced the oxidative burst capacity of monocytes *in vivo*, impairing the host immune response and improving immune evasion (Li *et al.*, 2014).

Collectively, the findings presented in this study illustrate that *L. rhamnosus* R0011 and *L. helveticus* R0389 secrete bioactive molecules retaining immunomodulatory activity, and that these bioactive molecules can down-regulate IL-8 production by human HT-29 IECs stimulated through varied PRRs or by pro-inflammatory cytokines. However, the two secretomes showed some differences in the ability to modulate TLR agonist-induced chemokine secretion; only the *L. rhamnosus* R0011 secretome was able to down-regulate CINC-1 production from rat IEC-6 cells challenged with flagellin or LPS, and neither the R0011 or the R0389 secretome significantly decreased CINC-1 production induced by poly (I:C). The bioactive secreted molecule appears to be less than 10 kDa in size and is sensitive to pH neutralisation with sodium hydroxide.

Milk fermented with *L. rhamnosus* R0011 has differential effects on pro-atherogenic and regulatory cytokine production from U937 and THP-1 human monocytes and macrophages

Probiotic-derived fermented products have become attractive functional foods for the delivery of probiotics and their biologically active products. The consumption of fermented dairy products has also been associated with immune system modulation, improved lactose digestion, alleviation of antibiotic-induced diarrhea, and reductions in serum cholesterol levels (Nagpal *et al.*, 2012). It is currently unknown how these fermented dairy products confer their health benefits. Therefore, it is important to understand and elucidate the mechanisms behind these effects by examining the interactions between fermented dairy products and cells of the innate immune system.

This study shows that milk fermented with *L. rhamnosus* R0011 has differential effects on cytokine production from challenged U937 and THP-1 human monocytes. Pre-incubating U937 human monocyte cells with the filtered *L. rhamnosus* R0011 ferment and unfermented milk control prior to LPS challenge resulted in significantly higher levels of IL-8 production. This effect was not seen with THP-1 monocyte cells exposed to the same treatment. Previous studies have illustrated an immunostimulatory role of fermented milk consumption on human monocytes *in vivo*. Milk fermented with *Lactobacillus casei* DN114001 was shown to increase the oxidative burst capacity of monocytes in a cohort of healthy middle-aged adults (Parra *et al.*, 2004). However, there were no changes in sCD54 or IL-1Ra production in either U937 or THP-1 monocytes in response to pre-incubation with *L. rhamnosus* R0011 milk ferments or milk controls.

When U937 monocytes were cultured with the whole *L. rhamnosus* R0011 milk ferment and LPS, there was a significant down-regulation of sCD54 production. sCD54 is a soluble form of CD54 (ICAM-1) which has been associated with increased cardiovascular disease risk and has been recently used as a marker of chronic inflammation in atherosclerotic plaques (Witkowska and Borawska, 2004). This down-regulation of sCD54 was also observed for U937 monocytes incubated with the whole lactic acid control and unfermented milk controls. Conversely, there was no effect of the *L. rhamnosus* R0011 ferment on LPS-induced sCD54 production by THP-1 monocytes. Further, there was no modulation of IL-1Ra or IL-8 production by either THP-1 or U937 monocytes cultured with LPS and the *L. rhamnosus* R0011 ferments or milk controls.

U937 and THP-1 monocytes were differentiated into macrophages in order to examine the effects of *L. rhamnosus* R0011 fermented milk on these key cell mediators

in atherosclerotic lesions. Previous work has indicated differential effects of milk ferments on cytokine production and adhesion molecule expression by U937 cells at the monocyte and macrophage stages (Masotti *et al.*, 2011). Interestingly, there was an increase in IL-6 production by LPS-stimulated U937 macrophages cultured with the *L. rhamnosus* R0011 milk ferment which was not observed when LPS-stimulated cells were cultured with the filtered *L. rhamnosus* R0011 milk ferment or milk controls. Further, there was a significant increase production of the regulatory cytokine IL-10 by LPS-stimulated U937 macrophages cultured with the *L. rhamnosus* R0011 milk ferment relative to those cells cultured with the whole lactic acid milk control and the unfermented milk control. Culturing of LPS-stimulated U937 macrophages with either the whole or filtered *L. rhamnosus* R0011 milk ferments also resulted in an increase in IL-1Ra production relative to the negative control. However, there was no modulation of sCD54, IL-8, IL-1 β , TNF- α , IL-12p70 or TGF- β production from LPS-stimulated U937 macrophages cultured with the *L. rhamnosus* R0011 milk ferments or milk controls and LPS. THP-1 macrophages cultured with the whole *L. rhamnosus* R0011 milk ferment demonstrated an increase in LPS-induced IL-10 and IL-1 β production. There were no changes in the production of LPS-induced IL-6, sCD54, IL-8, IL-1 β , TNF- α , IL-12p70 or TGF- β .

Taken together, it appears that milk fermented with *L. rhamnosus* R0011 increases regulatory cytokine production from LPS challenged U937 and THP-1 macrophages. The majority of these modulations in cytokine production were only observed in macrophages cultured with the whole *L. rhamnosus* R0011 ferment, and not with the filtered ferment. This suggests that a direct bacteria to host cell interaction is

required for the up-regulation of regulatory cytokine production. Indeed, there have been studies suggesting that probiotic Lactobacilli increase IL-10 and IL-6 production by murine macrophages through direct cell interactions (Morita *et al.*, 2002) and these effects may be mediated through TLR-2 dependent activation of ERK (Kaji *et al.*, 2010).

Observed differences between THP-1 and U937 cells in response to culture with milk ferments and non-fermented milk controls may be explained by the differences in the tissue that these cells originate from and stage of maturation of cells at time of harvesting. THP-1 cells were isolated from a 1 year old patient suffering from an acute monocytic leukemia while U937 cells were isolated from a 37 year old suffering from a histiocytic lymphoma. As such, THP-1 cells often exhibit characteristics of less mature human peripheral blood monocyte cells while U937 cells are more representative of mature monocytic cells (Baek *et al.*, 2009). Further, *in vitro* differentiation of THP-1 cells has been shown to induce changes in gene expression which render THP-1 macrophages more susceptible to food-derived compounds (Chanput *et al.*, 2014). Further differences observed in the responses to the milk ferments between the two cell types could be explained by the different *in vitro* differentiation protocols used in this study. PMA differentiation of THP-1 cells *in vitro* increases NF- κ B activation potentially resulting in a heightened sensitivity to stimuli (Chanput *et al.*, 2014).

These findings indicate that milk fermented with *L. rhamnosus* R0011 may polarise THP-1 and U937 macrophages into the immunoregulatory and atheroprotective M2b phenotype. This phenotype is characterised by the increased transcription of the regulatory cytokines IL-10 and IL-1Ra, with simultaneous transcription of pro-inflammatory cytokines such as IL-6 and IL-1 β (Colin *et al.*, 2014). Indeed, both

commensal and pathogenic strains of bacteria can induce cytokine transcription profiles within resting macrophages suggesting polarization towards either the M1 or M2 macrophage phenotype (Christoffersen *et al.*, 2013; Isidro *et al.*, 2014). However, further characterisation using cell surface markers is required to confirm polarization.

Conclusion

L. rhamnosus R0011 and *L. helveticus* R0389 secrete a bioactive molecule (or molecules) with immunomodulatory properties. This bioactive molecule was shown to down-regulate IL-8 production from challenged human HT-29 IEC and was shown to have differential effects on CINC-1 production from challenged rat IEC-6 cells, depending on the type of TLR agonist challenge.

Milk fermented with *L. rhamnosus* R0011 appears to polarise resting THP-1 and U937 macrophages into the immunoregulatory M2b macrophage phenotype. This was characterised by the increased production of IL-10, IL-1Ra, IL-6, and IL-1 β from both LPS-stimulated THP-1 and U937 macrophages cultured with *L. rhamnosus* R0011 milk ferments. These effects were only observed when cells were cultured with ferments containing live bacteria and not the filtered ferments containing the secreted products from *L. rhamnosus* R0011 following growth in milk.

Taken together, *L. rhamnosus* R0011 has immunomodulatory activity on two distinct cell types involved in innate immunity. Although these effects are observed when R0011 is grown in different substrates, the requirement for microbe-cell contact observed for R0011 cultured in milk suggest a difference in the production of soluble bioactive components. Indeed, growth in more complex medium such as milk can alter

gene and subsequent protein expression in lactic acid bacteria (Siragusa *et al.*, 2014). It is currently unknown whether or not *L. rhamnosus* R0011 only produces the secreted bioactive molecule when grown in RPMI or if the THP-1/U937 macrophages are not affected by the secreted bioactive molecule. Filtered *L. rhamnosus* R0011 milk ferments have been shown to modulate prostaglandin production by IECs suggesting that bioactive molecules are released during the fermentation process (Fiander *et al.*, 2003). This necessitates the need for cross-over studies examining the effects of RPMI-grown *L. rhamnosus* R0011 secreted products on challenged monocytes and macrophages and whole *L. rhamnosus* R0011 milk ferments on challenged IECs.

Future Directions

In order to elucidate the molecular pathways which are being modulated by the *L. rhamnosus* and *L. helveticus* R0389 secretomes, transcriptional profiling of IECs exposed to the secretomes in response to pro-inflammatory challenge using whole-genome microarrays should be performed. Although effects on gene expression in resting HT-29 IEC exposed to live *L. rhamnosus* R0011 have been reported with effects on genes involved in TLR and MAPK signalling (Audy *et al.*, 2013), effects on TLR-agonist induced stimuli have not been examined to date. This will potentially allow for the delineation of the mechanisms of action of the secretomes and should focus on negative regulators of the innate immune system such as the A20 protein. Further characterisation of the bioactive constituents of the secretomes should also be carried out using a combination of high-performance liquid chromatography and matrix-assisted laser desorption/ionization time of flight mass spectrometry to identify any potential proteins which may be eliciting the anti-inflammatory effect. Although there have been 132

unique proteins in *L. rhamnosus* R0011 which have been hypothesized to be secreted by the cell, only some have been annotated. These annotated proteins have been thought to be involved in binding to the mucin layer or fibronectin within the host (Kant *et al.*, 2014). This presents an intriguing area of future research to identify and annotate the remaining secreted proteins. If no proteins are found, gas chromatography-mass spectrometry should be used to identify other secreted products retaining immunomodulatory activity.

Although this study has suggested that the *L. rhamnosus* R0011 milk ferments may polarize resting macrophages into the immunoregulatory and atheroprotective M2b phenotype, these experiments were done using established, transformed cell lines. In order to gain a more comprehensive understanding of the range of the bioactivity of the milk ferments on monocytes and macrophages, effects of the milk ferments should be tested on peripheral blood monocytes isolated from healthy individuals.

Evaluation of the bioactivity of milk fermented with *L. helveticus* R0389 on the expression of pro-atherogenic biomarkers and macrophage polarization will also be useful. Milk fermented with *L. helveticus* R0389 has been shown to have immunomodulatory activity. Peptide fractions from milk fermented with *L. helveticus* R0389 promoted a Th2-mediated immunological response, characterised by an increase in IL-4, *in vivo* in rats infected with *E. coli* 0157:H7 (LeBlanc *et al.*, 2004). Vinderola *et al.*, (2007) have also shown that the milk fermented with *L. helveticus* R0389 modulates host immune responses within the gut by activating calcineurin (Vinderola *et al.*, 2007). Additional studies will allow for direct comparisons between secreted products retaining immunomodulatory capacities on LPS challenged human monocytes and macrophages.

Further characterisation of the bioactivity of *L. rhamnosus* R0011 and *L. helveticus* R0389 derived milk ferments should be carried out using a trans-well system consisting of human IECs and human monocytes or macrophages. This approach will allow for a more ideal simulation of the potential interactions between the *L. rhamnosus* R0011 and *L. helveticus* R0389 milk ferments and cells of the innate immune system. This system would also allow for the identification of bioactive proteins and molecules liberated during the fermentation process which can be transported across the IEC layer and modulate immune responses in the underlying monocytes or macrophages. It will also be interesting to see if the *L. rhamnosus* R0011 milk ferments can condition the IECs to produce regulatory cytokines which could down-regulate pro-inflammatory cytokine production from activated monocytes or macrophages.

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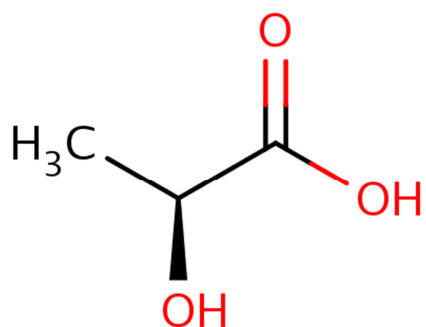
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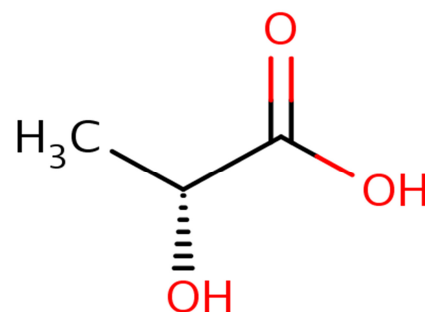
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Appendix



L-(+)-Lactic Acid



D (-)-Lactic Acid

Figure A1. Chemical configuration of L (+)-lactic acid and D(-) lactic acid. Images obtained from the Chemical Entities of Biological Interest database.