Immunomodulatory Effects of Lactic Acid Bacteria on Human Intestinal Epithelial Cells and Macrophages in the Context of a Pro-inflammatory Challenge

By

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ABSTRACT

Immunomodulatory effects of lactic acid bacteria vary with strain and may vary with growth phase and medium. The ability of different lactobacilli strains (*Lactobacillus helveticus* R0052, *L. rhamnosus* R0011, *L. rhamnosus* GG) at different growth phases to modulate macrophage and intestinal epithelial cell cytokine production following a pro-inflammatory challenge was examined. Modulation of cytokine production by human macrophage cell lines (U-937) and intestinal epithelial cells (HT-29) induced by Tumor Necrosis Factor α was assayed by ELISA for interleukin-8 (IL-8). Granulocyte-macrophage colony stimulating factor (GM-CSF) production was assayed by ELISA in the HT-29 cell line. Strain-dependent differences were observed in the ability of viable bacteria and spent de Mann-Rogosa-Sharpe (MRS) broths from log versus stationary growth phase in HT-29 and U-937 cells. Overall, variation in the immunomodulatory activity of these lactic acid bacteria and spent broths reflects not only strain variation but potentially also differences in growth phase and substrate.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

broths at 1/10, 1/50 and 1/100 dilutions. Culture supernatants were collected 24 hrs post challenge for IL-8 measurement by ELISA. Data are shown as mean (±SE) percent of TNFα-challenged control response. A trend toward decreased IL-8 production was observed for U-937 cells treated with stationary phase RO011 broth relative to controls (One-way ANOVA, $p = 0.082$; n=5).

Figure 8. IL-8 production by TNFα-challenged U-937 macrophages (TNFα at 40 ng/mL) concurrently treated with RO052 log, RO052 stationary or unspent MRS broths at 1/10, 1/50 and 1/100 dilutions. Culture supernatants were 24 hrs post challenge for IL-8 measurement by ELISA. Data are shown as mean (±SE) percent of TNFα-challenged control response (One-way ANOVA, $p = 0.59$; n = 5).

Figure 9. IL-8 production by TNFα-challenged U-937 macrophages (TNFα at 40 ng/mL) concurrently treated with GG log, GG stationary or unspent MRS broths at 1/10, 1/50 and 1/100 dilutions. Culture supernatants were 24 hrs post challenge for IL-8 measurement by ELISA. Data are shown as mean (±SE) percent of TNFα-challenged control response (One-way ANOVA, $p = 0.64$; n = 4).

Figure 10. Effect of spent *Lactobacillus* MRS broths (10% by total volume) on interleukin-8 production by TNFα-challenged HT-29 intestinal epithelial cells (5 x 10^5 cells/mL) challenged with 200 ng/mL TNFα following concurrent incubation. IL-8 was measured 6 hours after TNFα challenge. Data are shown as mean concentration in pg/mL ±SE. Asterisks denote statistical significance (One-way ANOVA; $p < 0.0001$; n = 6).

Figure 11. Effect of acidifed media broths and freeze-dried stationary phase *L. rhamnosus* RO011 bacteria and spent broth (0.01 g/mL; 10% by total volume) on interleukin-8 production by U-937 monocytic macrophage cells (5 x 10^5 cells/mL) in the presence of TNFα (40 ng/mL) following concurrent incubation. IL-8 was measured 24 hours after TNFα challenge and data are shown as mean concentration in pg/mL ±SE. Asterisks denote significant difference from control (One-way ANOVA; $p < 0.0001$; n = 3).

Figure 12. Effects of acidified media broths (10% by total volume) and freeze-dried stationary phase *L. rhamnosus* RO011 bacteria (5 x 10^6 cells/mL) and spent broth (0.01 g/mL; 10% by total volume) on interleukin-8 production by HT-29 intestinal epithelial cells (5 x 10^5 cells/mL) in the presence of TNFα (200 ng/mL) following concurrent incubation. IL-8 was measured 24 hours after TNFα challenge and data are shown as mean concentration in pg/mL ±SE. Asterisks denote statistical significance (One-way ANOVA; $p < 0.0001$; n = 3).

Figure 13. Effect of three strains of *Lactobacillus* at both growth phases, separately (5 x 10^6 cfu/mL) on interleukin-8 production by U-937 monocytic macrophage cells (5 x 10^5 cells/mL) in the absence of TNFα following concurrent incubation. IL-8 was measured 24 hours after TNFα challenge and data are shown as mean concentration in pg/mL ±SE (One-way ANOVA; p = 0.16; n = 4).

Figure 14. Effect of three strains of *Lactobacillus* at both growth phases, separately (5 x 10^6 cfu/mL) on interleukin-8 production by U-937 monocytic macrophage cells (5 x 10^5 cells/mL) in the presence of TNFα (40 ng/mL) following concurrent incubation. IL-8 was measured 24 hours after TNFα challenge and data are shown as mean concentration in pg/mL ±SE (One-way ANOVA; p = 0.69; n = 4).

Figure 15. Effect of three strains of *Lactobacillus* at both growth phases, separately (5 x 10^6 cfu/mL) on interleukin-8 production by HT-29 intestinal epithelial cells (5 x 10^5 cells/mL) in the absence of TNFα following concurrent incubation. IL-8 was measured 6 hours after TNFα challenge and data are shown as mean concentration in pg/mL ±SE (One-way ANOVA; p = 0.58; n = 4).

Figure 16. Effect of three strains of *Lactobacillus* at both growth phases, separately (5 x 10^6 cfu/mL) on interleukin-8 production by HT-29 intestinal epithelial cells (5 x 10^5 cells/mL) in the presence of TNFα (200 ng/mL) following concurrent incubation. IL-8 was measured 6 hours after TNFα challenge and data are shown as mean concentration in pg/mL ±SE (One-way ANOVA; p = 0.32; n = 4).

Figure 17. Effect of three strains of *Lactobacillus* at both growth phases, separately (5 x 10^6 cfu/mL) on interleukin-8 production by HT-29 intestinal epithelial cells (5 x 10^5 cells/mL) in the presence of TNFα (200 ng/mL) following concurrent incubation. IL-8 was measured 6 hours after TNFα challenge and data are shown as mean (±SE) percent of TNFα-challenged control response (One-way ANOVA; p = 0.12; n = 4).

LIST OF ABBREVIATIONS

ADCC – Antibody-dependent cellular cytotoxicity

ANOVA – Analysis of variance

APC – Antigen presenting cell

ATCC – American Type Culture Collection

BSA – Bovine serum albumin

CFU – Colony forming unit

DNA – Deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

FAO – Food and Agriculture Organization

FBS – Fetal bovine serum

FcR – Fragment crystallizable receptor

GALT – Gut-associated lymphoid tissue

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GI - Gastrointestinal

GM-CSF – Granulocyte-macrophage colony stimulating factor

GSK3 – Glycogen synthase kinase-3

HIV – Human immunodeficiency virus

IFN – Interferon

IL-1β – Interleukin-1-beta

IL-10 – Interleukin-10

IL-8 – Interleukin-8

LAB – Lactic acid bacteria

LPS – Lipopolysaccharide

M - cell – Microfold cell

MALT – Mucosa-associated lymphoid tissue

MAPK – Mitogen-activated protein kinases

MHC – Major histocompatiblity complex

MRS – de Man, Rogosa and Sharpe (bacterial media)

NF-κB - Nuclear factor kappa-light-chain-enhancer of B-cells

NK – Natural killer

PBMC – Peripheral blood mononuclear cell

PBS – Phosphate-buffered saline

PI3K – Phosphatidylinositol 3-kinase

PMA - Phorbol 12-myristate 13-acetate

QS – Quorum sensing

RANTES - Regulated upon activation, normal T-cell expressed, and secreted

ROS – Reactive oxygen species

RPM – Revolutions per minute

RPMI 1640 – Roswell Park Memorial Institute (balanced salt solution)

SCFA – Short-chain fatty acid

SEM – Standard error of the mean

Strep-HRP – Streptavidin-Horseradish peroxidase

Th or TH – T-helper

TLR – Toll-like receptors

TMB – 3,3',5,5' - tetramethylbenzidine

TNF - Tumour necrosis factor

WHO – World Health Organization

INTRODUCTION

Part 1: The Immune System

One of the most complex biological systems in vertebrate species is the immune system. As organisms began to occupy unexploited evolutionary and ecological niches, an evolution that observed the need for further tissue differentiation and tissue-tissue interactions occurred. Consequently, the need for an organism to distinguish foreign material from its own tissue was required to ensure proper physiological function and viability in the context of new external environments. It is logical to assume that individual organisms that were best immunologically suited for their respective environments would go on to become the ancestors of modern day vertebrates, since the variation in immune 'tuning' would provide a significant, unbalanced selective pressure. Since then, natural selection has 'shaped' immune responses to be just as intricate as evolution has allowed other physiological and biochemical processes to become.

Generally, vertebrate immune responses can be broken down into two classifications: innate and adaptive. Innate immune responses are those which can be considered constitutive and non-specific. Innate responses are usually quite basic, immediate, lack memory and have common mechanistic elements. For example, a bacterial infection may elicit an inflammatory response that utilizes part or all of a set of biochemical pathways also utilized in other types of tissue damage that require an immediate response – such as a skin burn (Ishii *et*

al., 2008). This also illustrates why innate responses are referred to as 'non-specific' (Bradley, 2008). In humans, the most common cells involved in innate responses to bacterial and foreign material are neutrophils, monocytes, macrophages and dendritic cells (Coombes and Powrie, 2008). All of the aforementioned cells are capable of phagocytosing and digesting foreign antigen in some capacity. These cells also possess other defence mechanisms such as the production of reactive oxygen intermediates and nitric oxide (Witthoft *et al*., 1998; Korhonen *et al*., 2001). These products are then effective in eliminating invasive pathogens by causing membrane, protein and DNA (Deoxyribonucleic acid) damage as well as the degradation of iron-sulfur centers involved in vital cellular processes (Salzman *et al*., 1998; Bertholet *et al*., 1999). These defences are occasionally, but not necessarily, dependent on signaling from other cells and tissues (Witthoft *et al*., 1998; Korhonen *et al*., 2004).

On the viral side of innate immunity, Natural Killer (NK) cells are capable of receiving signals from cells infected with viral material and are cytotoxic towards these cells despite not being required to specifically recognize Major Histocompatibility Complex Class 1 (MHC-1) cell surface proteins. NK cells produce cell-signaling molecules, known as cytokines, as well as granzymes and perforins involved in target-cell killing (Ward, 2004). NK cells also possess a Fragment Crystallizable Receptor (FcRyIII) – a cell-surface protein that allows for class-specific antibody binding and allows them to carry out antibody-dependent cellular cytotoxicity (ADCC). FcR proteins are a conserved portion of antibody

molecules thus allowing NK cells to participate indirectly as an important component of adaptive immunity (Ward, 2004).

As previously mentioned, many of the mechanisms involved in the initiation of innate immune responses are very similar. In the context of this thesis, the most relevant innate immune mechanism is the activation of a protein complex known as Nuclear Factor Kappa-Light-Chain-Enhancer of B-cells, or NF-κB. This complex is usually found floating freely in the cytoplasm in an inactivated state. There are three NF-κB subunits which are integral to end-product transcription. These subunits are NF-κB1 p50, NF-κB2 p52 and RelA p65 (Perkins, 2004). In the cytoplasm, the NF-κB1 p50 is sequestered and inhibited by IκBα/β/ε, while the NF-κB2 p52 and RelA p65 complexes are sequestered in tandem with IκBα (Petrof *et al*., 2004). Depending on the dynamics of upstream signaling, ubiquitin-tagging of the IκB sequestering factor will result in proteosomal degradation, at which point one of the NF-κB1 p50 or NF-κB2 p52 will bind to RelA p65 along with a variety of other potential protein chaperones which are integral in allowing nuclear translocation to occur (Huxford *et al*., 1998; Perkins, 2004). Dimers of any combination of the p50, p52 or RelA p65 will result in the transcription of numerous genes associated with innate pro-inflammatory responses, as well as tissue proliferation and differentiation (Suk *et al*., 2001; Yasumoto *et al*., 1992).

Due to the fact that this mechanism is quite complex and there are many forms in which NF-κB subunits can enter the nucleus, there is a debate over the consequences of NF-κB activation, as many of its supposed consequences are

physiologically contradictory in similar tissues (Perkins, 2004). Despite this, there is a strong consensus that NF-κB activation and translocation is a convergent endpoint reached through many different upstream mechanisms and cascades within an innate-immunity context (Hong *et al*., 2009). Initiation of NF-κB translocation and activation occurs through signal transduction initiated by the activation of the following receptors: Tumor-Necrosis Factor (TNFR1), Toll-like Receptors (TLR) and Interferon-Gamma Receptor complexes (Balkwill, 2009; Fayol-Messaoudi *et al*., 2005; Nareika *et al*., 2005) (Figure 1).

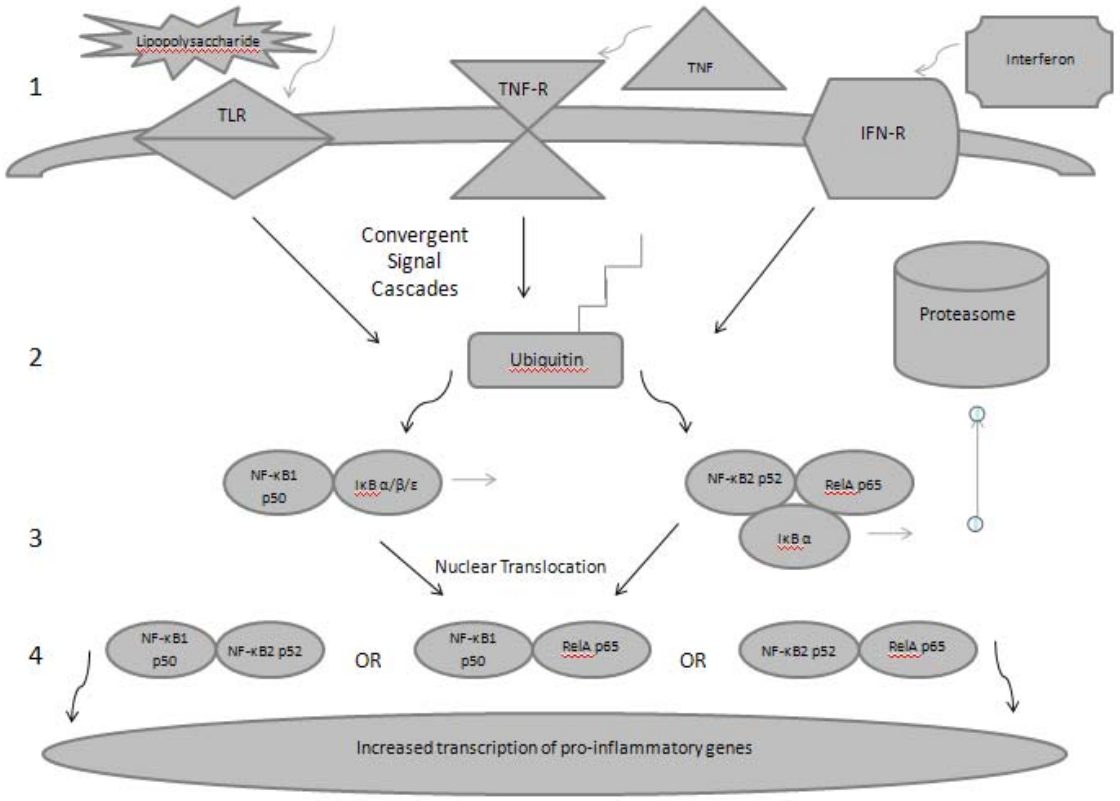

Figure 1. Generalized representation of the fundamental mechanism of NF-κB activation. The process can be broken down into four stages: 1) Ligand binding amongst a variety of ligand-specific transmembrane receptors, which activate signal cascades that converge upon 2) the ubiquitination of IκB complexes and liberation of essential protein subunits which 3) recombine to form dimers capable of nuclear translocation, which may result in the transcription of genes associated with pro-inflammatory responses (As adapted from Perkins, 2004).

The adaptive immune response often interacts with the innate immune response and boundaries between the two types of response are not always clear. Adaptive immune responses often require more time and physiological investment to initiate as further complex intercellular signaling and processing is often necessary (Byrne *et al*., 2002). The dogmatic crux of adaptive immunity can be summarized in the following sequence of events: 1) antigen presentation by a cell capable of processing and presenting antigen on an MHC Class II protein. 2) Specific recognition of the MHC-antigen complex on the Antigen Presenting Cell (APC) and binding by T-lymphocytes, which in turn produce cytokines that promote B-cell proliferation and antibody production (Haller *et al*. 2002) 3) Retention of small populations of specific antibody producing memory B-cells as a permanent adaptation against a specific antigen, providing the immune system with "memory", allowing for more rapid and effective responses with the same antigen in subsequent encounters. Cells capable of antigen presentation were once thought to be limited to dendritic cells, macrophages and B-cells, but have now been proven to include certain epithelial tissues (Galli *et al*., 2008; Byrne *et al*., 2002).

Naïve and mature T-lymphocytes are not capable of detecting independent antigen. Recognition is only possible after processing within APC's. Although not fully understood, it is believed that T-cells evolved as an intermediate step in the process of antibody production to protect the organism from autoimmunity. This notion is further strengthened by the fact that matured T-cells can be divided into two distinct CD4+ subgroups – Th1 cells and Th2

cells. Th1 cells take part in cell-mediated adaptive immunity through the recruitment of immunological interceders, such as macrophages, neutrophils and even CD8+ T-cells.

Some of the aforementioned interceders have the characteristic of producing apoptosis-inducing factors that can target infected cells. The second type of CD4+ T-cell, Th2, produces cytokines that promote antibody class switching and B-cell maturation (Cerrutti, 2008). This degree of differentiation into different subgroups of cells has allowed for decreased errors in the immune system, decreasing the likelihood of erroneous self-attack (So *et al*., 2008). Although it takes a great deal of time for these specific and lasting responses to be elicited in the host, the benefit of such a mechanism is beneficial - as natural selection has proven.

Interestingly, most diseases in which the body recognizes its own tissues (in whole or in part) as foreign are the result of a failure or flaw in the adaptive immune response. Perhaps the best illustration of such a flaw is what we refer to as flesh-eating disease. In this case, bacteria such as *Staphylococcus pyogenes*, which have the ability to produce potent endotoxins, grow in human tissue (Saito and Miyake, 2009). Through the ability of these endotoxins to force ‘bridging’ between APCs and T-cells, these endotoxins stimulate excessive Th cell responses, which usually result in a cytokine storm that triggers an overwhelming, immediate response to these toxins (Saito and Miyake, 2009). Adding to this response is respiratory burst by neutrophils and macrophages. Unfortunately, this response is usually about 1000-fold excessive and places

host tissues at risk of DNA damage and self-induced necrosis, and the whole organism is at put at risk of toxic shock (Saito and Miyake, 2009).

The above example illustrates the point that not all pathogenic bacteria cause damage through exploiting or parasitizing human tissue which results in damage. Instead, some pathogens disrupt the immune system through more sophisticated mechanisms.

It should now be quite clear how precise and intricate the mechanisms of both innate and adaptive immunity must be to ensure autoimmune responses do not occur while still allowing the host to detect invading pathogens. However, the immune system must also be discriminatory toward many of the endogenous bacteria that are encountered by the organism on a constant basis. It is with this in mind that the human intestinal tract is a logical model for observing the mechanisms and interactions of innate and adaptive immunity in humans. This tissue experiences continual exposure to chemical irritants, antigenic stimuli including food components and invasive bacteria from the external environment (Smith and Nagler-Anderson, 2005). Commensal bacteria have also been shown to inhabit these gut mucosal tissues, implying that a discriminatory factor must exist within both innate and adaptive immunity (Smith and Nagler-Anderson, 2005). Improving the understanding of this miniature ecological niche is currently of primary importance to many immunologists.

Part 2: The Intestinal Ecosystem

Currently, it is believed that up to 70% of the human body's immune system (by mass) is in close contact and associated with intestinal tissue (Artis, 2008). The majority of this mass can further be classified as what is known as MALT (Mucosa-Associated Lymphoid Tissue). Within this, another classification of immune tissue can be established: GALT (Gut-Associated Lymphoid Tissue) (Artis, 2008). Although MALT is primarily a dynamic cluster of tissues often associated with the gastrointestinal system, this tissue-formation is also found in the eyes, skin and some glands (Brandtzaeg and Pabst, 2004). This tissue is mainly composed of T and B lymphocytes, as well as some macrophages and microfold (M)-cells, which are often embedded in epithelial tissues with the purpose of presenting antigen to subcutaneous tissues, especially when amongst epithelial cells that do not possess this ability (Artis, 2008; Schulzke *et al*., 2009). Interestingly, many intestinal epithelial cells possess the ability to present antigen on MHCII proteins with the accompaniment of other co-stimulatory molecules, such as CD80(B7-1) and CD86(B7-2), which improve adhesion between the IEC and associated T-cell (Zou and Chen, 2008).

On a separate note, many epithelial cells in the intestine have the ability to secrete a variety of proteins designed for either 'flushing' away foreign material (such as the mucin family of glycosylated proteins) or for destruction of phagocytized bacteria in other cells through the production of cysteine-rich β-defensins (Mack *et al*., 2003; Schlee *et al*., 2008). Both of these characteristics fall under the branch of innate immunity (Schlee *et al*., 2007).

More specifically, GALT is composed of cell clusters that utilize both innate and adaptive immune responses to deal with foreign antigen. One of the most intriguing and complex components of the GALT are structures known as Peyer's Patches – the anatomy of which has been known for years, while specific immune functions are still being discovered (Artis, 2008). These patches are located beneath intestinal epithelial tissues within the lamina propria as a loose connective tissue (Artis, 2008). Peyer's Patches also have the distinction of being the reason for differences in the nomenclature of intestinal regions, where the ileum is distinguished from both the jejunum and duodenum based on an increasing density of patches as the intestinal tract descends (Artis, 2008). These patches are also useful in differentiating where the small intestine ends and colonic functions are observed.

In general, mammalian digestive tissues harbour many different types of bacteria and form one of the most densely populated microbial habitats in biology (Artis, 2008). As mentioned earlier, this environment hosts many environmental niches. Colonization of the intestinal tract can occur within minutes of mammalian birth, at which point colonizing bacteria race to exploit the environmental niches a newborn provides (Hrncir *et al*., 2008). Bacteria commonly found in the human digestive tract include: *Eubacterium* spp., *Bifidobacterium* spp., *Fusobacterium* spp., *Peptostreptococcus* spp., *Escherichia coli* spp., *Lactobacillus* spp., and *Enterobacter* spp (Pedron and Sansonetti, 2008).

It is logical to assume that the benefit conferred upon any bacterial species inhabiting the gut is related to the provision of optimum conditions for growth, survival and reproduction. With the gastrointestinal tract fundamentally acting as an 'internalized' external environment, the aforementioned microbes are provided with warmth and a constant exposure to foods ingested by the host, which may or may not be exploited by the bacteria. However, with this in mind, one must ask the reciprocate question: What benefit do the bacteria confer upon the host? Although digestion can occur across intestinal tissues in the absence of the normal gut flora, mice-models produced with the goal of absolute intestinal sterility ('germ-free') have consistently been shown to have difficulty in developing the same immune tissues and physiology associated with wild-type (control) organisms (Hrncir *et al*., 2008).

The mechanisms behind this vast developmental discrepancy have been investigated and debated for many years now, and from this a variety of new questions have arisen. Many have pondered what other effects these populations of organisms, in whole or in part, have on fully developed hosts in other contexts (such as infection, for example). For the most part, the research and development that goes into answering questions surrounding the host microflora and its beneficial effects on the immune system have been draped in a field broadly referred to as 'probiotics', which is derived from the Latin language, meaning 'for life' (Lilley and Stillwell, 1965). This is in clear reference to the biotechnological potential for using or modifying the host microflora to improve immunity, general health or other life processes. However, with so many

variables surrounding the activity and processes of the normal gut flora, scientists and researchers are struggling with the single most fundamental question that encompasses the entire field: What defines a “probiotic”?

Part 3: Probiotics and Biomarkers

Many definitions of a probiotic have developed throughout the second half of the 20th Century. The definitions have concerned specific bacterial strains, viability, quantity and benefits conferred (Sanders, 2008). The most widely accepted definition of probiotics is currently employed through an emphasis on the manipulation or alteration of the natural microflora (Defined by the Food and Agriculture Organization and the World Health Organization as “live organisms that when administered in adequate amounts confer a health benefit on the host”) (FAO and WHO, 2002). Unfortunately, according to this definition the use of the “probiotic” term now hinges on what is considered a health benefit. To this end, some in the community of probiotic research have very liberal definitions of a probiotic effect, while many others will not concede the title of ‘probiotic’ upon an organism until an overwhelming number of perceived health benefits have been proven specific to the respective organism.

Dependent on the standardization of a definition probiotics is the emerging field of ‘prebiotics’, which can be defined as “nondigestible substances that provide a beneficial physiological effect on the host by selectively stimulating the favorable growth or activity of a limited number indigenous bacteria” (Reid, 2008). It is important to note that unlike the definition of probiotics, the prebiotic

definition seems tied to physiological effects, which in and of themselves may or may not necessarily be responsible for beneficial health effects.

Some of the beneficial health effects that have been generally accepted as well established within the probiotic field, according to de Vrese and Schrezenmeir (2008) are: a) a reduction in antibiotic induced diarrhea; b) a reduction in the concentration of cancer-promoting enzymes and putrefactive enzymes in the gut; c) an alleviation of inflammatory diseases and *H. pylori* infections and d) a prevention of allergies and infections.

Since most of the beneficial health effects claimed attributable to any so-called probiotic have a basis in cell biology and immunology, it is the instruments of these fields that provide the basis for studying these effects (Reid and Burton, 2002). The fields of cell biology and immunology are both reliant on the use of biomarkers. Effective biomarkers are those molecules which are indicative of some condition and specific to only that condition and very few others (Lebeer *et al*., 2008; Johnson-Henry *et al*., 2004). This has been an effective method for gaining an improved knowledge of cellular signaling and extracellular events. In other contexts, such as molecular biology, genome-wide microarrays are capable of taking instantaneous gene-expression ‘snap-shots’ (Peterson *et al*., 2008). Unfortunately, as many intracellular and extracellular pathways are currently too complex to instantaneously map or trace in their entirety at the enzymatic level, select markers from these pathways are taken and used as indicative of the involvement of those pathways in specific conditions (Peterson *et al*., 2008).

In a purely immunological sense, many biomarkers have been selected as indicative of specific conditions. The best practical example of a biomarker is a Human Immunodeficiency Virus (HIV) test, whereby the standalone virions are not the target of detection. Instead, a rise in HIV-specific antibodies by the host is used to indicate infection (Kwong and Wilson, 2009). In much the same way, biomarkers traditionally used in pure immunological studies are of primary importance in probiotic studies, and there have recently been many studies conducted on many of these biomarkers (Cross *et al*., 2004). These studies are conducted in the same manner as many pure immunological studies: through animal models and isolated cell culture.

Part 4: Immunological Research Investigating Probiotic Bioactivity and Immunomodulation

Section A: Empirical Evidence of Probiotic Modulation of Innate Immunity

The most common model systems in the field of probiotic and immunological research are *in vitro* cell culture and *in situ* animal studies. In the former, a standardized cell line is established from a sample tissue that is taken from a transformed tissue sample. The deregulation of cell cycle control allows for these cells to be easily used in culture and to establish permanent cell lines. For example, the HT-29 intestinal epithelial cell line was derived from glandular adenocarcinomas and the U-937 monocytic cell line was isolated from a patient

with a hematological malignancy (Sundstrom and Nilsson, 1976; Chen *et al*., 1987). The U-937 cell line can be further differentiated into a more mature macrophage stage using PMA (phorbol 12-myristate 13-acetate), Interferon-gamma or vitamin D (Kitamura *et al*., 2004).

Although these models are simple, they provide a 'first step' in evaluating the probable effect of an antigen, growth factor or other stimulant before moving to more complex and costly models. Interestingly, tissue culture has existed since the early 1900's and has since provided the basis for one of the most significant events in biotechnological history: the discovery of hybridomas and monoclonal antibody production.

It is important to consider the fact that many of the biomarkers currently used in immunological research have multiple roles which are often conflicting between different tissues and cell types. An excellent example of this is provided by the cytokine Tumour Necrosis Factor-α (TNFα), which not only acts as an excellent challenge/inducer of NF-kB activation, but it was originally discovered as possessing potent tumour cytotoxicity in earlier animal models (Bradley, 2008).

Investigations into probiotic activity have also complicated the notion that commensal bacteria are only beneficial to the health of the host. This is best exemplified by the reporting of NF-kB pathway induction by *L. casei*, given the pro-inflammatory context of the upregulated biomarkers (Kim *et al*., 2006). Usually, NF-kB activation is regarded as a pro-inflammatory response, and

inhibition of IEC NF-kB activation by gut commensals has been suggested as a mechanism for preventing excessive inflammatory responses to commensal microflora (Neish *et al*., 2000; Riedel *et al*., 2006; Borruel *et al*., 2003).

In general, most gram-positive Lactobacilli are believed to be neutral organisms in the human gut, if not conferring some immunomodulatory health benefit (Vizoso Pinto *et al*., 2007). However, this is not necessarily true. When introduced to the blood stream, numerous Lactobacilli strains have been shown to cause endocarditis, a chronic active infection of the heart (Amrikachi *et al*., 1997). This not only illustrates that the term 'probiotic' is context-dependent, but also that the regulation of the microflora in the gut is of extreme importance given its proximity to the bloodstream, as utilized in normal digestive functions. This also introduces a cautionary note regarding the use of probiotics in certain therapeutic applications where there is risk of spread via the bloodstream (Hessle *et al*., 1999).

Of particular interest in probiotic immunology is the mechanism and degree of gastrointestinal adhesion by commensal bacteria, and whether or not this adhesion or other biological effects are dependent on viability. There are two reasons for this. First, if the mechanism of adhesion is understood, it may be possible to determine how this affects interactions with epithelial tissues, as well as other cross-talk properties. Secondly, determining whether or not a specific characteristic of a commensal bacterial strain is dependent on viability is significant in light of practical applications and commercial implications. There is an ambiguity regarding whether or not ingested commensal bacteria will colonize

in the gut, as the environmental stressors encountered upon oral ingestion change rapidly. These include changes in pH between the stomach and intestinal tract, the thick mucous layer covering the intestine, as well as variable interactions between bacterial species.

As the degree of true gastrointestinal colonization through oral consumption of probiotics is controversial, the future of commensal probiotic foods and supplements may hinge on isolating bioactive components which are produced or released by these bacteria. An excellent example of this was provided by Ghadimi *et al*. (2008) in which a wide array of diverse gut-derived bacteria and their respective genomic DNA were tested on the peripheral blood mononuclear cells of healthy subjects as well as those with chronic atopic dermatitis. The genomic DNA of the following bacterial strains was used: *Lactobacillus rhamnosus* GG, *Lactobacillus gasseri* (PA16/8), *Bifidobacterium bifidum* (MP20/5), and *Bifidobacterium longum* (SP07/3). Interestingly, the genomic DNA of each individual strain was shown to decrease the secretion of *Dermatophagoides pteronyssinus*-induced Th2 cytokines as effectively as the live bacteria. Although it would probably be erroneous to believe that it is solely the DNA of the live bacteria that is responsible for the change in cytokine profile, the message resulting from the experiment is clear: any single intracellular or extracellular component of a bacterial species able to exert some “probiotic” biological activity could be responsible for viability-independent immunomodulatory effects.

One of the most striking examples of viability-dependent immunomodulation was provided by Ma *et al*. (2004), where viable *L. reuteri* was found to decrease TNFα-induced interleukin-8 (IL-8) expression by HT-29 IEC at the molecular and secretory levels. This was not the case however when the *L. reuteri* were heat killed, illustrating a requirement for viability for the immunomodulatory effects of this strain. IL-8 is a chemotactic cytokine (chemokine) which recruits macrophages and NK cells from the blood stream and/or Peyer's patches into epithelial gut tissues resulting in improved antigen sampling (Candela *et al*., 2008). The IL-8 secretory response is a consequence of NF-kB activation, and is initiated by a variety of stimulants, including toll-like receptor (TLR) activation by lipopolysaccharide and teichoic acid, and cytokine receptor activation, such as the tumor necrosis factor receptor (TNF-R) and Interferon-gamma receptor (IFN-R). Although this cytokine has long been associated with leukocyte recruitment and inflammation (Baggiolini *et al*., 1995), the rhodopsin-type receptors it acts upon are also affected by other chemotaxins, suggesting ambiguity in the current understanding of specific leukocyte recruitment (Baggiolini *et al*., 1995).

An improved understanding of bacterial adhesion within the intestinal mucosa is essential in shaping the direction of future probiotic research. Assays have been developed which can determine the amount of adhesion through fluorescence in simulated mucosal conditions. To date, one of the most advanced assays of intestinal bacterial adhesion was conducted by Izquierdo *et al*. (2008). The goal of this study was to determine the tolerance of eight

Bifidobacterium longum strains to stomach acid and bile within porcine digestive mucous. The strains most effective at adhering to mucin were discovered to be *B. longum* NCC 2705 and BIF 53. However, upon treatment with lysozyme, it was discovered that these strains were not nearly as effective at adhering to mucin, thus suggesting that cell-wall components are involved in the adhesion mechanism (Izquierdo *et al*. 2008). This supports the notion that differences in bacterial cell-wall hydrophobicity may be responsible for the adhesive properties observed in many gram positive strains (Vizoso Pinto *et al*. 2007).

Although there is still much activity in the area of cytokine profiling in the intestinal ecosystem, the use of co-culture systems to further mimic *in vivo* conditions is becoming more prevalent (Haller *et al*., 2000). The impact of probiotics and commensals on dendritic cells has also been the focus of recent research (Drakes *et al*., 2004).

Section B: Other Approaches to Probiotic-related Immunomodulation

Although it may appear that most of the research investigating probiotic immunomodulatory activity is entirely based on bacterial:host cell interactions, interest in bacterial supernatant:host cell interactions has increased. Several recent studies have examined the effects of spent bacterial broths and media and analyzed them for secreted proteins, metabolites and enzymes. An example of this is provided by research conducted by Hoarau *et al*. (2008), where it was discovered that *Bifidobacterium breve* BbC50sn supernatant modulated signal

transduction involved in the immune functions of human dendritic cells. In this experiment, pathway blocking and multiple stimuli were used to determine how *B. breve* BbC50sn is capable of prolonging survival and modulating cytokine secretion.

To determine the pathways involved, multiple combinations of *B. breve* BbC50sn supernatant, lipopolysaccharide and zymosan with inhibitors of p38 mitogen activated protein kinases (MAPK), glycogen synthase kinase-3 (GSK3) and phosphatidylinositol 3-kinase (PI3K) pathways were tested and, IL-10 secretion was assayed. IL-10 secretion was greatest when the PI3K pathway was positively involved in prolonged DC survival (as induced by BbC50sn). It was determined that the BbC50sn must be influencing the dendritic cells through a Toll-like Receptor 2 mechanism, as PI3K is one of the first pathways initiated through TLR2 activation. Although the identity of the TLR2 agonist released by BbC50 into the culture media was not determined, this study provides evidence for potential immunomodulation by secreted bacterial products or metabolites.

Another example of supernatant-based immunomodulatory investigation has been provided by Sougioultzis *et al*., (2006). In this study, it was discovered that *Saccharomyces bouldardii* (*Sb*) produced an anti-inflammatory factor that significantly inhibited NF-kB-mediated IL-8 gene and cytokine expression in HT-29 cells stimulated by both TNFα and IL-1β. This effect was lost when the experiment was repeated and the *Sb* supernatant had been passed through a <10kDa molecular mass cut-off filter. As these studies illustrate, the isolation and

characterization of secreted products and factors has recently become an area of interest for many immunologists studying probiotics.

Although the previous study involved a factor produced by yeast, the probiotic field is primarily focused on bacterial products. Expanding on the empirical technique employed by Sougioultzis *et al*., (2006) was the work of Yan *et al*., (2007). *L. rhamnosus* GG broth culture supernatants were 'stripped' of their soluble protein factors, which were then purified using SDS-PAGE, Western blot analysis and mass spectroscopy. Next, two soluble factors were isolated from *L. rhamnosus* GG media, p75 and p40. The p75 protein was identified as a cell wall hydrolase, while the function of p40 is unknown despite having a close relation to a *L. casei* homolog. Isolated human and mouse colonic epithelial cells were independently treated with each of the two isolated proteins under TNFα challenge. Interestingly, Akt protein activity displayed an increase under treated challenge conditions relative to cells that were untreated, yet challenged. The Akt protein family is a set of enzymes which inhibit cytokine-mediated apoptosis through the activation of the PI3K pathway. This result is consistent with that of Hoarau *et al*., (2008), albeit through the use of a different genus of bacteria. Yan *et al*., (2007) also has a more direct objective in determining the properties of proteins secreted by *L. rhamnosus* GG than those utilized in Sougioultzis *et al*., (2006). Both of the aforementioned proteins, p40 and p75 were shown to protect the intestinal epithelial barrier from hydrogen peroxide damage (Seth *et al*., 2008).

More recently, the use of mass spectroscopy has become widespread among immunologists that have an interest in determining key molecules in bacterial cross-talk (Yang *et al*., 2007). Recently, a more comprehensive investigation into the identification of novel proteins secreted by *L. rhamnosus* GG was undertaken by Sanchez *et al*., (2009). Although many of the proteins previously found in media conditioned by *L. rhamnosus* GG are still being characterized for function, it was determined that cell wall hydrolases and a glyceraldehydes 3-phosphate dehydrogenase (GAPDH) are definitively present in *L. rhamnosus* GG-cultured media. Sanchez *et al*. (2009) further hypothesized that the extracellular GAPDH may retain enzymatic function and even play a role in bacterial adhesion to the intestinal epithelium. This has yet to be tested.

The most recent literature in supernatant-based probiotic research has provided evidence for the possibility that immunomodulation is actually determined by the secretion of much simpler molecules such as short chain fatty acids (SCFA) (Kumar *et al*., 2008). It has been suggested that the ubiquitination of the IκB sequestering factor (which activates the transcription of pro-inflammatory NF-κB-dependent genes) is inhibited by changes in the neddylation (a process analogous to ubiquitination) of cullin-1, a key player in the tagging of the liberation of the aforementioned sequestering factor (Kumar *et al*., 2008). However, this change is mediated by processes upstream of this step, where reactive oxygen species (ROS) are blocked from promoting proper neddylation of cullin-1 to initiate the pro-inflammatory response. This is largely due to reactivity between ROS with extracellular short-chain fatty acids, such as

butyrate and possibly other terminal products such as lactate from bacterial fermentations. This process is rendered a rate-limiting step, and consequently dictates the dynamics of NF-κB activation (Kumar *et al*., 2008). An intriguing issue arising from these findings is that SCFA are capable of freely crossing mammalian membranes. If SCFA are truly responsible for certain immunomodulatory effects of probiotics, this would either complicate the belief that bacterial cross-talk is intricate and tightly-regulated in conjunction with complex cellular mechanisms, such as NF-κB regulation, or suggest that there are larger, more complex physiological processes involved.

A key and often neglected aspect related to studies of both secreted products from spent-culture media and whole bacterial cells is the effect of bacterial growth phase. Growth phase could be important in determining the root cause of any observed effect of probiotics. There are two plausible, non-mutually exclusive reasons for this.

The first possibility concerns variables intrinsic to bacterial growth. A bacterial growth phase is not just an indication of the proportion of viable bacterial cells at any given time in the life cycle of a population. It is also a gradient of altered gene expression, as the enzymology of viable cells must change to consume waste products or secondary substrates as primary media is depleted (Rydzak *et al*., 2009). This change in enzymology and metabolites may yield different products not secreted at earlier growth phases, and these factors may in turn be responsible for any immunomodulatory or adhesive properties

observed. Other fluctuating factors may also play a role, such as altered oxygen availability and changes in pH as cells lyse and die (Fu and Mathews, 1999).

An example of an effect observed with respect to growth phase is provided by Haller *et al*. (1999), in which human peripheral blood monocytes (PBMC) were shown to produce more TNFα under stimulation when in contact with viable log-phase LAB strains than those that were heat-killed. However, this effect was reversed in the stationary phase. The authors hypothesized that these differences reflected changes in cell wall structure at different growth phases, and in the ability of viable bacteria to secrete waste products under these conditions (Haller *et al*., 1999). Another possibility could be that the stationary phase bacteria were secreting an immunomodulatory factor which was released upon heat killing. Growth phase has also been shown to alter the enzyme profile of pyruvate catabolism in other types of bacteria and is thus a variable that must not be overlooked in cross-talk research.

The second possibility involves density-dependent effects. There is no doubt that the intestinal ecosystem is, literally, a biological battlefield. Many different species of bacteria are attempting to occupy the same ecological niches, and have evolved mechanisms which enable them to strive for a competitive advantage for survival. One of these mechanisms is known as Quorum Sensing (QS) which can be defined as density-dependent effects that result in altered gene expression, and often concern the production of bacteriocins or other products that give populations collective advantages over other species vying for the same niche (Maldonado-Barragan *et al*., 2009). The

evolutionary benefit of quorum sensing is that it enables an all or nothing attempt for the population. Synergistic effects may be present in a total population as opposed to individual control which may be more costly on a transient, per-cell basis.

The significance of QS in probiotic studies concerns the ability of probiotics to outcompete enterinvasive bacteria, whereby secreted bacteriocins or other defensive molecules may have immunomodulatory potential or decrease inflammatory responses (Maldonado-Barragan *et al*., 2009). However, cross-talk may not be necessary for reducing inflammatory effects, as simply decreasing enteroinvasive bacterial viability (due to competition) may result in a significant decrease in functionally effective irritants, such as LPS. However, a decrease in viability may also result in an increase of LPS due to lysis.

Aside from determining what is responsible for the immunomodulatory effects of bacteria in the intestinal epithelium, many questions surrounding other, more specific variables still exist in probiotic research. Given that bacteria have to respond and adapt to their external environments in the same fashion as mammalian cells, the fluctuation of variables in probiotic models must be considered and incorporated into experiments. In a separate field of microbiological research related to probiotics, it is important to note that numerous experiments investigating the effect of cytokines and foreign endotoxins on bacterial growth have been conducted (Kanangat *et al*., 1999). There have also been attempts to artificially create synthetic intestinal models for bacterial culture, as well as pure bacterial experiments in which the effects of

bacterial supernatants assumed to be probiotic are observed upon enteroinvasive strains (Medellin-Pena and Griffiths, 2009).

There is a clear commercial benefit for discovering functional immunomodulatory products released by probiotics. The most significant benefit may be that organism viability may no longer be as much of a concern, especially in food products where viability decreases during storage (Champagne *et al*., 2008). As a growing area of biotechnology with practical and industrial potential, it only seems logical that both bacterial- and supernatant-based approaches to probiotic research will be conducted as the field continues to grow and move forward.

Objectives and Experimental Approach

The interactions between the intestinal microflora and innate immunity are too significant to overlook for an improved understanding of mammalian digestion. The intricacy of these interactions are important to note and worthy of research. The knowledge gained from an improved understanding of these interactions may be implicated in other contexts, such as chronic disease or bacterial function (Uronis *et al*., 2009).

The objective of this study was to compare responses in innate immunity between two distinct cell types: 1) IECs, using a human colorectal adenocarcinoma (HT-29) and 2) monocytes, using a human monocytic lymphoma (U-937). With an abundance of literature showing numerous strains of Lactic Acid Bacteria with immunomodulatory potential, it was noted that many

studies do not consider the fundamental mechanisms or factors that are responsible for these effects. Consequently, two bacterial strains, *L. rhamnosus* and *L. helveticus*, were tested for immunomodulatory effects which, in turn, would be further empirically scrutinized as both the bacteria and bacterial products were assessed for their isolated effects on cell lines challenged with TNFα. These strains were chosen based on earlier work indicating their ability to influence cytokine production by IEC (Wallace *et al*., 2003; Wood *et al*., 2007). *L. rhamnosus* RO011 and *L. helveticus* RO052 were shown to down-regulate constitutive production of the pro-inflammatory cytokines IL-8 and TNFα, and strain RO011 also down-regulates constitutive expression of the chemokine RANTES and the regulatory cytokine TGFβ, by HT-29 IEC (Wallace *et al*., 2003). *L. rhamnosus* RO011 was also shown to down-regulate IL-8 production by LPS-challenged HT-29 IEC (Wood *et al*., 2007). *L. rhamnosus* GG was also included as it is one of the most widely studied strains available. The effects upon the IEC and monocytic cell lines were indicated by the chemotactic cytokine IL-8 and GM-CSF biomarkers. It was hypothesized that the mechanisms underlying the secretion of both TNFα-induced pro-inflammatory IL-8 and monocyte-differentiating GM-CSF by either cell line would be influenced by the products secreted as a result of continued bacterial growth.

Furthermore, it was hypothesized that the media and cells obtained during the stationary phase of bacterial growth (as opposed to logarithmic growth) would show a greater down-regulatory effect on IL-8 and GM-CSF for at least one of, if not all three strains.

MATERIALS AND METHODS

Cell Culture

The HT-29 human colorectal adenocarcinoma adherent cell line (ATCC # HTB-38) was obtained from the American Type Culture Collection (ATCC). HT-29 cultures were maintained in media consisting of RPMI 1640 medium supplemented by 10% Fetal Bovine Serum (FBS, Sigma-Aldrich Canada) and 0.05 mg/mL gentamicin sulphate (Sigma). Cells were grown in 250 mL, 75 cm^2 tissue culture flasks (Cellstar, Greiner Bio-One, Mississauga, Ontario). Sub-culturing of cells was carried out every 3-4 days by rinsing the monolayers with Phosphate Buffered Saline (PBS) followed by incubation with 3 mL 20% trypsin EDTA (Sigma) diluted in RPMI 1640 at 37°C and 5% CO_2 for 5 minutes. Adherent cells were removed by washing with RPMI 1640 media by centrifugation at 300 x *g* at 4°C for 10 minutes. The trypsin-RPMI 1640 solution was removed and the pellet was resuspended in 2 mL of RPMI 1640 growth medium for passaging or FBS-free RPMI 1640 for use in challenge-assays. Cell cultures were incubated in a humidified incubator (Thermo Corporation, Toronto) at 37°C and 5% CO_2.

The U-937 histiocytic lymphoma suspension cell line (ATCC# CRL-1593.2) was obtained from the American Type Culture Collection (ATCC). Cultures of U-937 cells were maintained in media consisting of RPMI 1640 medium supplemented by 10% FBS (Sigma-Aldrich Canada), 1% Sodium

Pyruvate (Sigma), 1% HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma) and 0.05 mg/mL gentamicin sulphate (Sigma). Sub-culturing of cells was carried out every 3-4 days by rinsing the bottom face of the flask with the already-present growth media. Approximately 20% of the media in the flask was transferred to new flasks, at an inoculation of approximately 20% by volume. All other characteristics of HT-29 passaging, except for the trypsinization, were shared with the U-937 cell line.

Preparation of Bacterial Strains

Industrially packaged lyophilized *Lactobacillus rhamnosus* (strain RO011) and *Lactobacillus helveticus* (strain RO052) were obtained from Institut Rosell Inc. (Montreal, Quebec). Both strains were reconstituted in PBS at a concentration of 1 x 10^9 cfu/mL. Prior to culture, cells were washed by centrifugation at 3000 x *g* for 20 minutes at 4°C. The supernatant was discarded and the pellets were washed in PBS twice more. *Lactobacillus rhamnosus* GG (ATCC 53103) was obtained from the ATCC. The vial contents were resuspended in 2 mL of PBS, which was in turn dissolved in 6 mL of de Man-Rogosa-Sharpe broth (MRS, Oxoid, United Kingdom; pH 6.2) and grown for 20 hours. Pellets from all bacterial strains were then mixed and 19.4 mL of MRS broth were inoculated with 400 µL of culture and incubated in a shaking incubator for 20 hours at 37°C at a speed of 200-220 RPM. At 20 hours, the bacteria were stored at 4°C and served as refrigerator stocks.

Bacterial Growth Curves

Bacterial growth curves were established with the use of a correlation between plate reader (Bio-tek Instrumentation, Nepean, Ontario) absorbance over a 24-hour period at a wavelength of 625 nm and MRS agar (Oxoid, United Kingdom; pH 6.2) plate counts. Volumes of 19.4 mL of MRS broth were inoculated with 400 µL of refrigerator stock bacteria and optical densities were taken hourly for each strain in triplicate using 96-well culture plates. Readings continued over a period of 24 hours. Subsequent refrigerator stocks were derived from established stationary phase, to a maximum of 4 passages.

Preparation of Freeze-Dried Bacteria and Broths

Bacteria were grown in accordance with the established growth curve and isolated at the appropriate growth phase. Volumes of MRS broth of 98 mL were inoculated with 2 mL into Erlenmeyer flasks and grown to the appropriate growth phase, as verified by optical density. The contents of each Erlenmeyer flask were distributed to smaller centrifuge tubes. Bacteria were collected by centrifugation at 3000 x *g* for 20 minutes at 4°C. The broths and remaining bacterial pellets were removed and distributed equally into separate centrifuge tubes (half-full) and frozen to -80°C. Upon freezing, the tubes were placed in freeze dryer flasks (Thermo Corporation – ModulyoD, Toronto, Ontario). Freeze drying was carried out at a pressure of 30 mbar and a temperature of -50°C for 24 hours, or until full lyophilization of both the broth and bacterial pellets occurred. Lyophilized broths

and bacteria were frozen at -20°C until further utilized. For media acidification controls, pH of unspent media was adjusted to the average of spent stationary phase broth (pH = 4.7).

Challenge of Epithelial Cells

Intestinal epithelial cell HT-29's were seeded at a concentration of 1 x 10^6 cells/mL and challenged at a final concentration of 5 x 10^5 cells/mL at initial viabilities greater than 80% (as determined by a Trypan-blue exclusion count (Sigma-Aldrich Canada)). Cells were treated with either bacteria or broth at selected concentrations shortly before the introduction of challenge. Bacteria were washed twice in PBS and once in RPMI 1640 before being diluted in RPMI 1640 for addition to cell cultures. All non-freeze dried broths were filtered using 0.2 μm filters (Sigma-Aldrich). Epithelial cells were challenged for 6 hours at a TNFα concentration of 200 ng/mL in a humidified incubator at 37°C and 5% CO_2. Supernatants were collected and frozen at -80°C until cytokine content was assessed by ELISA.

Challenge of Macrophages

Monocytic U-937's were treated and challenged with TNFα exactly the same as the HT-29 cell line. However, the final concentration of TNFα was 40 ng/mL and incubation of challenge occurred for 24 hours.

ELISA Quantification of IL-8 and GM-CSF

All buffers and reagents were produced and used in accordance with the manufacturer's protocol for both IL-8 (BioSource, San Diego, California) and GM-CSF (R&D Systems, Minneapolis, Minnesota) assays. For the IL-8 ELISA (Enzyme-linked Immunosorbent Assay), coating antibody was plated at a concentration of 1 µg/mL in coating buffer. The concentration of biotinylated anti-IL-8 detection antibody was 0.08 µg/mL. The horseradish peroxidase (HRP)-conjugated streptavidin was used at a 1:2500 dilution for the IL-8 assay. The GM-CSF coating antibody working concentration in PBS was 2.0 µg/mL. Detection antibody was diluted to a working concentration of 0.5 µg/mL while Streptavidin-HRP was diluted to 1:200. Both recombinant human IL-8 and GM-CSF served as standards and all plates were read at a wavelength of 450 nm. The limit of detection for the IL-8 and GM-CSF ELISA were 12.5 pg/mL and 15.625 pg/mL, respectively. The composition of the buffers used in each assay are summarized and listed in Table 1.

Table 1. Concentrations of each respective assay solution for both IL-8 and GM-CSF ELISA kits.

Solution	IL-8 Assay	GM-CSF Assay
Coating Buffer	137 mM NaCl, 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7mM KCl, pH 7.4	Phosphate-buffered Saline (PBS) – 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4 (0.2 µm filtered)
Assay Buffer	137 mM NaCl, 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 5.0g Bovine Serum Albumin (Fracton V), 1mL Tween 20, pH 7.4	1% Bovine Serum Albumin in PBS, pH 7.2-7.4 (0.2 µm filtered)
Wash Buffer	1.5 mM KH_2PO_4, 10 mM K_2HPO_4-$3H_20$, 0.4g EDTA, 0.5mL Tween 20, pH 7.4	0.05% Tween 20 in PBS pH 7.2-7.4
Substrate Solution	1:1 H_2O_2:Tetramethylbenzidine	1:1 H_2O_2:Tetramethylbenzidine
Stop Solution	1.8M H_2SO_4 (Excess)	2M H_2SO_4 (Excess)

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA). Results indicating significance ($p < 0.05$) were then analyzed using Tukey's multiple range test for further comparison of means. Results are presented as means +/- standard error of the mean (S.E.M.). Each sample was run in triplicate and assigned an average value. Therefore, a value represented as n=1 is equivalent to a triplicate average for any single sample.

RESULTS

Lactobacilli growth phases and characteristics

In order to determine the concentrations and pH of spent media of *Lactobacillus rhamnosus* RO011, *L. helveticus* RO052 and *L. rhamnosus* GG, a 24-hour growth phase for each respective strain was performed (Figures 2, 3, 4). From these curves, distinct log and stationary growth phases were determined based on the consistency of absorbance, concentration and acidity (pH) of spent media (Table 2).

Spent MRS broth does not influence baseline IL-8 *secretion levels in monocytic* U-937 *cells*

No spent broth from a specific strain or growth phase displayed a significant increase or decrease of baseline IL-8 secretion levels in monocytic U-937 cells. However, a Tukey post-hoc test indicates that the MRS broth control ("unspent" medium) displayed a trend toward increased constitutive IL-8 production by U-937 cells relative to all other treatments and controls, with the exception of spent log phase RO052 broth (Figure 5).

Effects of spent MRS broth on TNFα*-challenged monocytic* U-937 *cells*

No spent broth from a specific strain or growth phase displayed a significant increase or decrease in IL-8 production from baseline (Figure 6). However, when inter-assay variation was removed by means of percent of control conversion, a trend toward decreased IL-8 production was observed in stationary phase RO011 spent broth at a 1/10 media dilution (Figure 7). The same property was not observed after treatment of either the RO052 (Figure 8) or GG (Figure 9) strains with spent MRS broths.

Effects of spent MRS broth on TNFα*-challenged* HT-29 *intestinal epithelial cells*

Unchallenged HT-29 cells displayed a significant increase in IL-8 production upon treatment with unspent MRS broth (Table 3). A significant reduction of IL-8 was present in TNFα-challenged cells treated with strain RO011, relative to all other challenge conditions, as indicated by a Tukey post-hoc test (Figure 10).

Effects of freeze-dried spent stationary RO011 *broth and bacteria and acidified controls on* TNFα*-challenged* U-937 *monocytic macrophages*

A significant reduction in IL-8 production was observed for U-937 cells treated with freeze-dried, spent RO011 broths (Figure 11). A significant increase

in IL-8 production was observed in U-937 cells treated with freeze-dried RO011 bacteria (Figure 11). No significant difference was observed between acidified media and broth controls (Figure 11).

Effects of freeze-dried spent stationary RO011 *broth and bacteria and acidified controls on TNFα-challenged* HT-29 *intestinal epithelial cells*

A significant reduction in IL-8 production was observed for U-937 cells treated with freeze-dried, spent RO011 broths (Figure 12). No significant difference was observed between freeze-dried stationary RO011 bacteria acidified media and broth controls (Figure 12).

Effects of log and stationary growth phase bacteria on unchallenged and TNFα-*challenged* U-937 *monocytic macrophages*

No significant difference in baseline IL-8 production was observed for any strain or growth phase in unchallenged U-937 cells (Figure 13). No significant difference in IL-8 production was observed for any strain or growth phase in TNFα-challenged U-937 cells (Figure 14).

Effects of log and stationary growth phase bacteria on unchallenged and TNFα-*challenged* HT-29 *intestinal epithelial cells*

No significant difference in baseline IL-8 production was observed for any strain or growth phase in unchallenged HT-29 cells (Figure 15). No significant difference in IL-8 production was observed for any strain or growth phase in TNFα-challenged HT-29 cells (Figure 16). A percent-control representation of the challenged HT-29 cells approaches trend-like status (Figure 17).

Effects of freeze-dried spent stationary RO011 *broth over a dilution gradient in* TNFα-*challenged* HT-29 *intestinal epithelial cells*

A significant threshold of detection for IL-8 production was present between dilutions of freeze-dried RO011 MRS broth at 12.5% and 6.25% (by volume) (Table 4).

Effects of spent RO011 *stationary broth on the production of Granulocyte Macrophage-Colony Stimulating Factor* (GM-CSF) *in* HT-29 *intestinal epithelial cells*

Unchallenged and TNFα-challenged HT-29 cells did not produce any GM-CSF above the level of detection, regardless of RO011 broth presence.

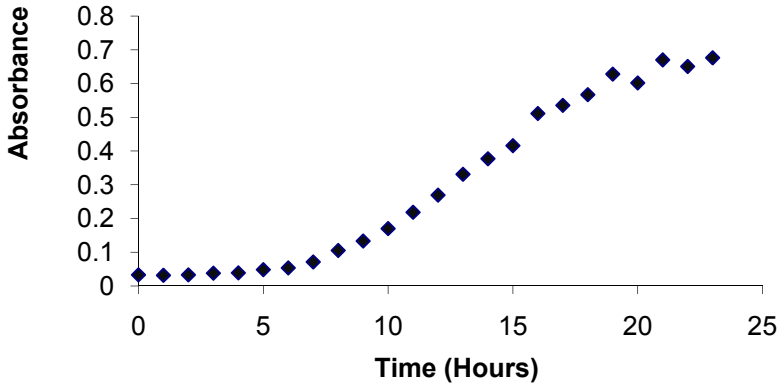

Figure 2. Representative growth curve for *L. rhamnosus* RO011 over a 24-hour period. The log phase occurred between the 12 and 15 hour time points (O.D. = 0.27 - 0.42 at 625 nm). The stationary phase occurred between the 20 and 24 hour time points (O.D. = 0.58 - 0.71 at 625 nm).

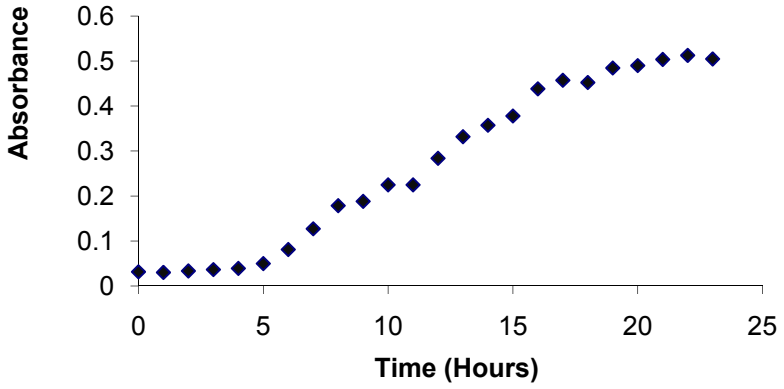

Figure 3. Representative growth curve for *L. helveticus* RO052 over a 24-hour period. The log phase occurred between the 9 and 15 hour time points (O.D. = 0.22 - 0.42 at 625 nm). The stationary phase occurred between the 20 and 24 hour time points (O.D. = 0.48 - 0.52 at 625 nm).

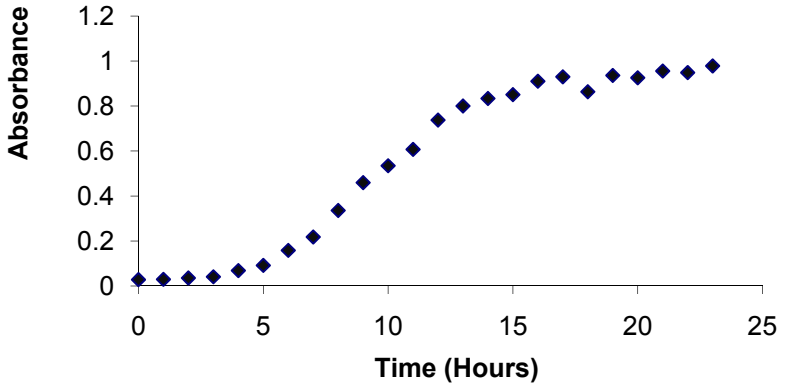

Figure 4. Representative growth curve for *L. rhamnosus* GG over a 24-hour period. The log phase occurred between the 6 and 12 hour time points (O.D. = 0.22 - 0.77 at 625 nm). The stationary phase occurred between the 19 and 24 hour time points (O.D. = 0.92 - 0.978 at 625 nm).

Table 2. Quantitative information pertaining to properties of the growth phases of various strains of *Lactobacillus* (n = 6).

Strain	Log Phase Absorbance at 625 nm	Stationary Phase Absorbance at 625 nm	Average Log Phase Concentration (cfu/mL ±S.D.)	Average Stationary Phase Concentration (cfu/mL ±S.D.)	Average Log Phase pH (±SE)	Average Stationary Phase pH (±SE)
L. rhamnosus RO011	12-15 Hours (O.D. = 0.27-0.42)	20-24 Hours (O.D. = 0.58-0.79)	4.3×10^7 +/- 1.3×10^7	2.0×10^7 +/- 8.1×10^6	4.81+/- 0.08	4.72 +/- 0.03
L. helveticus RO052	9-15 Hours (O.D. = 0.22-0.42)	20-24 Hours (O.D. = 0.48-0.75)	2.3×10^8 +/- 1.2×10^7	6.8×10^7 +/- 1.1×10^7	4.83 +/- 0.046	4.73 +/- 0.046
L. rhamnosus GG	6-12 Hours (O.D. = 0.22-0.77)	19-24 Hours (O.D. = 0.82-1.021)	6.3×10^8 +/- 2.2×10^8	1.2×10^8 +/- 8.8×10^7	4.81+/- 0.044	4.69+/- 0.032

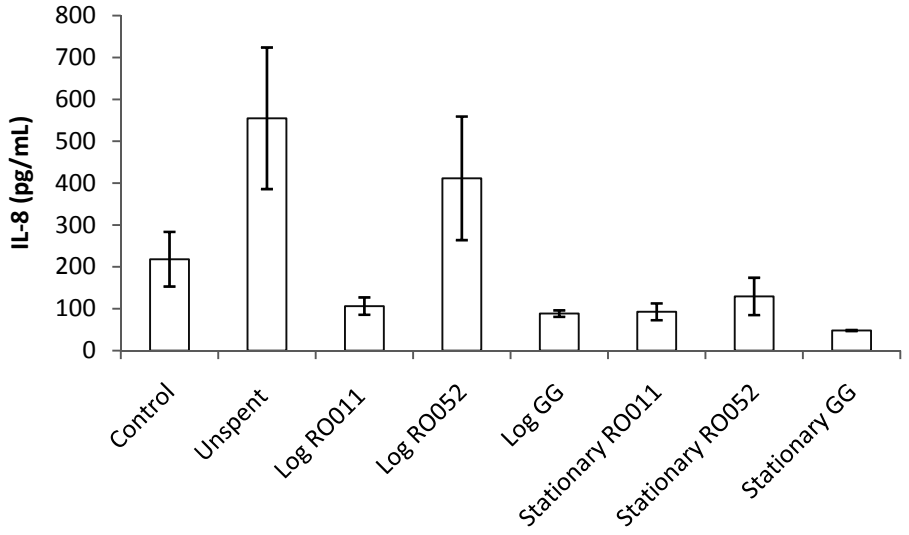

Figure 5. Effect of spent *Lactobacillus* MRS broths (10% by total volume) on interleukin-8 production by U-937 monocytic macrophage cells (5 x 10^5 cells/mL) in the absence of TNFα following concurrent incubation. U-937 cells were incubated for 24 hours before supernatants were collected and assayed (data are shown as mean concentration in pg/mL ±SE) (One-way ANOVA; p = 0.064; n = 5 for RO011 and RO052; n = 4 for GG; n = 14 for control and unspent value).

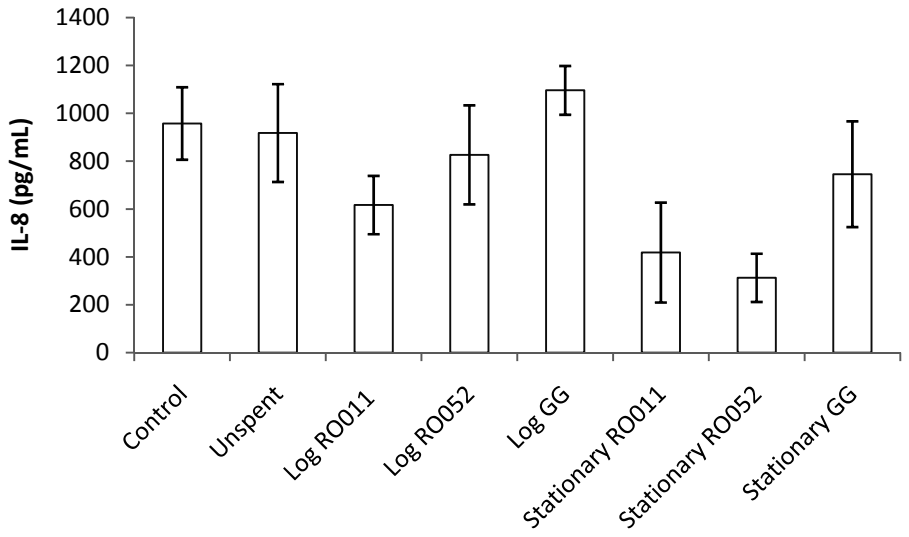

Figure 6. Effect of spent *Lactobacillus* MRS broths (10% by total volume) on interleukin-8 production by U-937 monocytic macrophage cells (5 x 10^5 cells/mL) in the presence of concurrent TNFα (40 ng/mL) challenge. U-937 cells were incubated for 24 hours before supernatants were collected and assayed (mean concentration in pg/mL ±SE) (One-way ANOVA; p = 0.39; n = 5 for RO011 and RO052; n = 4 for GG; n = 14 for control and unspent value).

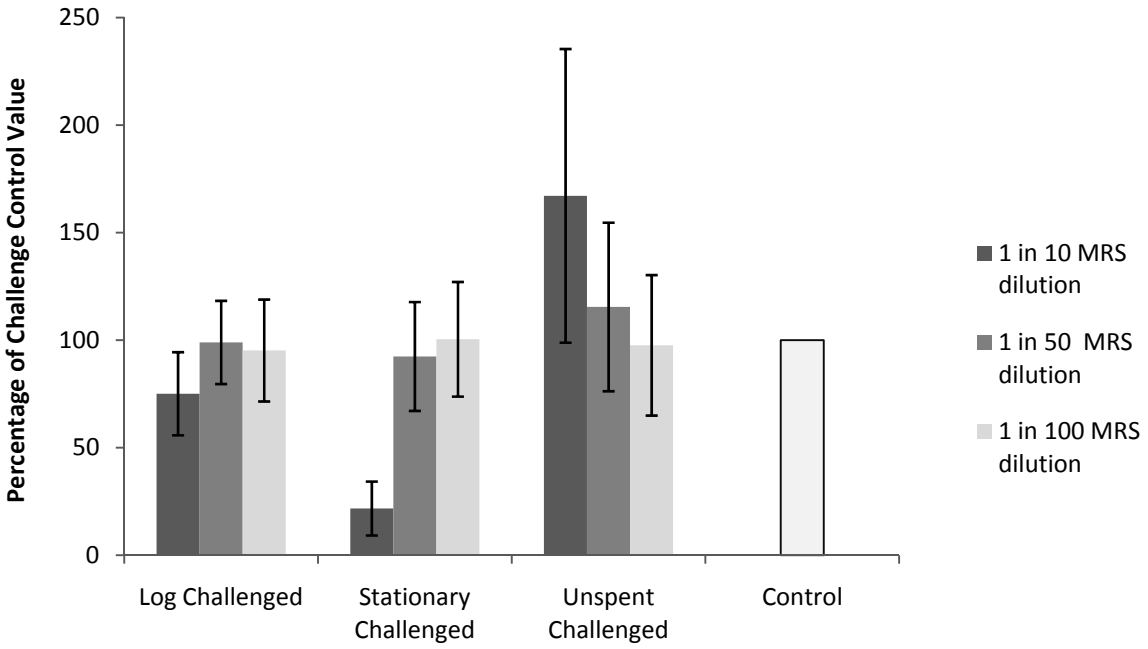

Figure 7. IL-8 production by TNFα-challenged U-937 macrophages (TNFα at 40 ng/mL) concurrently treated with RO011 log, RO011 stationary or unspent MRS broths at 1/10, 1/50 and 1/100 dilutions. Culture supernatants were collected 24 hrs post challenge for IL-8 measurement by ELISA. Data are shown as mean (±SE) percent of TNFα-challenged control response. A trend toward decreased IL-8 production was observed for U-937 cells treated with stationary phase RO011 broth relative to controls (One-way ANOVA, $p = 0.082$; $n=5$).

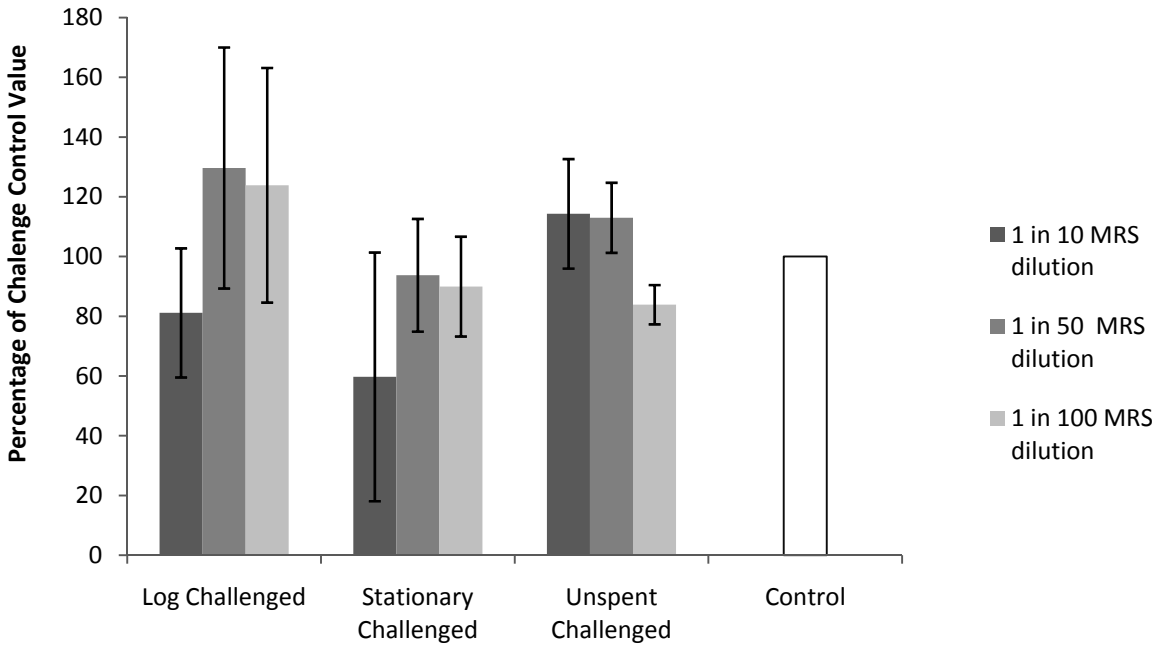

Figure 8. IL-8 production by TNFα-challenged U-937 macrophages (TNFα at 40 ng/mL) concurrently treated with RO052 log, RO052 stationary or unspent MRS broths at 1/10, 1/50 and 1/100 dilutions. Culture supernatants were 24 hrs post challenge for IL-8 measurement by ELISA. Data are shown as mean (±SE) percent of TNFα-challenged control response (One-way ANOVA, $p = 0.59$; $n = 5$).

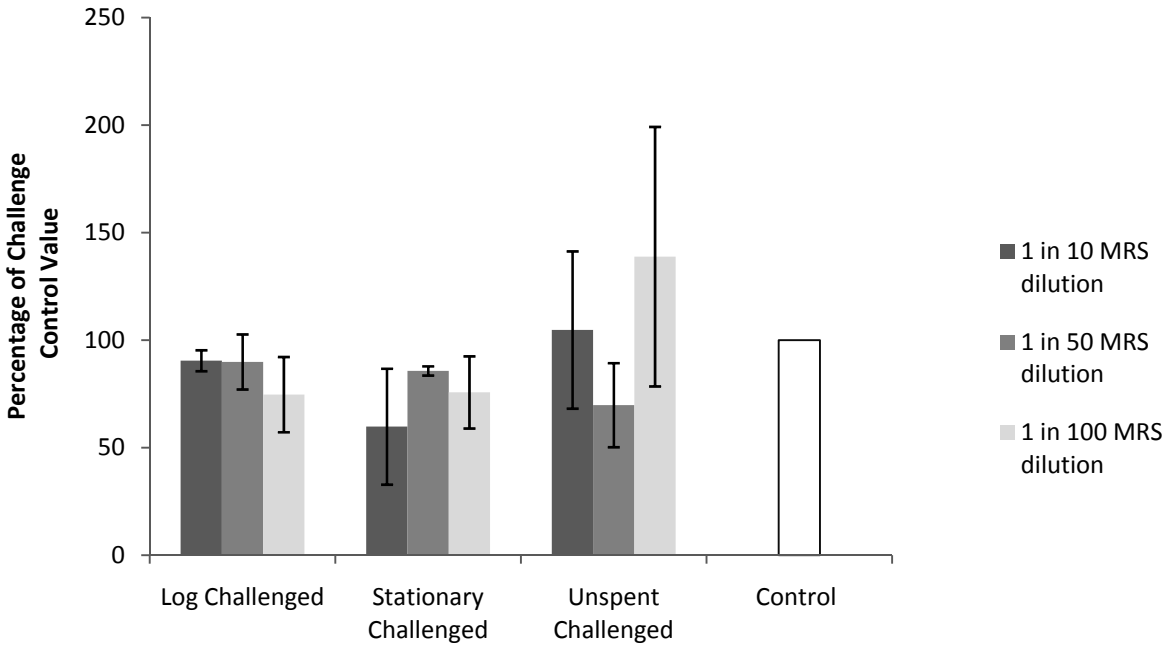

Figure 9. IL-8 production by TNFα-challenged U-937 macrophages (TNFα at 40 ng/mL) concurrently treated with GG log, GG stationary or unspent MRS broths at 1/10, 1/50 and 1/100 dilutions. Culture supernatants were 24 hrs post challenge for IL-8 measurement by ELISA. Data are shown as mean (±SE) percent of TNFα-challenged control response (One-way ANOVA, $p = 0.64$; $n = 4$).

Table 3. Effect of spent *Lactobacillus* MRS broths (10% by volume) on interleukin-8 production by HT-29 intestinal epithelial cells (5 x 10^5 cells/mL) in the absence of TNFα following concurrent incubation. HT-29 cells were incubated for 6 hours before supernatants were collected and assayed (mean concentration in pg/mL ±SE). The increase in IL-8 production observed with unspent MRS broth was significantly different from the control and every other treatment shown. Asterisks denote significant difference from control (One-way ANOVA; $p < 0.0001$; $n = 6$).

Condition	Mean IL-8 Production (pg/mL)	Standard Error
Control	84.2	22.8
Unspent MRS*	1123.3	146.9
Log RO011	29.9	6.1
Log RO052	32.9	7.1
Log GG	25.8	6.3
Stationary RO011	64.8	15.9
Stationary RO052	25.3	2.6
Stationary GG	32.9	2.2

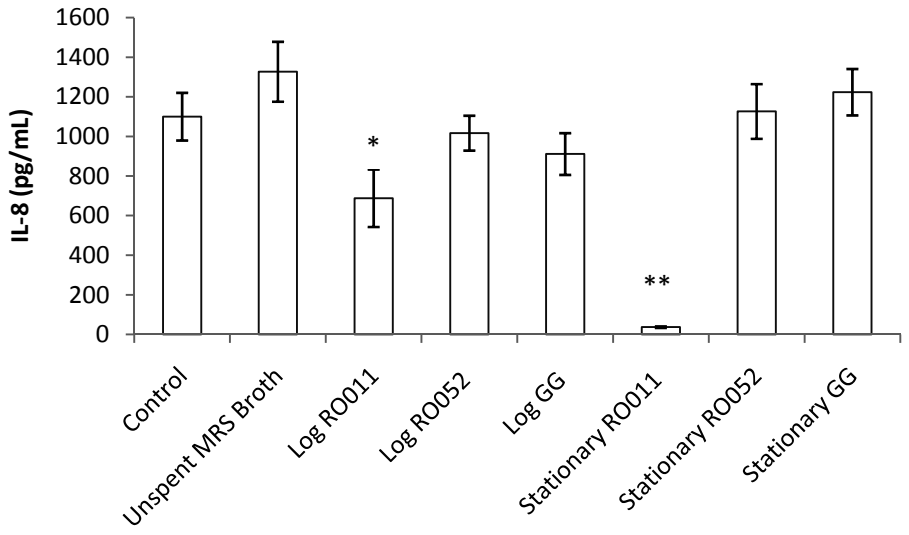

Figure 10. Effect of spent *Lactobacillus* MRS broths (10% by total volume) on interleukin-8 production by TNFα-challenged HT-29 intestinal epithelial cells (5 x 10^5 cells/mL) challenged with 200 ng/mL TNFα following concurrent incubation. IL-8 was measured 6 hours after TNFα challenge. Data are shown as mean concentration in pg/mL ±SE. Asterisks denote statistical significance (One-way ANOVA; $p < 0.0001$; $n = 6$).

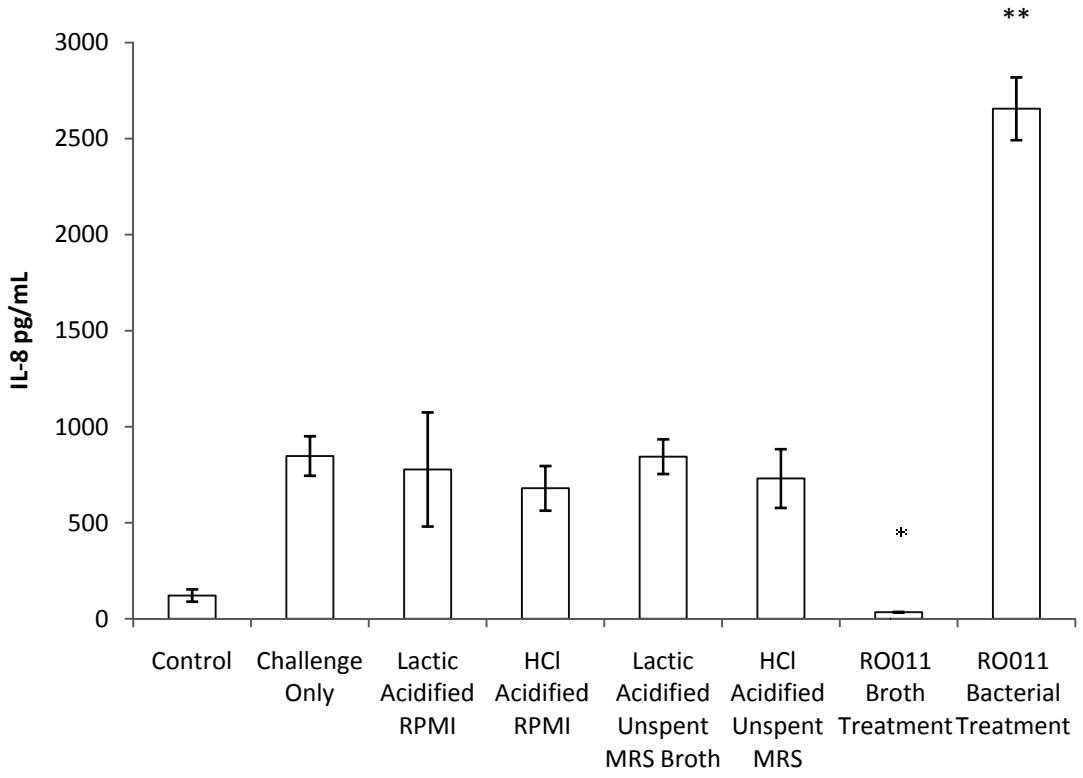

Figure 11. Effect of acidifed media broths and freeze-dried stationary phase *L. rhamnosus* RO011 bacteria and spent broth (0.01 g/mL; 10% by total volume) on interleukin-8 production by U-937 monocytic macrophage cells (5 x 10^5 cells/mL) in the presence of TNFα (40 ng/mL) following concurrent incubation. IL-8 was measured 24 hours after TNFα challenge and data are shown as mean concentration in pg/mL ±SE. Asterisks denote significant difference from control (One-way ANOVA; $p < 0.0001$; $n = 3$).

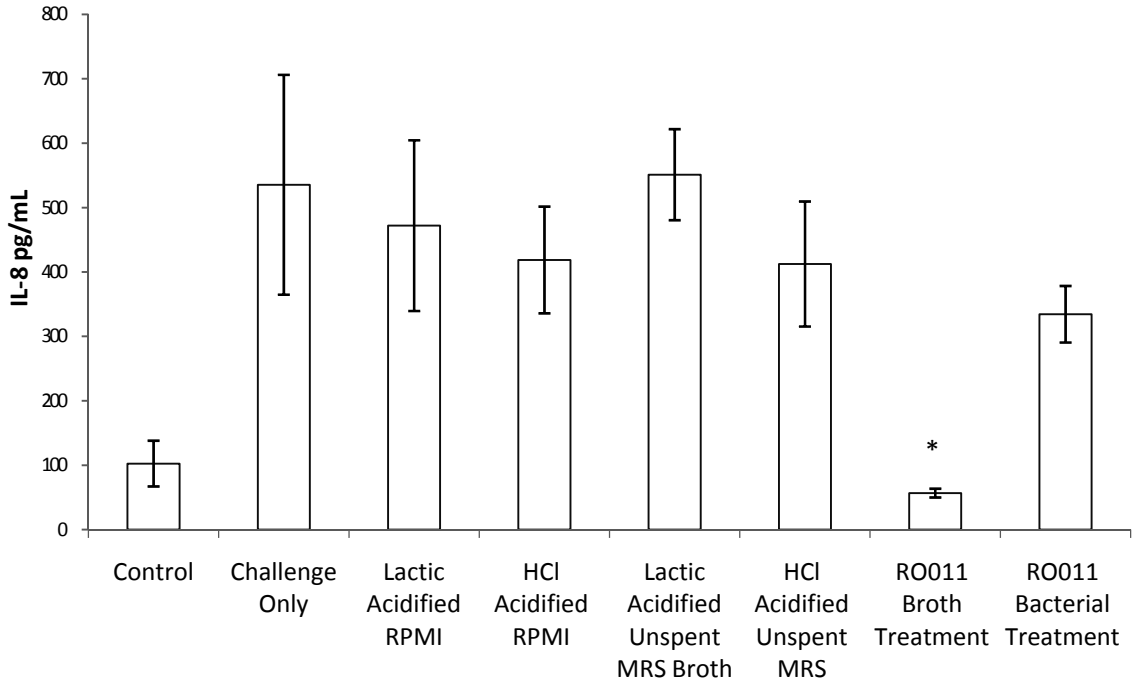

Figure 12. Effects of acidified media broths (10% by total volume) and freeze-dried stationary phase *L. rhamnosus* RO011 bacteria (5 x 10^6 cells/mL) and spent broth (0.01 g/mL; 10% by total volume) on interleukin-8 production by HT-29 intestinal epithelial cells (5 x 10^5 cells/mL) in the presence of TNFα (200 ng/mL) following concurrent incubation. IL-8 was measured 24 hours after TNFα challenge and data are shown as mean concentration in pg/mL ±SE. Asterisks denote statistical significance (One-way ANOVA; $p < 0.0001$; $n = 3$).

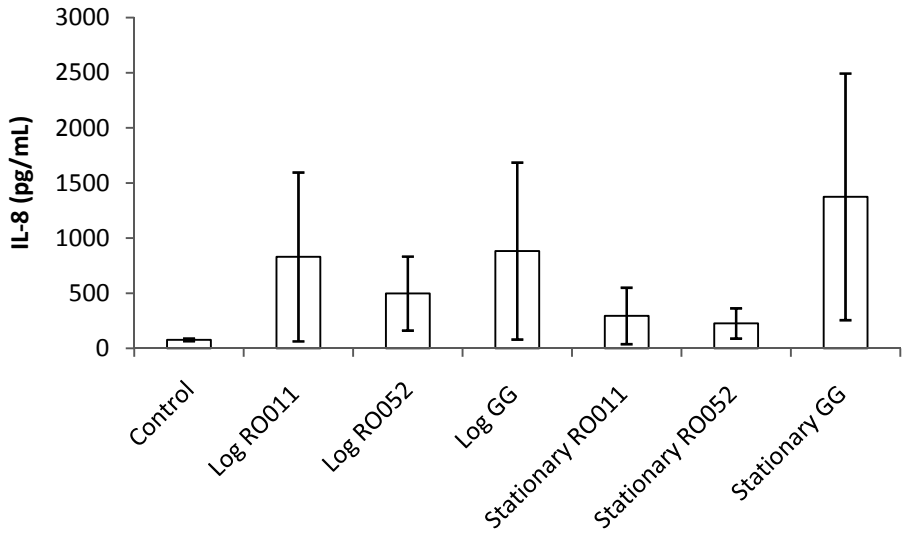

Figure 13. Effect of three strains of *Lactobacillus* at both growth phases, separately (5 x 10^6 cfu/mL) on interleukin-8 production by U-937 monocytic macrophage cells (5 x 10^5 cells/mL) in the absence of TNFα following concurrent incubation. IL-8 was measured 24 hours after TNFα challenge and data are shown as mean concentration in pg/mL ±SE (One-way ANOVA; p = 0.16; n = 4).

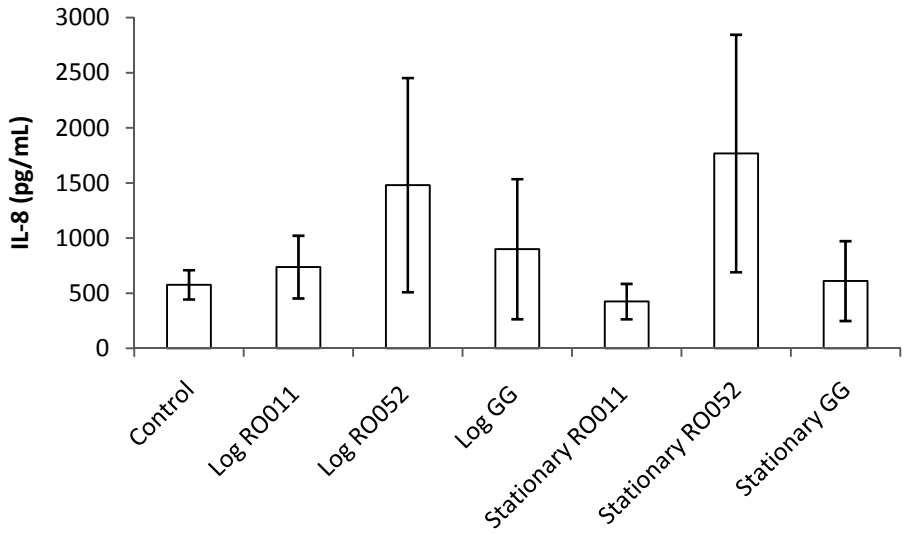

Figure 14. Effect of three strains of *Lactobacillus* at both growth phases, separately (5 x 10^6 cfu/mL) on interleukin-8 production by U-937 monocytic macrophage cells (5 x 10^5 cells/mL) in the presence of TNFα (40 ng/mL) following concurrent incubation. IL-8 was measured 24 hours after TNFα challenge and data are shown as mean concentration in pg/mL ±SE (One-way ANOVA; p = 0.69; n = 4).

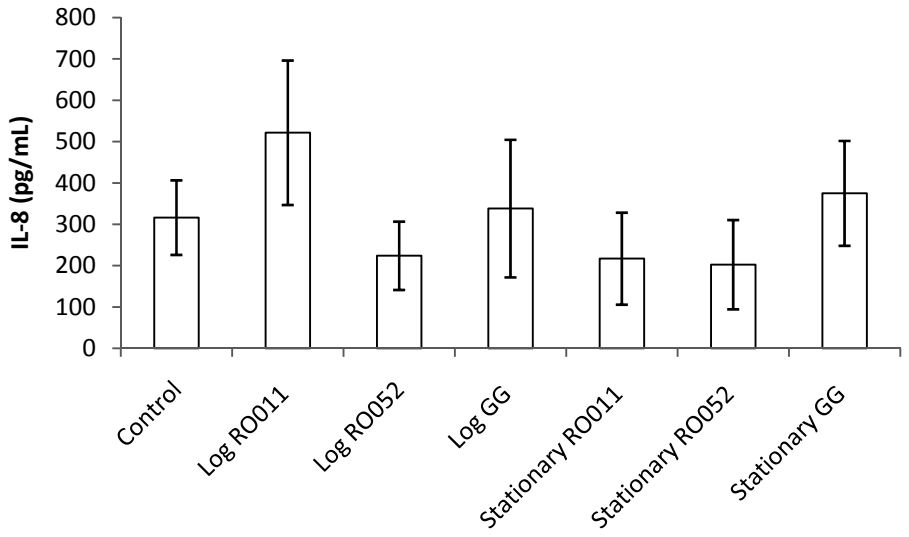

Figure 15. Effect of three strains of *Lactobacillus* at both growth phases, separately (5 x 10^6 cfu/mL) on interleukin-8 production by HT-29 intestinal epithelial cells (5 x 10^5 cells/mL) in the absence of TNFα following concurrent incubation. IL-8 was measured 6 hours after TNFα challenge and data are shown as mean concentration in pg/mL ±SE (One-way ANOVA; p = 0.58; n = 4).

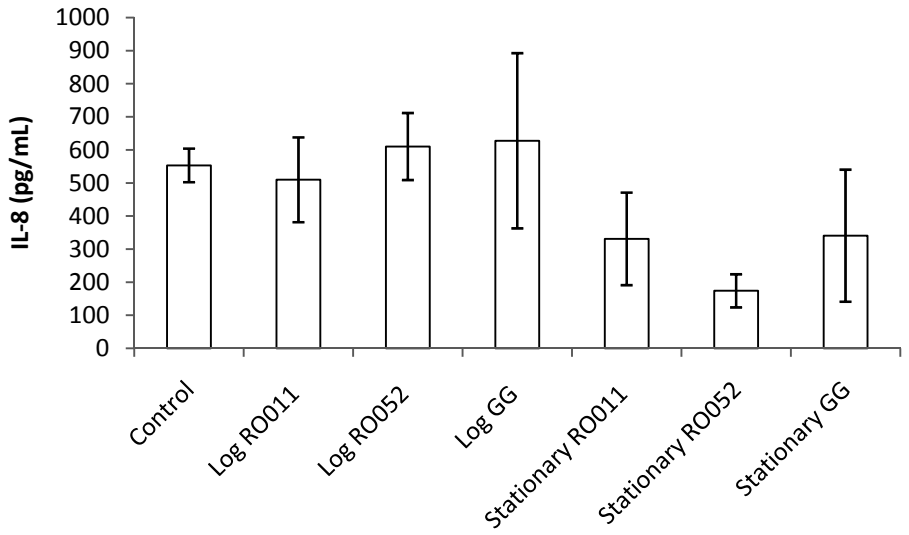

Figure 16. Effect of three strains of *Lactobacillus* at both growth phases, separately (5 x 10^6 cfu/mL) on interleukin-8 production by HT-29 intestinal epithelial cells (5 x 10^5 cells/mL) in the presence of TNFα (200 ng/mL) following concurrent incubation. IL-8 was measured 6 hours after TNFα challenge and data are shown as mean concentration in pg/mL ±SE (One-way ANOVA; p = 0.32; n = 4).

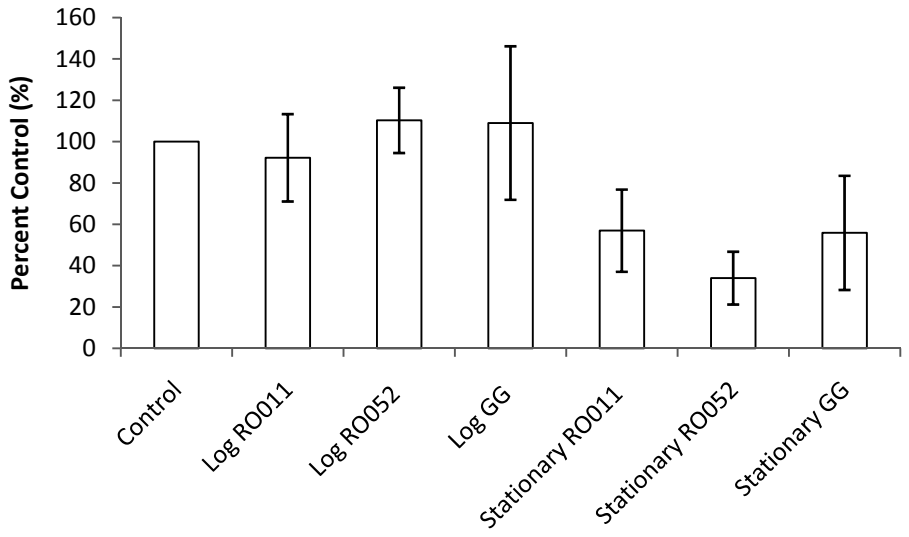

Figure 17. Effect of three strains of *Lactobacillus* at both growth phases, separately (5 x 10^6 cfu/mL) on interleukin-8 production by HT-29 intestinal epithelial cells (5 x 10^5 cells/mL) in the presence of TNFα (200 ng/mL) following concurrent incubation. IL-8 was measured 6 hours after TNFα challenge and data are shown as mean (±SE) percent of TNFα-challenged control response (One-way ANOVA; p = 0.12; n = 4).

Table 4. Effect of freeze-dried stationary phase *L. rhamnosus* RO011 broth on interleukin-8 production by TNFα challenged HT-29 IEC across a dilution series. IL-8 was measured 6 hours after TNFα challenge and data are shown as mean concentration in pg/mL ±SE. Asterisks denote significance (One-way ANOVA; $p << 0.005$; n=3).

Freeze-Dried MRS Broth Dilution (by Volume)	Mean IL-8 Secretion (pg/mL)	Standard Error
0% RO011 (Challenge Control)	761.93	124.79
75% RO011	BELOW DETECTION LIMIT	NOT APPLICABLE
50% RO011	BELOW DETECTION LIMIT	NOT APPLICABLE
25% RO011	BELOW DETECTION LIMIT	NOT APPLICABLE
12.5% RO011	BELOW DETECTION LIMIT	NOT APPLICABLE
6.25% RO011*	4.50	1.94
3.125% RO011	717.06	166.18
1.563% RO011	796.50	195.19
0.781% RO011	640.82	75.59
0.391% RO011	544.10	38.59
0.20% RO011	523.83	25.67
0.10% RO011	552.10	47.87
0.05% RO011	565.02	126.56
1/10 Unspent Broth Dilution	466.86	38.57
0%(Unchallenged)	7.49	1.90

DISCUSSION

Recently, the field of probiotic research has seen an increasing number of studies examining effects of products released into culture supernatants. Investigations concerning the immune responses of a specific tissue to bacterial growth products and cellular components are becoming as widespread as interactions concerning viable bacteria. At the core of most supernatant-based experiments is a desire to elucidate and determine the key players involved in bacterial cross-talk. Determining which components are responsible for the observed effects is often difficult *in vitro* due to the fact that most model systems are not capable of isolating synergies that occur between excluded variables that would otherwise exist *in vivo*, such as other tissue-tissue interactions.

A common criticism of probiotic research performed *in vitro* often concerns the relevance and accuracy of variables surrounding environmental and physiological challenges. In the context of this thesis, the primary challenge, TNFα, is pleiotropically characteristic of numerous autoimmune intestinal diseases. Specifically, an excess of TNFα production is a hallmark of Crohn's disease and a property of intermittent ulcerative colitis (Cho, 2008). TNFα is also produced by the MALT in response to many gastrointestinal pathogens and can contribute both to host defence and host damage (Jung *et al*., 1995). Within the MALT, it is important that both IECs and macrophages are capable of producing TNFα, as it has numerous regulatory functions. The deregulation of TNFα

production in either of these cell types could cause the intestinal epithelium to experience an excessive amount of TNFα exposure, greatly increasing basal physiological concentrations and contributing to host damage through inflammatory responses.

As mentioned previously, supernatant-based approaches to probiotic immunology are now being contrasted with the more traditional approach of using whole bacterial cells. However, within both approaches, literature concerning the effect of growth phase on the bioactivity of lactic acid bacteria and supernatants is uncommon. The development of a functional and reproducible growth curve was required to accurately determine the timings for log and stationary growth phases of the lactic acid bacteria used in this study and to prepare the corresponding spent culture supernatants.

Lactic acid bacteria growth curves and other properties

The optical density (O.D.) ranges for each growth phase varied between bacterial strains. However, for all strains, the log and stationary phases of growth were clearly distinct. Each time period, as determined by O.D. patterns, for every strain and growth phase yielded consistent bacterial concentrations and broth pH. However, for each *Lactobacillus* strain the log phase concentrations were greater than for strain-respective stationary phase concentrations. Although this was not expected, there is a plausible reason for this observation: as bacterial growth continues, waste-products accumulate at disproportionate rates relative

to earlier time points. As a result, slight changes in population decreases may be represented by increased light absorption at later growth phases. This may also be compounded by the fact that the O.D. curves misleadingly appear similar to theoretical growth curves. This has been a point of contention in the establishment of growth curves for other strains of lactic acid bacteria (Fu and Mathews, 1999; Delley and Germond, 2002).

Since the growth curves were determined on the basis of O.D., as opposed to concentration and viability, it is possible that the curve may have been misrepresenting the actual growth phases, even if only slightly. At the end of a stationary growth phase, populations are expected to plateau, if not begin to experience increased mortality as media is depleted of nutrients. Therefore, it is possible that the accepted stationary growth phase is actually a plateau or death spiral. This could also explain the increased absorbance relative to concentration, especially in light of the fact that cells begin to lyse and distribute cellular debris as the media is fully consumed. This possibility is further strengthened by the fact that other strains of lactic acid bacteria have had the end of stationary growth phases established at a period of 16 hours (Haller *et al*. 1999), in contrast to the times of 19 hours for the GG stationary phase, and 20 hours for the RO011 and RO052 strains observed in this study.

For each strain, the pH of spent broths was always lowest during stationary phase. This characteristic was expected for two reasons: First, lactic acid bacteria produce lactic acid as a product of their metabolism, which in turn should continue to decrease the pH of surrounding media as growth continues.

Secondly, the greater quantity of lysing cells at stationary phase may release numerous other acidic metabolic components into the surrounding media, further decreasing pH (De Angelis and Gobbetti, 2004).

Unspent and Spent Broth Effects

Initially, the intention of the research conducted for this thesis concerned the immunomodulatory effects of LAB on a variety of challenges with a focus on the effects of whole bacteria. However, in response to studies suggesting biological activity in spent culture media (Yan *et al*., 2007; Coconnier *et al*., 1992; Coconnier *et al*., 2000) this approach was tested and found to be effective. Initial testing of spent culture broths was carried out on the U-937 cell line and indicated that RO011 stationary phase broth was most effective at decreasing TNFα-induced IL-8 production. Log phase broth from RO011 and log or stationary broth from RO052 or *L*. GG were less effective.

The same experiment was repeated using the HT-29 IEC line. Interestingly, the same pattern of activity was present yet the effects were much more striking. TNFα -challenged HT-29 cells displayed a significant decrease in IL-8 production when treated with a 1 in 10 dilution of spent, stationary phase RO011 MRS broth.

To determine whether or not the decrease in IL-8 production was a consequence of the unique compounds within the spent broth or simply due to a change in pH, a variety of pH controls were run on both challenged cell lines.

These controls were designed to determine whether the decrease in IL-8 production was genuine or a consequence of a low pH, leading to the death of the cell line before any IL-8 could be produced. The acidified controls (prepared with either HCl or lactic acid) had no significant effects on IL-8 production in either unchallenged or TNFα-challenged cells. The results suggest that the observed effects of RO011 stationary phase broth were not due to lactic acid, a mediator that has recently been suggested as responsible for inhibition of NF-κB activation and IL-8 production in IEC (Kumar *et al*., 2009).

Initial testing of spent culture supernatants carried out on U-937 macrophage cells indicated that supernatants from RO011 stationary phase broth were most effective at decreasing TNFα-induced IL-8 production using broth at a 1 in 10 dilution. However, a percent-control conversion was used to examine the data as the variation in IL-8 production between TNFα challenges was high. This appears to be a property of the U-937 response to TNFα, as the variation in responses to TNFα was much less pronounced for the HT-29 IEC line.

The same significant decrease in IL-8 production was observed for both cell lines after treatment with freeze-dried, spent stationary phase RO011 phase broth. For the most part, the IL-8 production was the same among all iterations of acidified broth controls. To determine the point at which the freeze-dried, stationary phase RO011broth was most effective at decreasing IL-8 production under challenge, a dilution gradient was constructed. Interestingly, the IL-8 production was not detectable until a freeze-dried broth concentration of 6.25%

by volume was reached. Despite a low IL-8 secretion level, the TNFα-challenged cells remained viable beyond the challenge incubation. The broth dilution of 3% resulted in IL-8 production return to levels that were not significantly different from those of TNFα-challenged control cells. This suggests there is an optimal dilution “breakpoint” or threshold level for the immunomodulatory effects of the spent RO011 broth. The method of freeze-drying was used to allow for a gradient of spent broth concentrations, as well as determine whether or not the broth retained its effect after being frozen (Broekaert *et al*., 2007).

It is interesting to note that unspent MRS broth increased IL-8 production in unchallenged cells. To determine the cause of this effect, the components of the broth have to be considered. This effect is particularly perplexing because every component of the broth is encountered by human IECs, especially cells involved in regular digestion, although this does not apply to the U-937 monocytes. One possible explanation for this effect would be that the surfactants within the broth are irritants to cells, causing a disruption to membrane structure, eliciting a pro-inflammatory response. Changes in membrane structure and exposure to various irritants have been shown to prolong NF-κB nuclear localization, and this effect could be present when cells experience unspent MRS broth (Enesa *et al*., 2008). However, as these compounds catabolize during bacterial metabolic processes, the effect is not present in spent broth.

Another point of interest is the significant increase observed in IL-8 production in challenged HT-29 cells when treated with freeze-dried, stationary phase RO011 bacteria. It is plausible that some irritating cellular component may

have been released from lysed bacterial cells, thus leading to NF-κB activation and subsequent IL-8 production (Iyer *et al*., 2008). This was the only time any LAB or LAB-based supernatant-product was seen to significantly increase IL-8 levels above challenge-control values. However, this was only observed once with one sample of RO011 broth, it is possibly an aberration. Further comparison of bacteria and broth effects will help clarify this.

Although the GM-CSF assays yielded very little information in the HT-29 cell line responses to spent-culture broth treatments, the ability of this cell line to produce GM-CSF has been documented before, albeit under the influence of another challenge type (Long *et al*., 2001). However, as the level of GM-CSF induced in our system was below the level of detection, this analysis was not pursued further.

Although much remains to be determined regarding the underlying cause of the immunomodulatory effect of spent broth, they are highly suggestive that RO011 releases an active component from the cell into the culture media. This is in agreement with some of the most recent studies examining LAB-derived secreted molecules and culture broths (Table 5).

Table 5. Summary of the current literature concerning the immunomodulatory abilities of *Lactobacillus* supernatants and growth phases.

Authors	Cell Line	Challenge	Commensal or Probiotic	Target/Biomarker	Highlights
Coconnier *et al*. (2000)	Caco-2/TC-7	*S. enteric* serovar Typhimurium	*L. acidophilus* LB	Intracellular pathogen viability and IEC IL-8 production	Spent *L. acidophilus* LB supernatant decreases intracellular growth of *S. enteric* serovar Typhimurium in Caco-2/TC-7 cells. IL-8 production was also decreased.
Haller *et al*. (1999)	Human blood monocytes	3 LAB strains, *L. monocytogenes* and enterohaemorrhagic *E. coli*	*L. johnsonii* La 1 and *L. sakei* LTH 681	TNFα production	Viable LAB stimulates maximal TNFα less effectively than dead LAB. Log phase bacterial cells walls stimulated TNFα more effectively than stationary phase. LPS stimulated TNFα much more effectively than bacterial cell walls.
Kelly *et al*., (2005)	N/A	N/A	*L. salivarius UCC118*	Supernatant proteins	The progression from log to lag to stationary phase growth phases correlates with an increasing prominence in 84 kD protein presence. This band was further separated into 20 protein spots at differing isoelectric points. Mass spectrometry displayed these proteins to be analogous with the *Listeria monocytogenes* cell-wall proteome, thus suggesting the probiotic benefits of lactobacilli are attributable to nonpathogenic mimicry of

					pathogens.
Kumar *et al*. (2008)	T-84, Caco-2, HeLa and IEC-6	Butyrate, SCFAs, Flagellin	N/A	ROS production	Short-chain fatty acids and other membrane soluble chemicals may be responsible for regulating epithelial inflammatory mechanisms.
Seth *et al*. (2008)	Caco-2	Hydrogen peroxide	*L. rhamnosus* GG	Inulin flux; tight junction organization	Two novel proteins (p75 and p40) seem to reduce the damage of intestinal tight junctions after hydrogen peroxide treatment, possibly through a MAP-kinase-dependent mechanism.
Sanchez *et al*. (2009)	N/A	N/A	*L. rhamnosus* GG	N/A	Two novel proteins secreted by stationary-phase *L. rhamnosus* GG grown in MRS broth were identified by mass spectrometry, furthering the work put forward by Yan *et al*. (2007).
Yan *et al*. (2007)	Mouse colonic epithelial and HT-29	TNFα	*L. rhamnosus* GG	Akt activation, cytokine-indeuced epithelial cell apoptosis, epithelial cell growth	Two novel proteins (p75 and p40) secreted by *L. rhamnosus* GG inhibited cytokine-induced apoptosis, activated Akt and promoted cell growth in human and mouse colonic epithelial cells.

Bacterial effects

The immunomodulatory effects of viable LAB were also examined, especially in light of the increase in HT-29 IL-8 production observed following treatment with freeze-dried, stationary phase RO011 bacteria. The 3 strains tested had no statistically significant effects on IL-8 production by either HT-29 or U-937 cell lines, unchallenged or TNFα-challenged. When results were analyzed as a percent-control, in order to normalize variation in responses to TNFα, the effects of stationary phase RO052 on IL-8 production by TNFα-challenged HT-29 cells approaches a trend toward statistical significance. This could suggest that RO052 is more effective when whole cells are in contact with HT-29 IEC, rather than mediating effects through a released component, in contrast to the RO011 strain. This would also be in keeping with the current literature evaluating the immunomodulatory effects of LAB on IL-8 production in a variety of cell lines as some strains have been reported to be effective only when whole viable cells are tested, while others have been reported to produce biologically active components released into spent culture media (Table 6).

Table 6. Summary of current literature concerning the immunomodulatory abilities of viable *Lactobacillus* strains at the intestinal epithelial surface.

Authors	Cell Line	Challenge	Commensal or Probiotic	Target/Biomarker	Highlights
Candela *et al*. (2008)	Caco-2 and HT-29 IEC	*E. coli* and *S. cholerasuis*	*Bifidobacterium* and *Lactobacillus*	Cell Adhesion	Specific strains of *Bifidobacteria* and *Lactobacillus* displace *E. coli* and *S. cholerasuis* while decreasing IL-8 production.
Ma *et al*. (2004)	T-84, HT-29 and Caco-2	TNFα	*L. reuteri*	IL-8 gene expression and secretion; IkB degradation	Live *L. reuteri* was shown to be essential for the inhibitory effect of IL-8 expression and production when challenged with TNFα.
McCracken *et al*. (2002)	HT-29	TNFα	*L. plantarum* 299v	IL-8 gene expression and secretion	Heat-killed *L. plantarum* 299v did not increase IL-8 expression levels. IL-8 secretion by challenged cells treated with *L. plantarum* was lower than those treated with only TNFα.
O'Hara *et al*. (2006)	HT-29	*S. typhimurium* flagellin	*B. infantis* and *L. salicarus*	NF-κB activation, IL-8, IL-10 and TNFα secretion, pathogen adherence, mucin-3 and E-cadherin gene expression	Neither commensal increased gene expression of any of the 847 genes analyzed. Both commensal strains decreased unchallenged and challenged IL-8 secretion levels. The commensal strains did not interfere with pathogen binding to HT-29 cells, although they did increase both IL-10 and TNFα secretion.
Schlee *et al*. (2008)	Caco-2	N/A	VSL#3 bacterial cocktail	Human beta-Defensin-2	The VSL#3 bacterial cocktail strengthens the intestinal barrier

					functions through increased hBD-2 production.
Tien *et al*. (2006)	Caco-2 and HEK293T IECs	*S. flexneri*	*L.casei*	NF-κB activation; Global gene expression by microarray	Net effect of decreased pro-inflammatory gene expression associated with the stabilization of I-κBα upon treatment with *L. casei*.
Wood *et al*. (2007)	HT-29	LPS	*L. rhamnosus*	IL-8 secretion, cAMP production	Suggestion put forward that intestinal vasoactive peptides may block the ability of *L. rhamnosus* to down-regulate IL-8 production in IECs.

Work in Progress and Future Directions

The work contained within this thesis has branched off into several, related endeavours. The first branch concerns improving knowledge of the properties of the *L. rhamnosus* RO011 component in spent culture broth. Currently, freeze-dried stationary phase broth from RO011 is being tested to determine the nature of the biologically active component responsible for the decrease in IL-8 production by TNFα-challenged HT-29 IEC and U-937 monocytes.

The second branch of work in progress concerns characterization of the range of immunomodulatory activity of the RO011 stationary phase broth components by expanding both the range of challenges used and the range of parameters measured. The main focus of this research will revolve around determining whether other biomarkers of inflammation and activation are influenced by strain RO011. The impact on cell-surface molecule expression is also being evaluated, focusing on surface markers related to co-stimulatory activity (CD80 and CD86) and strengthened adhesion in professional APC's (CD58-CD2 junction) (Ebert *et al*., 2009). The basis for this is not only to evaluate the immunomodulation of cells by LAB through this pathway, but also as a means of comparing the involvement of this form of signaling between two distinct cell types.

Characterizations of the effects on the same cell lines under a variety of new challenges (such as interferon-γ) will also provide insight into effects on

pathways induced by other cytokines (Beaurepaire *et al*., 2009). Effects on gene expression of pro-inflammatory biomarkers is also being established to complement the secretory-level protein quantification in an attempt to provide a more holistic understanding of the underlying mechanisms involved in immunomodulation and bacterial cross-talk, as suggested by the results of by Li *et al*. (1998).

Evaluating the effect of these LAB strains and their respective spent culture broths on apoptosis induction is also a relevant future direction, as anti-apoptotic activity of peptides produced by *L. rhamnosus* GG has been reported, especially in the wake of pro-apoptotic TNFα effects (Yan *et al*., 2007; Iyer *et al*., 2008)

This research can be carried further into numerous directions. As a field, there are many underlying issues which must be resolved before a complete understanding of the role of probiotics in immunology can be reached. For example, further elucidation of the kinetics and dynamics of NF-κB mechanism will assist the current understanding of cellular responses to autoimmune and pathogenic challenges. Breakthroughs in the field of pure immunology are poised to benefit fields of applied immunology, especially probiotic research.

Although there are many questions that must still be answered using the current *in vitro* model, the next logical step in advancing the experiments outlined in this thesis would be to investigate the applicability of co-culture studies. Co-culture studies are not a novel technique in immunology, but could prove useful

in further evaluating such measures as the IL-8 macrophage recruitment ability of intestinal epithelial cells (Haller *et al*., 2000).

Effects of lactic acid bacteria on measures of macrophage and IEC activity, including phagocytosis, oxidative burst and nitric oxide production assays could be observed upon treating HT-29 IECs with U-937 monocytic cells post-challenge and LAB (broth or bacteria) treatment. Examining the effect of the LAB on macrophage activity in the presence of HT-29 could provide insight towards how the LAB is immunomodulating any cell-cell interaction. Also, if two challenges were separately used, one pathogenic (such as LPS) and the other endogenous (TNFα) – both strong inducers of the NF-κB pathway - the aforementioned assays could be useful in helping establish whether or not LAB are more useful in enhancing immunity in response to a pathogen. Other pathogenic challenges could also be utilized in the future, such as soluble egg antigens from parasites known to infect the human gastrointestinal tract (van Liempt, 2007).

In the practical sense, the knowledge obtained from gains in the understanding of commensal and probiotic bacteria will help create healthier, improved foods with longer shelf lives and, ultimately, improved health and disease prevention (Champagne *et al*., 2009; Resta-Lenert and Barrett, 2006; 2009). Unfortunately, due to the fact that the composition of the intestinal microflora is quite varied between individuals from different environments and habitats, the potential applications of probiotics remain controversial and much remains to be determined to ensure results are relevant and representative

(Denou *et al*., 2008). A proposed solution to this dilemma could lie in *in situ* global assays of host gene expression correlated with microflora composition. Although such a process would be many years in the making, it is not unrealistic to believe this type of diagnostic technology is on the horizon because the framework is already taking shape (Denou *et al*., 2008).

Conclusion

This work further characterized immunomodulatory effects of 3 strains of lactic acid bacteria. *L. rhamnosus* RO011 was found to have growth phase-dependent effects on two different cell types involved in innate immunity (IECs and monocytes). These effects appear to be mediated by a component released by *L. rhamnosus* RO011 unto spent culture medium. Although the underlying mechanisms responsible for the observed effects require more investigation and observation, these results are in keeping with current models for bioactivity of probiotics and commensals at the level of mucosal immune system in the GI tract.

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