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MODULATION OF *ESCHERICHIA COLI* O157:H7 MEDIATED PRODUCTION OF PROINFLAMMATORY MEDIATORS BY TWO SPECIES OF *LACTOBACILLI* IN TWO CONDITIONALLY IMMORTAL COLON EPITHELIAL CELL LINES

presented by

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Major Professor's Signature

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By

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ABSTRACT

MODULATION OF *ESCHERICHIA COLI* O157:H7 MEDIATED PRODUCTION OF PROINFLAMMATORY MEDIATORS BY TWO SPECIES OF *LACTOBACILLI* IN TWO CONDIDTIONALLY IMMORTAL COLON EPITHELIAL CELL LINES

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We hypothesized that probiotic bacteria, *Lactobacillus casei* (LC) and *Lactobacillus reuteri* (LR) would decrease production of proinflammatory mediators (e.g. nitric oxide [NO], chemotactic cytokines [MIP-2, TNF-a by ELISA]) in response to exposure to bacterial pathogen *E .coli* O157:H7 (EC). Two non-tumorigenic murine colon epithelial cell lines (i.e. Young Adult Mouse Colon [YAMC, Apc +/+]; Immortomouse/Min Colon Epithelial [IMCE], ApcMin/+ cells) were used to assess the production of NO and cytokines when treated with bacteria, spent medium or both.

EC caused a concentration-dependent increase in NO and MIP-2 production compared to control ($p < 0.001$). LC and LR co-treatment with EC caused a decrease ($p < 0.001$) in NO production compared to EC treatment in both cell types. EC/LC co-treatment also attenuated ($p < 0.001$) MIP-2 production compared to EC treatment.

The use of inhibitors of NF-kB, p38 MAPK, and JNK individually and p38 MAPK/JNK in combination accomplished partial inhibition ($p < 0.001$) of EC induced NO and MIP-2 production. The use of hemoglobin indicated an NO-independent mechanism was activated in the presence of EC in potentiation of MIP-2 production. These results suggest that probiotic bacteria influence proinflammatory mediator production in colon-epithelial cells in a genus- and species- specific fashion, affecting both quantity of immune cells and type attracted under inflammatory conditions.

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ABBREVIATIONS

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ANOVA	Analysis of variance
APC	Ademnotous polyposis coli
ATCC	American type culture collection
BLP	Bio-lactics powder
BSA	Bovine serum albumin
CDC	Center for disease control
CFU	Colony forming units
DMSO	Dimethyl sulfoxide
DNA	Deoxiribonucleic acid
EC	*E.coli*
EHEC	Enterohemmoragic *E.coli*
ELISA	Enzyme linked immunosorbent assay
GALT	Gut associated lymphoid tissue
GRAS	General recognized as safe
HACCP	Hazard analysis critical control point
HCL	Hydrochloride
HUS	Hemolytic uremic syndrome
IBS	Irritable bowel syndrome
IEL	Intra epithelial lymphocytes
Ig	Immunoglobulin
IL	Interlukin

IMCE	Immortomouse colon epithelial
IFN	Interferon
JNK	c- Jun N terminal kinase
LAB	Lactic acid bacteria
LC	*Lactobacillus casei*
LR	*Lactobacillus reuteri*
L-NAME	NG-nitro-L-arginine-methyl-ester
LPS	Lipopolysaccharide
MAPK	MAP kinase
MIP-2	Macrophage Inflammatory Protein-2
MRS	De Man, Rogosa, Sharpe medium
MTT	3-(4,5 dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide
NF-kB	Nuclear factor kB
NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthase
OD	Optical density
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with 0.05% Tween-20
RPM	Rotations per minute
RPMI	Roswell Park Memorial Institute
STAT	Signal transducers and activators of transcription
STEC	Shiga toxin *E.coli*

Stx	Shiga toxin
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethylkbenzidine
TNF-α	Tumor necrosis factor alpha
TSB-YE	Trypticase soy broth-yeast extract
YAMC	Young adult mouse colon

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

Colorectal cancer, caused by interaction of environmental and genetic susceptibility factors, is the second leading cause of cancer death in the United States (Brady et al., 2000). Inflammatory bowel disease, i.e. Crohn's disease or ulcerative colitis, increases a person's risk for developing colon cancer. Dietary factors, including foodborne pathogens, play a role in influencing the level of inflammation in the colon by affecting the growth of gastrointestinal cells and the activation of the inflammatory immune response in lymphoid tissue associated with epithelial cells (Brandtzaeg et al., 1989).

Foodborne pathogens cause approximately 76 million illnesses, in the United States each year (Mead, 1995, CDC 2004). *Escherichia coli O157:H7*, also known as hemorrhagic *E.coli (*EHEC), is a gastrointestinal pathogen that is generally non-invasive for intestinal epithelial cells, yet causes acute gastroenteritis, intestinal inflammation, diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (Berin et al., 2002). This particular form of *E.coli* produces Stx (shiga-toxin) 1 and Stx 2, which are thought to be important in the pathogenesis induced by this form of *E.coli.* The long-term goal of this research is to identify epithelial cell mediators of the inflammatory immune response caused by exposure to bacterial pathogens, like *E.coli* O157:H7.

Probiotic bacteria are microorganisms that have a favorable influence on the host in part by their effect on the intestinal microflora. They are found in foods such as fermented dairy products and potentially modulate the gut inflammation. The specific mechanisms behind the observed changes in immune function that have been observed

with the use of probiotics remain unclear. It has been hypothesized that probiotics effect several aspects of immune function including humoral, cellular, or non-specific immunity; and that probiotic bacteria can alter the inflammatory response that occurs in cells when in the presence of pathogens (Erickson, 2000). Probiotic bacteria have been shown to reinforce the different lines of gut defense, which are immune exclusion, immune elimination, and immune regulation (Isolauri, 2001). One current and significant question to be answered about probiotics is whether they work at the local, or systemic levels, or both, in modulating the immune response.

In response to threatening factors, epithelial cells and immune cells of the host produce inflammatory mediators such as nitric oxide (NO) and cytokines/chemokines. Nitric oxide (NO) is a local mediator and has been implicated in intestinal mucosal protection (Nathan et al., 1994)). It plays many roles in the body including an endothelium derived relaxing factor, a mediator of immune responses, a neurotransmitter, a cytotoxic free radical, a proangiogenic factor, and a signaling molecule (Nathan et al., 1994). NO plays a crucial role in virtually every cellular and organ function in the body (Nathan et al., 1994). NO is one activator of nuclear kappa B (NF-kB) which is an important transcription factor involved in the expression of inflammatory proteins. Recent evidence indicates that NF-kB and the signaling pathway involved in its activation are also important in tumor development (Karin et al., 2002). Another pathway recently identified in pro-inflammatory signaling is up-regulation of macrophage inflammatory protein-2 (MIP-2) by NO generated after administration of *E. coli* (Skidgel et al., 2002).

Therefore it would be beneficial to examine these pro-inflammatory indicators and the effect that probiotic bacteria have on these signaling pathways. We have chosen two mouse colon epithelial cell lines, the young adult mouse colon epithelial cell line (YAMC, Apc $^{+/+}$), and the Immortomouse/Min colon epithelial cell line (IMCE, Apc$^{Min/+}$), which are considered a good model system to examine the effects of probiotic bacteria on normal cells. We have utilized these cells to assess the effect of specific probiotic bacteria on the modulation of inflammatory mediator production caused by *E.coli* O157:H7 (*E.coli*).

This research was formulated around four hypotheses. The first hypothesis was that *E.coli* mediates the production of proinflammatory mediators in two conditionally immortalized cell lines of mouse colon epithelial cells, a normal mouse colon epithelial cell (YAMC Apc $^{+/+}$) and a pre-cancerous colon epithelial cell (IMCE, Apc $^{Min/+}$). The first objective of this hypothesis was to quantify production of proinflammatory mediators in response to exposure to colon epithelial cells to *E.coli*. The second objective was to verify a concentration-dependent pro-inflammatory response in the two lines of colon epithelial cells in response to *E.coli*. The third objective of the first hypothesis looked to delineate which component in the *E.coli* is producing the pro-inflammatory mediated response in the two cell models.

Our second hypothesis of this research was that *Lactobacilli reuteri (L.reuteri)* and *Lactobacilli casei* (*L.casei)* would attenuate the production of proinflammatory mediators in colon epithelial cells exposed to *E.coli*. The first objective of this hypothesis was to determine the capacity of *L.casei* and *L.reuteri* to downregulate the proinflammatory response mediated by *E.coli*. The second hypothesis was to identify

concentrations of *L.casei/E.coli* and *L.reuteri/E.coli* that has the most significant effect on the pro-inflammatory response mediated by *E.coli*.

The third hypothesis was *E.coli* mediated production of proinflammatory mediators occurs through the activation of multiple cell signaling pathways. The objective was to survey potential pathways through which *E.coli* causes production of NO and MIP-2.

The final hypothesis looked to determine whether the production of pro-inflammatory mediators was different between our model of normal compared to preneoplastic cells when exposed to *E.coli*, *L.casei*, *L.reuteri* and co-treatments.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

2.1 Gastrointestinal ecosystem

The gastrointestinal ecosystem is a stable alliance among the resident microflora, immune mediators, and the epithelial barrier (Vance et al., 2001). Imbalances in these components are associated with increased risk of inflammatory bowel disease. The gastrointestinal tract is a highly specialized organ that connects the food we consume with the rest of the body. Today there is an array of factors that can alter the normal ecosystem of the gastrointestinal tract including stress, changes in dietary patterns and eating habits, consumption of pharmaceutical compounds (i.e. antibiotics), and increased immune system demands (Fooks et al., 2002). These changes in the ecosystem of the gut can make the host more susceptible to pathogenic infection by throwing off the balance of the gut microflora, increasing growth of pathogenic micro-organisms. This literature review will examine the context in which foodborne bacterial pathogens, the mucosal immune system, and probiotic bacteria interact to modulate mucosal immune function and related immunological conditions.

2.1.1 The burden of foodborne bacterial pathogens

Foodborne illness is a serious public health problem affecting an estimated 76 million people in the United States each year; of which 324,000 are hospitalized, and 5,000 occur in death (CDC, 2004). Foodborne illness is caused by the consumption of contaminated food or beverages. There are more than 250 foodborne diseases that have been identified (CDC, 2004). When the balance of the gastrointestinal tract is

compromised infection can occur by allowing normally transient enteropathogens to colonize and multiply. Infection by these bacteria can lead to flu-like symptoms, organ damage, and even death. The inflammation caused by infection from these organisms can also increase susceptibility to disease states such as colitis and Crohn's disease. Entero-adherent strains of *Escherichia coli* in the ileal mucosa have been found in patients with Crohn's disease (Masseret et al., 2001). Patients with inflammatory bowel diseases have increased intestinal mucosal secretion of IgG type antibodies. IgG mediates immunoinflammatory responses which can lead to damage of the intestinal mucosa by activating the complement and the cascade of inflammatory mediators (Brandtzaeg, 1989). These disease states can likewise increase the risk for and early onset of colorectal cancer (Newman et al., 2001).

The most commonly recognized foodborne infections are those caused by the bacteria *Campylobacter, Listeria, Salmonella*, and certain species of *Escherichia coli* (Fooks et al., 2002). These bacteria all cause foodborne illness but have varying vehicles of transmission, incidence, symptoms, risk groups, and possible side-effects. *Campylobacter* is a gram-negative, microaerophilic bacterium and is the most common bacterial cause of diarrheal illness. It affects 2.4 million people each year through contaminated food (particularly poultry), water, or contact with infected animals (CDC, 2004). Clinical features include fever, abdominal cramps, and diarrhea typically lasting one week. All age groups are at risk; it can lead to life threatening sepsis in persons with compromised immune systems, and 1 in every 1000 diagnosed infections leads to Guillian-Barre syndrome (CDC, 2004).

Listeria monocytogenes is a gram-positive rod shaped bacterium that causes *Listeriosis* when consumed in contaminated food. There are approximately 2500 cases annually in the United States leading to 500 fatal cases (CDC, 2004). Those at risk include the elderly, immunocompromised, and pregnant women. Clinical features vary in the elderly and immunocompromised they include sepsis and meningitis. In pregnant women they have mild, flu-like symptoms followed by fetal loss or bacterimia and meningitis of the newborn (CDC, 2004). Immunocompromised persons are at increased risk for febrile gastroenteritis (CDC, 2004).

Salmonella is a gram-negative rod-shaped bacillus with approximately 2000 serotypes that cause human disease. This bacterium causes *Salmonellosis* in approximately 1.4 million people annually leading to approximately 500 deaths and chronic arthritis in 2% of the cases (CDC, 2004). Like *Campylobacter* the disease is spread through contaminated food, water, or contact with infected animals and affects all age groups. Symptoms include fever, abdominal cramps, and diarrhea. Occasionally it can lead to localized infection or progress to sepsis (CDC, 2004).

Escherichia coli is a gram-negative rod-shaped bacterium. It has hundreds of strains most of which are harmless and live in the intestines of healthy humans and animals (CDC, 2004). However, one strain *E.coli* O157:H7 causes an estimated 73,000 cases in the United States, 61 fatal cases, and 2,100 hospitalizations annually (CDC, 2004). The major source is ground beef, other sources include unpasteurized milk, juice, sprouts, lettuce, and salami, and contact with cattle. Waterborne transmission can also occur in contaminated lakes, pools, or drinking inadequately chlorinated water (CDC, 2004). All persons are susceptible and children under 5 years of age and the elderly are

at increased risk (CDC, 2004). Three to five percent (3-5%) of cases develop hemolytic uremic syndrome leading to prolonged hospitalization, dialysis, and long-term follow-up (CDC, 2004).

2.1.2 Importance for the modulation of foodborne illness

Foodborne illness is a serious health burden. It leads to increased medical care expenses, lost work days, long lasting side effects, and even death. It is important for us to find ways to lessen this burden and protect people from developing these illnesses. With changes in areas such as food preferences, food production, food distribution systems, and microbial adaptation, there is an emergence of novel as well as traditional foodborne diseases (CDC, 2004). Therefore, it is rational to identify strategies to maintain the normal microflora of the gut while strengthening the immune system to combat current and emerging foodborne pathogens.

2.1.3 *Escherichia coli* O157:H7

Escherichia coli (E.coli) O157:H7 was first recognized as a cause of illness in 1982 during an outbreak of severe bloody diarrhea; the outbreak was traced to contaminated hamburgers (CDC, 2004). The majority of cases of *E.coli* infections have occurred from consumption of undercooked ground beef. Infections with *E.coli* are diagnosed by detecting the bacterium in the stool. Most persons are treated with antibiotics or other specific treatment and clear up in 5-10 days (CDC, 2004). However, there is no evidence that antibiotics improve the course of the disease and some believe the treatment may precipitate the kidney complications (CDC, 2004). The virulence of this strain of *E.coli* comes from its production of factors including Stx 1 and Stx 2 (shiga toxin I and II), intimin, and lipopolysaccharide (LPS) (Kurioka et al., 1998). Stx has a

direct cytotoxic effect on neurons and is paralytic-lethal for mice (Kurioka et al., 1998). Stx and LPS in particular seem to be involved in the pathogenesis of hemolytic uremic syndrome (HUS). HUS is the major complication of *E.coli* infection; it is a life threatening condition that usually requires blood transfusions, and kidney dialysis (CDC, 2004).

As long as *E.coli* O157:H7 is contaminating food and water supplies it will be an important health concern. Knowledge about the ecology of this organism can assist in devising methods to decrease its prevalence in food and animals. These important steps are critical to modulate this foodborne illness caused by *E.coli* (CDC, 2004). Using irradiation methods to increase the safety of ground beef has been proposed (CDC, 2004). Identifying ways to control the organism's ability to grow and infect the gastrointestinal tract will aid in the management of this pathogen. Decreasing the incidence of these infections would decrease HUS, the major cause of kidney failure in children in the United States (CDC, 2004).

2.1.4. Gastrointestinal tract

The organs of the gastrointestinal tract include the mouth, esophagus, stomach, small intestine, and large intestine; in addition, the pancreas and liver secrete into the small intestine (Schneeman, 2002). The gastrointestinal tract is the body's connection to the external environment. It is a highly specialized organ system that allows man to consume food and foodstuffs to meet the body's nutrient needs (Scheenman, 2002). The main functions of the gastrointestinal tract include digestion of food, the absorption of nutrients, and a series of activates aimed at establishing a strong defense against aggressions from the external environment (Bourlioux et al., 2003).

Digestion the main function of the gastrointestinal tract begins in the mouth with chewing and the production of saliva. This allows the food to move smoothly through the esophagus to the stomach. The stomach continues the digestion with gastric secretions and motility. The stomach regulates the rate of digestion through the production of chime (Shneeman, 2002). After the food has been broken down in the stomach it enters the small intestine here nutrients are absorbed and digestion continues. It then enters the large intestine were nutrients continue to be absorbed, and microorganisms work on the food particles that were not digestible by the stomach or small intestine, such as oligofructose and other non-digestible carbohydrates. Those foodstuffs which can not be utilized by the body are moved to the final stage of the large intestine the colon and excreted (Shneeman, 2002). In healthy persons, the transit time from mouth to anus is between 55 and 72 hrs (4-6 h is from the mouth to the cecum and 54-56 h is in the colon) (Cummings et al., 1992).

2.2 Gastrointestinal immune system

2.2.1. Components of the gastrointestinal immune system

The gastrointestinal immune system is composed of three main components the microflora, the mucosal barrier, and the gut associate lymphoid tissue (GALT) (Bourlioux et al., 2003). These factors work together to protect the host from pathogenic invasion, disease, and illness. Each plays an important role in protecting the host. The flora of the gastrointestinal tract is a complex combination of bacterial species estimated to be near 400 and has been considered as a functionally active organ, the full potential of which remains to be elucidated (Falk et al., 1998, Simon et al., 1984). The bacterial distribution varies throughout the gastrointestinal tract with $< 10^3$ colony forming

units/ml (cfu/ml) in the stomach (due to gastric acid and short storage time), to 10^{11} – 10^{12} cfu/ml, within the colon, where anaerobes outnumber aerobes by a ratio of 1000:1 (Hart et al., 2002). The indigenous microflora of the gastrointestinal tract participate in the development and maturation of the gut (Hooper et al, 2001), and the regulation of intestinal function, including host innate and adaptive immunity (i.e. systemic antibody response) (Schiffrin et al., 2002). Establishment and maintenance of the intestinal microbiota is a complex process which is influenced by diet, method of birth, and microbe-microbe and microbe-host interactions (Savage, 1999).

The most dominant flora in the human intestine include the genera *Bacteroides, Bifidobacterium, Eubacterium, Clostridium, Peptococcus, Peptostreptococcus*, and *Ruminococcus*, where as *Escherichia, Enterobacter, Enterococcus, Klebsiella, Lactobacillus,* and *Proteus* are among the subdominant genera (Guarner et al., 2003). The main functions of the microflora of the gut are metabolic, trophic, and protective (Guarner et al., 2003). The microflora is involved in fermentation of non-digestible dietary residue and endogenous mucus, salvage of short-chain fatty acids for energy, production of vitamin K, and absorption of ions. The flora control epithelial proliferation and differentiation and the development and homeostasis of the immune system (Guarner et al., 2003). The microflora plays a crucial role in the protection of the gastrointestinal tract against pathogens (Guarner et al., 2003).

The composition of the microflora of the gastrointestinal tract can have a large impact on the health of an individual. Changes in the microflora can lead to infection, inflammatory conditions, and immune suppression. There are two main categories in which different bacteria can be placed based on their impact on the body, pathogenic or

commensal. Pathogenic bacteria are those that elicit a strong defense response and have a potentially harmful impact on the host. Colonization of the intestinal mucosa by a pathogen may result in cell damage and initiate a host response to eliminate the noxious agent, mounting an inflammatory reaction (Schiffrin et al., 2002). Pathogenic bacteria include *Escherichia coli* O157:H7, *Campylobacter jejuni, and Salmonella typhi.*

Commensal bacteria are those that live in harmony with the gastrointestinal tract. In some cases there can be a symbiotic existence between the two in which both gain from the relationship. They can be autochthonous (stationary to the gut) or allocthonous (transient, must be consumed continuously to have an effect). Commensal colonization in the gut affects nutritional and defensive functions of the intestine by modulating gene expression (Hooper et al, 2001). They do not induce a strong epithelial defensive response but instead exert some type of immune-modulation on the host (Schiffrin, 2002). *Bifidobacterium* and *Lactobacilli* are two genera of bacteria that act as commensal bacteria and appear to have beneficial effects on the host.

While microflora is not essential to live, as seen through the survival of germ-free mice, humans do not live in a sterile world (Bourlioux et al., 2002). Therefore it is important to have an appropriate balance of microflora in the gastrointestinal tract so that the ecosystem is in equilibrium. A shift in this equilibrium toward an increase in harmful or pathogenic microorganisms can increase the risk for a number of clinical disorders, including colon cancer, inflammatory bowel diseases such as ulcerative colitis, and infections from transient pathogens such as E.*coli 0157:H7, Salmonella, Listeria*, and *Campylobacter* (Fooks et al., 2002).

The mucosal barrier is a complex physiochemical structure that separates the tissues from the luminal environment; it consists of cellular and stromal components from the vascular endothelium to the epithelial cell lining, and the mucous layer (Bourlioux et al., 2003). Mucosal surfaces are exposed to both pathogenic and commensal microorganisms; the ability of the mucosa to distinguish between the two is crucial (Schiffrin et al., 2002). Ultimately, the mucosal barrier function depends on the physical integrity of the mucosa and the reactivity dynamic defensive factors such as mucosal blood flow, mucosal secretions, and epithelial cell function (Schiffrin et al., 2002).

Epithelial cells line the walls of the gastrointestinal tract and are the first to come in contact with the microflora. Intestinal epithelial cells protect the host by providing a strong physical barrier and producing a variety of innate antimicrobial defenses (McCracken et al., 2001). These cells play a key role in integrating the signals from luminal microorganisms with host development and local mucosal defense (Kagnoff and Eckmann, 1997).

The GALT is the local immune system of the gastrointestinal tract. It is the primary immune organ in the body; it contains 60% of the total immunoglobulin and > 10^6 lymphphocytes/g tissue (Salminene, et al., 2002). It is divided into two areas: Peyer's patches and the mesenteric lymph nodes. The mesenteric lymph nodes are where antigen presentation and affinity maturation occur (McGhee et al, 1999). The GALT is able to tolerate a massive load of dietary antigen and commensal microorganisms that colonize the gastrointestinal tract, while identifying and rejecting enteropathogenic microorganisms that may challenge the body's defenses (Bourlioux, 2002).

In addition to these fixed organs of the mucosal immune system there are diverse motile cells of the immune system, which play a significant role in the immune response and protection of the human body. These cells include dendritic cells, macrophages, neutrophils, natural killer cells, and intraepithelial lymphocytes (Bourlioux et al., 2002). These cells play a crucial role in eliminating pathogens from the body. Dendritic cells act as the major antigen presenting cells; they present antigens to naive T cells which can invoke an immune response (Parham, 2000).

Macrophages are phagocytes. The primary function of a macrophage is to clear the blood of particles, including bacteria. They work by engulfing whatever they don't recognize as healthy tissue, including pathogens and the organism's own dead cells. They present fragments of what they have engulfed, called antigens, on their outer surface where eventually a helper T cell will notice it and release a lymphokine notification to the B cells. The B cells then create and release antibodies specific to the particular antigen, and hence to the pathogens (Parham, 2000). Neutrophils are active phagocytes, unlike macrophages they are only capable of one phagocytic event, expending all of their glucose reserves in an extremely vigorous respiratory burst. Being highly motile neutrophils quickly congregate at a focus of infection, attracted by cytokines and chemokines (Parham, 2000). They are much more numerous than the longer-lived macrophages. The first phagocyte a pathogen is likely to encounter is a neutrophil.

Natural killer cells (NK) are a type of lymphocyte (a white blood cell) and a component of nonspecific immune defense. These cells do not destroy the attacking microorganisms directly; they attack infected cells and cells that appear that they don't

recognize. NK cells are not phagocytic; they weaken the target cell's plasma membrane, causing water and ions to diffuse into the cell and expanding it. Under this large pressure, the target cell lyses (Parham, 2000).

Finally, a distinct population of lymphocytes located between enterocytes in the epithelium above the basement membrane are called intraepithelial lymphocytes (IEL). These lymphocytes are phenotypically and functionally distinct from lymphocytes in the underlying lamina propria, lymph nodes, and peripheral blood. Due to their close and intimate contact with the epithelial cells and the environment, IEL play an important role in mucosal immunity (Mattapallil et al., 1998). These motile cells together elicit immune responses necessary to the protection and survival of the host.

2.2.2 Immune response: the immune components at work

All the components that play a role in the immune responses in the body fall broadly into two categories innate or adaptive immunity. The immune system uses innate mechanisms that are fast but limited, and adaptive mechanisms that are slow to start but eventually become both powerful and quick to recall (Parham, 2000). The site as well as the type of pathogen determines largely which type of immune response will occur (Parham, 2000). The immune response involves recognition of the pathogen or foreign material and the mounting of a reaction to eliminate it (Parham, 2000).

Innate immunity is the first response to exposure to a foreign pathogen it is nonspecific. Innate immunity can lead to the production and release of mediators such as cytokines. Innate responses are mediated by white blood cells (i.e. neutrophils and macrophages) and by intestinal epithelial cells (Bourlioux et al., 2002). The white blood

cells act to engulf and kill pathogens, while the epithelial cells coordinate host responses (Bourlioux et al., 2002). For example, intestinal epithelial cells can synthesize a wide range of inflammatory mediators and transmit signals to underlying cells in the mucosa (Bourlioux et al., 2002). Epithelial cells are the first host cell in contact with luminal antigens and microorganisms and were proven to be antigen presenting cells (Bland et al., 1986). The epithelial cells actively participate in the local recognition against pathogens exerting a form of innate immunity (Blum et al., 2000).

Innate immunity is the first line of defense and must discriminate between commensal bacteria and pathogenic using a restricted number of receptors (Bourlioux et al., 2002). These receptors are toll-like receptors that recognize motifs conserved by bacteria but that are not found in eukaryotes (Aderem et al. 2000). The immediate protection incurred by innate immunity via different toll-like receptors that recognize critical molecules on the bacterial surface is expression of a series of proinflammatory cytokines and inducible proinflammatory enzymes activated in many cases by nuclear transcription factor kB (NF-kB) (Elewaut et al., 1999). Different bacteria elicit different types of cytokine responses from epithelial cells, which are transduced to the underlying tissue and promote changes in the phenotype of lamina propria lymphocytes (Borruel et al., 2002). This innate mechanism of defense plays a major role in the regulation of intestinal homeostasis and contributes to the control of the inflammatory reaction (Schiffrin et al., 2002).

Adaptive immunity, unlike innate immunity is specific to the particular pathogen and leads to a conditioned long-lived protection specific to that pathogen (Parham, 2000). Adaptive immunity involves lymphocytes with receptors for a specific antigen and

presentation of that antigen in the context of the major histocompatability complex (MHC) of which there are two classes that activate subsets of helper T cells (Parham, 2000). Cytokines secreted by the helper T-cells of Type 2 (Th2) subset activate B cells for the antigen, while Type 1 (Th1) subset is involved mainly in inflammation and the activation of cytotoxic T cells (Parham, 2000). The surface of mucosal membranes is protected by a local adaptive immune system; the gut associate lymphoid tissue (GALT) which represents the largest mass of lymphoid tissue in the human body (Isolauri et al., 2001). An immune response initiated in the GALT can affect immune response at other mucosal surfaces (Isolauri et al. 2001). One of the major adaptive responses in mucosal immunity is the production of sIgA (Parham, 2000). This adaptive response is produced to try to alleviate the pathogen from the body and produces memory of this exposure which can be remounted if the pathogen returns.

These components of immunity are being challenged daily. There is an array of factors challenging the immune system including new bacteria strains and environmental changes. This is why strategies need to be developed to decrease the burden of foodborne illness.

2.3 Strategies to decrease the burden of foodborne illness

The burden that foodborne illness has put on society has led to the need for strategies to alleviate this burden. Four of the major strategies being implemented today include the use of HACCP, antibiotics, probiotics, prebiotics, and a combination of these strategies. Foodborne diseases are largely preventable through a combination of steps from the farm to table (CDC, 2004). The hazard analysis critical control point (HACCP) is a formal system for evaluating the control of risk in foods (CDC, 2004). It was first

developed by Pillsbury for NASA to make sure food eaten by astronauts was safe. "HACCP is dedicated to determine and monitor locations, practices, procedures or processes (defined as 'critical control points', CCPs) at which control can be exercised over one or more factors which, if controlled, could minimize (CCP2) or prevent (CCP1) a hazard" (Sinell et al., 1995). The control measures are set up to 1) prevent microorganisms from contaminating food and involve all hygiene production measures; 2) prevent microorganisms both from growing or forming toxins, e.g. through chilling, freezing or other processes that do not destroy microbes, such as reduction of a_w or pH; and 3) eliminate microorganisms, e.g. through thermal processing (Sinell et al., 1995). Antibiotics singly or in multiple have been used for preventing and treating infections caused by bacteria, which can come from contaminated food. Antibiotics have been used as a pharmaceutical compound designed to destroy bacteria. However, they can have harmful effects on the balance of the gut microflora away from potentially beneficial or health promoting bacteria such as *Lactobacilli* and *Bifidobacteria* towards an increase in harmful or pathogenic micro-organisms (Fooks et al., 2002). Also, bacteria have become resistant to many of the commonly used antibiotics and stronger and stronger forms are needed to combat the bacterial pathogens (Fooks et al., 2002).

Probiotics as defined by the National Yogurt Association and the International Life Science Institute in the United States are "Living micro-organisms which, upon ingestion in sufficient number exert health benefits beyond basic nutrition." Metchnikoff introduced the concept of probiotics in the early 1900's (Fooks et al, 2002). Metchnikoff found that the Bulgarian peasants, who consumed large quantities of fermented milk, experienced longer life spans. Probiotics are provided in products in one

of three basic ways: as a culture concentrate added to a food (usually a dairy product), inoculated into a milk-based food (usually a dairy product), or as concentrated or dried cells packaged as dietary supplements such as powders, capsules, or tablets (usprobiotics.org, 2004).

The proposed beneficial effects of probiotic consumption include: improved intestinal tract health, enhanced immune function, increased synthesis and bioavailability of nutrients, reduced symptoms of lactose intolerance, decreased prevalence of allergy, and reduced risk of cancers. Currently probiotics are only substantiated for use in the alleviation of diarrhea and lactose intolerance (Marteau et al., 2001). A supplement VSL-3 is currently being tested in patients with inflammatory bowel diseases (Bourlioux et al., 2002) and may be added to this list of substantiated uses of probiotics. The probiotic species that show the most promise in treating diarrheal diseases in children include *Lactobacillus GG, L. reuteri, L. casei, Saccharomyces boulardii, B. bifidum* and *Streptococcus thermophilus*. Lactic acid bacteria are believed to produce lactase when in the presence of bile aiding in the digestion of lactose in the gut lumen (de Vrese et al., 2001).

Other research areas that have shown benefits but have not yet been substantiated include but are not limited to the role probiotics play in cancer prevention, blood lipid levels, and allergy. *Lactobacillus acidophilus, Lactobacillus GG, Lactobacillus casei*, and *B. longum* have all showed promise in treating and preventing cancer growth and reoccurrence (Bourlioux et al., 2002). *L. casei* consumption was found to increase the recurrence free period among subjects with bladder cancer compared to control group (Aso et al., 1992). *Lactobacillus acidophilus* significantly suppressed the total number of

colon cancer cells in rats in concentration-dependent manner (Rao et al., 1999). Evidence is accumulating that probiotics have a beneficial effect on blood cholesterol and triglyceride levels. *L. reuteri* taken for seven days was found to decrease total cholesterol and triglyceride levels by 38% and 40% (Taranto, 1998). Finally probiotics may modulate allergy. *Lactobacillus GG* (LGG) added to the diet of infants on hydrolyzed whey formula decreased the symptoms of atopic dermatitis (Majamaa et al., 1997). Probiotics hold great promise for the prevention and treatment of clinical conditions associated with impaired gut mucosal barrier functions and sustained inflammatory responses (Isolauri et al., 2001).

The mechanisms by which probiotics exert their health benefits are still speculative. Probiotics may work by 1) antagonizing pathogens directly through production of antimicrobial and antibacterial compounds such as bacteriocins and butyric acid (Collins et al., 1999); 2) reducing gut pH by stimulating lactic acid producing microflora (Langhendries et al., 1995); 3) competing for binding and receptor sites that pathogens occupy (Kailasapathy et al., 2000, Fujiwara et al., 1997); 4) improving immune function and stimulating immunomodulatory cells (Rolfe et al., 2000); 5) competing with pathogens for available nutrients and other growth factors (Rolfe et al., 2000); or 6) producing lactase which aids in lactose digestion (Kopp-Hoolihan, 2001). The probiotic bacteria have been found to reinforce the different lines of gut defense including, immune exclusion, immune elimination, immune regulation, and non-specific host resistance to microbial pathogens (Isolauri et al., 2001).

The most commonly used and researched species include: *Lactobacillus* and *Bifidobacterium* (Bourlioux et al., 2003). *Lactobacilli* are Gram-positive, non-spore

forming rods, catalase negative, usually non-motile and do not reduce nitrate. *Lactobacilli* have GRAS (generally recognized as safe) status (Salminen et al. 1998). The most commonly used species of *Lactobacilli* as probiotics are *L.acidophilus, L.casei, L. rhamnosus, L. reuteri*, and *L. planatarum* (Fooks et al. 2002).

Bifidobacterium are Gram-positive, non-spore forming rods, with distinct cellular bifurcating or club-shaped morphologies. They make up 25% of the gut microflora and play a significant role in fermentation of carbohydrate in the colon (Fooks et al. 2002). The most commonly used *Bifidobacterium* species as probiotics include: *B.longum, B. bifidum, B. breve,* and *B. infantis* (Fooks, 2002). Different strains, species, and genera of bacteria have been shown to have different effects; therefore it is important to look at each species differently and not to generalize an effect seen with one species to all probiotic microorganisms.

A prebiotic is a 'non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve the host health (Gibson et al. 1995). Non-digestible carbohydrates like oligosaccharides are the most likely prebiotics, but any dietary ingredient that reaches the colon is a candidate (Fooks et al., 2002). The fructans have been the most thoroughly investigated form of prebiotic (Fooks et al., 2002). Usage of prebiotics is a way of maintaining mucosal growth, mucosal function, water and electrolyte balance, providing the host with energy and nutrients, and increasing resistance against invading pathogens (Fooks et al., 2002). Prebiotics likely stimulate the growth of non-pathogenic gut microflora.

Synbiotics is the use of probiotics and prebiotics in combination. The end result is the survival of the probiotic, which has a readily available substrate for its fermentation, as well as the individual advantages that each may offer (Fooks et al., 2002). Many of the lactic acid bacteria found to be stronger in the presence of plants are expected to exhibit stronger health-promoting abilities (Bengmark et al., 2003).

2.4 Mucosal inflammation

Inflammation is a protective response of the host to infectious/injurious factors (Korhonen et al., 2002). The purpose of inflammation is to eliminate the cause of the response, and to repair and/or regenerate the injured tissue (Korhonen et al., 2002). Inflammation enables cells and molecules of the immune system to be brought rapidly and in large numbers into infected tissues (Parham, 2000). The accumulation of cells and fluid at the site of infections causes swelling, redness, heat and pain the collective signs of inflammation (Parham, 2000).

In response to threatening factors, immune cells of the host produce inflammatory mediators such as nitric oxide (NO), cytokines, and eicosanoids, which regulate the course of the inflammation (Korhonen et al., 2002). Mucosal inflammation is characterized by the up-regulation of a specific array of epithelial gene products, including secreted cytokines with chemoattractant or proinflammatory function (Hauf et al., 2003).

2.4.2 Effects of inflammation

Normal inflammation is self limiting with the pro-inflammatory mediators being followed by anti-inflammatory cytokines. It helps to remove the foreign substance from the body and is short lived. However, chronic inflammation appears to be due to

persistent proinflammatory stimulation (Coussens et al. 2002). This chronic inflammation is what leads to severally detrimental effects. There is a growing body of evidence that many cancers are initiated by infections, upwards of 15% of malignancies worldwide can be attributed to infections, a global total of 1.2 million cases per year (Kuper et al., 2000). Persistent infections in the host lead to chronic inflammation, and in turn stimulate cytokines and chemokines that contribute to the development of malignant disease (Hauf et al. 2003, Balkwill et al., 2001). Leukocytes and other phagocytic cells induce DNA damage in proliferating cells, through generation of reactive oxygen and nitrogen species that are produced normally by these cells to fight infection (Maeda et al., 1998). Experimental and clinical observations have shown links between cancer and inflammation. Many bacteria such as *E.coli* cause severe gastrointestinal diseases, finding ways to bypass the normal inflammatory system, disrupting the normal sequence and prolonging the inflammatory process.

2.4.3 Major players in the inflammatory response

In response to inflammation and tissue injury, multifactorial networks of chemical signals initiate and maintain a host response designed to 'heal' the afflicted tissue (Coussens et al., 2002). NF-kB plays a key role in the expression of genes involved in inflammation and immune responses (Hauf et al., 2003). NF-kB compromises a family of closely related transcription factors that bind a common sequence motif known as kB site (Karin et al., 2002). NF-kB becomes activated in response to inflammatory stimuli and its constitutive activation has been linked to cancer (Karin et al., 2002). NF-kB regulates the transcription of numerous genes involved in varied inflammatory and immune responses, including nitric oxide (NO), tumor necrosis factor alpha (TNF-α),

ICAM-1, VCAM-1, and macrophage inflammatory protein-2 (MIP-2) (Liu et al., 1999). Four mediators of inflammation will be measured in various stages of this project NO, MIP-2, TNF-α, and TGF-β.

Nitric oxide (NO) is a crucial mediator of the inflammatory response. Generally, NO is synthesized by the conversion of the amino acid L-arginine to L-citrulline by the action of NO synthase (NOS), a highly reactive radical gas that regulates cellular functions in both physiological and pathologic conditions (Skidgel et al., 2002). NO synthase exists in three isoforms, each encoded by a separate gene (Witthoft et al., 1998). The three types are nNOS, eNOS, and iNOS (which will be the primary type discussed). nNOS is neuronal (encoded by NOS1) and eNOS is endothelial (encoded by NOS3) both of which are usually constitutively expressed (Witthoft et al., 1998). iNOS or inducible nitric oxide synthase (iNOS) is encoded by NOS2 and is regulated in various cell types (Witthoft et al. 1998). iNOS is produced in response to infectious and injurious agents and proinflammatory cytokines (i.e. NO) by the host (Korhonen et al., 2002). Increased production of iNOS expression and NO production are involved in many chronic inflammatory diseases such as asthma, rheumatoid arthritis and inflammatory bowel disease. In physiologic states NO can serve a protective function, but under conditions of high output NO may contribute to tissue damage by reacting with superoxide to from peroxynitrite, a strong oxidant (Ischiropoulos et al., 1992).

NO can act as a proinflammatory signal and up-regulate cytokines and chemokines such as MIP-2 in response to pathogens such as *E.coli* (Skidgel, 2002). Elevated iNOS activity has been linked to colon cancer and NO is thought to contribute to the progression of adenoma to carcinoma by damaging DNA, increasing gene

expression of COX-2, or generating posttranslation modifications via nitrosylation of proteins (Barrett et al., 1995).

Other mediators of the immune response include cytokines. Cytokines are humoral immunomodulatory proteins or glycoproteins, which control or modulate the activities of target cells (Bidwell et al., 1999). The pathologies of many infectious, autoimmune and malignant diseases are influenced by the profiles of cytokine production in pro-inflammatory (TH1) and anti-inflammatory (TH2) T cells (Bidwell et al., 1999). They can activate signal transduction and secondary messenger pathways within target cells that lead to gene activation, leading to mitotic division, growth and differentiation, migration, or apoptosis (Bidwell et al., 1999).

Th1 responses are characterized by secretion of interleukin (IL)-2, TNF-α, MIP-2, lymphotoxin, and interferon (IFN)-γ and are associated with delayed-type hypersensitivity reactions, whereas Th2 responses, which are characterized by secretion of IL-4, IL-5, and IL-10 have been associated with humoral immune responses and allergy (Camoglio, 1998).

One cytokine, TNF-α is reported to be a multifunctional cytokine with antitumor activity. TNF-α is believed to mediate pathogenic shock and tissue injury associated with endotoxemia (Balkwill et al. 2001). It mediates part of the cell mediated immunity against obligate and facultative bacteria and parasites (Balkwill et al., 2001). Proinflammatory cytokines like TNF-α and IFN-γ illicit strong inflammatory responses and are major inducers of a family of chemoattractant cytokines called chemokines that play a central role in leucocyte recruitment to sites of inflammation (Balkwill et al., 2001).

Chemokines are the largest family of cytokines. Epithelial chemokines may help determine the character of local immune responses and contribute to the systemic organization of the immune system (Kunkel et al., 2002). Chemokines have been implicated as important mediators in the pathogenesis of endotoxin injury by controlling the nature and magnitude of inflammatory cell infiltration (Skidgel et al., 2002). Macrophage inflammatory protein-2 (MIP-2) is a C-X-C chemokine generated by macrophages in response to LPS in mice (similar to IL-8 in humans) and studies indicate that it plays a significant role in the LPS-induced inflammatory response (Kopydlowski et al., 1999). The composition of chemokines produced at sites of tissue wounding effect the duration of the inflammatory response, often with the net affect being the switch from a Th1 type to a Th2 type response (Coussens et al., 2002).

Tumor growth factor-β (TGF-β) is a growth factor involved in growth inhibition in most cell types. TGF-β is highly protective against cancer, and the genetic or epigenetic loss of TGF-β signaling would lead to tumor outgrowth and progression (Akhurst et al., 2001). However, once a lesion has developed (premalignant stages) TGF-β acts as a promoter for progression, invasion, and metastasis (Cui et al. 1996).

2.5 The role of lactic acid bacteria on the immune system

Recent studies are summarized in Table 2.1.

2.5.1 *In vitro* studies

Using enterocyte-like Caco-2 cells Jacobsen et al. (1999) looked at the efficacy of forty-seven strains of *Lactobacillus* to resist pH 2.5 and adhere to the cells. Of the forty-seven they found five strains that showed good viability at pH 2.5 and appeared to

adhere; *L.reuteri, L. rhamnosus, LGG, L.delbrueki, and L. casei.* There screening found that these five strains showed promise as having probiotic activity.

Wallace et al. (2003) used seven strains of heat killed *Lactobacillus* and one strain of heat killed *Bif idobacterium* to stimulate HT 29 human intestinal epithelial cells. They reported that certain strains of *Lactobacillus*: *L. rhamnonsus, L. delrueckii, and L. acidophilus* were able to suppress the production of the chemokine RANTES when added to the cell line. They also found that certain strains could also suppress the production of IL-8. TNF-α production was also down-regulated by specific strains mainly *L. rhamnosus, B.longum, and L. delbrueki. L. rhamnosus* had the greatest effect on down regulation of TGF-β. Overall *L. rhamnosus* had the greatest effect on chemokine production and the strongest binding capabilities to the HT-29 cell line.

Yan and Polk (2002) also used the HT-29 cell line as well as the YAMC epithelial cell lines to look at the effects of probiotics on cytokine induced apoptosis. *LGG, L.casei* and *L.acidophilus* were used to treat the cells at 10^7 cfu/ml. They reported that YAMC cells had inhibited TNF-stimulated apoptosis when co-cultured with *LGG.* They also reported *LGG* activated the anti-apoptotic Akt/protein kinase B pathway and inhibited the activation of the pro-apoptotic p38/mitogen-activated protein kinase. Overall they saw products with *LGG* culture that show concentration dependent activation of Akt and inhibition of cytokine induced apoptosis.

Table 2.1 The Effect of Lactic Acid Bacteria on Immune Function

Model	Experimental Design	Results	Reference
HT 29 Intestinal epithelial cell line	Seven strains of *lactobacillus* and one strain of *bifdobacterium* were heat killed and added to cells at a final concentration of 5 x 10^6 cfu/ml	The bacteria had variable effects on down-regulation of several chemokines and cytokines production. They also varied in their ability to bind the intestinal epithelial cells.	Wallace, 2003
YAMC and HT 29 cells	Cells treated with *LGG, L.casei, L.acidphophilus* at 10^7 cfu/ml	LGG prevented cytokine induced apoptosis in a mouse and a human epithelial cell line	Yan and Polk, 2002
PBMC human macrophages	Macrophages stimulated with *L. rhamnosus GG* and *Streptococcus pyogenes* in a 1:1 ratio	Both bacteria activated NF-kB and STAT DNA binding activity	Miettinen et al., 2000
Caco-2 cells	47 strains of *Lactobacillus* examined for resistance to pH 2.5, and adhesion to Caco-2 cells	5 strains showed strong resistance at pH 2.5 and adhesion to the cells	Jacobsen et al., 1999
Male Fischer Rats	Yogurt containing 4 strains of *Lactobacilli*	LAB were highly protective against the genotoxic effects of HCA's	Zsivkovits et al., 2003
Male BALB/c Mice	Oral and intragastric administration of three *lactobacillus* and one *lactococcus* strain at 10^9 cfu/ml	*L. plantarum* NCIM B8826 showed the most persistence in the digestive tract and is a good candidate for prevention and treatment of chronic inflammation	Pavan et al., 2003

Table 2.1 (cont.)

Model	Experimental Design	Results	References
BALB/c Mice	Oral administration of 5 strains of lactobacillus, 1 strain of *lactococus,* and 1 strain of *streptococcus*	*L. casei* and *Lb. planterum* interacted with peyer's patches and increased IgA and CD 4+ cells and Ab. *Lb. acidophilus* induced gut mucosal activation by interaction with epithelial cells. *Lactococcus lactis* and *Lb. delbrueckii* increased IgA but not CD 4+ cells.	Perdigon et al. 1998
Male BALB/c Mice	Biolactis powder with viable *Lactobacillus casei* YIT 9018 at 2.3 x 10^{11}/g	*L. casei* inhibited tumor growth	Kato et al., 1994
Nasal colonization of Human volunteers	Consumption of a probiotic fermented milk drink or yogurt daily for 3 weeks	Showed a significant decrease in potential pathogenic bacteria in the upper respiratory tract.	Gluck et al., 2003

Finally, Miettian et al. (2000) found that PBMC human macrophages stimulated with *L. rhamnosus GG* and *Streptococcus pyogenes* in a 1:1 ratios activated NF-kB and STAT DNA binding activity. Both are involved in inflammation, stimulating the activation of an array of cytokines during the immune response. The two bacteria did differ in the type of cytokines they produced. This indicates the differential way in which lactic acid bacteria modulate the immune response. For example, the streptococcus induced IFN-α a strong inflammatory stimulator, while *L. rhamnosus GG* did not.

2.5.2 Animal and human studies

Delineating the strain-specific effects of probiotics is an active area of investigation. Many of the current studies in animals and a few in humans have looked at the efficacy of specific probiotic bacteria. While others have looked at the up-regulation of immune markers in mostly animal models but a few recent human studies have been conducting. Feeding studies in animals and humans show the enhanced immune effects of consumed lactic acid bacteria. Recent studies with animal models and human models are summarized in Table 2.1.

Pavan et al. orally and gastrically administered three *Lactobacillus* strains and one *Lactococcus* at 1 x 10^9 cfu/ml. They saw strain-specific effects in the ability of the four bacteria to remain in the gastrointestinal tract. *L. planatarum* showed the most persistence in the gastrointestinal tract and had the highest bacterial levels in the feces (10^4-10^6 cfu/g). The other strains were able to be detected but in much smaller amounts and for a shorter duration. They also found that there was no adverse effect from repeated oral administration of the *L. plantarum*, indicating *L. planatarum* was a good

candidate for a probiotic. Jacobson et al. (1999) also looked at lactic acid bacteria viability. They measured the cfu/g of different mixtures of probiotics in the feces of twelve healthy men. They found L. *rhamnosus, L. reuteri, and L. rhamnosus LGG* most frequently in the feces.

Other recent studies specifically examined the effect of lactic acid bacteria in various disease states or at susceptible points for the formation of disease. Niedzielin et al. (2001) found that in humans with irritable bowel syndrome, the oral consumption of L. *plantarum 299v* improved IBS symptoms 95% compared to control. Zskiovities et al. (2003) feed male Fischer rats yogurt containing four strains of *Lactobacilli.* After or in conjunction with the probiotics the rats where orally given heterocyclic aromatic amines, which have been linked to the etiology of human cancer. They found a concentration dependent effect of the probiotics in reducing the DNA damage.

Kato et al. (1993) orally administered biolactis powder (BLP) a preparation *of Lactobacillus casei YIT 9018* to male BALB/c mice to look at its effect on tumor growth. Mice were injected with Colon 26 cancer cells to induce tumor development, after which the tumors were excised. The BLP solution was administered orally followed by another injection of Colon 26. They observed a decline in tumor growth in those mice who consumed a concentration of 100 or 200 mg/kg/ day of the BLP. They concluded that oral BLP potentiated the systemic immune responses through modified T cell functions.

Gluck and Gebbers (2003) orally administered a probiotic containing fermented milk drink to 209 human volunteers daily for three weeks. They observed marked declines in the numbers of potentially pathogenic bacteria in the nasal passages. They

speculated a linkage between the lymphoid tissue of the gut and the upper respiratory tract.

Finally, Perdigon et al. (1998) focused on specific cells involved in the immune response and the effect on their production by consumption of probiotics. They orally administered five strains of *Lactobacillus*, one strain of *Lactoccocus*, and one strain of *Streptococcus* to BALB/c mice. They found strain specific responses in the production of adaptive immune markers CD 4^+ T cells and IgA. *L. casei and Lb. planterum* interacted with Peyer's patches and increased IgA and CD 4+ cells. *Lb. acidophilus* induced gut mucosal activation by interaction with epithelial cells. *Lactococcus lactis* and *Lb. delbrueckii* increased IgA but not CD 4+ T cells.

2.6 The pathogenesis of *E.coli* mediated inflammation

Table 2.2 summarizes the recent studies on the inflammatory pathogenesis of *E.coli.*

2.6.1 In vitro studies

The difficulty in studying a pathogen like *E.coli* is that it is very harmful to the host and can illicit detrimental effects. Therefore it is necessary to use appropriate cell models to look at the pathogens' effects. Hauf et al. used epithelioid human cervix carcinoma cells (HeLa) to analyze the impact of Stx producing *Escherichia coli* (STEC) on the NF-kB binding activity in the cell line. They found that STEC interfered with the NF-kB activation initiated by TNF-α. They concluded that this may be a commonality to several attaching and effacing bacteria allowing them to colonize the gut and attach to the epithelial lining. McKee et al. using human laryngeal epithelial cells (HEp-2) looked at

the adherence properties of Enterohemorragic *E.coli* O157:H7 (EHEC). They found that intimin was a necessity for attachment of bacteria to the epithelial cell.

Other recent studies have looked at specific inflammatory markers through which *E.coli* elicits its immune response. Witthoft et al. (1998) used HT-29 and Caco-2 cells infected with entroinvasive *E.coli and S. Dublin* to look at the activation of pro-inflammatory markers. They found *E.coli and S. Dublin* increased iNOS expression and epithelial NO production. They saw a larger production of NO with *E.coli* compared to S. Dublin. However, when they measured IL-8 production they found S. Dublin was a stronger stimulus. They concluded that their results show the importance NO plays in the intestinal epithelial response to microbial infection. Finally, Berin et al. used Caco-2 cells infected with EHEC to study the invasive nature of this pathogen. They found that infection with EHEC activated p38 and ERK MAP kinases and the nuclear translocation of the transcription factor NF-kB, which is a precursor to many pro-inflammatory mediators. They also found an increased expression of mRNA and protein for the neutrophil chemoattractant IL-8 which will illicit increases in inflammation in the area. They did associate the proinflammatory activation to the H7 flagellin on the *E.coli* indicating again the bacteria's structure as an important component of its pathogenisity.

2.6.2 Animal studies

The invasive nature of *E.coli* O157:H7 has left many of the recent studies to cell models where the mechanistic aspects of its pathogenicity can be worked out before sacrificing animals. Kurikoka et al. (1997) inoculated gastrically C57BL/6 mice on a protein calorie malnourished diet with Stx producing *E.coli* O157:H7. They found LPS in the stool indicate the bacteria were present in the gastrointestinal tract. They found

Table 2.2 The Pathogenesis of *E.coli* Mediated Inflammation

Model	Experimental Design	Results	Reference
HeLa cells	Cells were infected with 3 strains of STEC, 1 strain of EPEC and 1 strain of EHEC	STEC suppressed NF-kB activation and initiated decreased mRNA levels of IL-8, IL-6, and IL1-alpha	Hauf et al., 2003
CaCo 2 cells	Infected cells with EHEC O157:H7	Activated p38 and ERK Map kinases and nuclear translocation of NF-kB. Increased IL-8 mRNA expression.	Berin et al. 2002
HT-29 and CaCo 2 cells	Cells were infected with enteroinvasive *E.coli* and *S. Dublin*	*E.coli* increased epithelial NO production to a greater extent than *S. dublin*. *S.dublin* increased IL-8 production to a greater extent than *E.coli*. Both bacteria increased iNOS expression and NO production in both cell lines.	Witthoft et al. 1998
HE p-2 cells	Cells infected with strains of EHEC O157:H7	Intimin is a requirement for adhesion of EHEC to epithelial cells	McKee et al., 1996
C57BL/6 Mice	Infected with Stx producing *E.coli* O157:H7	Protein calorie malnutrition increased susceptibility of mice to intragastric infection with Stx producing *E.coli* 0157:H7	Kurioka, 1991

that the mice developed increased intragastric infection due to their protein calorie malnourished diet when exposed to a relatively low concentration of Stx-producing *E.coli* O157:H7.

2.7 Effects of lactic acid bacteria on the modulation of pathogenesis of the gastrointestinal tract

Table 2.3 summarizes the recent studies in this area.

2.7.1 *In vitro* studies

Recent research in this area has looked at how lactic acid bacteria can work as probiotics. Many studies have looked at strain specific effects of lactic acid bacteria on the adherence, colonization, internalization, and the overall pathogenesis of bacteria such as *E.coli* O157:H7 in different epithelial models. Hirano et al. (2003) infected C2BBe1 a human colon epithelial cell line with EHEC and four strains of *Lactobacillus*. They found that L. rhamnosus was effective in suppressing the internalization of EHEC into the cell line while the other three *Lactobacilli* were not, indicating a strain specific effect. Lee et al. (2003) found similar results infecting Caco-2 cells a human colon epithelial cell line with eight strains of *E.coli* and *Salmonella* and *L. rhamnossus GG* and *L. casei shirota.* Both strains were able to compete with, exclude, and displace the pathogenic bacteria when incubated together. The degree of inhibition was strain dependent (certain *E.coli* bacterial strains were stronger adherers than others). Finally, Mack et al. looked at the efficacy of *L. plantarum 299v and L. rhamnosus GG* to inhibit the adherence of *E.coli* O157:H7. Both *Lactobacilli* stains were able to quantitatively inhibit the adherence,

Table 2.3 Effects of Lactic Acid Bacteria on Modulation of Pathogenesis in The Gastrointestinal Tract

Model	Experimental Design	Results	Reference
C2BBe1 human colon epithelial cell line	Infection of cells with EHEC and 4 strains of *lactobacillus*	*L. rhamnosus* decreased internalization of EHEC into the cell line	Hirano et al., 2003
CaCo 2 cells and human intestinal mucus glycoproteins	Cells infected with 8 strains of *E.coli* and *Salmonella*, and co-incubated with 2 strains of *lactobacillus*	*Lactobacillus* were able to compete with exclude and displace pathogenic GI bacteria when incubated together. Degree of inhibition of adhesion was strain dependant.	Lee et al., 2003
HT-29 cells	EHEC 0157:H7 was coincubated with *L. planatrum 299v* and *L. rhamnosus GG* on cells	The *lactobacilli* quantitatively inhibited the adherence and attaching of the pathogenic *E.coli.*	Mack et al., 1999
Wild Type Mice and Mice deficient in different TLR	Unmethylated and unmethylated genomic DNA isolated form probiotics (VSL-3), DNAse-treated probiotics, and E.coli (DH5alpha were administered intragastricly or subcutaneously.	Probiotic and *E.coli* DNA together decreased severity of DSS-induced colitis. TLR-2 and 4 deficient mice had decreased colitis. TLR-9 was found to be essential in anti-inflammatory effect of probiotic.	Rachmilewitz et al., 2004
Male BALB/c mice	Colonization with *L. casei shirota* then treated with *Salmonella, E.coli*, or *L. monocytogenes.*	Increased resistance to lethal infection of *Salmonella, E.coli,* and *L. monocytogenes.*	Nomoto et al., 1989
Male Rat	Consumption of Yoghurt bacteria then infection with *Salmonellosis*	Increased resistance to *Salmonellosis* infection.	Hitchins et al. 1986

Table 2.3 (cont.)

Model	Experimental Design	Results	Reference
Human model	5 week oral administration of *Lactobacillus rhamnosus GG* then stimulated with either *Bacteroides fragilis* or *E.coli*	Increased CD 4+ lymphocytes, IL-10, and IL-4, and decreased TNF-alpha and IL-6 production.	Schultz et al. 2003
Human model	Consumptin of *Lactobacillus GG* in patients with *Shigellosis*	Decreased *shigelosis*-associated diarrhea.	Sepp et al. 1995

attachment and efficacy of EHEC. These three papers point to the physical effect bacteria like probiotics can have on the colonization of the gastrointestinal tract.

2.7.2 Animal and human studies

Hitchins et al. (1986) looked at the effect of yogurt known to carry lactic acid bacteria on infection of male rats with *Salmonella*. They found that the yogurt bacteria increased the resistance of the animals to *Salmonellosis* infection. Like-wise Nomoto et al. (1989) looked at the effect of *L. casei Shirota* consumption on a lethal injection of *Salmonella, E. coli,* and *L. monocytogenes* in BALB/c mice. They found that *L. casei shirota* increased resistance to lethal infection of all three of these pathogens.

Sepp et al. (1995) looked at consumption of *Lactobacillus GG* in humans infected with *Shigella* and the duration of the symptom diarrhea. They found a significant decrease in the shigellosis associated diarrhea in those infected who consumed the *Lactobacillus GG*. Rachmilewitz et al. (2004) looked at unmethylated and methylated DNA from *Lactobacilli* strains in the VSL-3 (a mixture of 8 strains of lactic acid bacteria being used in the treatment of inflammatory bowel disease) and *E. coli's* effects on DSS-induced colitis. They found a decrease in the severity of the colitis with the probiotic and *E.coli* DNA together. They also looked at toll-like receptor (TLR) deficient mice and saw that TLR-2 and TLR-4 deficient mice had decreased colitis. They also saw that TLR-9 was essential in mediating the anti-inflammatory effect of the probiotics. This indicated that probiotic bacteria may be mediated by their own DNA.

Schultz et al. (2003) examined the effect of oral consumption of *L. rhamnosus GG* in ten healthy volunteers on immune response to intestinal microorganisms. They found effects on the production of various cytokine both pro and anti inflammatory. They found increased production of CD 4+ lymphocytes and a decrease in TNF-α and IL-6, along with an increase in IL-10 and IL-4 after probiotic treatment. This indicated a protective affect of the probiotic in limiting the inflammatory response. These recent studies are important because they look at the benefits that probiotics have on the immune response.

2.8 Rational for the use of cell models

In order to look for the mechanistic effects of probiotics on the immune system one needs to be able to control the environment. The cell model allows for a reductionist view and the ability to measure and quantify particular aspects of the immune response. Since epithelial cells are the first line of defense and the first cell type in which bacteria will come in contact it is fitting to use these types of cells. Also, since the majority of the interaction will occur in the colon due to the fact that this is were they will spend the majority of their time and possibly be able to colonize it is fitting to look at cells from this region.

2.8.2 Conditionally immortal colonic epithelial cells

The two cell types used in this research are mouse colon epithelial cells developed by and obtained from Robert Whitehead from the Ludwig Institute for Cancer Research in Melbourne, Australia. The cells bear a temperature sensitive mutation of the simian virus 40 large tumor antigen gene (tsA58) which enables the cells to be conditionally immortal. The cells proliferate continuously at the permissive temperature (33° C), but

proliferation ceases at the nonpermissive temperature (39.5° C). Growth of these cells is enhanced by γ-interferon.

The first cell type used in this research was designated young adult mouse colon (YAMC Apc $^{+/+}$) by Whitehead et al. (1993). It has characteristics similar to a normal mouse colon epithelial cell. These cells appear phenotypically and morphologically immature, and they do not differentiate (Whitehead, 1993). They grow in confluent mono-layers on collagen-coated surfaces and spread to form islands of epithelial cells (Whitehead, 1993).

The second cell type used in this research was designated "Immortomouse"/ Min hybrid (IMCE, Apc $^{Min/+}$) by Whitehead et al. (1993). Besides carrying the temperature sensitive mutant of the SV40 large T gene like the YAMC cells, the IMCE cells also have a Min mutation. This is the homologue of the human APC gene and is a model of familial adenomatous polyposis. This model was derived through the mating of a heterozygous male Min mouse with a heterozygous female "Immortomouse". The IMCE cells grow as flat cuboidal cells in monolayer culture. These cells do not form colonies in soft agar and do not form tumors in nude mice. The Min mutation alone has not been seen to be sufficient to transform the cells. Therefore they are pre-neoplastic in nature and have been described as a model for Familial Adenomatous Polyposis (Moser et al., 1992). The IMCE cells have been demonstrated to have several properties of preneoplastic cells, including decreased cell migration and decreased intracellular communication (Fenton, 2002). These nontumorogenic colon epithelial cells offer an excellent model system to determine/analyze the effect of probiotic bacteria on inflammatory mediator production caused by bacterial pathogens.

2.8.3 Benefits of this model

By using these two models one can look not only at the effect different bacteria will have on a normal cell line but can contrast it with a precancerous cell line. Because the second cell line IMCE is the same in almost all respects to the normal YAMC, except for the Min mutation they are vary comparable. By using a precancerous cell line instead of a cancerous or tumor cell line as many previous studies have done, one can look at prevention, before the cells become tumors. Also tumor cells behave very differently from normal cells so what you see in a tumor cell may not relate to a normal cell.

2.8.4 Limitations

The limitations of this model are those with most cell lines. The results of experiments in cell lines are just the first step. Because the body is a complex organism there is more than just one component. Therefore the epithelial cells of the gut will come in contact with other components of the gastrointestinal tract (i.e. macrophages, lymphocytes, and mucus).

2.9 Rationale for this research

The roles probiotics play in modulating the immune system still needs to be answered. Research needs to address how probiotics have their effects, whether at the local and/or systemic level. The burden that pathogenic bacteria have caused and the fact that probiotics have been seen to elevate the severity of their infection needs to be understood to be utilized effectively. There are a limited number of studies looking at the effects of probiotics on the immune system, let alone their effects on pathogenic induced

inflammation. This in part is due to the difficulty in finding an appropriate model. The model we have chosen allows a more preventative outlook (normal versus preneoplastic) on disease. Much of the research to this point has been done in models that are already cancerous.

The working hypothesis for this research is that two strains of lactic acid bacteria can differentially alter the immune function of colon epithelial cells in response to *E. coli* O157:H7. The two probiotic strains were chosen based on previous research conducted by other labs (Wong, 2002) and their ability to stimulate cytokine production. The probiotics and the *E.coli* were grown in the lab and separated using a washing procedure, irradiated and stored at -80° C prior to use. Studies were conducted to look at the production of pro-inflammatory mediators from the cells in response to various concentrations and co-treatments of the probiotics and *E.coli*. Further, studies were conducted with enzymatic inhibitors to look at signaling molecules roles in the regulation of the various pro-inflammatory mediators observed with *E.coli* exposure.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

3.1 Culture preparation

Lactobacillus casei, American Type Culture Collection (ATCC) 39539 (Rockville, MD; LC), and *Lactobacillus reuteri,* ATCC 23272 (Rockville, MD; LR) were grown in De Man, Rogosa, Sharpe (MRS) broth (Difco Laboratories, Detroit, MI). *Escherichia coli* O157:H7 AR (EC) was grown in Trypticase Soy Broth containing 0.6% (w/v) yeast extract (TSB-YE; Becton Dickinson, Sparks, MD). All bacteria were irradiated and plated to ensure cultures were no longer viable.

In preliminary experiments irradiated bacterial cultures were grown to maximal colony forming units per milliliter (cfu/ml) and irradiated. Bacteria in spent media, bacteria alone, and spent media alone were diluted for experimentation at 1:10, 1:100, and 1:1000 of original volume. In early experiments separation of bacteria from spent media was done by centrifuging the culture at 2600 x g (gravity), 4 °C for 15 min, the media was then removed. The bacteria was reconstituted in sterile phosphate buffered saline (PBS) (GIBCO, Rockville, MD) to original volume and centrifuged at 2600 x g for 10 min and repeated two times to wash media components from bacteria.

The bacteria was then reconstituted in IFN-γ free media (RPMI 1640 supplemented with 1% neonatal calf serum, 1% ITS ® (BD Biosciences, Bedford, MA; insulin 625 µg/L, transferin 625 µg/L and selenous acid 625 ng/L) and 1% penicillin-streptomycin (Sigma, St. Louis, MO; 100,000 IU/L penicillin and 100 mg/L

streptomycin) to original volume, aliquot into 10 ml tubes and frozen for treatment use When the stock of EC, LC, and LR was consumed a new method was developed which was used for all subsequent experimentation.

Appendix 1 provides a schematic diagram of bacterial preparation. There was an additional step in the preparation of the EC compared to the lactic acid bacteria (LAB; LC and LR); one loop full of frozen EC was inoculated into 10 ml of TSB-YE for 24 hrs at 37 °C. One and a half ml (1.5 ml) of thawed stock of the LAB (stored at -80 °C) was inoculated in 25 ml MRS media, while, 1.5 ml of the TSB- with EC (inoculated the previous day) was pipette into 25 ml of fresh TSB-YE, all bacteria were incubated for 24 hrs at 37 °C. Bacteria were then centrifuged at 18,773 x g, 4 °C, for 10 min. The supernatant (spend media) was removed and cultures were washed with 20ml 1XPBS by centrifugation (18,773 x g, 4 °C, 15 min). Twenty-five milliliters (25 ml) of their respective growth media as mentioned above was added to each bacterium and they were again incubated at 37 °C for 24 hrs. This growth procedure was repeated twice.

On the third wash and incubation, all bacterial incubations where shortened to 15 hrs at 37 °C. Ten milliliters (10 ml) of each culture was transferred into an Erlenmeyer flask of 250 ml of fresh media incubated at 37 °C until late log phase in shaker (6-10 hrs for LAB, 24 hrs for EC). This was done in duplicate for all bacteria. Optical density (OD) was used to determine growth phase based on standard curves generated in previous experimentation (Wong, 2002). ODs of cultures in their respective spent media (1ml each) were measured on a Spectronic 1001 Plus (Milton Roy, Rochester, NY) at 650 nm using uninoculated growth media as blank.

Culture samples were then diluted and plated to estimate cell numbers by OD. Bacteria in media were diluted using 0.1% (w/v; weight/volume) bacto-peptone dilution buffer (Difco) to obtain ten-fold dilutions of 10^{-1} to 10^{-8} w/v. One milliliter (1 ml) samples were plated using the spread plate method. Media containing 1.5 % (w/v) agar was used respectively using the pour plate method. Plates were incubated for 48 hrs. at 37 °C and then counted.

After bacteria reached late log phase they were aliquoted into sterile tubes and centrifuged at 18,773 x g, 4 °C, 15 min. Bacteria were then washed three times with PBS, centrifuged and aspirated as above after each wash. Bacteria were then resuspended at one-tenth their original volume in sterile PBS (1/ 10^{th} of 500 ml or 50 ml) and frozen immediately at -80 °C. Frozen bacteria were then taken to the Phoenix Memorial Laboratory (University of Michigan, Ann Arbor, MI) and inactivated by gamma irradiation (1 Mrad). Inactivation was determined by spread plate method for all bacteria. Dry weights of cultures were then determined by speed vacuuming 500 µl aliquots, PBS samples were also dried to determine the contribution of salt to find original bacterial weight. Weights of the dried samples were measured using a Mettler balance. Bacterial weight was determined by subtracting tube and dried PBS weight from total tube weight. Table 3.2 represents bacterial numbers in the growth stages described above as OD, cfu/ml, and weight per volume for each bacterial culture.

3.2 Cells and Cell Culture Condition

Experiments were carried out using two cell lines, a non-tumorogenic murine colon epithelial cell line (i.e. Young Adult Mouse Colon or YAMC; $Apc^{+/+}$, a model of "normal" cells) and Immortomouse/Min Colon Epithelial (IMCE; $Apc^{Min/+}$ cells, a model

Table 3.1 Bacterial growth amount per milliliter of predried, reconstituted samples

Type of Bacteria	Absorbance (650 nm)	CFU/ml	µg/ml *
Escherichia coli 0157:H7	1.363	5.5×10^9	15,935
Lactobacillus casei	1.387	2.8×10^9	18,700
Lactobacillus reuteri	1.673	2.3×10^{10}	25,700

* Based on Speed Vacuum, 500 ul sample, 1/10th original volume of bacteria

of "preneoplastic" cells) both developed by Dr. Robert Whitehead (Ludwig Institute for Cancer Research, Melbourne, Australia) and grown in RPMI 1640 media (GIBCO) supplemented with 5 % neonatal calf serum (NCS), ITS® (BD Biosciences; insulin 625 μg/L, transferring 625 μg/L and selenous acid 625 ng/L), 500 IU/L of murine IFN- γ (Sigma), 100,000 IU/L penicillin and 100mg/L streptomycin (Sigma) (Complete media). Cells were first grown in 75 cm^2 (T-75) tissue culture flasks (Fischer, Pittsburgh, PA) coated with 5 $\mu g/cm^2$ type 1 rat tail collagen (BD Biosciences) at 33 °C with 5% CO_2 in media plus aforementioned supplements until they reached 100% confluence.

At 100% confluence cells were detached from the flask using Trypsin-EDTA (5ml per flask, Sigma) and harvested by centrifugation 1800 x g for 5 min. Cells $5x10^5$ cell/ml for YAMC and 1 x 10^6 cells/ml for IMCE cells (1ml per well) were transferred to either 24 well tissue culture plates (Falcon, San Jose, CA) or 96 well plates (Sigma) (200 μl media per well) previously coated with 5 $\mu g/cm^2$ type 1 rat tail collagen, at 33 °C until they reached 80% confluence. At 80% confluence, cells were transferred to 39 °C under non-transforming conditions with 1ml per well (24 well plates) or 200 μl per well (96 well plates) of 1640 RPMI media supplemented with low serum 1% NCS, IFN-γ free media for 24 hrs before use in experiments.

3.3 Stimulation of proinflammatory mediators

Irradiated bacterial samples as described in section 3.1 were added to cells in low serum, IFN-γ free media for 72 hrs at varying concentrations. Early experiments included *E.coli* O157:H7 (EC), *L. casei* (LC), and *L.reuteri* (LR) complete (spent media and bacteria), bacteria alone, or spent media at 1:10, 1:100, 1:100 dilutions as well as co-

treatments of the components. Experiments were also conducted using EC, LC, and LR at varying concentrations of bacteria from 1000μg/ml to 1 μg/ml. Supernatant from cells treated with sterile culture medium was used as a negative control. Supernatant was collected and pooled at 72 hrs for most experiments (experiments were conducted at 24 hrs and 72 hrs to establish the temporality of these effects). The supernatant were collected form six wells (96 well plate) or 12 wells (96 well plate) per treatment, pooled and analyzed in triplicate. The supernatant was centrifuged at 2600 x g, 15 min, aliquot into 1.5 ml tubes, 500μl per tube, and frozen at -80 °C until analyzed for MIP-2, TNF-α, TGF-β by enzyme linked immunosorbent assays (ELISA) or for nitric oxide (NO) by the Greiss reaction. All experiments were conducted at least three times.

3.4 Cell viability

3-(4, 5 dimethylthiazol-2γl)-2, 5 diphenyl tetrazolium bromide (MTT) was used to measure cell viability. After supernatants were removed from treated plates they were washed with 1ml PBS in 24 well plates, or 200 μl in 96 well plates and aspirated. Then, one ml of IFN-γ free low serum medium and 100 μl of MTT was added to each well of the 24 well plates, or 200 μl of IFN-γ free low serum medium and 25 μl of MTT was added to each well of the 96 well plates (in low light) and incubated at 39 °C overnight. The medium/MTT was then aspirated off and 500μl of dimethyl sulfoxide (DMSO) (J.T. Baker, Phillipsburg, NJ) (24 well plate) or 150 μl DMSO (96 well plate) was added to each well (in low light). Plates were allowed to sit at room temperature for 15 min to allow crystals to detach. 100 μl aliquot from each well were then added to a 96 well plate. Absorbance was read at 570 nm using the Spectra Max 300® plate reader

(Molecular Devices, Sunnyvale, CA). Results were calculated using the negative control as 100% cell viability.

3.5 Nitric oxide (NO) quantification

Nitrite, a stable end product of NO metabolism, was measured in conditioned media using the Greiss reaction and sodium nitrate (J.T. Baker, Phillipsburg, NJ) as a standard. In brief, 150 µl of standard was aliquot into two wells of a 96 well plate and serial diluted 1:2, eight times (112 to 0.875 µM) in low serum, IFN-γ free media. Seventy-five microliters (75 µl) of samples were then added in triplicate to the 96 well plate. Seventy-five microliters (75 µl) of media was then added to two wells to serve as blanks. Reactant (75 µl) was then added to each well. Reactant contained 0.5 g sulfanilamide (Sigma), 0.05 g N-1napthylethylendiamide hydrochloride (Sigma) in 37.5ml ddH_20 and 12.5 ml phosphoric acid (concentrated; J.T. Baker). Absorbance at 540 nm was determined using the Spectra Max 300® plate reader. Results are expressed as µmol of NO/well.

3.6 MIP-2, TNF- α, and TGF-β quantification

MIP-2, TNF- α and TGF-β were measured by using ELISA. MIP-2 detection, standard, and biotinylated antibody were purchased from Peprotech (Rocky Hill, NJ). Briefly, plate was coated with 50 µl/ well (overnight at 4 °C) containing IL coating buffer (4.2 g $NaHCO_3$ [pH 8.2]; Sigma and anti-mouse polyclonal MIP-2 capture antibody at 1µg/ml final concentration. Plate was washed three times in a tub of PBS-containing 0.05% Tween 20 (PBST; Sigma), discarding into the sink after each wash. Each well was then blocked with 300 µl of 3% bovine serum albumin (BSA) (Sigma) in PBST for 30 min at 37° C. Plate was washed four times as previously mentioned. Standards were

then added to the plate in duplicate at 50µl/well (10ng to 0ng/ml). Followed by samples in triplicate at 50 µl/well and incubated at 37 °C for 1 hr. Plate again washed four times as previously described. Fifty microliters (50 µl) of biotinylated anti-murine MIP-2 detection antibody in 3% BSA-PBST was added to each well at 1µg/ml final concentration for 1hr at room temperature. Plate was washed 6 times with PBST and 1 time with dH_20. Streptavidin-HRP (1.5µg/ml diluted in 3% BSA-PBST) (Sigma) was then added to the plate at 50µg/well for 1 hr at room temperature. Plate was washed eight times with PBST and two times with dH20. TMB substrate (100 µg/ml; Neogen, Lansing, MI) was added to each well and color was allowed to develop. One-hundered microliters (100 µl/well) of 6N H_2SO_4 (J.T. Baker) was added to stop the reaction. Absorbance at 450 nm was determined using SpectraMax 300® plate reader. Results were expressed as pg/ml.

TNF-α was quantified using the BD Opt EIA® ELISA set from BD Biosciences (BD Pharmigen, San Diego, CA). Procedures were followed as outlined in the kit. Briefly, 100 µl diluted capture antibody was added to each well of a 96 well plate and incubated overnight at 4 °C. The plate was aspirated and washed three times with PBST. Next, the plate was blocked with 300 µl/ well of 10% NCS in PBST. Plate was washed again three times with PBST. Standards were added in duplicate at concentrations ranging from 1000 pg/ml to 15pg/ml, samples were added in triplicate 100 µl/well. Plate was than incubated 2 hrs at room temperature. Plates were then aspirated and washed five times with PBST. One hundred microliters (100µl) horseradish peroxidase (HRP) was added to each well and incubated 30 min at room temperature. The plate was washed 7 more times with 30 sec between washes. One hundred microliters (100 µl) of

TMB substrate solution was added to each well and incubated for 30 min at room temperature in low light. The reaction was stopped with 50 µl of 1M H_3PO_4. Absorbance at 450-570 nm was determined using SpectraMax 300® plate reader (Molecular Devices, Sunnyvale, CA). Results are expressed as pg/ml.

TGF-β was quantified using the TGF β $_1$ E_{max}® ImmunoAssay System from Promega (Madison, WI). Procedures were followed as outlined in the kit. Briefly, 100 µl/well of carbonate coating buffer with mouse antibody (mAb) was added to a 96 well plate and incubated overnight at 4 °C. Plate was blocked with 270µl/well of 1X Buffer for 35 min at 37 °C. The plate was washed one time with PBST. Standard was prepared in duplicate on plate in a serial dilution 1:2 with 100 µl/ well final volume (0 pg/ml to 1000 pg/ml). One-hundered microliters (100µl) of samples were then added in triplicate to the plate and incubated with shaking for 2 hrs at room temperature. Next, plate was washed five times with PBS-T. One-hundred microliters/well (100 µl/well) of the anti-TGF- β 1 pAb was then added in 1 x Buffer to each well for 2hrs at room temperature. Plate again washed five times with PBST. TGF- β HRP conjugate was then added 100µl/well and incubated with shaking for two hrs at room temperature. The plate was washed five times. TMB One Solution (100 µl/ ml) at room temperature was then added to each well and color was allowed to develop at room temperature without shaking for 15 min. Reaction was stopped with 1N HCL (J.T. Baker). Absorbance at 450 nm was determined using SpectraMax 300 plate reader. Results are expressed as pg/ml.

3.7 Inhibition of NO and Other Cell Signaling Pathways

Four inhibitors and one NO chelator were used to survey for the mechanism by which EC bacteria was altering the production of proinflammatory mediators in the two colon epithelial cell lines. NG-nitro-L-arginine-methyl ester (L-NAME; Cayman Chemical, Ann Arbor, MI) was used at 50 μM as an enzymatic inhibitor of iNOS. Hemoglobin (Sigma, St. Louis, MO) was added at 500 μM as a binder of NO. It was added to 96 well plates to bind the NO and look at the role the precursors to NO had on the two cell lines (exposed to the EC) production of MIP-2.

Pyrrolidinedithiocarbamate ammonium ([PDTC];TOCRIS, Ellisville, MO) was used at 10 μM to inhibit NF-kB translocation. It was used to look at the effect EC had on NF-kB activation on NO and MIP-2 production in the two cell models. SB 202190 (SB; TOCRIS) was used at 10 μM and 0.1 μM to inhibit the p38 MAPK (MAP kinase) pathway to assess the contribution of this pathway to MIP-2 and NO production in the two cell models treated with EC. SP 600125 (SP; TOCRIS) was used at 20 μM and 0.2 μM to inhibit JNK (c-Jun N-terminal kinase) pathway to determine the contribution of this pathway to MIP-2 and NO production in the two cell models treated with *E.coli* bacteria. Finally, SP 20 μM and SB 10 μM in combination was used to control for the possible up regulation of the p38 pathway by the SP inhibitor.

Table 3.2 Inhibitors of Signaling Pathways

Inhibitor	Concentration	Source
L-name	50μM	Cayman Chemical, Ann Arbor, MI
Hemoglobin	500μM	Sigma, St. Louis, MO
PDTC	10 μM	TOCRIS, Ellisville, MO
SB 202190	10 or 0.1 μM	TOCRIS, Ellisville, MO
SP 600125	20 or 0.2 μM	TOCRIS, Ellisville, MO
SB 202190 and SP 600125	10 μM and 20 μM	TOCRIS, Ellisville, MO

3.8 Antibody Microarrays

Supernatants from control, EC, LC, LR, EC-LC, EC-LR, treated cells and low serum, IFN-γ freemedia alone were exposed to antibody microarrays (Raybiotech, Inc., Atlanta, GA; Appendix III) containing antibodies against 62 cytokines/ chemokines. See Table 3.3 for the list of cytokines/chemokine included on this array. Briefly, supernatants from cells treated 72 hrs. and media were exposed to the antibody micro arrays. Detection was carried out using biotinylated primary antibodies, streptavidin- HRP, and chemiluminescence detection using the methodology supplied by the manufacturer. Densitometric analysis of cytokine signals were quantified with Molecular Analyst software by Bio Rad (Hercules, CA). Preliminary data was graphed with PRISM 4 software.

3.9 Statistical Analysis

Experiments were run in duplicate or triplicate on a pooled sample with an n = 6 (24 well plates) n = 12 (96 well plates). Data was analyzed using Graph Pad PRISM 4 statistical software (Graph Pad Software, San Diego, CA). One-way or Two-way ANOVA were used with Bonferonni post tests to compare between treatments within experiments. A $p \leq 0.05$ was used as the level of significance.

Chemokines	Cytokines	Growth factors, adhesion molecules, other protiens
BLC	CRG-2	AXL
CTACK	IFN-gamma	CD30L
CXCL16	IL-1 alpha	CD30T
Eotaxin	IL-1B	CD 40
Eotaxin-2	IL-2	Fas Ligand
KC	IL-3	Fractalkine
LIX	IL-3Rb	GCSF
Lymphotactin	IL-4	GM-CSF
MCP-1	IL-5	IGFBP-3
MCP-5	IL-6	IGFBP-5
M-CSF	IL-9	IGFPBP-6
MIG	IL-10	Leptin R
MIP-1 alpha	IL-12 p40/p70	LEPTIN (OB)
MIP-1 gamma	IL-12 p70	L-selectin
MIP-2	IL-13	L-selectin
MIP-3 alpha	IL-17	PF-4
MIP-3 B	MIP-1 alpha	P-selectin
SDF-1alpha	RANTES	SCF
TARC	TNF-alpha	TCA-3
TECK		TIMP-1
		TNF-RI and RII
		TPO
		VCAM-1
		VEGF

Figure 3.4 Inflammatory Antibody Array Cytokines and Other Proteins Measured

CHAPTER 4

RESULTS AND DISCUSSION

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Effect of *E.coli* O157:H7 (EC), *L. casei* (LC), and *L.reuteri* (LR) in spent media on proinflammatory mediator production (NO and TGF-B) and cell viability in YAMC and IMCE cells

Twelve bacterial cultures (4 pathogens, 1 commensal, and 7 probiotic) were prepared to evaluate the hypothesis that probiotic bacteria, but not commensal bacteria, could decrease the production of inflammatory mediators in colon epithelial cells (Table 4.1). Two probiotic organisms and one pathogenic organism were selected from the list of cultures grown to more specifically analyze the hypotheses. The organisms were chosen because of their strong characteristics including there presence as part of the natural gut microflora, their gut colonizing capabilities, their induction or attenuation of inflammatory mediators, and the results of their use in previous experiments, by collaborating laboratories (Bourlioux et al., 2002, Wong, 2002).

The data are presented with NO production and the corresponding cell viability as a % of control (control set as baseline) for each cell type. Concentrations of nitrite which were below detection limit of this assay are noted as zero values on each graph. EC induced NO production in a concentration-dependent manner in both cell types ($p < 0.001$; Figure 4.1 and Figure 4.2; YAMC > IMCE). LC and LR alone caused no nitric oxide production in either cell types. Similarly, the growth media for each bacterium (EC –Trypticase Soy-Yeast Extract, LC and LR- MRS) did not elicit NO production. Both lactic acid bacteria at the higher concentration (10^6) were able to decrease ($p < 0.001$) NO production compared to the higher concentration of EC (10^6) treated cells alone in both cell types (Figure 4.3 and Figure 4.4).

Table 4.1. List of probiotic, commensal, and pathogenic bacteria cultures

Bacteria	Strain	Source/ Location
Bifidobacterium	Bf-6	Sanofi Bio-Industries, Waukesah, WI
Bifidobacterium adolescentis	M101-4	Japan Bifidus Foundation, Tokyo, Japan
Lactobacillus acidophilus	La-2	Sanofi Bio-Industries, Waukesah, WI
Lactobacillus bulgaricus	NCK 231	North Carolina State, NC
Lactobacillus casei	ATCC 39539	American Type Culture Collection, Rockville, MD
Lactobacillus reuteri	ATCC 23272	American Type Culture Collection, Rockville, MD
Streptococcus thermophilus	St 133	Sanofi Bio-Industries, Waukesah, WI
Salmonella typhimurium	DT104	CDC, Atlanta, GA
E.coli O157:H7	AR	U. Vermont, Burlington, Vt
Bacteroides thetaiotaomicron	ATCC 29148	American Type Culture Collection, Rockville, MD
Campylobacter jejuni	ATCC 33292	American Type Culture Collection, Rockville, MD
Campylobacter jejuni	ATCC 81176	American Type Culture Collection, Rockville, MD

Cell viability of YAMC cells and IMCE cells was fairly consistent among cell type and within the treatments (Figure 4.5 and Figure 4.6). EC appeared to have a positive effect on cell viability in YAMC cells, while LC appeared to have a positive effect on cell viability in IMCE cells. Co-treatments did not appear to adversely affect the cell viability.

In measuring TGF-β only the higher concentration of each bacteria was used (1 x 10^6 cfu/ml) in treatment of the two cell types for 72 hrs. Bacterial treatments did not alter TGF-β production in YAMC and IMCE cells. Production of TGF-β was consistent across treatment and between cell type. (Data not shown).

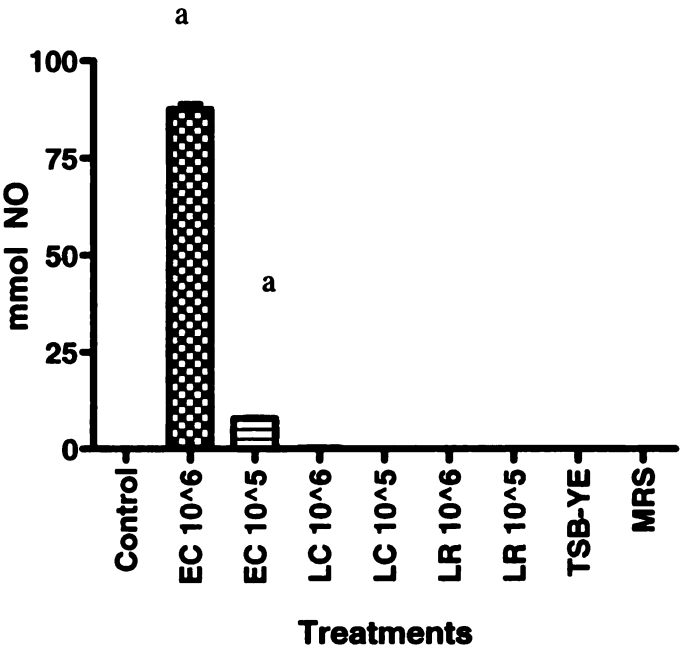


Figure 4.1 Nitric oxide (Mean +/- SEM) production in YAMC cells treated with 10^6 or 10^5 cfu/ml of *E.coli* (EC), *L.casei* (LC), *L.reuteri* (LR) bacteria in their spent media and their sterile culture medium for 72 hrs. a- Different compared to control, $p < 0.001$

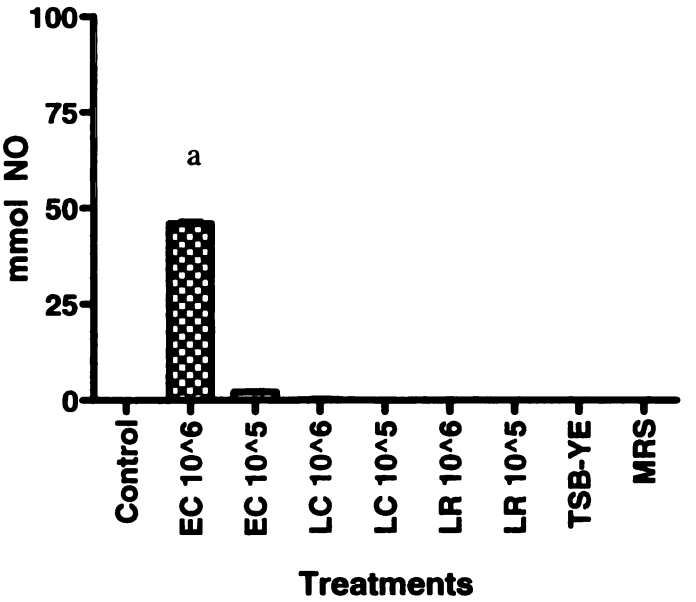


Figure 4.2 Nitric oxide (Mean +/- SEM) production in IMCE cells treated with 10^6 or 10^5 cfu/ml of *E.coli* (EC), *L.casei* (LC), *L.reuteri* (LR) bacteria in their spent media and their sterile culture medium for 72 hrs. a- Different compared to control, $p < 0.001$

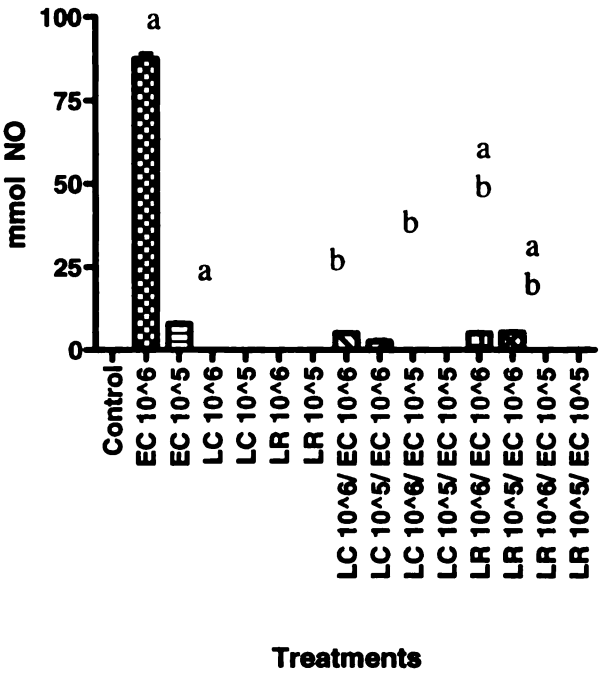


Figure 4.3 Nitric oxide (Mean +/- SEM) production in YAMC cells treated with 10^6 or 10^5 cfu/ml of *E.coli* (EC), *L.casei* (LC), *L.reuteri* (LR) bacteria in their spent medium and co-treatments for 72 hrs. a- Different compared to control, $p < 0.001$. Different compared to EC $p < 0.001$.

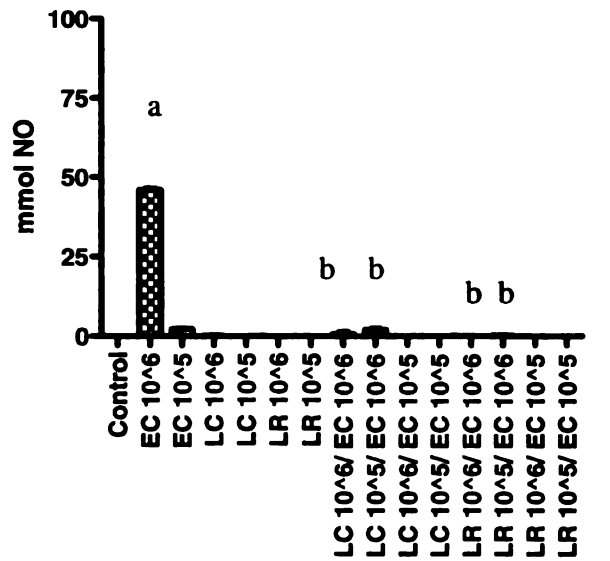


Figure 4.4 Nitric oxide (Mean +/- SEM) production in IMCE cells treated with 10^6 or 10^5 cfu/ml of *E.coli* (EC), *L.casei* (LC), *L.reuteri* (LR) bacteria in their spent medium and co-treatments for 72 hrs. a- Different compared to control, $p < 0.001$. Different compared to EC $p < 0.001$.

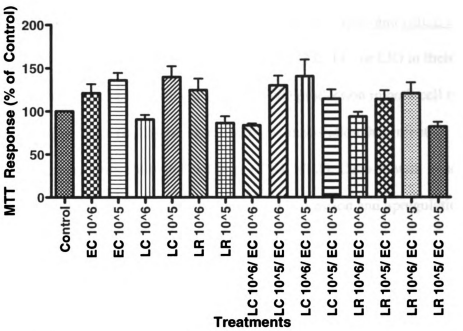


Figure 4.5 Cell viability (Mean +/- SEM) compared to control in YAMC cells treated with 10^6 or 10^5 cfu/ml of *E.coli* (EC), *L.casei* (LC), *L.reuteri* (LR) bacteria in their spent medium and co-treatments for 72 hrs. a- Different compared to control, $p < 0.001$.

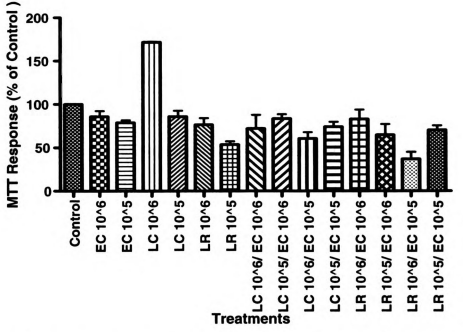


Figure 4.6 Cell viability (Mean +/- SEM) compared to control in IMCE cells treated with 10^6 or 10^5 cfu/ml of *E.coli* (EC), *L.casei* (LC), *L.reuteri* (LR) bacteria in their spent medium and co-treatments for 72 hrs. a- Different compared to control, $p < 0.001$.

4.1.2 Discussion of the effect of irradiated *E.coli* (EC), *L.casei* (LC) and *L.reuteri* (LR) bacteria in spent media on production of NO and TGF-β in YAMC and IMCE cells.

The results of initial experiments using bacteria (EC, LC, or LR) in their spent medium demonstrated that EC treatment increased NO production in both cell types (YAMC and IMCE). This finding is supported by demonstration that infection of monolayers of human colon epithelial cell lines (T 84, HT29, Caco-2) with invasive strains of bacteria (like EC) resulted in a coordinated expression and upregulation of proinflammatory cytokines IL-8 and TNF-α (Jung et al, 1994). Witthoft and others (1998) also found that human colon epithelial cells (CaCo2 and HT 29) rapidly unregulated NO production after infection with EC supporting our findings.

The ability of the probiotic bacteria to differentially attenuate the production of proinflammatory mediators such as NO, points to their use as anti-inflammatory agents to protect the host and improve health. These results are supported by Wallace and others (2000) who found strain-specific growth condition-dependent suppression of IL-8, TNF-α, and TGF-β production in HT-29 cells. The fact that LC and LR did not induce NO production indicates they may act as discrete immunomodulators. Probiotics may induce a heightened immune response by directly activating cells such as macrophages, B-cells, and natural killer cells (Wallace et al., 2002).

4.2 Effect of separation of bacteria from its spent media on proinflammatory mediator production (NO) and cell viability in YAMC and IMCE cells.

In order to further deduce the active component responsible for the effect of nitric oxide production, the bacteria were removed from their spent medias, washed three times with PBS and reconstituted in low serum, IFN-γ free media. The results below reflect the

response of YAMC and IMCE cells to the aforementioned bacteria or their respective bacterial spent medium.

EC bacteria caused a concentration-dependent production of NO in both cell types compared to control (Figure 4.7 and Figure 4.8). The higher concentration of EC bacteria (10^6) produced ($p < 0.001$) more nitric oxide than the lower concentration (10^5). There was no production of nitric oxide with either LC or LR bacteria.

In both cell types spent medium isolated from EC caused a concentration-dependent increase ($p < 0.001$) in production of NO in comparison with control (Figure 4.9 and 4.10). The higher concentration of EC spent medium produced more nitric oxide than the lower concentration. In the YAMC cells the LR broth also significantly ($p < 0.001$) produced nitric oxide in comparison with control. Our results indicate that EC bacteria and its spent media additively impact NO production in both cell types.

Overall cell viability was fairly consistent among treatment groups and between cell types. The cell viability for both cell types with LC and LR treatments was slightly lower than it had been with the bacteria and spent media treatments in both cell types (Figure 4.11 and Figure 4. 12). The cell viabilities of both cell types appeared to be similar with slightly less cell viability in the cells treated with spent media compared to cells exposed to bacteria plus spent media.

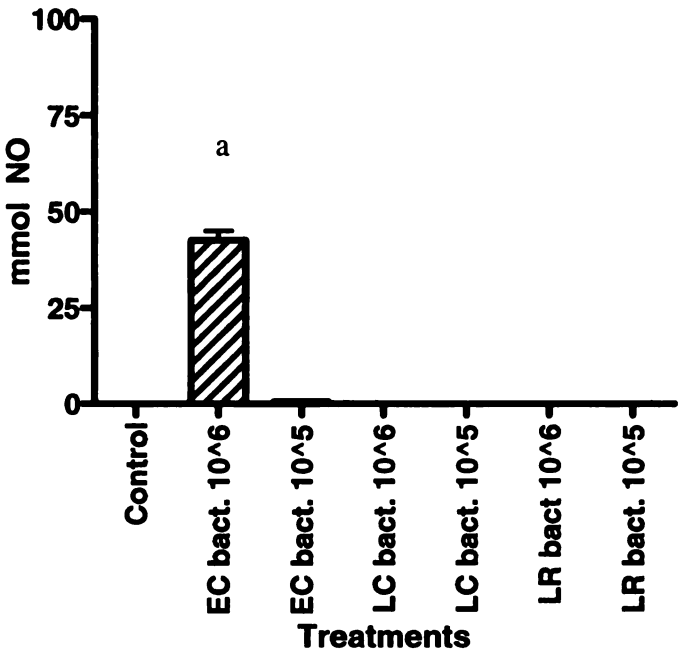


Figure 4.7 Nitric oxide (Mean +/- SEM) production in YAMC cells exposed to 10^6 and 10^5 cfu/ml of *E.coli*(EC), *L.casei*(LC), and *L.reuteri* (LR) bacteria for 72 hrs. a- Different compared to control $p < 0.001$

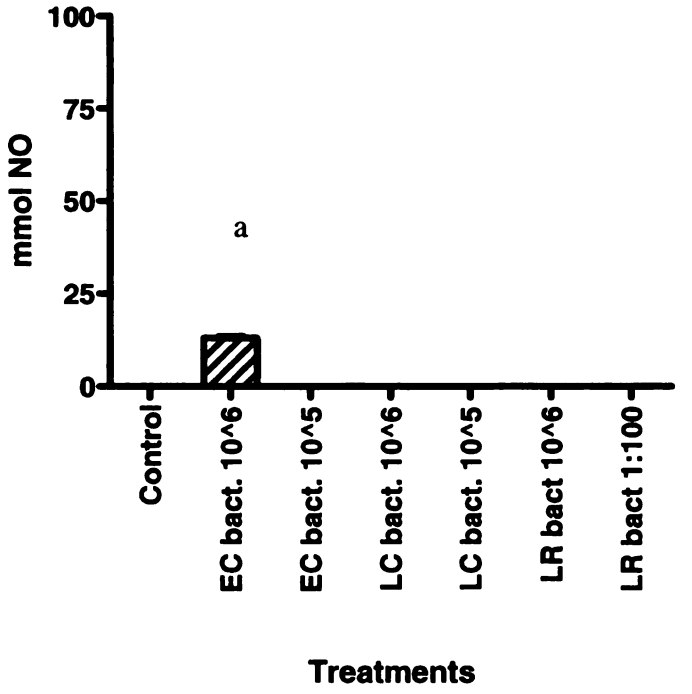


Figure 4.8 Nitric oxide (Mean +/- SEM) production in IMCE cells exposed to 10^6 and 10^5 cfu/ml of *E.coli*(EC), *L.casei*(LC), and *L.reuteri* (LR) bacteria for 72 hrs. a- Different compared to control $p < 0.001$

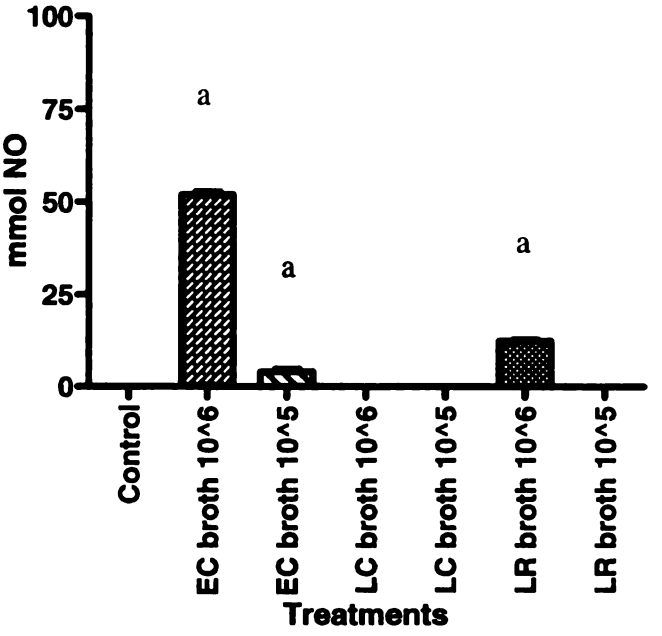


Figure 4.9 Nitric oxide (Mean +/- SEM) production in YAMC cells exposed to 10^6 and 10^5 cfu/ml of *E.coli*(EC), *L.casei*(LC), and *L.reuteri* (LR) spent medium (broth) for 72 hrs. a- Different compared to control $p < 0.001$

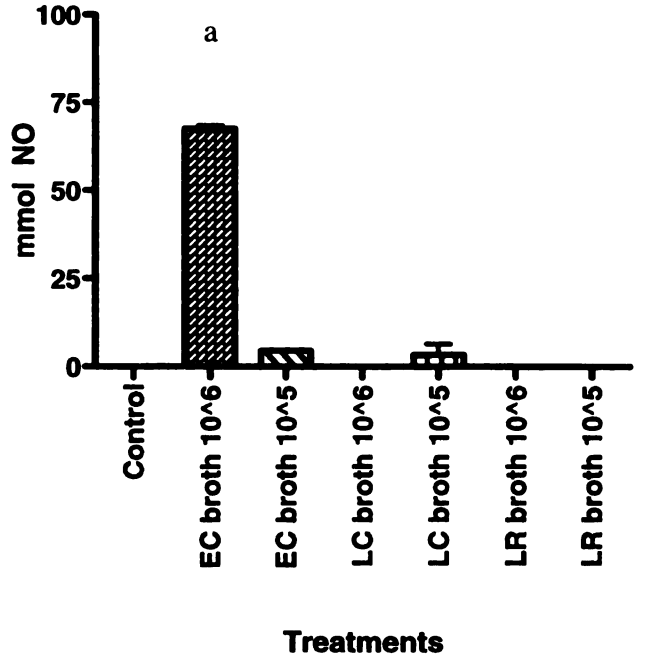


Figure 4.10 Nitric oxide (Mean +/- SEM) production in IMCE cells exposed to 10^6 and 10^5 cfu/ml of *E.coli*(EC), *L.casei*(LC), and *L.reuteri* (LR) spent medium (broth) for 72 hrs. a- Different compared to control $p < 0.001$

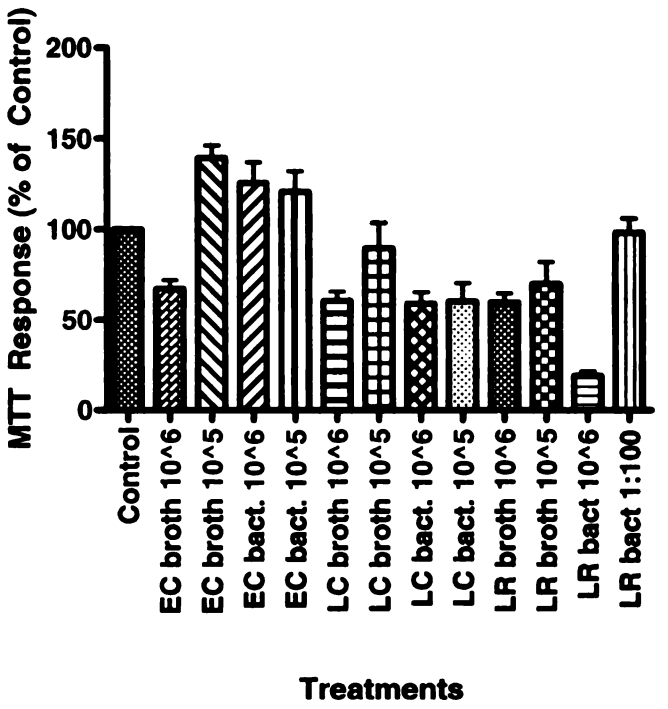


Figure 4.11 Cell viability (Mean +/- SEM) in YAMC exposed to 10^6 and 10^5 cfu/ml of *E.coli*(EC), *L.casei*(LC), and *L.reuteri* (LR) bacteria or spent medium (broth) for 72 hrs. a- Different compared to control $p < 0.001$

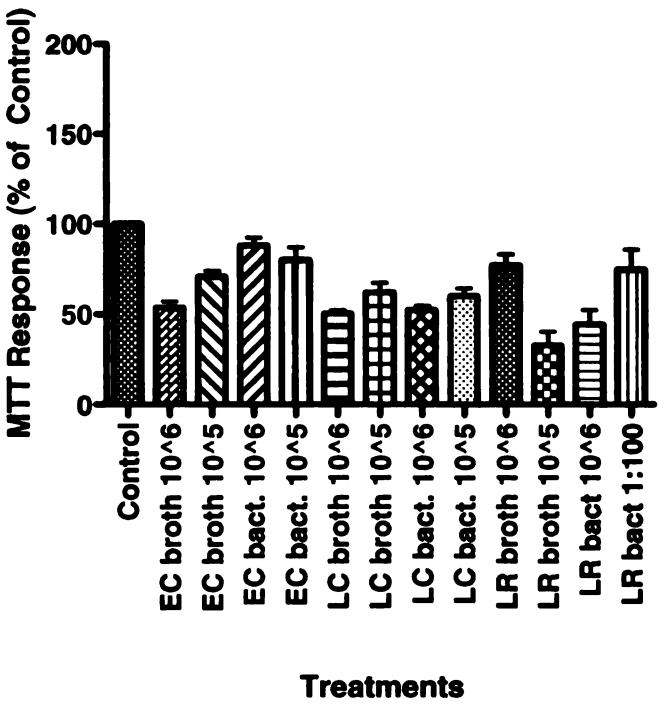


Figure 4.12 Cell viability (Mean +/- SEM) of IMCE cells exposed to 10^6 and 10^5 cfu/ml of *E.coli*(EC), *L.casei*(LC), and *L.reuteri* (LR) bacteria or spent medium (broth) for 72 hrs. a- Different compared to control $p < 0.001$

4.2.2 Discussion of the production of NO and cell viability in YAMC and IMCE cells exposed to washed bacteria or spent growth media

The efficacy of both the EC spent medium and bacteria to stimulate NO production indicates there are potentiating factors in both components. EC secretes factors such as intimin and Stx which would be present in the media and perhaps account for the spent medium's induction of NO in both cell types. Thorpe and others (1999) showed that purified Stxs stimulate low level production of the neutrophil chemoattractant IL-8 by epithelial cells. This indicates that Stxs can have an immunomodulatory role. Also, EC contains the flagellar protein, H7 flagellin, on its surface which may bind TLR 5, activating induction of iNOS and NO production (Berlin, 2002).

4.3 Effect of Stx 1 on proinflammatory mediator production (NO, TGF-β) and cell viability in YAMC and IMCE cells

We hypothesized that Stx 1 (formerly shiga-like toxin) a secreted component of EC, maybe responsible for the elevated NO production seen in EC spent medium exposed cells. Stx 1 was added in nanogram/ml quantities (10ng to 0.01 nanograms/ml) to the low serum, IFN-γ free media. As well as co-treatment with either *L.casei* or *L.reuteri* at 1 x 10^6 cfu/ml and the highest concentration of Stx 1 (10 ng/ml).

Stx 1 exposure did not result in NO production in either cell type (Figure 4.13 and Figure 4.14). The cell viability of the two cell types remained very similar to control. (Figure 4.15 and Figure 4.16). Stx concentrations also had no effect on overall production of TGF-β compared to control in both cell types (Data not shown).

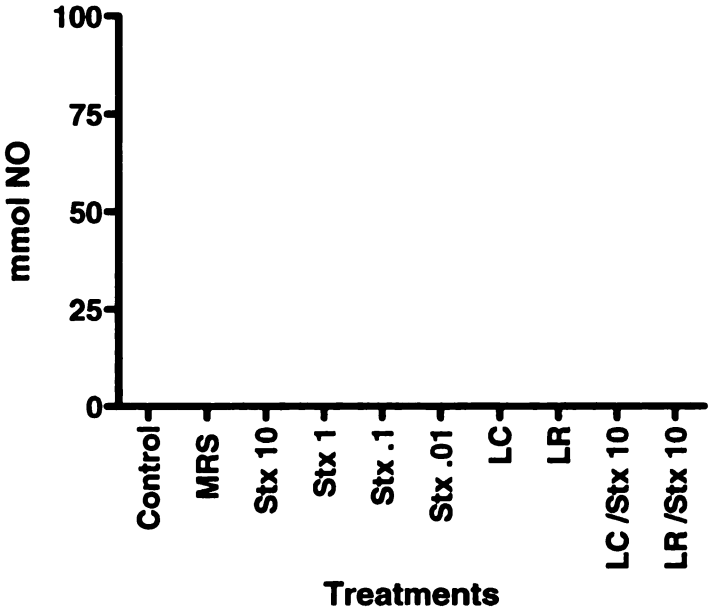


Figure 4.13 Nitric oxide (Mean +/- SEM) production in YAMC cells treated with Stx 1 (0.01 to 10 ng/ml) and Stx 1 (10 ng/ml) with LC or LR for 72 hrs.

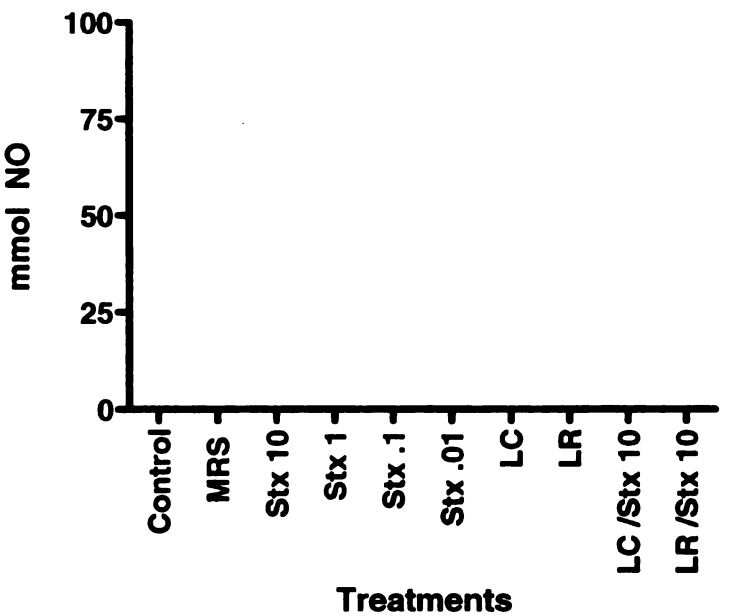


Figure 4.14 Nitric oxide (Mean +/- SEM) production in IMCE cells treated with Stx 1 (0.1 to 10 ng/ml) and Stx 1 (10ng/ml) with LC or LR for 72 hrs.

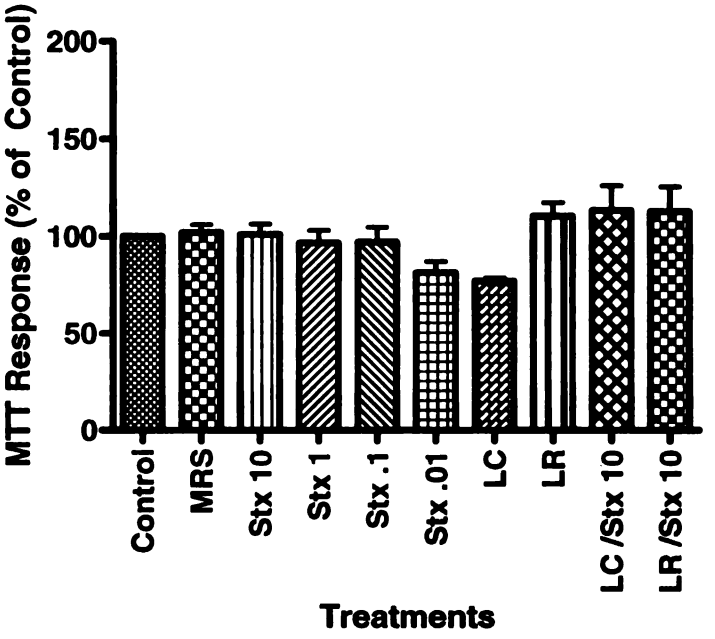


Figure 4.15 Cell viability (Mean +/- SEM) in YAMC cells treated with Stx 1 (0.1 to 10 ng/ml) and Stx 1 (10ng/ml) with LC or LR for 72 hrs.

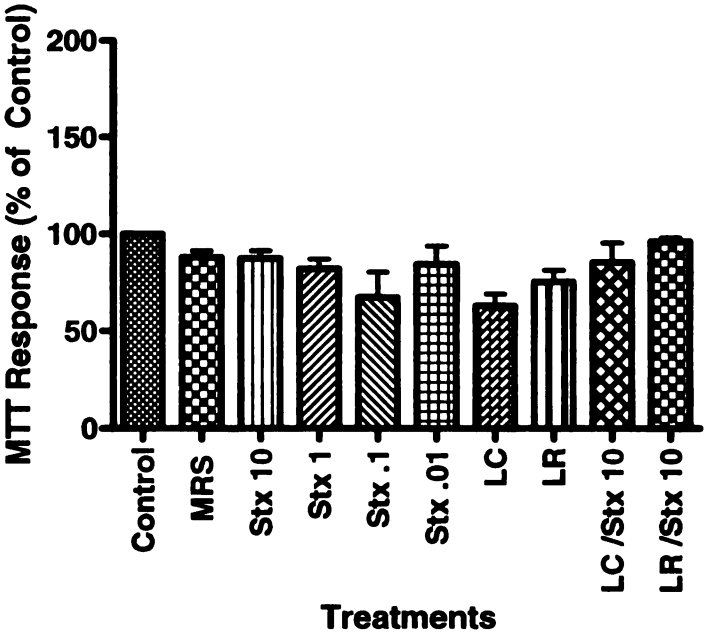


Figure 4.16 Cell viability (Mean +/- SEM) compared to control in IMCE cells treated with Stx 1 (0.1 to 10 ng/ml) and Stx 1 (10ng/ml) with LC or LR for 72 hrs.

4.3.2 Discussion of the effect of Stx 1 on proinflammatory mediator production (NO, TGF-β) and cell viability in YAMC and IMCE cells

The fact that Stx did not cause NO production made us wonder if perhaps it was evading the cell in some way. Perhaps by up-regulating TGF- β by mediated inhibition of NF-kB recruitment. Jobin and others (2003) found that TGF- β 1/ Smad signaling pathway helps maintain normal intestinal homeostasis to commensal luminal enteric bacteria by regulating NF-kB signaling in intestinal epithelial cells through histone acetylation. However, our results showed Stx had no effect on TGF- β production either. Our results were supported by Berin and others (2002) who found that EC was causing a proinflammatory response in epithelial cells independently of either Stx or intimin. They determined that this response was dependent to a significant extent, on the presence of H7 flagellin.

4.4 Rationale for the use of washed, irradiated *E.coli* (EC), *L.casei* (LC), and *L.reuteri* (LR) bacteria in YAMC and IMCE cell culture

Due to the nature of the gastrointestinal tract and the digestive process we concluded that the spent media components would be absorbed or degraded before reaching the colon. It is rational that bacteria alone would reach the colon. Therefore, we utilized washed, irradiated bacteria to determine their effect on NO and MIP-2 production in YAMC and IMCE cells.

Because of the difficulty in achieving reproducible concentrations of bacteria for treatments, a new method was devised for preparation, isolation, and quantization of bacteria. Bacteria were grown as previously described and quantified on a weight per volume basis as discussed in Material and Methods. This allowed for identical

concentrations of each bacterium to be added to cells in culture. Each experiment was run at least three times to examine the effect of bacteria and bacterial co-cultures on NO and MIP-2 production. Bacteria were added at various concentrations (1μg/ml to 1000 μg/ml) either as individual bacterium or in bacterial co-treatments.

The effect of (EC, LC, and LR) isolated, washed, bacteria on NO and MIP-2 production was demonstrated to be time- and concentration- dependent (Data not shown). We established that EC bacterial concentrations of 1 to 1000 μg/ml alone or in co-treatments with LC or LR (1000μg/ml) resulted in a concentration-dependent effect on NO. The 1000 μg/ml bacterial cultures at 72 hrs incubation showed the highest ($p < 0.001$) production of NO and MIP-2 in both cell types compared to control. As such, the 72 hour time point using 1000 μg/ml concentrations of each bacterium was used in all subsequent experiments.

TNF-α was assayed because previous research in tumor cells indicated it was produced in large quantities in response to infection. So we thought it might be produced in our cells. However, our results did not yield interpretable patterns of NO and MIP-2 expression. Therefore, TNF-α did not appear to be a relevant proinflammatory mediator influenced by exposure to *E.coli, L.casei,* or *L.reuteri* in our cell models.

4.4.2 Effect of bacterial constituent of *E.coli* (EC), *L.casei* (LC), and *L.reuteri* (LR) on proinflammatory mediator production (NO and MIP-2) in YAMC and IMCE cells

EC (1000 μg/ml) consistently caused increased ($p < 0.001$) NO production compared to untreated control cells in both cell types (Figure 4.17 and Figure 4.18). Co-treatment of EC with LC or LR caused a cell type- and bacterial species- dependent decrease ($p < 0.001$) in NO production. LC co-treatments decreased EC- induced NO

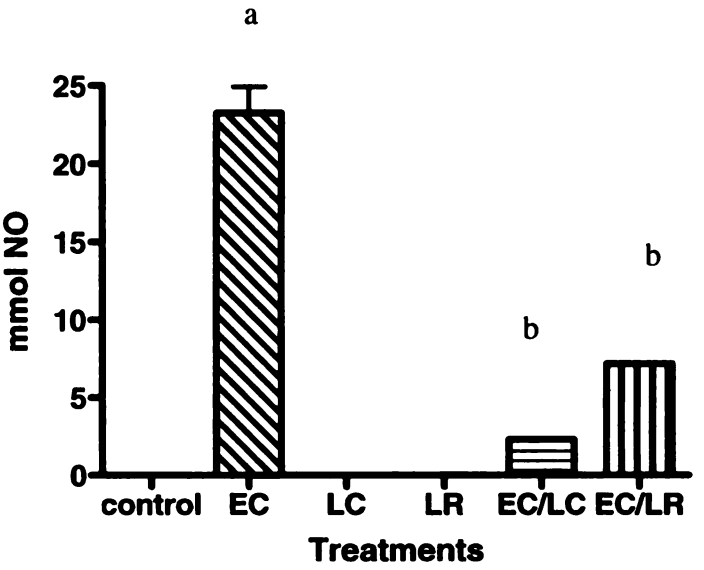


Figure 4.17 Nitric oxide (Mean +/- SEM) production in YAMC cells treated with *E.coli* (EC), *L.casei* (LC), *L.reuteri* (LR) or co-treatments of bacteria (1000 μg/ml) for 72 hrs. a- Different compared to control $p < 0.001$, b- Different compared to EC treatment, $p < 0.001$.

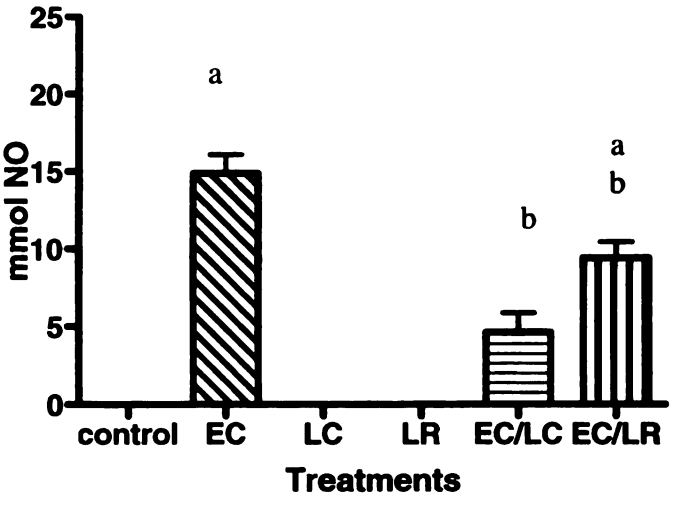


Figure 4.18 Nitric oxide (Mean +/- SEM) production in IMCE cells treated with *E.coli* (EC), *L.casei* (LC), *L.reuteri* (LR) or co-treatments of bacteria (1000 μg/ml) for 72 hrs. a- Different compared to control $p < 0.001$, b- Different compared to EC treatment, $p < 0.001$.

production in both cell types ($p < 0.001$). LR co-treatment decreased EC- induced NO production in both cell types as well. Cell viability was consistent across treatment and cell type (Figure 4.19 and Figure 4.20). Neither LC nor LR co-treatment with EC negatively affected cell viability compared to untreated control cells.

Production of MIP-2 was increased ($p < 0.001$) compared to untreated control cells with bacterial treatments and co-treatments in both cell types (Figure 4.21 and Figure 4.22). EC/LC co-treatment decreased ($p < 0.01$) MIP-2 production in both cell types compared to EC-treated cells. The pattern amongst the treatments was consistent in both cell types. Concentrations of MIP-2 which were below detection limit of this assay are noted as zero values on each graph.

4.4.3 Discussion of bacterial effects on NO and MIP-2 production in YAMC and IMCE cells

As seen previously EC-induces large quanities of NO. Here we see the bacterial component also causes a large production of MIP-2 as well, indicating EC's strong impact on the proinflammatory process. *E.coli* generated NO may serve as an important pro-inflammatory signal to up- regulate MIP-2 expression as was found in Caco-2 cells. (Skidgel et al., 2002). Interestingly, Witthoft (1998) found that infection with *E.coli* in Caco-2 and HT-29 cells caused NO release into apical compartments early after infection, where as IL-8 was released in parallel into the basolateral compartment.

The data indicate that LC and LR produced MIP-2, had the ability to differentially down regulate NO and MIP-2 production compared to EC treatment and hence may act as immunomodulators. This is supported by results in many other cell types. *Lactobacilli* have been demonstrated to induce cytokine production in HT-29 cells

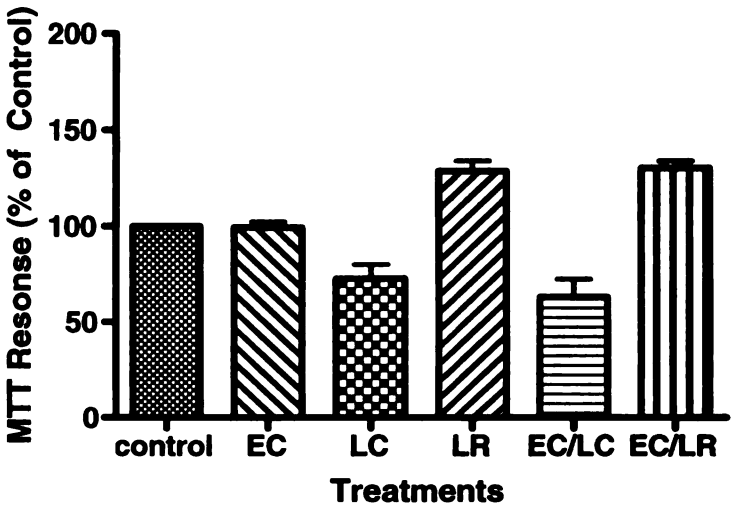


Figure 4.19 Cell viability (Mean +/- SEM) of YAMC cells treated with *E.coli* (EC), *L.casei* (LC), *L.reuteri* (LR) or co-treatments of bacteria (1000 μg/ml) for 72 hrs. a- Different compared to control $p < 0.001$.

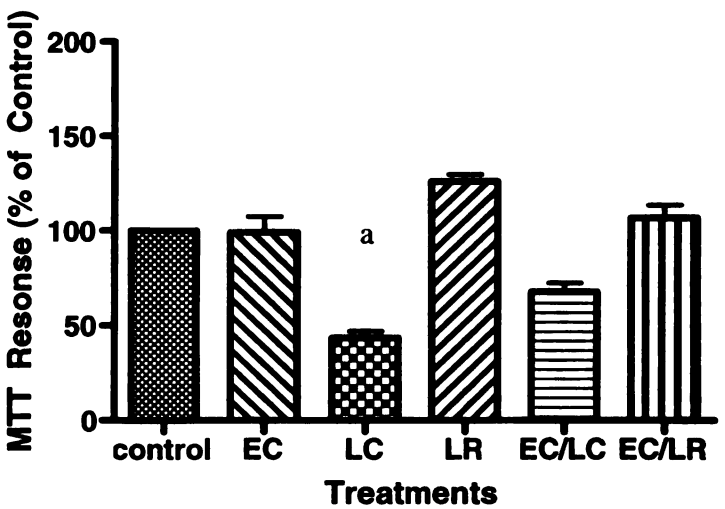


Figure 4.20 Cell viability (Mean +/- SEM) of IMCE cells treated with *E.coli* (EC), *L.casei* (LC), *L.reuteri* (LR) or co-treatments of bacteria (1000 μg/ml) for 72 hrs. a- Different compared to control $p < 0.001$

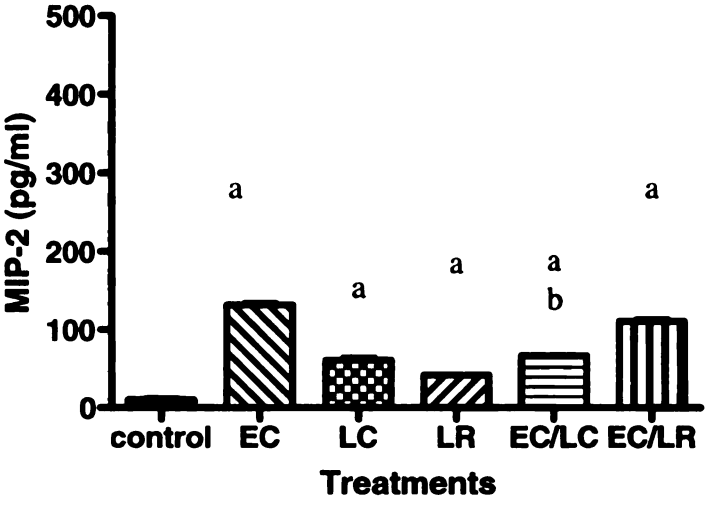


Figure 4. 21 Macrophage Inflammatory Protein-2 (MIP-2; Mean +/- SEM) production in YAMC cells treated with *E.coli* (EC), *L.casei* (LC), *L.reuteri* (LR) or co-treatments of bacteria (1000 µg/ml) for 72 hrs. a- Different compared to control $p < 0.001$, b- Different compared to EC treatment, $p < 0.001$.

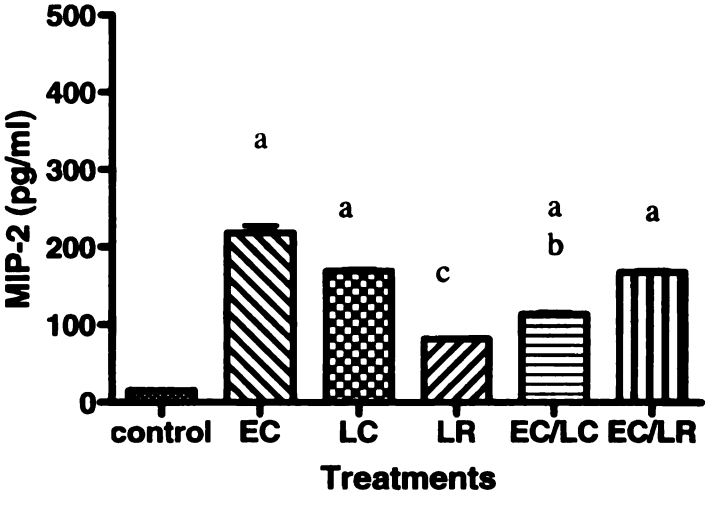


Figure 4.22 Macrophage Inflammatory Protein-2 (MIP-2; Mean +/- SEM) production in IMCE cells treated with *E.coli* (EC), *L.casei* (LC), *L.reuteri* (LR) or co-treatments of bacteria (1000 µg/ml) for 72 hrs. a- Different compared to control $p < 0.001$, b- Different compared to EC treatment, $p < 0.001$.

(Wallace et al, 2002). This effect was strain-dependent. Likewise, Nader and others (1999) showed the ability of LC to inhibit colonization of ingested *Shigella* to liver and spleen and improve survival. Continuous feeding of LC was able to suppress EC colonization in an infant rat model (Oganwa et al., 2001). These findings indicate that LC maybe an excellent candidate for use as a probiotic.

4.4.3 Implications for future research

The differential production of the chemokine MIP-2 by different co-treatments in both cell types indicates it may serve as an important chemoattractant for neutrophils *in vivo*. The ability of LC to downregulate MIP-2 and NO production indicates that this bacterium may modulate inflammatory mediators produced by epithelial cells. Future research could utilize assays to determine if bacterial exposure to epithelial cells results in differences in macrophage, dendritic cell, or neutrophil migration in a dual chamber chemotaxis assay.

Antibody microarrays have been used to quantify chemokines and cytokines in cell culture supernatants produced as a result of exposure of YAMC and IMCE cells to EC, LC, LR and co-treatments (Appendix III). These results provide preliminary evidence for a role of epithelial cells in regulating mucosal inflammation. Further analysis and repetition need to be done.

4.5 Effect of inhibition or chelation of iNOS, NO, NF-kB, p38 MAPK, and JNK on proinflammatory mediator production (NO and MIP-2) in YAMC and IMCE cells exposed to EC.

In order to access the relative contribution of specific signaling pathways initiated by EC exposure on the production of pro-inflammatory mediators (NO and MIP-2), inhibitors were employed. Attenuation of NO or MIP-2 production resulting from exposure to these inhibitors in the presence of EC treatment was taken as possible evidence for the involvement of these pathways in the production of these mediators.

The first inhibitor used was NG-nitro-L-arginine-methyl ester (L-name) at 50 μM as a potent inhibitor of iNOS. However, this inhibitor was extremely toxic to the cells and measurements of NO and MIP-2 was not possible. Hemoglobin was used at 500 μM to chelate NO in order to establish a possible connection between the presence of NO and MIP-2 production in cells exposed to EC. Hemoglobin inhibited ($p< 0.001$) EC-induced NO production in both cell types (Figure 4.23 and Figure 4.24, Appendix IV). However, hemoglobin increased MIP-2 production ($p< 0.001$) compared to EC treatment in both cell types (Figure 4.25 and Figure 4.26; Appendix IV).

PDTC, an NF-kB inhibitor, was used at a concentration of 10 μM along with EC (1000 μg/ml) (Figure 4.23 and Figure 4.24; Appendix IV). Co-treatment of YAMC and IMCE cells with NF-kB inhibitor (PDTC) and EC caused a decrease ($p < 0.001$) in NO production compared to EC-treated cells. PDTC decreased ($p< 0.001$) EC potentiated ($p < 0.001$) MIP-2 production in IMCE cells (Figure 4.25 and Figure 4.26; Appendix IV).

p38 MAPK inhibitor (SB 202190 or SB) in a concentration-dependent fashion decreased EC-induced NO production in both cell types (Figure 4.23 and Figure 4.24;

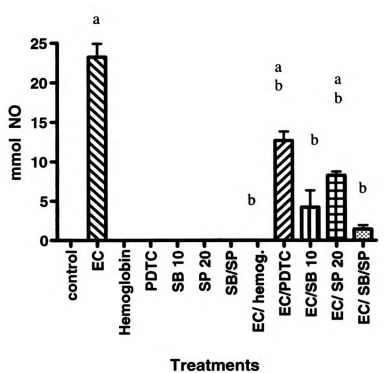


Figure 4. 23 Nitric oxide (Mean +/- SEM) production in YAMC cells treated with *E.coli (EC,* 1000μg/ml*)* bacteria and Hemoglobin, PDTC, SB, SP, SB/SP, or both for 72 hrs. a- Different compared to control $p < 0.001$, b- Different compared to EC treatment $p < 0.001$

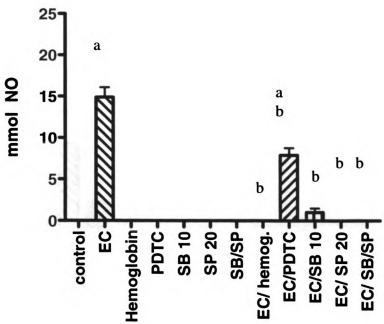


Figure 4. 24 Nitric oxide(Mean +/- SEM) production in IMCE cells treated with *E.coli (EC,* 1000μg/ml*)* bacteria and Hemoglobin, PDTC, SB, SP, SB/SP, or both for 72 hrs. . a- Different compared to control $p < 0.001$, b- Different compared to EC treatment $p < 0.001$

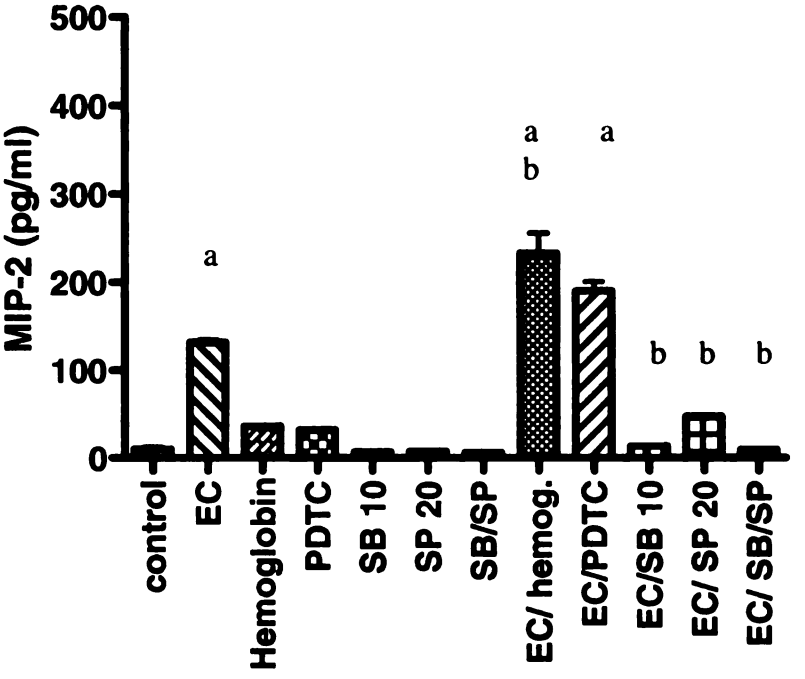


Figure 4.25 Macrophage Inflammatory Protein-2 (MIP-2; Mean +/- SEM) production in YAMC cells treated with *E.coli (EC,* 1000µg/ml*)* bacteria and Hemoglobin, PDTC, SB, SP, SB/SP, or both for 72 hrs.

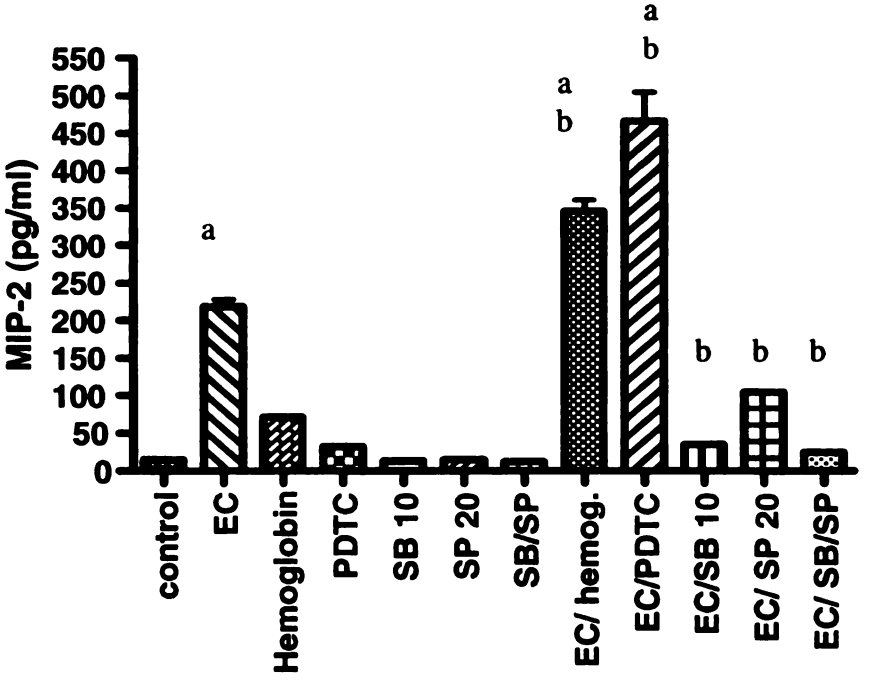


Figure 4. 26 Macrophage Inflammatory Protein-2 (MIP-2; Mean +/- SEM) production in IMCE cells treated with *E.coli (EC,* 1000µg/ml*)* bacteria and Hemoglobin, PDTC, SB, SP, SB/SP, or both for 72 hrs.

Appendix IV). SB at 10 μM partially inhibited ($p < 0.05$) MIP-2 production of EC-treated cells (Figure 4.25 and Figure 4.26; Appendix IV)

The JNK inhibitor, SP 600125 (SP), also decreased ($p < 0.001$) EC-induced NO production in both cell types in a concentration-dependent fashion (Figure 4.23 and Figure 4.24; Appendix IV). MIP-2 was also decreased ($p < 0.001$) in a concentration-dependent fashion in both cell types (Figure 4.25 and Figure 4.26; Appendix IV). SP (20μM) decreased ($p<0.01$) EC induced MIP-2 production compared to EC treatment alone. Use of SB and SP with EC caused near total inhibition of EC-induced ($p < 0.001$) NO production compared to EC treatment (Figure 4.23 and Figure 4.24; Appendix IV). The use of inhibitors of JNK (SP 20 μM) and p38 (10 μM) together accomplished near total inhibition of EC-induced MIP-2 production, respectively compared to EC treatment in both cell types (Figure 4.25 and Figure 4.26; Appendix IV).

4.5.2 Discussion on the use inhibitors on the production of NO and MIP-2

These data indicate that EC-induced NO is being initiated through signals which activate NF-kB, p38 MAPK, and JNK signaling pathways. This is supported by various lines of research. Bacterial DNA induced iNOS expression through MyD88-p38 MAP kinase activation in mouse primary cultured glial cells (Hosoi et al., 2002). NF-kB and p38 MAPK-dependent pathways were seen to play a role in NO production in mesangial cells (Chang et al., 2004). Gram negative bacteria were found to activate Nod1 which signals through p38 to induce NF-kB activation. Production of NO in IMCE cells due to EC treatments was decreased to a greater extent by SB compared to YAMC cells. This indicates important differences in bacteria-induced signaling between cell types. The fact that co-treatment of SB/SP with bacteria caused near complete inhibition of EC-induced

NO indicating that JNK and p38 are potentially responsible for the EC-induced NO production.

NF-kB inhibition using PDTC reduced MIP-2 production in EC-treated YAMC cells but potentiated MIP-2 production in EC-treated IMCE cells. The decrease in MIP-2 is support for our hypothesis that NO may regulate MIP-2 production. Berin and others (2002) found that EHEC H7 flagellin activated NF-kB and MAP kinase pathways leading to IL-8 (MIP-2 in mice) secretion by Caco2 cells. These findings support our hypothesis and findings. The IMCE data indicates that inhibition of NF-kB may potentiate the activation of other signaling pathways initiated by bacterial exposure.

This phenomenon appears to be specific to our pre-neoplastic colon epithelial cells. This may indicate that pre-neoplastic cells sense and respond to bacterial stimuli differently than normal cells. These data implicate a potential for differential cell signaling pathway activation by EC exposure that may have important ramifications for monocyte/ neutrophil chemoattraction and inflammatory processes in the colon.

There was partial or near total inhibition of EC-induced MIP-2 with the use of p38 and JNK inhibitors individually and in combination. This indicates that as with NO, JNK and p38 MAPK may play a large role in conveying bacterial signals leading to MIP-2 production. This is supported by Keates and others (1999) who found that p38 and c-JNK play a role in the upregulation of IL-8 in epithelial cells in response to infection with bacterial pathogens.

The increase in MIP-2 production resulting from co-treatmentof cells with EC and hemoglobin co-treated cells was paradoxical. These data indicate there maybe NO-independent mechanisms activated in the presence of EC and/or hemoglobin which result

in potentiation of MIP-2 production. Indicating that perhaps inhibiting NO through chelation upregulates another regulator of MIP-2 production.

4.5.3 Implications for future research

The fact that NF-kB, p38 MAPK, and JNK all played a role in EC-induced NO and MIP-2 production was noteworthy. The effect of bacterial co-treatment and cell-type specific effects of bacterial treatment is an area for future and continued research. It will be necessary to utilize iNOS inhibitors that are not toxic to the cells in order to thoroughly understand the mechanism by which EC effects NO and MIP-2 production.

CHAPTER 5

SUMMARY AND CONCLUSIONS

CHAPTER 5

SUMMARY AND CONCLUSION

In this research, we have assessed the potential immunomodulatory roles of LC and LR via their ability to attenuate the EC- mediated production of proinflammatory mediators by two conditionally immortal colon epithelial cells. Elucidation of the signaling pathways initiated by probiotic bacteria is essential to understanding their role in modulating EC-induced inflammatory events. Our research examined cell culture models of normal and preneoplastic epithelial cells preventative approaches to inflammatory conditions. The growing burden of foodborne illness by such pathogens as EC leading to gastroenteritis, intestinal inflammation, diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome indicates the necessity to find ways to modulate the pro-inflammatory response. Therefore, finding strategies to decrease foodborne illness is necessary to help control the damage that can occur with inflammation by modulating one of the major culprits.

The results of this investigation indicate that EC (bacteria, spent media, and both) cause a concentration-dependent increase ($p < 0.001$) in NO and MIP-2 production compared to control in YAMC and IMCE cells. EC bacteria and its spent medium additively increased NO production. Our results indicated a differential effect caused by two probiotics in their ability to modulate the proinflammatory immune response to EC treatment. LC and LR caused a cell type- and bacterial species- dependent decrease (YAMC > IMCE, $p < 0.001$) in NO production compared to EC (bacteria, spent media, and combination) treatment in both cell types. EC/LC (bacteria) co-treatment also inhibited ($p < 0.001$) MIP-2 production compared to EC treatment.

The use of enzymatic inhibitors of NF-kB, p38 MAPK, and JNK individually and p38 MAPK/JNK in combination accomplished partial or near total inhibition ($p < 0.001$) of EC-induced NO and MIP-2 production, respectively. The ability of PDTC to increase NO in EC-induced IMCE treatments above EC-treatment alone indicates the potential for preneoplastic cells to sense and respond to bacterial stimuli differentially than normal cells.

In conclusion, these data implicate a potential for differential cell signaling pathway activation by bacterial exposure that may have important ramifications for monocyte/neutrophil chemoattraction and inflammatory processes in the colon. The differential effect of LC and LR on the modulation of the EC-induced production of proinflammatory mediators indicates that the *Lactobacilli* have strain specific effects. Our data suggest that the consumption of specific *Lactobacilli* has the potential to impact the ability of the host to respond to foodborne pathogens like *E.coli* O157:H7.

APPENDIX I

E. coli O157: H7 → One frozen loop inoculated into 10 ml TSB-YE media and incubated at 37° C for 24 hrs.

↓

E.coli O157:H7
Lactobacillus casei
Lactobacillus reuteri

↓

1.5 ml of frozen stock of *Lactobacilli* and 1.5 ml of *E.coli* was grown overnight in TSB-YE inoculated in 25 ml of media (Lactobacilli in MRS media, *E. coli* in TSB-YE) at 37° C for 24 hrs.

↓

Bacteria were centrifuged at 10,000 rpm, 4° C, 15 min. Media was aspirated and bacteria washed with 1X PBS. 25 ml fresh media was added to bacteria and incubated at 37° C for 24 hrs. (Repeated 3 X, on third time incubation shortened to 15 hrs.)

↓

10 ml of each culture was transferred to 250 ml fresh media and incubated at 37° C in a shaker until mid- to late log phase (6- 12 hrs). Repeated 2 times for each bacterium.

↓

After the bacterias reached log phase (calculated by absorbance reading) dilution blanks were prepared to run a standard plate count.

↓

Spent medium was removed and 200 ml aliquoted and frozen at -80° C.
Bacteria were washed three times in 1X PBS. Centrifugation as above after each wash step.

↓

Bacteria were resuspended at one-tenth their original volume in sterile PBS and frozen immediately at -80° C

↓

Frozen bacteria was inactivated by gamma irradiation (1 Mrad; at the Phoenix Memorial Laboratory, University of Michigan)

↓

Weight of bacteria was determined by drying (speed vacuum) 500 µl aliquots of bacteria in PBS and correcting for buffer salt content

↓

Irradiated bacteria were plated to insure bacteria were no longer viable.

↓

Irradiated bacteria were added to cells at concentrations of 1 to 1000 µg/ml in low serum, IFN-γ free media

Protocol used for preparation of bacteria for experimentation.

APPENDIX II

University of Michigan

Ford Nuclear Reactor
Phoenix Memorial Laboratory
Ann Arbor, Michigan 48109-2100
(734)764-6220

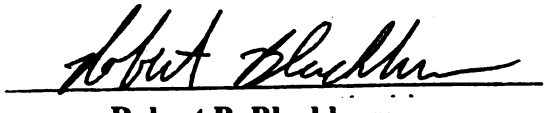
CERTIFICATE OF COMPLIANCE

Version 3

This is to certify that the following specimens were irradiated in the facility's cobalt-60 irradiator. Dose rates were measured with Reuter-Stokes ion chamber model RS-C4-1606-207, serial number I-8943, which is calibrated annually by the manufacturer or Phoenix against a National Institute of Standards and Technology source. The specimens were rotated 180 degrees half-way through the irradiation to achieve a uniform dose. Irradiation was continuous except for the interruption while the specimens were rotated.

Organization:	**Michigan State University-Food Science and Human Nutrition**
Irradiation Date:	**8/4/03**
Specimen Type:	**Bacteria cultures**
Specimen Identification:	**080403MSUFSHN02**
Distance from irradiator (cm):	**10**
Gamma Dose Rate: (rad hr):	**218341**
Irradiation Time (hr):	**4.583**
Interrupt Time: (min):	**23**
Gamma Dose (Mrad):	**1.00**

Aug. 4, 2004
Date



Robert B. Blackburn
Asst. Manager of Laboratory Operations

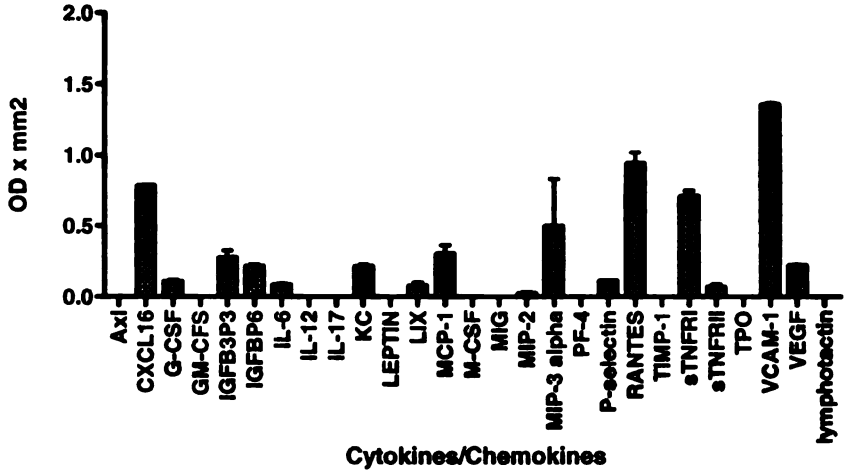
APPENDIX III

RayBio® Cytokine Antibody Array III and 3.1

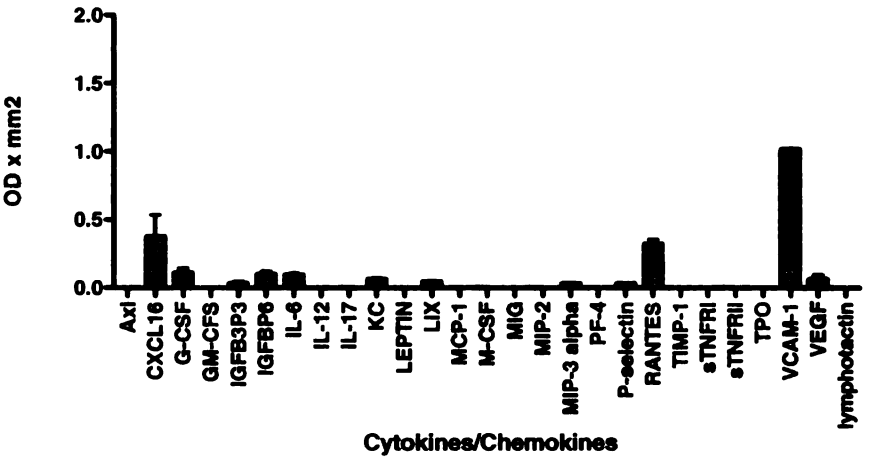
Detect 62 cytokines and other proteins in one experiment

POS	POS	POS	POS	Blank	Axl	BLC	CD30L	CD 30T	CD 40	CRG-2	CTAC K	CXCL16	Eotaxin
POS	POS	POS	POS	Blank	Axl	BLC	CD30L	CD 30T	CD 40	CRG-2	CTAC K	CXCL16	Eotaxin
Eotaxin -2	Fas Ligand	Fractal kine	GCSF	GM-CFS	IFNγ	IGFBP-3	IGFBP-5	IGFBP-6	IL1-α	IL-1B	IL-2	IL-3	IL-3Rb
Eotaxin -2	Fas Ligand	Fractal kine	GCSF	GM-CFS	IFNγ	IGFBP-3	IGFBP-5	IGFBP-6	IL1-α	IL-1B	IL-2	IL-3	IL-3Rb
IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40/p 70	IL-12 p70	IL-13	IL-17	KC	LeptinR	LEPTI N (OB)	LIX	L-selectin
IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40/p 70	IL-12 p70	IL-13	IL-17	KC	LeptinR	LEPTI N (OB)	LIX	L-selectin
Lymph otactin	MCP-1	MCP-5	M-CSF	MIG	MIP-1α	MIP-1γ	MIP-2	MIP-3B	MIP-3α	PF-4	P-selectin	RANTES	SCF
Lymph otactin	MCP-1	MCP-5	M-CSF	MIG	MIP-1α	MIP-1γ	MIP-2	MIP-3B	MIP-3α	PF-4	P-selectin	RANTES	SCF
SDF-1α	TARC	TCA-3	TECK	TIMP-1	TNFα	TNF RI	TNF RII	TPO	VCAM-1	VEGF	Blank	Blank	Blank
SDF-1α	TARC	TCA-3	TECK	TIMP-1	TNFα	TNF RI	TNF RII	TPO	VCAM-1	VEGF	Blank	POS	POS

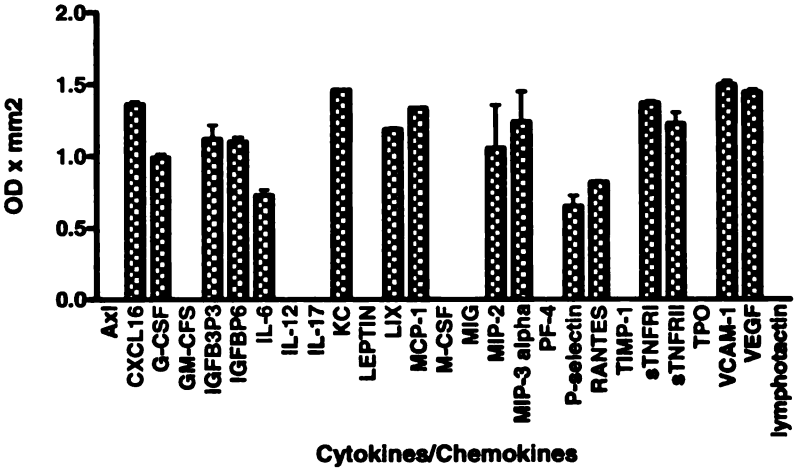
Cytokines and other proteins production in YAMC cells in Low serum/IFN free media



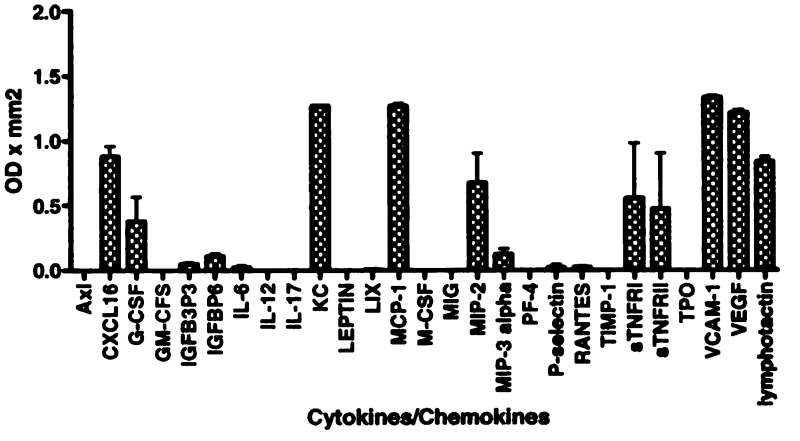
Cytokine and other proteinsproduction in IMCE cells treated with INF-free/low serum media



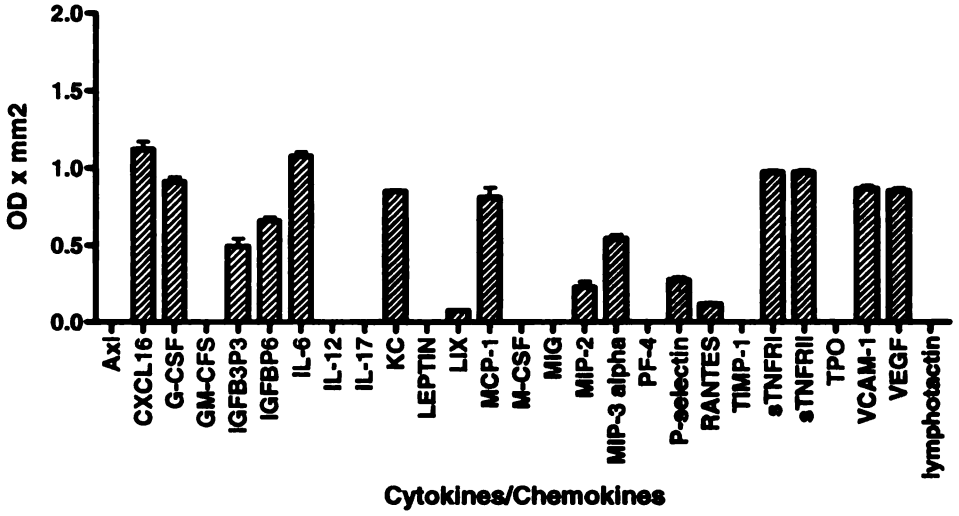
Cytokines and other proteins production in YAMC cells treated with EC 1000ug/ml



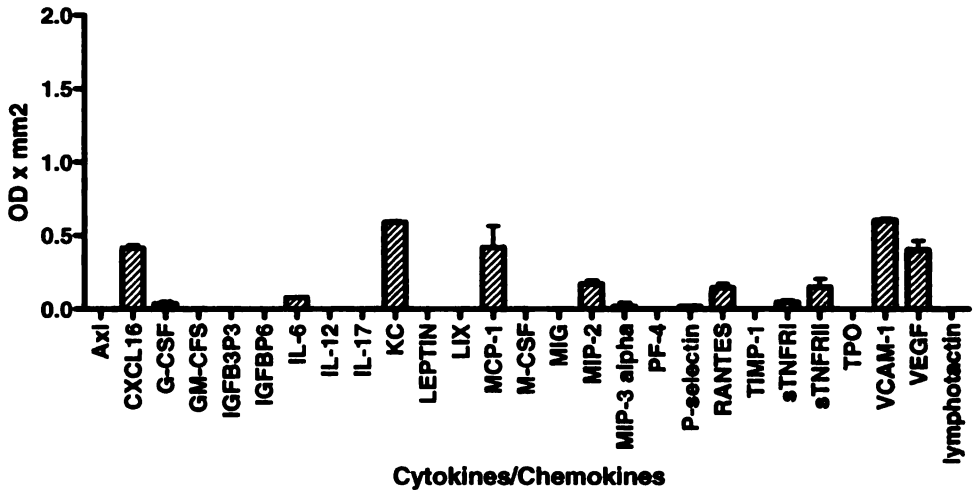
Cytokines and other proteins production in IMCE cells exposed to EC 1000 ug/ml



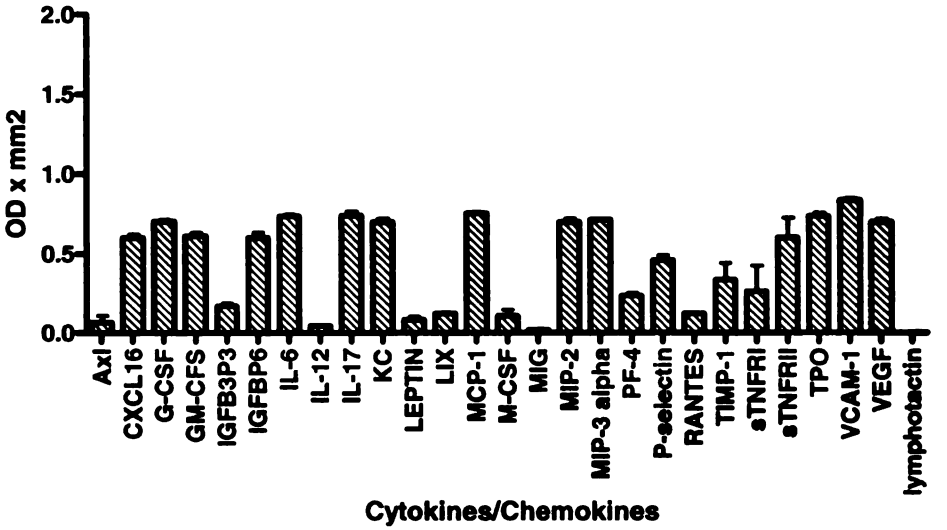
Cytokines and other proteins production in YAMC cells exposed to EC/LC



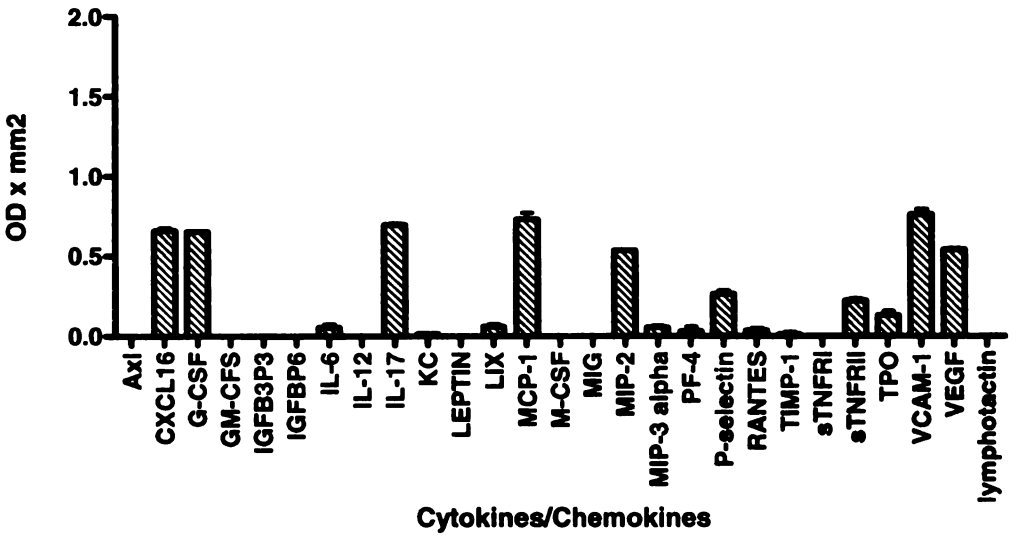
Cytokines and other proteins production in IMCE cells exposed to EC/LC



Cytokines and other proteins production in YAMC cells exposed to EC/LR

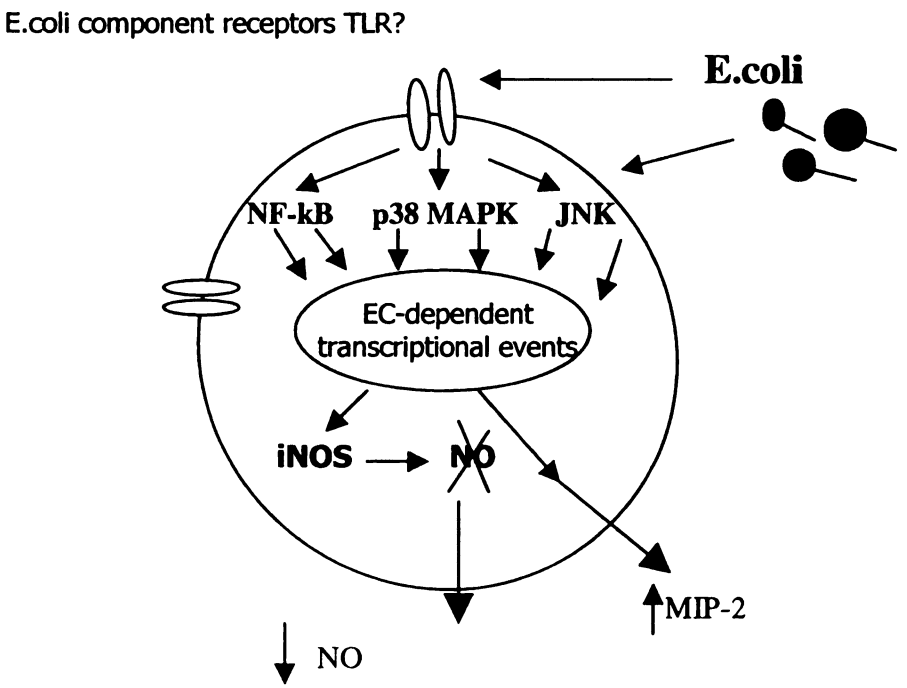


Cytokines and other proteins production in IMCE cells exposed to EC/LR

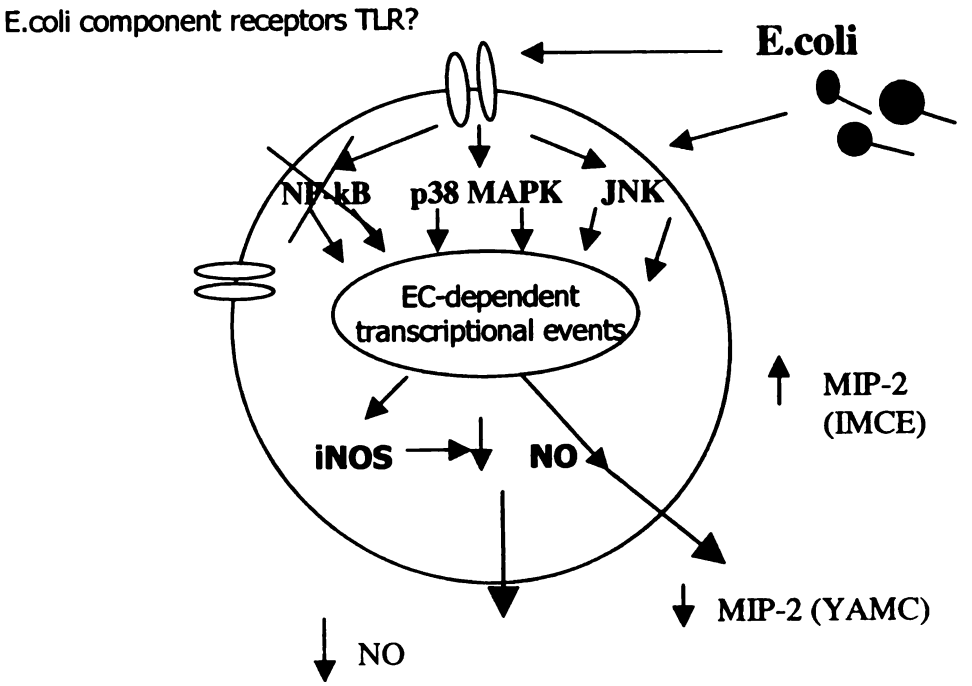


APPENDIX IV

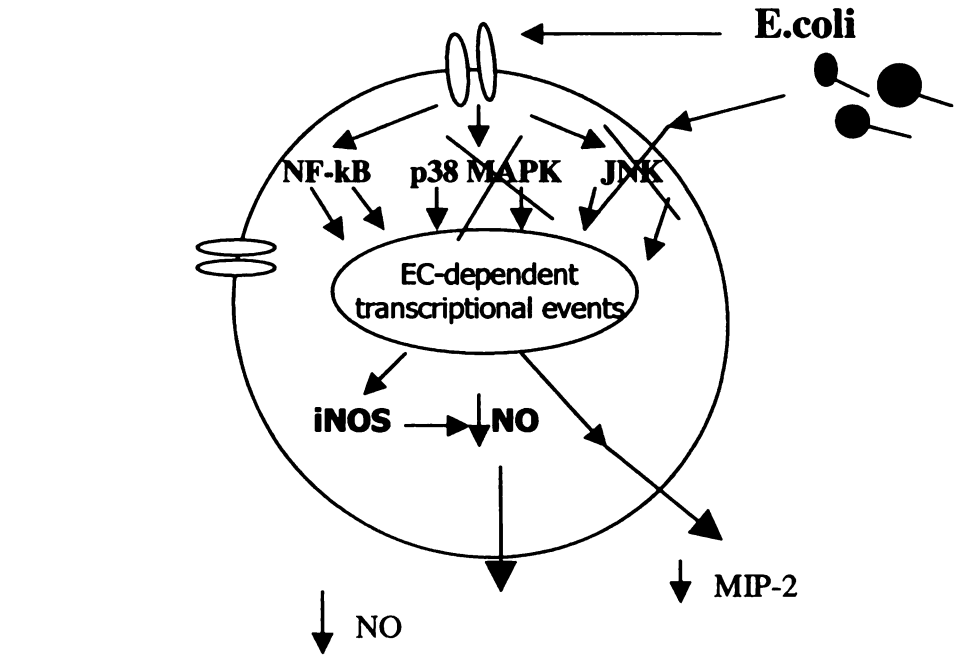
Use of (500 μM) hemoglobin to chelate NO



Use of (10uM) PDTC as NF-kB inhibitor



Use of (10 μM) SB and (20 μM) SP to inhibit p38 MAPK and JNK



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