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COLON EPITHELIAL CELLS EXPOSED TO PROBIOTIC BACTERIA MODIFY MACROPHAGE ACTIVATION AND CHEMOTAXIS IN RESPONSE TO A BACTERIAL PATHOGEN

presented by

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By

Amanda Doris Metz

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ABSTRACT

COLON EPITHELIAL CELLS EXPOSED TO PROBIOTIC BACTERIA MODIFY MACROPHAGE ACTIVATION AND CHEMOTAXIS IN RESPONSE TO A BACTERIAL PATHOGEN

By

Amanda Doris Metz

Bacteria may influence toll-like receptor (TLR)-dependent colonic epithelial cell (CEC) production of inflammatory mediators and subsequently activate immune cells implicated in carcinogenesis. We hypothesized that probiotic bacteria would decrease *Escherichia coli O157: H7* (*O157: H7*) induced production of NO and IL-6 as well as macrophage activation and chemotaxis in a genus- and species-specific manner.

O157: H7 induced production of NO and IL-6 compared to untreated YAMC and IMCE epithelial cells (p-value <0.001). CECs co-treated with *O157: H7* and probiotics showed a genus- and species-specific decrease in NO and IL-6 compared to *O157: H7* (p-value <0.001). Neutralizing antibodies against TLR-2 and -4 reduced NO and IL-6 compared to *O157: H7* (p-value <0.05).

Supernatants collected from CECs treated with *O157: H7* exposed to macrophages induced IL-6 (p<0.001) but not macrophage NO. Supernatants from CECs co-treated with *O157: H7* and probiotics exposed to macrophages caused a decrease in macrophage IL-6 compared to *O157: H7* in a genus- and species-specific manner (p-value <0.001). Supernatants from *O157: H7*-treated IMCE cells resulted in macrophage chemotaxis (p <0.01). These results suggest that *O157: H7*-induced NO and IL-6 occurs, in part, by activating TLR-2 and 4. The mechanisms by which probiotic bacteria ameliorate these TLR-mediated events require further research.

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Images in this thesis are presented in color.

ABBREVIATIONS COMMONLY USED

1. *Adenamatous polyposis coci* (Apc)
2. *Bifidobacterium breve* (BB)
3. *Bifidobacterium lactis* (BL)
4. Colony Forming Unit (CFU)
5. Colorectal Cancer (CRC)
6. DeMan, Rogosa, and Sharpe (MRS)
7. Dulbecco Modified Eagle Media (DMEM)
8. Enterohaemorrhagic Escherichia coli (EHEC)
9. *Escherichia Coli O157: H7* (EC)
10. Gastrointestinal (GI)
11. Gut Associated Lymphoid Tissue (GALT)
12. Immorto Mouse Colon Epithelial Cell (IMCE)
13. Inducible Nitric Oxide Synthase (iNOS or NOS2)
14. Inflammatory Bowel Disease (IBD)
15. Inhibitory Oligonucleotide (ODN 2088)
16. Inhibitory protein of kappa B (IkB)
17. Interleukin-6 (IL-6)
18. *Lactobacillus paracasei* (LPC)
19. *Lactobacillus plantarum* (LP)
20. *Lactobacillus reuteri* (LR)
21. *Lactobacillus rhamnosus* GG (LGG)
22. *Lactobacillus salivarius* (LS)

23. **Lipopolysaccharide (LPS)**

24. **Mucosa Associated Lymphoid Tissue (MALT)**

25. **Multiple intestinal neoplasia (Min)**

26. **Murabutide (MBT)**

27. **Nitric Oxide (NO)**

28. **Nuclear Factor kappa B (NF-kB)**

29. **Peptidoglycan (PGN)**

30. **Phosphate Buffer Saline (PBS)**

31. **Polymyxin B (PMB)**

32. **Pyrrolidine dithiocarbamate (PDTC)**

33. **Roswell Park Memorial Institute (RPMI)**

34. **Stimulatory Oligonucleotide (ODN 1826)**

35. **Stimulatory control Oligonucleotide (ODN 1826c)**

36. **Toll-like Receptor (TLR)**

37. **Tumor Growth Factor-ß (TGF-ß)**

38. **Trypticase Soy Broth-Yeast Extract (TSB-YE)**

39. **Young Adult Mouse Colon Epithelial Cell (YAMC)**

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

Colorectal Cancer

Colorectal cancer (CRC) is the third leading cause of cancer mortality for both men and women alike in the United States (Jemal et al, 2004). Colon cancers result from a succession of changes from the normal colon epithelium into an invasive tumor. A number of mutational events occur with each step in the adenoma-carcinoma sequence, such as *adenamatous polyposis coci* (*Apc*) mutations, ras oncogene mutations, or p53 mutations (Knudson, 2001). CRC is caused by a combination of environment, diet, and genetic factors; several nutritional factors highly influence the risk for this type of cancer (Giovannucci, 2003).

A Link Between Inflammation and Cancer

Cancers associated with chronic inflammation are caused by genomic alternations that arise due to repeated tissue damage and/or persistent infections; these cancers occur in areas of the body in close proximity to the external environment (Kagnoff et al, 1997). One such cancer with a strong association between chronic inflammation and malignancy is colon cancer, arising in individuals with inflammatory bowel diseases, such as ulcerative colitis (Coussens et al, 2002). Mucosal surfaces of the intestine, respiratory, and genitourinary tracts are the most common method of entry of pathogens into the host and are therefore more frequent sites for disease (Kagnoff et al, 1997).

Mucosal Barrier and Role of *Escherichia coli O157: H7 (O157: H7)*

The intestinal mucosal barrier has a large surface area comprised of epithelial cells aligned with an abundance of luminal agents and lymphoid tissues designed to protect the host against harmful foreign substances (Acheson et al, 2004). Upon the consumption of food-borne microorganisms, specialized epithelial cells called M cells and other mucosal epithelial cells detect these pathogens and respond by communicating to innate and adaptive immune cells. If the epithelial barrier is breached, these pathogens may be exposed to immune cells in the lamina propria including macrophages, dendritic cells and others. Food-borne pathogens, such as *O157: H7* play a role in the induction of inflammation in the colon by stimulating colon epithelial cells to activate and attract immune cells. This particular strain is a gastrointestinal pathogen that causes inflammatory conditions such as, acute gastroenteritis, intestinal inflammation, or chronic diarrhea (Slutsker et al, 1997). In the context of work proposed here, *O157: H7* exposure is not postulated to be a cancer risk factor. Rather, it is used as a prototype of pathogen, which stimulates innate immune responses.

Role of the Immune System

When the immune system is exposed to a foreign substance, such as a pathogen, the immune system responds by recognizing the pathogen and elicits a reaction to eliminate it (Erickson et al, 2000). Pathogenic bacteria elicit a strong immune response and have a potentially harmful effect on the host (Yan, 2002). Pathogens may drive intestinal inflammation in susceptible individuals by disrupting the mucosal barrier as well as by activating the innate mucosal immune system (Sheil et al, 2007). Intestinal epithelial cells sense the outside world and respond to environmental signals by releasing

chemokines and cytokines that recruit innate and/or adaptive immune cells to location (Rumbo et al, 2004). The intestine is an important immune organ consisting of a complex cellular network, secreted peptides and proteins and other host defenses (Yuan et al, 2004). Innate immunity plays a central role in intestinal immune defense against invading pathogens. It also serves as a bridge to the activation of the adaptive immune system.

Probiotic Bacteria

The transformation from normal mucosa to adenoma and subsequent development to carcinoma are prolonged events that present opportunities for preventive interventions (Gill et al, 2005). The importance of nonpathogenic, or "good" bacteria has led to the concept of probiotics as promoters of human health (Teitelbaum et al, 2002). Probiotic bacteria are viable microorganisms that when given in ample amounts, modify the microflora (by implantation or colonization) in the host and exert beneficial effects on the host (Teitelbaum et al, 2002). Intestinal bacteria maintain human health beyond basic nutrition, a fact first discovered by Elie Metchnikoff at the beginning of the 20th century (Hope et al, 2005). In addition, it has been clearly demonstrated that consuming fermented dairy foods, such as yogurt, that contain probiotic bacteria, decreases the risk for developing colon cancer (Rosman-Urbach et al, 2006).

Immunomodulatory Mechanisms of Probiotics

The specific mechanisms by which probiotics cause immune modulation remain unclear. It has been hypothesized that probiotic bacteria alter the inflammatory response that is induced in epithelial cells when pathogens are present (Boyle et al, 2006). Probiotics can prevent pathogenic strains of microbes, such as *O157:H7*, from mucosal adhesion, by competing with the pathogens for TLR-binding sites on the gut mucosa

(Chermesh et al, 2006). Alternatively, probiotics may trigger signaling through toll-like receptors, or TLRs, to enhance innate immune host defense in the intestinal mucosa (Cario 2005). Toll-like receptors are expressed by various cell types, including intestinal epithelial cells, and the epithelial expression of the receptors has been identified in an altered form in disease states such as ulcerative colitis and Crohn's disease (Shanahan, 2002).

Epithelial Cells: Accessory Cells to the Immune Response

Epithelial cells are the predominant cell type present in the gut mucosa that provide barrier function and, along with dendritic cells, provide necessary sensor mechanisms between luminal contents and mucosal immune cells (Cruickshank et al. 2004 and Huang et al. 1996). Epithelial cells can serve as accessory cells to the immune response to bacterial pathogens by producing signals important for the initiation and amplification of an acute mucosal immune response (Kagnoff, 1997). In addition to luminal exposure to bacterial pathogens, epithelial cells exposed to pro-inflammatory cytokines, such as TNF-α, in the mucosa, can also cause epithelial cells to produce signals that can amplify the immune response.

For example, in response to inflammation caused by *O157: H7*, epithelial cells and immune cells of the host produce inflammatory mediators such as nitric oxide (NO) as well as various cytokines and chemokines. Increased expression of NO is found in intestinal epithelial cells at times of chronic intestinal inflammation as well as in response to stimulation of epithelial cells with a combination of cytokines. The inducible nitric oxide synthase 2 (NOS2) is upregulated in breast, brain, colon, and gynecological tumors, which indicates that NO may have a role in tumorigenesis (Fransen et al. 2002).

Pro-Inflammatory Mediators of Interest

NO is produced in high concentrations by inducible nitric oxide synthase (iNOS or NOS2) after stimulation of bacterial products and cytokines as a product of Nuclear Factor-Kappa B (NF-kB) regulation of the iNOS gene (Mei et al, 2000). It is proposed that epithelial cells of the colon activate the iNOS gene through NF-kB regulation. Interleukin-6 (IL-6) is a multifunctional cytokine that plays a central role in host defense due to its wide range of immune activities (Simpson, et al. 1997). It is proposed that IL-6 may be over-expressed during inflammation in epithelial cells during inflammation-induced progression of colon cancer. NO and IL-6 have been chosen as specific indicators of pro-inflammatory mediator production.

Cell Culture Models

Two mouse colon epithelial cell lines will be used during the course of this research to examine the mechanisms these cells utilize when stimulated with bacteria to produce pro-inflammatory mediators. The cell models will also be used to analyze the inflammatory response during the transition between normal and pre-neoplastic cell phenotype. As such, these cell types are non-tumorigenic and, under our experimental conditions, are differentiated and quiescent (i.e., G_0 of the cell division cycle). The young adult mouse colon epithelial cell line (YAMC) is one that has a normal phenotype of the *Apc* gene ($Apc^{+/+}$) while the immortomouse/Min colon epithelial cell line (IMCE) is one that has an altered phenotype of the *Apc* gene ($Apc^{Min/+}$). Therefore, YAMC cells represent a model of a normal colon epithelial cell, while IMCE cells represent a model of a pre-neoplastic cell line. These cell lines will be used as models to analyze changes in

the inflammatory response when epithelial cells are exposed to pathogens and how this effect is attenuated when co-treated with probiotic bacteria.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

Cancer Statistics

Cancer is a leading cause of death in the United States; second only to heart disease (CDC, 2005). For men and women alike, heart disease and cancer are the two leading causes of death in the United States. Those individuals who are at a higher predisposition for developing cancer are: infants and children with genetic predisposition, elderly, immunocompromised individuals, and those having a positive family history (NCI, 2005).

Cancer Background

Under normal conditions, cells grow and divide, forming new cells, as the body needs them. When cells mature and die, new cells take their place. Sometimes this systematic cell growth and proliferation process becomes dysregulated. Under abnormal conditions, new cells may form when the body does need them or cells do not die when they are supposed to. These dysregulated cells can form a mass of tissue called a tumor (NCI, 2005).

The multiple hit theory of cancer describes the situation in which the first hit in carcinogenesis induces changes in the tissue environment, making second and subsequent hits more likely (Knudson, 2001). By identifying the cell type that experience these transformational events, such as an initiated mutation to a stem cell, stem cell progenitor, or cancer stem cell early enough, perhaps cancer progression can be diminished through preventative measures aimed at a specific cell type (Hord et al, 2007). Since cancer results from an interaction of environment and genetics, it is critical that the factors

affecting the neoplastic transformation of normal tissue be identified (Hord et al, 2007). It is these initiated cells within transformational events that drive tumorigenesis and thereby carcinogenesis. Since 80-90% of cancers are of epithelial origin suggests that these tissues are most susceptible to dietary compounds, immune cells, and tissue components that can influence the initiation and subsequent neoplastic transformation (Hord et al, 2007). The ability to identify the transformations from the early stages of neoplastic development would be a key factor in terms of cancer prevention.

Inflammation and Increased Cancer Risk

Inflammation is a defense mechanism that the body takes up against its surrounding environment (Coussens et al, 2002). Rudolf Virchow first suggested the connection between inflammation and cancer in 1863 when he observed the presence of immune cells in neoplastic tissues (Hope et al, 2005). Tumor cells produce various cytokines and chemokines that attract leukocytes and other immune cells (Coussens et al, 2002). Inflammatory cells influence the process of carcinogenesis by facilitating genomic instability, promoting angiogenesis, regulating the proliferation, migration, and differentiation of many cell types associated with tumor (Coussens et al, 2002). Cancers associated with chronic inflammation may be caused by genomic alternations from repeated tissue damage and/or persistent infections and are those cancers that pertain to areas of the body in close proximity to the external environment. Mucosal surfaces of the intestine, respiratory, and genitourinary tracts are the most common method of entry of pathogens into the host, and are more frequent sites for disease (Kagnoff et al, 1997). One such cancer with a strong association between chronic inflammation and malignancy is colorectal cancer, arising in individuals with inflammatory bowel diseases, such as

ulcerative colitis (Coussens et al, 2002).

Colon Cancer Background

Colorectal cancer is the third most common malignancy and the third leading cause of cancer death in the western world (Lal et al, 2000). Estimated new cases and deaths from colon cancer in the United States in 2006 are 106,680 and 55,170 (colon and rectal cancers combined) respectively (NCI, 2006). Colorectal cancer is caused by a number of mutational events that occur with each step in the adenoma-carcinoma sequence, such as *Apc* mutations, ras oncogene mutations, p53 mutations, and other chromosomal deviations (Knudson, 2001). In the initial stages of colon cancer, early changes occur as a result of cell overgrowth in the colonic crypts; these morphological structures are called aberrant crypts, which are considered preneoplastic structures. However, only a small fraction of these aberrant crypts progress to polyps and eventually malignancy (Teitelbaum et al, 2002). The pattern of mutagenic events is thought to be a two-step process. The first step for an adenoma to develop within a population of mutated cells is for a stem cell to undergo the first hit; because of the microenvironment of the colonic crypt, the odds of a second or subsequent hit are increased with the mutations of progenitor cells (Potter, 1999).

Conditions Associated With Predisposition of Colon Cancer

Colorectal cancer is known to be associated with a number of hereditary syndromes. One such inherited disease is familial adenomatous polyposis, or FAP. This syndrome is characterized by a number of mutations of the *Apc* gene and polyp formation on the colon and rectum during adolescence, predisposing one to colorectal cancer (Lal et al, 2000). Another hereditary syndrome predisposing one to the development of colon

cancer is Hereditary Nonpolyposis Colorectal Cancer, or HNPCC with features such as early onset of colon cancer and a pattern of other cancers such as stomach or endometrial cancer (Potter, 1999).

Risk factors for colon cancer include: age, family history, personal history of ulcerative colitis or Crohn's disease or other IBD, smoking, meat consumption, sedentary lifestyle, or low vegetable intake (Potter, 1999). Without regard to hereditary predisposition, colon cancer could largely be thought of as an environmental cancer as it can be prevented through environmental or dietary changes. It is well recognized that individuals with inflammatory bowel disease (IBD) are at an increased risk for the development of IBD-associated cancer (Hope et al, 2005). Genetic factors, immune system susceptibility, and environmental elements are all believed to play a role in these inflammatory conditions (Sheil et al, 2007). By interfering with the molecular events leading up to genetic alterations, chemoprevention could inhibit or reverse the development of adenoma to carcinoma (Mayer et al, 2000).

Genetics vs. Environment

The majority of colorectal cancers are preventable through lifestyle and diet alterations (Boyle et al, 2000). Risk factors that are associated with colorectal neoplasia include a positive family history, high meat intake, habitual smoking, central obesity, sedentary lifestyle, and excessive alcohol (Heavey et al, 2004). Epidemiological studies have identified beneficial factors, such as dietary factors, i.e., fruit, vegetables, and folate; non-steroidal anti-inflammatory drugs, or NSAID's; and physical activity. Diets highest in folate and fruit and vegetable consumption are inversely associated with the incidence of colorectal cancer (Heavey et al, 2004). Vegetables associated with the strongest

decrease in colorectal cancer risk are green leafy vegetables or cruciferous vegetables. Diets rich in red meat, particularly processed meat, are associated with an increased risk for developing colorectal cancer (Heavey et al, 2004).

Genetic alterations play a role in the development of all colorectal cancers (Strate et al, 2005). However, hereditary colorectal cancer only accounts for 10% of cases; the majority of colorectal cases follow a sporadic pattern highly influenced by lifestyle, diet, and environmental factors (Heavey et al, 2004). Colorectal cancer appears to be highly susceptible to the effects of diet (Giovannucci, 2003).

The Association Between Bacterial Exposure and Cancer Risk

There is growing evidence that infection with bacterial pathogens can play a role in cancer risk, indicating that inflammatory conditions can be linked to an increased susceptibility to certain cancers. One study that illustrates this connection with bacterial pathogens to inflammation-associated cancer and increased tumor burden dealt with the bacterial pathogen, *Citrobacter rodentium* (Newman et al. 2001). This particular pathogenic strain promoted colon tumorigenesis in $Apc^{Min/+}$ mice. *C. rodentium* infection is characterized by epithelial cell hyperproliferation, similar to that seen in inflammatory bowel disease (IBD) such as Crohn's disease or ulcerative colitis, and increases the likelihood for the onset of CRC. In this particular study, colonic adenomas developed in Min mice as a result of an induced hyperproliferative state in response to the pathogen (Newman et al, 2001).

Enterohemorrhagic *Escherichia coli O157:H7* (EHEC) is a gastrointestinal pathogen that is generally non-invasive for intestinal epithelial cells, yet chronic inflammation caused by persistent infection with bacterial pathogens such as *O157: H7,*

as well as parasites and viral pathogens, are major driving forces in neoplastic development (Berin, 2002 and Lin et al, 2007). *O157: H7* is not necessarily directly linked to colon cancer. The relevance of studies in colon cancer cell lines to the production of proinflammatory signals by normal epithelial cells in *O157: H7*-infected human colon is not known (Miyamoto et al, 2006). However, the induced inflammation that it does cause increases the risk for colon carcinogenesis. Therefore, it would be biologically plausible to associate *O157: H7* exposure with colon carcinogenesis.

Mucosal Microbiota: Autochthonous and Allochthonous Bacteria

Microbiota as an Organ

The human gastrointestinal ecosystem is sterile at birth and is colonized by maternal vaginal and fecal bacteria. More than 500 bacterial and archael species colonize the adult gastrointestinal tract (Noverr et al, 2005). The gut microbiota can be pictured as a microbial organ placed within a host organ (Bäckhed et al, 2005). The mucosal surface of the gastrointestinal tract is a complex ecosystem housing a variety of resident microbes, which communicate with one another as well as the host (Servin et al, 2006; Bäckhed et al 2005).

The intestinal microbiota is composed of a wide variety of microorganisms that carry out essential roles for the host and can have transiently altered environmental influences, such as with diet (Servin et al, 2006 and Schiffrin et al, 2002). This large and diverse microbiota contributes to gut maturation, host nutrition, and pathogen resistance (Dethlefsen et al, 2006).

The intestinal ecosystem can house numerous species of microorganisms. Some strains include commensal, or indigenous bacteria, while other strains can include foreign

bacteria, such as pathogenic or probiotic bacterial species (Yan, 2002). Commensal bacteria (natural or native microbiota) consist of those microorganisms, which are present on body surfaces covered by epithelial cells and are exposed to the external environment, such as the GI or respiratory tracts, or vagina, or skin (Tlaskalova-Hogenova et al, 2004). Two such species of commensal bacteria are *Bifidobacterium* and *Lactobacillus* and they appear to have beneficial effects on the host (Yan, 2002).

Pathogenic bacteria elicit a strong defense response and have a potentially harmful effect on the host (Yan, 2002). Pathogens may drive intestinal inflammation in susceptible individuals by disrupting the mucosal barrier as well as by activating the mucosal immune system (Sheil et al, 2007). A defective epithelial barrier could result in loss of tolerance to nonpathogenic bacteria, which may result in driving inflammation further (Sheil et al, 2007). Enteric bacterial products may alter immune homeostasis in the GI tract by inhibiting regulatory cytokine production, which could then contribute to bacterial pathogenesis (Acheson et al, 2004).

O157:H7 is one of hundreds of strains of the bacterium *Escherichia coli.* Although most strains are harmless and live in the intestines of healthy humans and animals, *O157: H7* produces a toxin and can cause severe illness (CDC, 2005). *O157:H7* is an important cause of food borne illness. In susceptible individuals, predominantly small children, elderly, and other immunocompromised individuals, the infection can also cause a complication called hemolytic uremic syndrome or HUS, in which the red blood cells are destroyed and the kidneys fail (CDC, 2005). One possible pathogenic mechanism receiving attention is the effect of *O157: H7* bacterial products on intestinal cells (Acheson et al, 2004). *O157: H7* can be thought of as a prototypical bacterial

pathogen, which has been shown to cause proinflammatory mediator production.

Roles of Intestinal Microbiota

There are two primary roles of the intestinal microbiota. One of the key functions is to recover calories from the diet that would be lost through excretion. Another important role of the resident microbiota is the ability to confront the colonization of new strains of bacteria and distinguishing them between pathogens and non-pathogens (Hope et al, 2005). There are three mechanisms that the resident microbiota utilize resistance to generate colonization by new bacteria. These mechanisms include competition for adhesion sites, alteration of the physiological environment including pH, and production of substances including bacteriocins that generate an environment preventing inhabitance of other bacteria (Hope et al, 2005). The control of the inflammatory response is an important element in prolonging the integrity of the mucosal boundary with the external environment (Schiffrin et al, 2002).

Probiotic Influence on the Mucosal Microbiota

The intestinal microbiota can stimulate both positive and negative effects on the host's well being and there has been increased interest in the modulation of the intestinal ecosystem in a beneficial way; thus, improving health (Alvaro et al, 2007). The importance of nonpathogenic bacteria has led to the concept of probiotics as mediators of human health (Teitelbaum et al, 2002). Probiotic bacteria are defined as live microbial supplements which benefit the host by improving the intestinal microbiota; and more so, as living microorganisms, which when ingested in ample amounts, exert health benefits beyond basic nutrition (Dunne et al, 2001). The term "probiotic" is derived from the Greek, meaning "for life" (Teitelbaum et al, 2002). Upon consumption, probiotics

benefit the host by preventing or reducing symptoms of disease (Yan et al, 2002). Increasing evidence shows that consumption of fermented dairy foods, such as yogurt, that contain probiotic bacteria, diminishes the risk for developing colon cancer (Rosman-Urbach et al, 2006). Probiotics prevent or improve diarrhea and/or inflammation through local effects on the immune system (Chermesh et al, 2006). In general, colonization and mucosal adhesion by ingested probiotic bacteria lasts no more than the time in which the probiotics are consumed (Teitelbaum et al, 2002). Where prebiotics and probiotics alone can provide health benefits in chemoprevention, a combination of the two (synbiotics) provides an even more pronounced benefit on the colon. Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and activity of the microbiota composition in the colon; thus improving host health (Femia et al, 2002 and Gibson, 1999).

Probiotics can prevent pathogenic strains of microbes, such as *O157:H7*, from mucosal adhesion, by competing with the pathogens for toll-like receptor (TLR) binding sites on the gut mucosa (Chermesh et al, 2006). This mechanism of probiotic action is governed by the fact that TLR dysregulation may be associated with increased or decreased susceptibility to infection (Cario et al, 2000). Another possible approach for probiotic protection is to modulate microbe-host cell signaling that could assist in renewing intestinal microbiota homeostasis and preventing colonization of bacterial pathogens in the GI tract (Altenhoefer et al, 2004). Specific interaction with pattern recognition molecules on pathogenic microorganisms, is a likely mechanism by which probiotics act (Boyle et al, 2006), and is studied throughout the course of this research.

Types of Probiotics

Genus- and Species-Dependent Criteria for Probiotics

A probiotic preparation may contain one or several different strains (Rolfe 2000). The most commonly used microorganisms for probiotics are strains of lactic acid bacteria (i.e. *Lactobacillus, Bifidobacterium,* and *Streptococcus*). Lactic acid bacteria have been shown *in vitro* to inhibit growth of several pathogens, including *Clostridium difficile*, *Clostridium perfringens*, and *Salmonella typhimerium* (Rolfe 2000). One specific example of a probiotic that is not bacterium is *Saccharomyces boulardii*, a patented yeast preparation used to treat diarrhea and other GI disorders caused by antimicrobial agents (Rolfe 2000). Examples of probiotic bacteria are *Lactobacillus rhamnosus GG, Lactobacillus reuteri, Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus salivarius, Bifidobacterium breve,* and *Bifidobacterium lactis,* all of which will be analyzed for immunomodulatory properties throughout the course of this research.

Criteria for ascribing probiotic activities to a specific microorganism are human origin, nonpathogenicity, resistance to stomach acids and bile, adherence to intestinal epithelial tissue, colonization within the GI tract, production of antimicrobial substances, modulation of immune responses, and metabolism influence (Teitelbaum et al, 2002). Commensal bacteria within the GI tract vary widely in function, with some having pro-inflammatory effects and others having anti-inflammatory effects. Optimal modification of the intestinal microbiota with probiotics has emerged as a realistic therapeutic opportunity for inflammatory bowel disease (Shanahan, 2005). Recolonization of the GI tract with appropriate strains of beneficial microbes can restore tolerance and can regain the subsequent development of a balanced immune phenotype (Cross, 2002).

Overview of the Immune System

The immune system is composed of numerous cells and mediators that dynamically interact to ensure host protection against foreign pathogenic invaders that one encounters (de Visser et al, 2005). The immune system has evolved to protect us from foreign substances, such as pathogens (Erickson et al, 2000). Innate immune cells, such as dendritic cells, macrophages, mast cells, and NK cells, express recognition patterns enabling them to distinguish self (host) from non-self (microorganism). Epithelial cells that line mucosal surfaces, especially within the intestine, are an essential component in the communication between commensal and pathogenic bacteria and immune and inflammatory cells in the underlying mucosa (Kagnoff et al, 1997).

Exposure to numerous microbes early in life may lead to immune tolerance and colonization of the intestine prior to adulthood enabling the intestine to respond more efficiently to microbial challenge. This hypothesis was coined the term 'Hygiene Hypothesis' (Schaub et al, 2006). The hygiene hypothesis suggests that microbiota is necessary for gut development and that insufficient or minimal exposure to environmental microbes may result in atopic diseases (Ouwehand, 2007). Humans evolved in an environment with a heavy bacterial population and our immune system has adapted to it (Ouwehand, 2007). When a foreign substance breaches the immune system, specifically a pathogen, the immune system responds by recognizing the pathogen followed by eliciting a reaction to eliminate it (Erickson et al, 2000).

Inflammatory Response in Carcinogenesis

Immune Response and Key Players

Immune responses fall broadly into two categories, innate or adaptive immune

responses (Erickson et al, 2000). At the gut mucosal level, the innate immune system not only provides the first line of defense against bacterial pathogens but also provides the appropriate signals that instruct the adaptive immune response to elicit a response (Galdeano et al, 2006). The immune system is composed of many types of cells and mediators that together interact in a complex and dynamic system to ensure protection against unwanted intruders (Coussens et al, 2005). The key players in our immune system, those that mediate immunity are T lymphocytes, B lymphocytes, macrophages, mast cells, neutrophils, dendritic cells, other lymphocytes, and epithelial cells lining the gastrointestinal tract.

A symbiotic relationship between the normal flora and the host immune system exists in that they mutually depend on and benefit the other (Hart et al. 2002). The host must suppress an immune response to tolerate the microbiota as favorable microorganisms may be lost, resulting in inflammation. In addition, the host also prevents the spread of pathogenic bacteria from intestinal lumen to neighboring tissues (Hart et al. 2002). The innate immune response to microorganisms occurs in three phases: 1) the detecting the bacterium; 2) transducing a cell signal; and 3) inducing an appropriate effector response (Kelly et al. 2005).

Macrophages are key immune cells in the inflammatory process. They are phagocytes that assist in eliminating unwanted invaders and present the antigen to T cells or B cells to stimulate the appropriate immune response. Intestinal macrophages are the first phagocytic cells of the innate immune system to interact with microorganisms that have entered the epithelium (Smythies et al, 2005). Intestinal epithelial cells (IEC) are another primary contact for enteric pathogens and may play a direct role in mucosal

immunity, particularly in the regulation of T-cell responses to enteric pathogens (Cruickshank et al, 2004). When T helper cells are activated, they differentiate into 2 subsets of cells; namely Type 1 T helper cells and Type 2 T helper cells (Xu et al, 2006). T lymphocytes are those that become active in cell-mediated immunity. T regulatory cells play an important role in suppressing normal immune responses secreting cytokines, such as IL-10 or TGF-ß (Schaub et al, 2006 and Strober, 1998). T regulatory cells assist in the inhibition of cell-mediated T helper cell immune responses.

Cross-Talk Between Immune Cells

Cytokines, chemokines, and growth factors mediate cross talk between different types of immune cells. Chemokines are crucial in controlling immune cell activation and recruitment into ttissues at inflammatory sites (Veckman et al, 2003). Chemotaxis can be defined as a directed movement and recruitment of cells towards the initiated problem; and in this case, it is inflammation. Cells recruited to sites of inflammation assist in strengthening the defense against infection; this involves a balance between pro-inflammatory and anti-inflammatory molecules. Th1 and Th2 responses counter regulate each other in that the cytokines produced by Th1 cells inhibit Th2 cell function and vice versa (Isolauri et al, 2003). Cytokines secreted by Th2 subset activate specific B cells for the antigen, whereas the Th1 subset is involved mainly in inflammation and activation of cytotoxic T cells (Erickson et al, 2000). Until recently, it was believed that there were only two types of T-helper cells: Th1 and Th2 (Bluestone, 2007). A new T-cell subset named "Th17," characterized by the production of IL-17, was identified as having an important role in inflammatory responses (Lin et al, 2007). IL-17 induces recruitment of immune cells to the site of infection as well as production of pro-inflammatory mediators,

such as TNF-alpha, IL-6, and IL-1ß. Induction of pro-inflammatory mediators suggests a role for IL-17 in localizing and amplifying inflammation (Lin et al, 2007). Recently, published papers demonstrated that IL-6 promotes the development of Th-17 cells by inhibiting T regulatory cells, resulting in disease (Bluestone, 2007). Inappropriate overexpression of the immunological response to Th1, Th2, or Th17 can result in disease.

Intestinal Epithelial Cells Cross-Talk with Other Immune Cells

Intestinal microbial populations influence intestinal functions by means of cross talk with the intestinal epithelial cells (Smythies et al, 2005). Upon binding to the intestinal epithelial surface, bacteria may colonize and create a permanent home in the gut (Lu et al, 2001). Competition between probiotic bacteria and bacterial pathogens for epithelial cell adhesion is one determinant of gut mucosa homeostasis. The first challenge to potential pathogenic bacteria is for such bacteria to successfully adhere to the intestinal mucosal surface (Lu et al, 2001). To uphold the integrity of the protective barrier capacity of the intestinal epithelium, the mucosal surfaces have a means for protection with immune components, such as the mucosa-associated lymphoid tissue, or MALT. Located in the subepithelial lamina propia, intestinal macrophages must defend the host against unwanted bacterial species and regulate mucosal responses to commensal bacteria (Smythies et al, 2005). The innate immune responses of intestinal epithelial cells are important in limiting infection by mucosal pathogens (Otte et al, 2004).

Epithelial Cells: Accessory Cells to the Immune Response

As cells which provide for gut barrier function, the intestinal epithelial cells lining the gastrointestinal tract play a role in sensing the environment of the outside world and communicating this information to their neighboring cells (Singh et al, 2005). Breakdown

of the mucosal barrier aids in the invasion of host cells by pathogens, some of which may not otherwise cause disease (Acheson et al, 2004). Moving astray from the normal homeostatic environment will often result in disease, such as inflammatory bowel disease (IBD). Intestinal epithelial cells may serve as targets for locally produced cytokines with IBD (Panja et al, 1998). Cytokines are produced even during times of homeostasis involved in epithelial growth and differentiation; but an imbalance in cytokine production as seen with IBD, may disrupt epithelial cell function (Panja et al, 1998).

It is crucial that the local response in the GI tract is tightly regulated to avoid an immune response against dietary antigens and commensal flora while still developing an efficient defense against pathogens (Iscue et al, 2006). The B cells of the immune system closely synchronize the microbiota environment by the production of IgA to help manage the microorganism volume and composition (Teitelbaum et al, 2002). The mucosal ecosystem of the gastrointestinal tract is quite complex with a combination of the GI epithelium, mucosal immune cells, and resident microflora (Moal et al, 2006).

The intestinal mucosa must efficiently recognize pathogenic threats to the colonic lumen to initiate controlled immune responses as well as maintain a down-regulated response to harmless commensal bacteria (Cario, 2005). The cornerstone of innate signaling at the epithelial cell level is initiated by a set of toll-like receptors, which assist in the recognition of pathogens or other foreign substances (Haller, 2006). Toll-like receptors, or TLRs, are emerging as key communicators of innate host defense in the intestinal mucosa (Cario, 2005). Toll-like receptors are expressed by various cell types, including intestinal epithelial cells, and the epithelial expression of the receptors has been identified in an altered form in disease states such as ulcerative colitis or Crohn's disease

(Shanahan, 2002). The mucosal epithelium along the gastrointestinal tract is in direct contact with the outside environment; therefore, the mucosa is exposed to a variety of pathogens with which it may come in contact. The surface of the colonic epithelium is lined with epithelial cells that are folded into what is called the crypt. This provides a physical protective barrier that protects the host against unwanted invaders. The intestinal microbial environment is composed of a broad mixture of bacteria that all carry out fundamental jobs for the host and can be adjusted by the environment which one lives, such as with diet (Moal et al, 2006).

There are three types of immunosensory cells that help to distinguish pathogen from commensal bacteria. The first type is surface enterocytes, which sense danger within the lumen by secreting chemokines and cytokines that direct immune responses to the infected area. Second are M cells, which transport luminal antigens to antigen-presenting cells, such as dendritic cells. Third are intestinal dendritic cells themselves, which sense immune responses by entering or extending dendrites between surface enterocytes without tight junction disruption (O'Hara et al, 2006). While the gut microflora is important in supporting a functional and balanced immune system, the processes that may lead to this balance can be mimicked by transiently colonizing the GI tract with appropriate strains of beneficial microbes, or probiotics (Cross, 2002). To modulate immunity, probiotic microorganisms must "talk" to other immune cells, specifically the intestinal epithelium; thereby triggering signaling cascades (Corthésy et al, 2007).

Intestinal epithelial cells play an important role in the recruitment of inflammatory cells to the site of infection through the secretion of chemokines (Huang et al, 1996).

Thus, the epithelial cells have a powerful accessory role in inflammation and may help in the promotion of cancer by the attraction of immune cells. The epithelial cells of the intestine participate in the onset and regulation of the mucosal immune response to bacteria, especially those of a pathogenic nature, by interacting with immune cells of the gut via toll-like receptor signaling (Vinderola et al. 2005 and Haller, 2006). The interaction between epithelial cells and immune cells of the gut occurs because intestinal epithelial cells are in constant contact with bacteria and bacterial products and are in close proximity to mucosal immune cells (Galdeano et al, 2006). Epithelial cells possess many characteristics of innate immune cells, including the ability to secrete chemokines and cytokines in response to toll-like receptor agonists (Herring et al, 2004). Cross talk between innate and adaptive immunity, allows the host to maintain homeostasis. T regulatory cells, or Th3 cells, help regulate the production of anti-inflammatory cytokines, expression of Type 1 T helper cell (inflammatory responses) and Type 2 T helper cell (allergic in nature) cytokines are pro-inflammatory (Herring et al. 2004). T regulatory cells help in maintaining the cytokine balance. Macrophage and other immune cells have been shown to be required for the promotional phase of carcinogenesis *in vivo* (Coussens et al. 2006). Intestinal epithelial cells produce a variety of chemoattractants when a pathogen is detected for recruiting macrophages, stimulating macrophage activation, proliferation, and additional cytokine production, which further perpetuates the inflammatory response; thus, increasing the risk for cancer progression (Mumy et al, 2005 and de Visser, 2005).

Beneficial Immunomodulatory Effects of Pre- and Probiotics

Probiotic Bacteria: Potential Mechanisms of Action

The use of probiotic bacteria can prevent or treat disease as well as promote health. Some mechanisms in which probiotic bacteria exert their beneficial effects on the host are: 1) preventing the colonization of host by pathogens by competing for nutrients and epithelial attachment site; 2) producing antimicrobial compounds and pH changes making the environment for pathogens unfavorable; 3) recruiting immune cells and activating appropriate immune and/or inflammatory responses by altering cytokine and chemokine release; and 4) secreting anti-microbial peptides (Lu et al, 2001 and Penner et al, 2005). My research proposal addresses this specific mechanism by utilizing gamma-irradiated bacteria to determine the effect of co-treatments of pathogen and probiotic bacteria in the production of inflammatory mediators by colonic epithelial cells. Probiotics can prevent or improve diarrhea and/or inflammation through local effects on the GI immune system; or they can prevent pathogenic strains of microbes from mucosal adhesion, by competing with the pathogens for binding sites on the gut mucosa (Chermesh et al. 2006). One particular pathogen that is able to cause inflammation in the colon is *Escherichia coli O157:H7* by producing shiga toxins, which are toxic to the host and cause symptoms such as bloody diarrhea and colonic inflammation (Slutsker et al. 1997). One consequence of *Escherichia coli* infection is activation of the nuclear transcription factor, NF-kB, which in turn promotes increased expression of pro-inflammatory cytokines (Sharma et al, 2005).

The balance between proinflammatory and anti-inflammatory cytokines, produced by epithelial cells and mucosal immune cells, may also explain probiotic effects (Isolauri

et al, 2002). Given the variety of inflammatory or immune responses that can be introduced to the intestinal epithelium, accompaniment of probiotics with epithelial cells might be enough to trigger signaling cascades that in due course will activate underlying immune cells of the gut (Corthésy et al, 2007).

Several clinical studies have analyzed probiotic bacteria, particularly *lactobacilli* and *bifidobacteria,* as dietary supplements for the prevention or treatment of various gastrointestinal infections or inflammatory conditions, such as in inflammatory bowel diseases (Altenhoefer et al, 2004). With the consumption of probiotic bacteria, the gut mucosa can be stabilized through the reduction of local proinflammatory cytokine secretion (Altenhoefer et al, 2004). One study investigated the use of non-pathogenic *Escherichia coli* strains in treating GI diseases. One of the most widely studied *E. coli* strains as a probiotic strain in the strain *Nissle 1917 (*Altenhoefer et al. 2004). One way in which probiotic bacteria exert benefit on the host is through their antagonistic activity against intestinal pathogens; also called bacterial interference. *E. Coli Nissle* 1917 was able to interfere with the invasiveness of pathogens, such as *Salmonella* by the secretion of a bacteriocidal product called microcin that may act on epithelial cells or invasive bacteria (Altenhoefer et al. 2004).

Lactobacillus rhamnosus GG Used as a Treatment Option

In one study, one group was given *Lactobacillus rhamnosus GG* (LGG) and the other group was given a placebo. The LGG treatment group had a shorter duration of illness vs. the placebo group. The LGG group had a greater number of immunoglobulin secreting cells in circulation, suggesting that the humoral immune system plays a considerable function in the defensive effects of probiotic bacteria (Teitelbaum et al,

2002). It has for some time been clear that bacterial infectious agents play a role in the pathogenesis of inflammatory bowel disease, or IBD. Pathogenic strains of *Escherichia coli* present in the colon may play a crucial role in the pathogenesis of IBD (Servin, 2004). Laboratory testing has indicated that IBD patients host an intestinal microflora containing few *lactobacilli* microorganisms and a reduction in *bifidobacteria* fecal concentration (Heyman et al, 2002). *Lactobacilli and Bifidobacterium*, which inhabit the GI microbiota, develop antimicrobial activities that participate in the host's GI defense system (Servin, 2004). *Lactobacillus* strains express competitive adhesion properties enabling them to inhibit the adhesion of bacterial pathogens to the host. After challenging *L. rhamnosus* fed mice infected with *E.coli O157:H7*, such mice showed signs of lower cumulative morbidity and bacterial translocation as well as an amplified intestinal anti-E.coli IgA responses and blood leukocyte phagocytic action; the beneficial effect of this probiotic strain probably was due to an enhanced local immune response (Servin, 2004). Early studies explored the effect of fermented milk products in tumor prevention in the colon. One such study found that the rats that were fed *bifidobacteium* developed fewer aberrant crypts in the colon than control fed rats. The probiotic bacteria reduced the aberrant crypt development by 50% (Teitelbaum et al, 2002).

The Effect of Prebiotics on Tumorigenesis

Among potentially protective foods, there has been given great attention dedicated to prebiotics and probiotics. Such food products have been shown to decrease induced colon cancer in animals. Rats treated with prebiotic Synergy I had a decreased number of AOM-induced colonic tumors when compared to untreated rats (Femia et al. 2002). One specific study was aimed at the antitumorigenic properties of prebiotics (e.g.,

inulin), probiotics (e.g., *Lactobacillus rhamnosus* and *Bifidobacterium lactis*), or a combination thereof (synbiotics) in the prevention of colon carcinogenesis in azoxymethane (AOM)-induced rats (Femia et al. 2002). Because the capability of live bacteria in food products and during transit time through the GI tract may be transiently variable, the notion of prebiotics has been developed (Gibson, 1999).

Bacterial Components as Toll-like Receptor Agonists

Bacteria possess toll-like receptor (TLR) ligands that modify mucosal immune responses (Table 2.1). TLR ligands play a powerful role in inflammation. The immune system discriminates between self and non-self by this system of toll-like receptors (Vinderola et al, 2005). In the healthy gut, TLR expression is functionally homeostatic, but with increased pathogenic threats, homeostasis could be altered to a disease state (O'Hara et al, 2006). In the normal uninflammed intestine, there is a low level of expression of both TLR2 and TLR4; levels of TLR2 and TLR4 become increased during inflammatory conditions (Abreu et al, 2005).

TLRs each participate in host defense against pathogens in at least 4 ways: 1) recognition of molecular patterns on pathogens; 2) expression at the interface with the "environment" of the GI lumen; 3) secretion of pro-inflammatory or anti-inflammatory cytokines and chemokines that connect to the adaptive immune system; and 4) induction of anti-microbial pathways (Cario, 2005). The colonic epithelium provides the first line of protection for newly introduced bacteria, such as pathogens or other antigens (O'Hara et al, 2006). TLR-4 recognizes lipopolysaccharide (LPS), TLR-2 recognizes peptidoglycan (PGN), TLR-5 recognizes flagellin, and TLR-9 recognizes ODN bacterial DNA (Vinderola et al. 2005). The role that epithelial cells of the gut play in cross talk

Table 2.1: Toll-Like Receptors

Receptor	Cellular Localization	Known Ligands
TLR1	Plasma membrane	Triacylated lipoproteins and mycobacterial products
TLR2	Plasma membrane	Peptidoglycan, lipoproteins, products of Gram-positive bacteria, zymosan, LPS, lipoarabinomannin
TLR3	Membrane of the endoplasmic reticulum	Double-stranded RNA
TLR4	Plasma membrane	LPS, taxol, fibronectin, HSP60
TLR5	Plasma membrane	Bacterial flagellin
TLR6	Plasma membrane	Bacterial lipopeptides and lipoteichoic acid
TLR7	Membrane of the endoplasmic reticulum	Imidazoquinolines (antiviral agents)
TLR8	Membrane of the lysosome	Single-stranded RNA
TLR9	Membrane of the endoplasmic reticulum	Bacterial DNA, CpG DNA, certain viruses
TLR10	Plasma membrane	Unknown

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with the microflora is that they express the TLRs, which play an important role in bacterial recognition, signal transduction, and mucosal immune modulation (Kelly et al, 2005).

In addition to being positive regulator of NF-kB activation, TLR ligands activate transcription factors AP1 and IRF3. Recent studies have also begun to identify proteins that negatively regulate these pathways. Two orphan receptors of the TLR superfamily, SIGIRR (single immunoglobulin IL-1R-related molecule; also known as TIR8) and ST2 have now been identified as negative regulators for the signaling pathways mediated by the members of this receptor superfamily. The other molecules that have been shown to negatively regulate the TLR signaling pathway include toll-interacting protein (tollip), IRAKM, MyD88, SOCS1 and Triad3A (Li et al, 2005).

Recent studies indicate that SIGIRR functions as a biologically important negative regulator of Toll-IL-1R signaling (Wald et al, 2003). SIGIRR-deficient mice had a reduced threshold for lethal endotoxin challenge and are more susceptible to destran sodium sulfate (DSS)-induced IBD. Therefore, the action of SIGIRR probably provides a novel mechanism by which the normal intestinal epithelium regulates innate immune response and inflammation (Wald et al, 2003). The mechanisms regulating the TLR response must be controlled tightly, first in responding appropriately to the pathogenic challenge and second in preventing excessive activation of the TLR signaling pathway, and thus controlling detrimental damage to host following TLR activation (Miggin et al, 2006). Hypothetically speaking, SIGIRR and the aforementioned negative regulators of TLR signaling may be involved in the response of epithelial cells to pathogenic and probiotic bacteria.

In order for the gut to maintain this homeostatic environment against tissue damage from potential pathogens, functional toll-like receptors are needed to help detect any imbalance in the system. Deficient toll-like receptor signaling may imbalance this homeostasis, making possible disease progression. The maintenance of a local homeostatic milieu in the intestinal mucosa to enteric bacteria and their antigens necessitates a close balance between immune activation and regulation, a course of action that may lead to intestinal inflammation (Parlesak et al, 2004).

Epithelial Cell Signaling in Inflammation-Associated Cancer Risk

Signal transduction leading to nuclear factor kappa B (NF-kB) activation is a key element in the intestinal epithelial cell signal transduction. In its inactive state, NF-kB is bound to the inhibitory protein IkB. Once IkB is phosphorylated, it can be degraded by ubiquitination, freeing NF-kB to translocate to the nucleus where pro-inflammatory gene activation is initiated (Claud et al, 2001 and Schiffrin et al, 2002). NF-kB activation by pathogens or stress is a pivotal event in defensive inflammatory reactions (Schiffrin et al, 2002). It has been recently reported that some non-pathogenic bacteria can prevent NF-kB activation through inhibition of IkB (Schiffrin et al, 2002). Recently, ß-catenin was found to interact with NF-kB and inhibit its activity. Nuclear NF-kB may not be an accurate marker for NF-kB when nuclear ß-catenin is also present (Deng et al, 2004). The *Apc* gene plays an important role in the regulation of ß-catenin. ß-catenin's role is to participate in both cell adhesion and proliferation. Inhibition or defects in either *APC*'s function can lead to activation of ß-catenin (Deng et al, 2004). Under normal conditions, the *APC* gene binds to ß-catenin, promoting ß-catenin's downregulation, thereby preventing signaling to the nucleus (Albuquerque et al, 2002). In the absence of a

functional *APC* gene, ß-catenin will build up in the cytoplasm and then translocated to the nucleus, where it associates with members of transcriptional activators, which can then alter transcription of the Wnt/ß-catenin pathway target genes (Albuquerque et al, 2002). Deregulated ß-catenin is involved in oncogenesis occurring through cross-regulating NF-kß. When ß-catenin forms a complex with NF-kB, a reduction of NF-kB DNA binding results (Deng et al, 2002). Recently ß-catenin has been found to interact with and inhibit nuclear factor kappa B (NF-kB) (Deng et al 2002). NF-kB is a transcriptional factor that is normally involved in a number of genes for immunity, inflammation, and apoptosis. Some observations that have shown some light on this is that by re-expressing the *APC* gene, NF-kB can be restored in *APC* mutations.

Immunomodulatory Protection Against Colon Cancer by T regulatory Cells

T-regulatory lymphocytes attenuate the inflammatory process possibly by their secretion of anti-inflammatory cytokines and chemokines, such as IL-10 and TGF-ß. This study provides evidence that by suppressing active inflammation by regulatory T lymphocytes, inflammation-associated cancers can be treated and/or prevented (Erdman et al. 2005). T regulatory cells have been shown to not only prevent the development of colitis in animal models but also cure established disease, acting both systemically and at the site of inflammation; that is, locally (Iscue et al, 2006). Specific immune cell products have been shown to influence tumorigenesis using adoptive transfer of T regulatory (CD4+CD25+) lymphocytes in $Apc^{Min/+}$ mice (Erdman et al. 2005). While other studies have shown that intestinal bacterial infections cause mucosal inflammation thereby facilitating intestinal adenomas in $Apc^{Min/+}$ mice, this particular study shows therapeutic benefits by suppressing inflammation throughout the host using T-regulatory cells.

Inhibition of TLR Signaling Provides Promising Evidence of Anti-inflammation

Toll-like receptors convert the recognition of pathogen-associated molecules in the gut into signals for antimicrobial peptide expression (Abreu et al, 2005). In states of inflammation as seen in inflammatory bowel disease, the normal TLR levels become overexpressed. Inhibitors of TLR signaling provide another means to reduce TLR signaling in the intestine (Abreu et al, 2005). Expression of TLR inhibitors help in the control of intestinal inflammation and reduced expression of TLR inhibitors may contribute to IBD. Intestinal epithelial cell (IEC) expression of TLR4 and TLR2 is muted and IEC poorly responsive to LPS; by cotreating IEC's with TLR4 and TLR2 antibodies, proinflammatory mediator production could be diminished.

Epithelial Derived Inflammatory Mediator Production

Overproduction of certain pro-inflammatory mediators during an immune challenge or inflammatory response can result in tissue injury and cellular death (Billack, 2006). It is estimated that >20% of all malignancies are initiated or exacerbated by inflammation. Until recently, the molecular basis of this process has not been clarified. However, recent studies have uncovered the molecular mechanism of intracellular signaling pathways of inflammatory cytokines or other pro-inflammatory mediators, such as tumor necrosis factor (TNF)-alpha and interleukin (IL)-6 or nitric oxide (NO) (Yoshimura, 2006; Billack, 2006). Due to the association between increased pro-inflammatory mediator production and carcinogenesis, we have chosen to quantitate NO and IL-6 production in epithelial cells exposed to bacteria as well as exposing bacterially treated epithelial supernatants to macrophages for macrophage activation and chemotaxis.

Nitric Oxide (NO)

NO is an important signaling molecule in numerous physiological and pathological conditions (Liu et al. 2003). NO and other reactive oxygen species, ROS, are considered to play a large role in inflammation-mediated carcinogenesis (Ding et al, 2005). Inducible nitric oxide, iNOS, is not expressed in most tissues under normal conditions, but can be induced under the influence of pathogens or various cytokines (Liu et al, 2003). Low concentrations of NO can stimulate cell growth and keep many cell types from programmed cell death, whereas high concentrations of NO can inhibit cell growth and persuade apoptosis (Liu et al, 2003). Although necessary for adequate immunity, the production of NO and other reactive nitrogen intermediates for the purpose of preventing the spread of infections can also have toxic effects on the host, including chronic inflammatory diseases or even cancer (Billack, 2006).

Increased expression of NO is found in intestinal epithelial cells at times of chronic intestinal inflammation as well as in response to stimulation of epithelial cells with a combination of cytokines. The inducible nitric oxide synthase 2 (NOS2) is upregulated in breast, brain, colon, and gynecological tumors, which indicate that NO may have a role in tumorigenesis (Fransen et al. 2002). NO is produced in high concentrations by iNOS after stimulation of bacterial products and cytokines as a product of NF-kB regulation of the NOS2 gene. NF-kB is a transcriptional factor involved in immune responses as well as inflammatory and cellular defense mechanisms (Fouad et al. 2004). NF-kB has been shown to be the most relevant transcriptional factor regulating the expression of human and rat iNOS in hepatocytes (Fouad et al. 2004). It is proposed that this same stimulation occurs in epithelial cells with NF-kB regulation of the iNOS gene.

Interleukin-6 (IL-6)

The pro-inflammatory cytokine, IL-6, plays an important role in the pathogenesis of the intestinal inflammatory process or IBD as well as of colon carcinogenesis. IL-6 is a multifunctional cytokine that plays a central role in host defense due to its wide range of immune activities (Simpson, et al. 1997). IL-6 is a cytokine that is practically expressed all the time, but is overexpressed in times of inflammation. IL-6 can be a pro-inflammatory cytokine that plays an important role in many chronic inflammatory conditions, such as in Crohn's disease and cancer (Gustot et al, 2005). However, IL-6 has many normal functions as well. It is proposed that IL-6 may be overexpressed during inflammation in epithelial cells during inflammation-induced progression of colon cancer.

Evidence for Research Models

A widely used model for human colorectal cancer is the multiple intestinal neoplasia (Min) mouse. The min mouse has a germ-line mutation in the *Apc* tumor suppressor gene ($Apc^{Min/+}$) (Erdman et al. 2005); the gatekeeper gene in colon cancer (Newman et al. 2001). Inactivation of this gene leads to intestinal adenoma development and early events in colon cancer development. These animals typically die at an average age 17 weeks due to anemia secondary to bleeding through adenomas. Immunoprevention can be effective in a variety of murine cell lines with spontaneous or carcinogen-induced cancer. $Apc^{Min/+}$ cells from murine mice provide us with a good cell model in studying the initiation and progression of intestinal carcinogenesis. The colon epithelial cells isolated from the Min mouse permit us to study the transition between normal and malignant growth in cell culture (Fenton et al, 2006).

Since there has been growing evidence that bacterial induced inflammation, such as that caused by the bacterial pathogen *O157:H7*, causes tumor formation in $Apc^{Min/+}$ mouse cells, we propose that food components, such as probiotic bacteria, will modulate proinflammatory mediator production.

Our Model System of Mucosal Inflammation

We will use two cell culture models to study the progression of neoplastic transformation in colonic epithelial cells. We propose that a specific set of non-tumorigenic conditionally immortal cell lines derived from C57/BL6 mice, YAMC (Young Adult Mouse Colon Cells $Apc^{+/+}$) and IMCE (Immortomouse-Min colonic epithelial cells $Apc^{Min/+}$) cells developed by Dr. Robert Whitehead have yielded important findings on early events in colorectal neoplasia development (Fenton et al. 2006). The cell lines will serve as a model to examine the effect of dietary compounds for colon cancer prevention on early preneoplastic stages with a mutation in the *Apc* gene, the gatekeeper gene of colon cancer. These cells are consistent with the normal to preneoplastic transition observed in colon epithelial carcinogenesis. YAMC cells mimic normal colonic epithelial cells while IMCE mimic preneoplastic colonic epithelial cells. We have characterized phenotypic changes in the IMCE cells that are consistent with human preneoplastic lesions, such as iNOS and IL-6 expression. We will make use of *O157:H7*, a bacterial pathogen, to model mucosal inflammation. Bacterial pathogens, such as *O157:H7*, bind to colon epithelial cells (CEC), promoting the production of proinflammatory mediators (i.e., NO, chemokines, and cytokines), which are produced in elevated amounts during times of inflammation. We will use various probiotic bacteria, such as *Lactobacillus rhamnosus GG, Lactobacillus reuteri, Lactobacillus salivarius,*

Lactobacillus plantarum, Lactobacillus paracasei, Bifidobacterium breve, and *Bifidobacterium lactis* from fermented dairy products to determine whether these dietary factors can block *O157:H7* induced inflammation.

Research Hypotheses and Aims

We will use a novel *in vitro* system to address the following hypotheses:

- Exposure of colon epithelial cells to a bacterial pathogen, *O157: H7* will cause an increase in the pro-inflammatory mediator production of NO and IL-6
- Specific probiotic bacteria will block the production of pro-inflammatory mediators NO and IL-6 by colonic epithelial cells resulting from exposure to *O157: H7*
- Exposure of colon epithelial cells to various toll-like receptor ligands (i.e., TLR-2, TLR-4, TLR-5, and TLR-9) will cause an increase the production of pro-inflammatory mediators NO and IL-6 and co-exposure of colon epithelial cells to *O157: H7* and antibodies against TLR-2 and TLR-4 will reduce the pro-inflammatory mediator production of NO and IL-6
- Exposure of colon epithelial cells to *O157: H7* will activate macrophages to produce increased amounts of pro-inflammatory mediators NO and IL-6 and co-exposure of colonic epithelial cells to specific probiotic bacteria and *O157: H7* will decrease macrophage production of NO and IL-6 activation
- Exposure of colon epithelial cells to *O157: H7* will cause increased macrophage chemotaxis and co-exposure of colonic epithelial cells to specific probiotic bacteria and *O157: H7* will decrease macrophage chemotaxis

The long-term goal of this research is to understand the role of dietary factors, like probiotic bacteria, in the modulation of mucosal immune responses relevant to inflammation and cancer risk. The short-term goal of this research is to assess the role of probiotic bacteria in the production of pro-inflammatory mediators and macrophage chemotaxis by colon epithelial cells exposed to *O157: H7*. This proposal will test these hypotheses by addressing three specific aims.

Specific Aim 1

Characterize the ability of probiotic bacteria to modulate the production of the pro-inflammatory mediators NO and IL-6 caused by exposure to *O157: H7*. Probiotic bacteria will modulate the inflammatory mediators produced by the colon epithelial cells in a genus- and species-specific manner. Preliminary data indicate that when colon epithelial cells are exposed to a pathogen, *O157: H7*, inflammatory mediators are increased in a dose response manner. Preliminary data also indicate that certain probiotic bacteria modulate the differential effects of *O157: H7* on inflammatory mediator production on a genus- and species-specific basis.

Specific Aim 2

Analyze possible intracellular mechanisms by which these cell types increase the production of NO and IL-6. This will be done by the application of inhibitors of toll-like-receptors (i.e., antibodies against TLR-2 and TLR-4) and inhibitors of NF-kB (PDTC). Preliminary data showed that the use of enzymatic inhibitors of NF-kB, such as PDTC and neutralizing antibodies against toll-like receptors TLR-2 and TLR-4, decreased the production of pro-inflammatory mediators NO and IL-6 by epithelial cells exposed to *O157: H7*.

Specific Aim 3

Characterize the ability of colonic epithelial cell supernatants from cells exposed to *O157: H7*, probiotics, or a combination thereof, to cause macrophage activation and chemotaxis. The working hypothesis of this aim is that probiotics will block the production of proinflammatory mediators that cause macrophage activation and chemotaxis. We expect to show that cells co-treated with *O157: H7* and probiotic but not cells treated with probiotics alone will cause macrophage activation and chemotaxis towards colon epithelial cells. Preliminary data indicates that upon treating macrophages with supernatants from bacterial-treated epithelial cells caused an increase of NO and IL-6 production in macrophages. These data imply that epithelial-derived products may activate macrophages dependent upon the genus and species of bacteria to which they were exposed.

The identification of dietary compounds that are efficient in blocking inflammation involved in the promotion of colon cancer will contribute to an effective strategy for diet-dependent cancer prevention. These studies will provide a mechanistic basis for diet recommendations to consume fermented dairy products containing probiotic bacteria, like yogurt, to reduce colon cancer risk.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

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Bacterial Growth and Preparation of ATCC Lactobacilli

Bacteria for these studies came from either American Type Culture Collection (ATCC) or Danisco (Madison, WI). *Lactobacillus rhamnosus GG, Lactobacillus reuteri* or *O157:H7* came from ATCC and *Lactobacillus paracasei, Lactobacillus salivarius, Lactobacillus plantarum, Bifidobacterium breve,* and *Bifidobacterium lactis* from Danisco. With the assistance of Ms. Allison Meldrum, the bacteria were grown based on Ms. Erica Block's protocol (Block, 2004). The freeze-dried powder form of the *lactobacilli* from ATCC were rehydrated in 10 ml of De Man, Rogosa and Sharpe (MRS) medium and held at 37°C for 24 hr. The following day 1 ml of sterile anhydrous glycerol was added to the bacteria-media mixture and vortexed to a homogenous mixture. Anhydrous glycerol was added to prohibit the bacteria from adherence. The bacteria was then added into 2 ml sterile test tubes and held at -80°C until needed.

The bacterial growth medium was prepared using the 2ml stock vile and adding it to 25 ml of sterile MRS media in a 50 ml sterile conical tube and holding it at 37°C for 24 hr. Day two of the bacterial growth preparation consisted of centrifuging the test tube at 10,000 RPM, 4°C, and for 15 min. The media was aspirated off and washed one time with sterile phosphate buffer saline (PBS). The PBS is aspirated off, 25 ml of fresh MRS media is added, and held at 37°C for 24 hr. Day three and four of the bacterial growth preparation was exactly like day two except that for day four, the bacteria is held for only 15 hr instead of 24 hr. Day five of the bacterial growth preparation consisted of

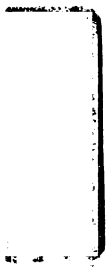
transferring 10ml of inoculated culture to a flask with 250 ml of fresh MRS media and held at 37°C. Optical densities were taken every hour via spectrometer until the late log phase (maximum growth) has been reached. Once the late log phase has been reached, a second transfer of 10 ml to a new flask with 250 ml of fresh MRS media and held at 37°C. A third transfer is completed after the allotted time for late log phase has passed, but this time 2 flasks with 500 ml of fresh MRS media with 20 ml of inoculated culture (10 ml per 250 ml media). The bacterium is held in the 37°C incubator. On day six of the bacterial growth preparation, the 1 L of inoculated culture is allocated into 50 ml conical test tubes with approximately 35 ml per tube. The procedure for centrifugation and washing is the same as for day two through four except that the process is repeated for a total of three times. After the third wash, 1/10th of the original volume or 3.5 ml of PBS is added and then the test tubes are combined (10 in 1 with a multiple of 3.5 ml PBS) and held at -80°C. Additional procedures for day six of the bacterial growth preparation consisted of pour and spread plate methods for cell counting purposes using peptone dilution with 10^1 to 10^8 (using only 10^6 to 10^8 for cell counting). Each plate was performed in duplicate and an average was taken for cell count. Once the plates solidified, they were placed in the 37°C incubator for 48 hr. On day eight of the bacterial growth protocol, the cells were counted under magnification and the growth preparation protocol is repeated for a second cycle with 1 L total for freezer stock.

Bacterial Growth and Preparation of O157: H7 and Danisco Strains

The bacterial growth preparation for *O157:H7* was the same as for *lactobacilli* except that trypticase soy broth with yeast extract (TSB-YE) was used instead of MRS medium. Day one for *O157: H7* growth consisted of taking a frozen loop from a frozen 2

ml vile and placing it in 10 ml of TSB-YE media using a 15 ml test tube and holding it at 37°C for 24 hr. Day two consisted of taking 1.5 ml from the overnight grown sample and placing it into 25 ml of TSB-YE media and holding it at 37°C for 24 hr. During the day, five transfers for *O157: H7*, the flask is held on a shaker instead of an incubator at 37°C. All other steps in the growth protocol for *O157: H7* were the same as for *lactobacilli* bacteria.

The bacterial growth preparation for Danisco strains was done differently during initial steps. Since Danisco strains came as greater than 400 billion colony forming units (CFU's) per gram instead of greater than 10^4 cfu's per vial as ATCC came, the Danisco strains had to be diluted. Since ATCC weight of the bacteria was about 0.2g, the Danisco strains were weighed out at 0.02g, which was $1/10^{th}$ of ATCC weight, and placed in 10 ml MRS medium and held at 37°C for 24 hr. One particular strain from both ATCC and Danisco were grown simultaneously and optical densities were run to determine the equivalence of Danisco with ATCC. To do this, Danisco bacteria was diluted in PBS until a similar number of CFU's was found. 550 μl of inoculated culture was diluted with 450 μl of sterile PBS and placed in 10ml MRS medium and incubated at 37°C for 24 hr. One ml of sterile anhydrous glycerol is added and vortexed. The solution is allocated into 2 ml tubes and held at -80°C. After this dilution was performed, all further steps remain the same as for ATCC *lactobacilli* bacterial growth procedures. Being that *bifidoabacterium* are facultative anaerobes, 0.05% cysteine was added to MRS media during the growth procedures to assist in *bifidobacterium* cell survival. Supernatants from each bacterial strain (all 7 probiotic strains and *O157: H7*) were kept as well to be able to



analyze if the bacterial cell signals are in the supernatants in addition to being on cell surface.

Figure 3.1

Growth and Preparation of ATCC Lactobacillus Bacteria

Take freeze-dried powder form, re-hydrate in 10ml of M.R.S. media, and incubate @ 37° C for 24°

↓

To the 10 ml of media/bacteria mixture, add 1 ml sterile anhydrous glycerol; vortex to make homogenous; then allocate into 6-2 ml sterile test tubes and freeze @ -80° C

↓

Day 1 of growth protocol: take a 2ml vile out of freezer and thaw it and place it into 25 ml of M.R.S. media in a 50 ml conical tube and incubate @ 37° C x 24°

↓

Day 2 of growth protocol: With centrifugation, spin down bacteria to form a nice and solid pellet, aspirate media, wash x1 with sterile PBS, spin and aspirate PBS, add fresh media of 25 ml, vortex to make homogenous and incubate @ 37° C X 24°

↓

Day 3 of growth protocol: Repeat day 2 x 24°

↓

Day 4 of growth protocol: Repeat day 3 x 15°

↓

Day 5 of growth protocol: Transfer 10 ml of inoculated culture into a flask with 250 ml of fresh MRS media and incubate @ 37° C; take optical densities every hour on the hour via a spectrometer until late log phase (or maximum growth) has been reached; once late log phase has been reached complete a second transfer with 10 ml from the flask from first transfer and adding it to a fresh flask with 250 ml MRS media and incubate @ 37° C; after the allotted time for late log phase has again passed, complete a third transfer with 10 ml per 250 ml media (this time: we want 1 L, so take 2 sterile flasks with 500 ml MRS and add to it 20 ml of inoculated culture) and place flasks in incubator until day 6

↓

Day 6 of growth protocol: Allocate the inoculated culture from the flasks into 50 ml tubes with approximately 35 ml per test tube; spin all test tubes down and wash with sterile PBS 3 times; after the third wash, add a multiple of 3.5 ml of PBS to 1 test tube and then combine test tubes into 1 for freezer stock (For example: if there were 30 test tubes, combine 10 test tubes into 1 test tube with 35 ml PBS and freeze at –80° C)

↓

Addition to Day 6: Perform pour and spread plate methods for cell counting purposes using peptone dilution with 10^1-10^8 (use only 10^6-10^8 for cell counting); perform duplicates; once plates have solidified, place in 37° C incubator for 48 hours

↓

Day 8 of growth protocol: count cells under magnification and repeat growth protocol for second cycle with 1 L total ending volume for freezer stock

↓

Additional Days: Irradiated Bacteria→Lyophilized bacteria in 1ml aliquots using a speed vacuum→Calculated resuspension volume per tube based on bacteria weight→freeze tubes at -80° C until needed for experimental analysis (0.1-1000 μg/ml)

Figure 3.1 (Continued)

Growth and Preparation of *E.coli* Bacteria

Day 1: Take 1 frozen loop from a frozen 2 ml stock out of the -80° C freezer and place in 10 ml of TSB-YE media using a 15 ml test tube and incubate at 37° C for 24°

↓

Day 2: Take 1.5 ml from the overnight *E.coli* growth and put in 25 ml of TSB-YE media and place in 37° C incubator for 24°

↓

Day 3: With centrifugation, spin down the test tube with parameters: 10000 RPM, 4° C, and 15 minutes; aspirate the media; wash x1 with sterile PBS; aspirate the PBS; add 25 ml fresh media; incubate @ 37° C for 24°

↓

Day 4: Repeat day 3 x 24°

↓

Day 5: Repeat day 4 x 15°

↓

Day 6: Transfer 10 ml of inoculated culture into a sterile flask with 250 ml fresh TSB-YE media and place in a 37° C shaker; Take optical densities every hour on the hour until maximum growth has been reached and construct a growth curve (in *E. coli* case, it was 24°)

↓

Day 7: Once maximum growth has been reached, do a second transfer with 10 ml from day 6 flask into a fresh flask with 250 ml media and place in shaker

↓

Day 8: Once 24° hours has past again, transfer 20 ml into 2-1 L flasks with 500 ml fresh media each for a total of IL volume and place in shaker

↓

Day 9: Allocate the 1L of inoculated culture into 50 ml test tubes with 35 ml per tube and then-spin down the test tubes, aspirate the media, wash x 3 with sterile PBS, aspirating PBS each time, and then add a multiple of $1/10^{th}$ of 35 ml or 3.5 ml to 1 test tube and combine 10 test tubes into 1 test tube by vortexing each pellet into the previous

↓

Addition to Day 9: Perform pour and spread plate methods by using TSA-YE and serial peptone dilutions (10^{1}-10^{8}) and only make plates with 10^{6}-10^{8} for cell counting purposes; once plates have solidified, place in 37° C incubator for 48°

↓

Day 11: Count cells using magnification; if there is a large quantity of cells, then count quadrants and multiply result by 4

↓

Repeat growth cycle x1 with 1L total ending volume

↓

Additional Days: Irradiated bacteria→Lyophilized 1ml aliquots tubes using a speed vacuum→Calculated resuspension volume based on bacteria weight→Freeze tubes at -80° C until needed for experimental analysis (0.1-1000 μg/ml)

Figure 3.1 (Continued)

Growth and Preparation of Danisco Cultures

Day 1: Weigh out 1/10th of weight of ATCC strains, which is approximately .02 grams of bacteria and place in a 15 ml test tube with 10 ml MRS broth media and incubate @ 37° C for 24°

↓

Day 2: Take 550 µl of inoculated culture and dilute it with 450 µl of sterile PBS and place total 1 ml in a fresh 15 ml test tube with 10 ml MRS broth media and incubate for 24° @ 37° C; do this x2 to end up with twice the # freezer stock in the end

↓

Day 3: Combine the 10 ml with inoculated culture with 1ml of sterile anhydrous glycerol; vortex to make a homogenous solution; allocate 1.5 ml into 12-15 2ml test tubes for freezer stock and freeze all but 1 test tube @ -80° C; take 1 2ml test tube and add to 25 ml of fresh MRS broth media in a 50 ml test tube and incubate @ 37° C for 24°

↓

Day 4: Spin down bacteria via centrifugation with parameters: 10000 RPM, 4° C, and 15 minutes; aspirate media and wash x1 with sterile PBS; spin down again with same parameters and aspirate PBS; add 25 ml fresh MRS broth media and incubate @ 37° C for 24°

↓

Day 5: Repeat day 4 x 24°

↓

Day 6: Repeat day 5 x 15°

↓

Day 7: Transfer 10 ml of inoculated culture into a sterile flask with 250 ml fresh MRS broth media and place in 37° C incubator; take optical densities via a spectrometer every hour on the hour, including time zero, until maximum growth has been reached; once maximum growth (or late log phase) has been reached, do a second transfer again with 10 ml of the culture in 250 ml of media and incubate; after the allotted time for maximum growth has passed again, complete a third transfer but this time combine 20ml per 500 ml of fresh media for a total of 1L and incubate overnight

↓

Day 8: Allocate the 1L of inoculated culture into 50 ml tubes with approximately 35 ml per tube; spin down via centrifugation with same parameters as before; and wash x3 with sterile PBS; after the third wash, combine 10 test tubes in 1 by vortexing homogenously each time so that the freezer stock is a smaller quantity; also perform spread and pour plate methods using MRS agar media; do peptone serial dilutions for 10^{1}-10^{8}, but only use 10^{6}-10^{8} for the agar plates to count cells

↓

Day 10: Count cells using magnification and record result in lab notebook with 1 decimal place

↓

Additional Days: Irradiated Bacteria→Lyophilized bacteria in 1ml aliquots with speed vacuum→Calculated resuspension volume based on bacteria weight→freeze tubes at -80° C until needed for experimental analysis (0.1-1000 µg/ml)

Irradiation

After all eight bacterial strains were grown, all bacteria-PBS mixtures and bacterial supernatants were transported on dry ice to the Phoenix Laboratory at the University of Michigan. Mr. Robert Blackburn administered 1Mrad gamma irradiation for about 7 hours, rotating the conical tubes each hour. After the irradiation took place, all tubes were stored at -80°C until needed.

Lyophilization and Reconstitution of Bacteria

After irradiation, 1 ml of irradiated bacteria was allocated into 1.5 ml microcentrifuge tubes and speed vacuumed with a Servant speed vacuum (40 tubes per cycle and each cycle took about 5 hours). There were between 5 and 6 cycles per strain. After the bacteria were lyophilized, each tube was weighed on an analytical Mettler balance to get a bacterial weight. A test run of PBS was done prior to speed vacuum cycles to get an average PBS weight to be able to subtract from bacterial weight as well as pre-bacteria tube weight. Each tube was reconstituted with low serum (1%) RPMI media. A concentration of 40 mg/ml was obtained per tube so that the treatments can be calculated in replication each time and a weight per ml was calculated.

Cell Lines and Cell Culture Conditions

Two cell culture models were used to study the progression of neoplastic transformation in colonic epithelial cells. It is proposed that a specific set of non-tumorigenic conditionally immortal cell lines derived from C57/BL6 mice, YAMC (Young Adult Mouse Colon Cells $Apc^{+/+}$) and IMCE (Immortomouse-Min colonic epithelial cells $Apc^{Min/+}$) cells developed by Dr. Robert Whitehead (Ludwig Institute for Cancer Research, Melbourne, Australia), have yielded important findings on early events

Table 3.1: Bacterial Growth Parameters as Optical Density and Colony Forming Units (CFU), as well as Weight per Volume (μg/ml) of Reconstituted Irradiated Samples

Quantitation of Bacterial Growth Amount per Milliliter			
Bacterial Strain	**Optical Density (650 nm)**	**Cfu/ml**	**Bacteria Weight (µg/ml)***
Eschericia coli O157:H7	1.743	2.7×10^{10}	3,497µg/ml
Lactobacillus reuteri	1.740	7.3×10^{9}	2,656µg/ml
Lactobacillus rhamnosus GG	1.188	2.2×10^{9}	**
Lactobacillus salivarius	1.659	4.8×10^{10}	2,921µg/ml
Lactobacillus plantarum	1.877	2.6×10^{10}	2,790µg/ml
Lactobacillus paracasei	1.563	2.7×10^{10}	2,225µg/ml
Bifidobacterium breve	1.683	3.0×10^{10}	3,091µg/ml
Bifidobacterium lactis	1.780	3.0×10^{10}	2,511µg/ml

*Denotes 1ml aliquoted dried bacteria sample and $1/10^{th}$ original volume of bacteria

**Denotes our guinea pig strain: Only grew 750ml vs. 2L and total weight is inaccurate due to freezer stock technique

in colorectal neoplasia development (Fenton et al. 2006). The cell lines will serve as a model to examine the effect of dietary compounds for colon cancer prevention on early preneoplastic stage with a mutation in the *Apc* gene, the gatekeeper gene of colon cancer. These cells are consistent with the normal to preneoplastic transition observed in colon epithelial carcinogenesis. YAMC cells mimic normal colonic epithelial cells while IMCE mimic preneoplastic colonic epithelial cells. We have characterized phenotypic changes in the IMCE cells that are consistent human preneoplastic lesions, such as iNOS and IL-6 expression.

Cells were grown on 75cm^2 (T-75) flasks (Fischer, Pittsburgh, PA) coated with type I rat tail collagen (5μg/cm^2) (BD Biosciences, San Diego, CA) at 33°C until they reached 80-100% confluency. Complete RPMI media (500mL RPMI 1640 media supplemented with 25ml newborn calf serum, 7mL pen-strep antibiotic, 5ml of insulin transferrin selenium (ITS), and 25μl of IFN-gamma) is the media that was used for carrying and splitting the cells. When the cells reached 100% confluency, they were detached from the flask using Trypsin-EDTA (Sigma, St. Louis, MO) and harvested by centrifugation (2000 RPM for 5 minutes).

For experimental purposes, one confluent flask was split into either 24-well plates (Falcon, San Jose, CA) coated with 5μg/cm^2 type I rat tail collagen (BD Biosciences, San Jose, CA) with one ml of complete RPMI media per well or coated 96 well plates (Sigma, St. Louis, MO) with 200 μl per well. All flasks and plates prior to treatment administration were held in the 33°C incubator until they reached confluency. Once the plates were confluent at 80%, they were transferred over to 39°C incubator with low serum RPMI (1%) non-permissive (without IFN-γ) media and the next day specified

treatments were administered. The cells were grown at 33° C, which is the temperature in which the temperature sensitive SV40 large T antigen is active. When the cells reached near 80-90% confluence, they were moved over to 39° C incubator for 24 hr in which they SV40 large T antigen protein becomes inactive.

Treatments

Cells were treated with various concentrations of irradiated bacteria (*O157: H7*: 1000 μg/ml to 0.5 μg/ml; *L. rhamnosus GG, L. reuteri, L. salivarius, L. paracasei, L. plantarum, B. breve,* and *B. lactis*: 1000 μg/ml to 25 μg/ml) or bacterial components at various concentrations. Flagellin (TLR-5 ligand), lipopolysaccharide (LPS) (TLR-4 ligand,) peptidoglycan (PGN) (TLR-2 ligand), murabutide (Nod II), stimulatory and inhibitory ODN (TLR-9 ligand), and E.coli ssDNA (TLR-9 ligand) were the bacterial components that were analyzed; all of the TLR-ligands were obtained from Invivogen (San Diego, CA). Cells were also treated with co-treatments of bacteria, bacterial components, monoclonal antibodies of TLR, NF-kB inhibitor (PDTC) (TOCRIS, Ellisville, MO), or pre-treated with LiCl. The cotreatments of *O157: H7* with LS, LGG, BB, or BL consisted of ratios of 1:1, 2:1, or 20:1 (500 μg/ml of *O157: H7* with either 500 μg/ml, 250 μg/ml, or 25 μg/ml of probiotic bacterial strain respectively). After the cells were in the 39° C incubator with non-permissive RPMI media for approximately 24 hr, the treatments were applied. Low-serum (1%) non-permissive RPMI media was used as a negative control for treatment analysis.

Pooling and Storing Treatment Samples

Approximately 48 hr later after treatments were administered; the treatments were pooled and collected in either 1.5 ml microcentrifuge tubes or 15 ml test tubes. The 1.5

Table 3.2: List of Probiotic and Pathogenic Bacteria Used

Bacteria Genus and Species	Pathogen	Probiotic	Bacterial Cell Wall Classification	Company
Lactobacillus reuteri	-	+	Gram Positive	ATCC
Lactobacillus rhamnosus GG	-	+	Gram Positive	ATCC
Lactobacillus plantarum	-	+	Gram Positive	Danisco
Lactobacillus salivarius	-	+	Gram Positive	Danisco
Lactobacillus paracasei	-	+	Gram Positive	Danisco
Bifidobacterium breve	-	+	Gram Positive	Danisco
Bifidobacterium lactis	-	+	Gram Positive	Danisco
Escherichia coli O157: H7	+	-	Gram Negative	ATCC

ml tubes allowed for minimization of numerous freezing, thawing, and refreezing episodes for experimental analysis. NO and IL-6 production were quantitatively analyzed as well as the MTT assay being performed to assess cell viability for the treatments. The samples were stored in -20° C freezer for additional analyses if necessary.

Experimental Analyses: Nitric Oxide

NO was quantified using the Griess reaction. Nitrite, a stable end product of NO metabolism, was measured in conditioned media using the Griess reaction and sodium nitrate (J.T. Baker, Phillipsburg, NJ) as a standard. 150 μl of standard was inserted into top and bottom left wells and serial dilution (1:2) was performed eight times (112-0.875 μM) in media. 75 μl of samples were added in quadruplicate to the 96 well plates and 75 μl of media was added to top and bottom right wells to serve as blanks; 75 μl of NO reagent was then added to each well of the 96 well plates. The NO reagent consisted of 0.5g sulfanilamide (Sigma, St. Louis, MO), 0.05g N-1napthylethylendiamide hydrochloride (Sigma) in 37.5 ml of deionized water and 12.5ml of concentrated phosphoric acid (J.T. baker). Absorbance was read on a Spectra Max® 300 plate reader (Molecular Devices, Sunnyvale, CA at 540nm. Results were expressed as μM of nitrite per well.

Experimental Analyses: IL-6

IL-6 was measured using a commercially available enzyme linked immunosorbent assay (ELISA) (BD Biosciences, San Diego, CA). Nunc Maxisorp 96 well plates (BD Biosciences, San Diego, CA) were coated overnight with anti-mouse IL-6 capture antibody in coating buffer (10 ml coating buffer per two 96-well plates + 40 μl of capture antibody). After the overnight plate coating, the plates were washed three

times with wash buffer (1L equals 900 ml deionized water, 100ml PBS 10x, 500 μl of Tween 20, discarding in the sink post wash. The plates were then blocked with 300 μl per well of assay diluent (fetal bovine serum (FBS) and PBS 1x solution) for 1 hr at room temperature. During that hour incubation, standards and samples were prepared. The standards were serial diluted (1000 pg/ml to 0 pg/ml). Since both pathogen and probiotic bacteria caused CEC's to produce increased IL-6, each sample excluding controls were diluted 1:10 with assay diluent. After the hour incubation of blocking, each standard and sample was placed at 50 μl per well in duplicate or quadruplicate (to minimize error bar) onto the 96 well plate according to IL-6 ELISA layout. The plates were incubated for 2 hr at room temperature. The plates were washed with wash buffer five times. After the series of 5 washes, the working detector (10 ml of assay diluent + 40 μl of IL-6 detection antibody + 40 μl of enzyme) was added to each well at 50 μl per well and incubated for 1 hr at room temperature. The plates were then washed 7x to allow for thorough washing with wash buffer. TMB substrate (Neogen, Lansing, MI) was then added at 100 μl per well and incubated for 30 min at room temperature in the dark to allow for color change. The darker the color, the more IL-6 the treatment caused the CEC's to produce. Stop solution (1M H_2PO_4) was added at 100 μl per well to stop the reaction. Absorbance was read using SpectraMax® 300 plate reader (Molecular Devices Sunnyvale, CA). IL-6 results are expressed as pg/ml.

Inhibition of NF-kB and TLR Expression To Decrease Pro-inflammatory Mediators

Inhibitors or antibodies were used to assess the mechanisms by which the production of inflammatory mediators was modified in the two CEC cell lines. Anti-TLR antibodies against TLR-2 and TLR-4 activity were used to assess the TLR expression on

the CEC's to allow for inflammatory production. Polymyxin B (PMB) (Invitrogen, Carlsbad, CA) was used to further assess TLR-4 action on inflammatory mediator production. Pyrrolidinedithiocarbamate ammonium (PDTC) (TOCRIS, Ellisville, MO) was used at 10 _M to inhibit NF-kB translocation. It was used to assess the effect that the bacterial treatments had on NF-kB activation on NO and IL-6 production in the two cell lines. LiCl 10 mM was used to assess how cytosolic ß-catenin ties up NF-kB prohibiting it from translocating to the nucleus to allow for gene transcription.

Macrophage Activation

RAW 264.7 murine macrophages (Dr. James Petska, Michigan State University; East Lansing, MI) were grown on non-collagen coated petri culture plates with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomyocin (pen-strep) antibiotic. The cells were either re-fed with fresh complete DMEM medium warmed to 37°C or split. Macrophages were harvested with a cell culture scraper vs. detachment with trypsin for epithelial cells. When the macrophages were split, they were either split into dishes for carrying purposes or into 24-well plates for experimental purposes. Supernatants from treated CEC's from both cell lines were exposed to macrophages on 24-well plates. One ml from bacterial treated epithelial supernatants was applied to non-coated 24-well plates. Pro-inflammatory mediators NO and IL-6 were then quantitatively measured to assess macrophage activation and chemotaxis.

Using supernatants from YAMC and IMCE epithelial cells facilitates macrophage activation experiments. Prior to adding 1ml of epithelial supernatants per well of uncoated 24-well plates, the supernatants were spun down via centrifugation. Both NO

and IL-6 were measured to analyze macrophage activation. The proposed goal of macrophage activation component of the research project is to see if certain probiotic bacteria can modify the induced effect of *O157: H7* on macrophage activation.

Macrophage Chemotaxis

RAW 264.7 murine macrophage cells were cultured in DMEM medium supplemented with 10% FBS and pen-strep antibiotic in a 5% CO_2-humidifed incubator at 37°C. Macrophage cell number was assessed by trypan blue dye exclusion using a hematocytometer. Cells were collected and prepared as per manufacturer instructions for the QCM™ chemotaxis (8μM) cell migration assay (Chemicon, Temecula, CA, USA). Briefly, 40000 RAW cells were seeded in the upper chamber of the provided 96-well plates. The lower chambers were filled with supernatants from the IMCE or YAMC treated cells. The plates were incubated overnight to allow for RAW 264.7 cell migration through the pores and into the lower chamber or to the outside bottom of the chamber. Any cells attached to the outside of the chamber were detached using the provided detachment buffer and collected according to manufacturer instructions. Any cells present were detected using a fluorescent compound activated by non-specific enzymes in live cells (provided with the kit). The plate was read at an excitation wavelength of 485nm and emission wavelength of 530nm using a Cytofluor fluorescent plate reader (Millipore, Bedford, MA, USA) and data were analyzed. Note that two-way ANOVA with Bonferoni's comparative analysis was used to analyze the macrophage chemotaxis data.

Table 3.3: Experimental Analyses

Nitric Oxide Assay	⌑ Measure nitrite, a stable end product of NO metabolism ⌑ Measure in conditioned media using Greiss reaction and NaNO3 as a standard ⌑ Absorbance read at 540 nm
MTT Assay	⌑ Measured cell viability ⌑ Absorbance read at 540 nm ⌑ Results calculated based on control readings ⌑ Baseline control is 100%
IL-6 ELISA	⌑ Plates were coated 24 hours prior to running ELISA with capture antibody ⌑ Measured at 450nm and 570nm to get wavelength correction ⌑ Run in duplicate per treatment (n of 4 common to cut down on error bar) ⌑ Compared to stock standard curve
Macrophage Activation	⌑ Measure nitrite, a stable end product of NO metabolism ⌑ Measure in conditioned media using Greiss reaction and NaNO3 as a standard ⌑ Absorbance read at 540 nm
Macrophage Chemotaxis	⌑ The Chemicon QCM™ 96-well 5_m Migration Assay ⌑ Measures chemotaxis cell migration ⌑ The 96-well insert and fluorescence detection format allows for large-scale screening and quantitative analysis of multiple samples ⌑ Read with a fluorescence plate reader using

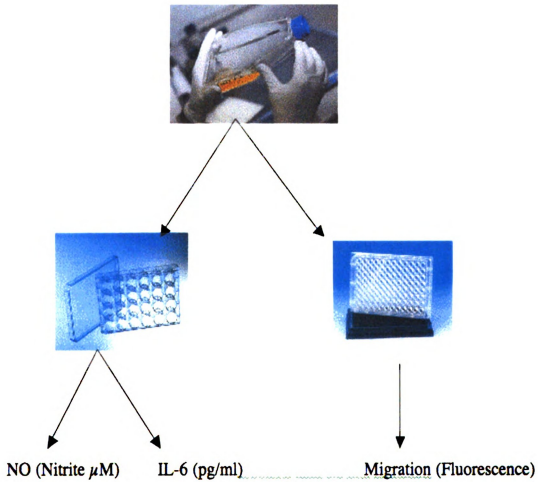
Cell Viability

3-(4, 5 dimethylthiazol-2_l)- 2, 5 diphenyl tetrazolium bromide (MTT) was used to measure cell viability. After each treatment was collected and pooled into labeled tubes, each well in the 24-well plate was washed with 1X PBS. One ml of 1% serum non-permissive RPMI media was added to each well followed by 100_l of MTT reagent was added to each well of the plates (in low light) and incubated at 33°C overnight. The following day, the wells were aspirated and 500 μl of dimethyl sulfoxide (DMSO). The plates sat at room temperature to allow for crystal detachment and 100 μl aliquots were added to a 96-well plate according to MTT assay layout. The plate was then read at 570nm using the SpectraMax 300 plate reader (Molecular Devices, Sunnyvale, CA). Results were calculated using a negative control as 100% cell viability.

Statistical Analysis

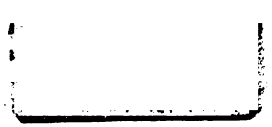
Experiments were run in quadruplicate or n of 8 for NO assay and in duplicate or quadruplicate for IL-6 ELISA. Data was analyzed using PRISM (Graphpad software, San Diego, CA). One-way or two-way ANOVA was used with Bonferonni posttests to compare treatments within experiments. A p-value less than or equal to 0.05 was used as the level of significance.

Figure 3.2 Procedure used for exposure of bacterial treated YAMC and IMCE supernatants to macrophages for macrophage activation and/or macrophage chemotaxis



CHAPTER 4

RESULTS



CHAPTER 4

RESULTS

4.1 Effect of *O157: H7* on proinflammatory mediator production (NO and IL-6) in YAMC and IMCE cells

We hypothesized that upon exposing YAMC and IMCE cells to *O157: H7*, pro-inflammatory mediator production of NO and IL-6 will increase in a concentration-dependent fashion. Irradiated *O157: H7* was incubated with either YAMC cells or IMCE cells for 24 hr. at various concentrations (50, 100, 250, 500, or 1000 μg/ml). Treatments included control low serum RPMI medium and *O157: H7* doses. The supernatants from the subsequent treatments were collected and pooled after 24 hr and analyzed. Nitric oxide (provided as μM of nitrite) was measured using the Greiss reaction. Interleukin-6 (IL-6 provided as pg/ml) was represented as a duplicate measured by ELISA.

O157: H7 induced NO production in a concentration-dependent manner in both cell types (p-value <0.001; Figure 4.1 and 4.2). IMCE and YAMC cells produced similar amounts of NO over 24 hr when treated with *O157: H7*. The 24 hr time period was chosen for *O157: H7* treatment as NO production for the highest dose of *O157: H7* (1000 μg/ml) decreased cell viability. The cell viability of both cell types, IMCE and YAMC, was consistent between concentrations with viability being 90% of control per treatment (Figure 4.3 and 4.4). IMCE cells produced a more significant amount of IL-6 compared to YAMC cells (p-value <0.001) (Figure 4.5 and 4.6) over 24 hr.

Figure 4.1 NO (Nitrite μM) production of YAMC cells treated with varying doses of *O157: H7* (μg/ml) for 24 hr. a-Different compared to control $p<0.001$. b-Different compared to *O157: H7* of 1000 μg/ml $p<0.001$

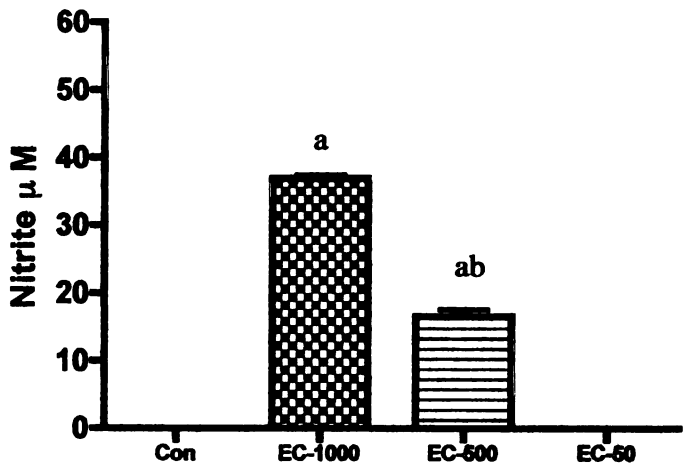


Figure 4.2 NO (Nitrite μM) production of IMCE cells treated with varying doses of *O157: H7* (μg/ml) for 24 hr. a-Different compared to control $p<0.001$ and b-Different compared to EC-1000 $p<0.001$

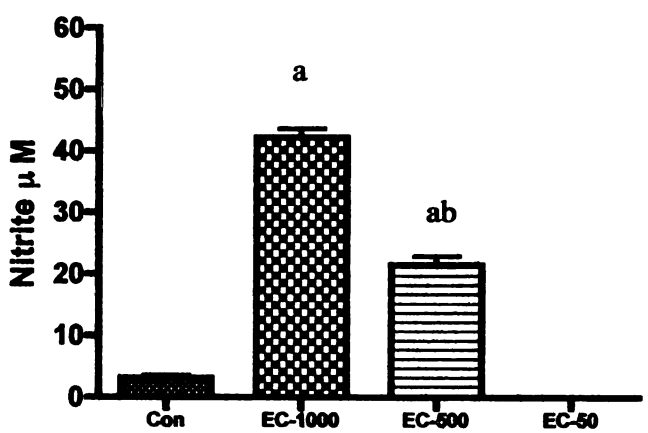


Figure 4.3 Representative cell viability compared to control of YAMC cells treated with varying doses of *O157: H7* (μg/ml) for 24 hr

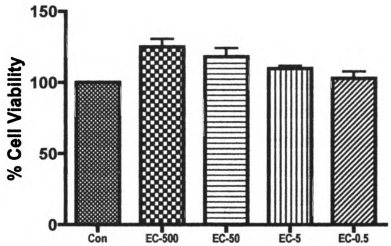


Figure 4.4 Representative cell viability compared to control of IMCE cells treated with varying doses of *O157: H7* (μg/ml) for 24 hr

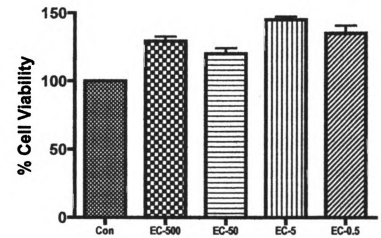


Figure 4.5 IL-6 production of YAMC cells treated with varying doses of *O157: H7* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* of 500 μg/ml (p-value <0.001)

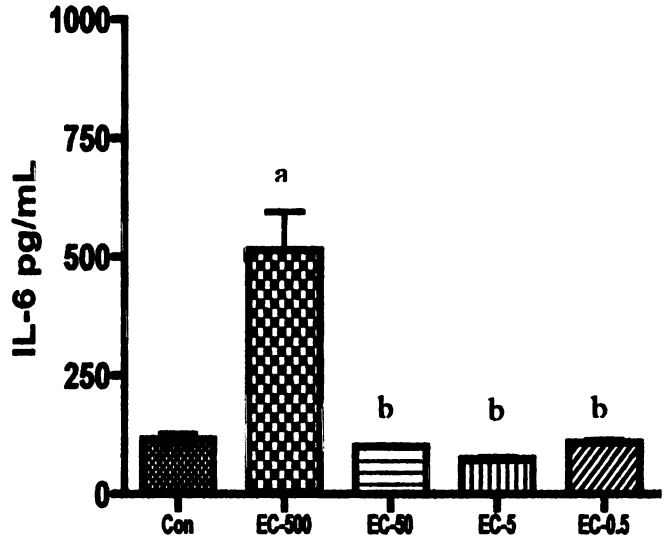
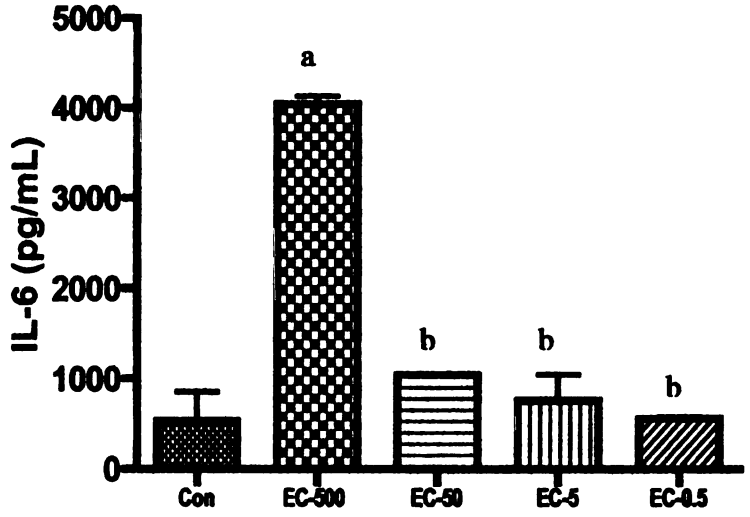


Figure 4.6 IL-6 production of IMCE cells treated with varying doses of *O157: H7* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* of 500 μg/ml (p-value <0.001)



4.2 Effect of co-treating *O157: H7* with probiotic bacteria on proinflammatory mediator production (NO and IL-6) in YAMC and IMCE cells

We hypothesized that upon co-exposing YAMC and IMCE cells to *O157: H7* and probiotic bacteria, pro-inflammatory mediator production of NO and IL-6 will be attenuated. Irradiated *O157: H7* was co-exposed with probiotic bacterial strains BB, LGG, LS, or BL and treated with either YAMC cells or IMCE cells for 48 hr. Treatments included control low serum RPMI medium and exposure to different concentrations of BB, BL, LS, or LGG (500 μg/ml, 250 μg/ml, or 25 μg/ml) co-treated with 500 μg/ml of *O157: H7*. The supernatants from the subsequent treatments were collected and pooled after 48 hr and analyzed for NO and IL-6 production.

NO production induced by *O157: H7* was reduced by probiotic bacterial strains BB, BL, LS, and LGG in a concentration-dependent manner as well as a genus- and species-dependent manner in both cell types (Figure 4.7 through 4.14). The cell viability among cell type and within treatments was consistently within 90% of control (Figure 4.15 and 4.16). No treatment appeared to adversely affect cell viability. IL-6 production induced by *O157: H7* was reduced by the probiotic bacterial strains BB, BL, LS, and LGG in a genus- and species-dependent manner; there was no evidence of a consistent concentration-dependent decrease in IL-6 production due these probiotic bacterial strains (Figure 4.17 through 4.24). We had also surveyed the production of NO and IL-6 upon exposing YAMC and IMCE cells to the probiotic bacterial strains BB, BL, LS, and LGG alone. No probiotic bacterial strain alone induced NO production; however, the probiotic bacterial strains did induce IL-6 production in a genus- and species-specific dependent manner with a greater significance in IMCE cells compared to YAMC cells (p-value

<0.001) and bifidobacterium species compared to lactobacillus species (p-value <0.001). The data for treating YAMC and IMCE cells with the probiotic bacterial strains BB, BL, LS, and LGG alone can be found in Appendix D.

Figure 4.7 NO (Nitrite μM) production of YAMC cells treated with *O157: H7* (500 μg/ml) and cotreatments *of O157: H7* and *BL* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* (p-value <0.001)

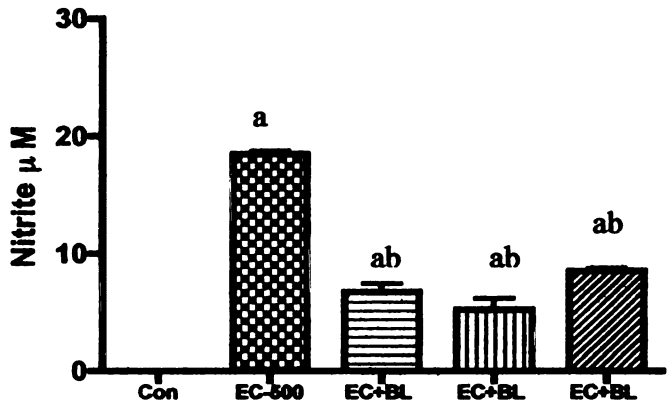
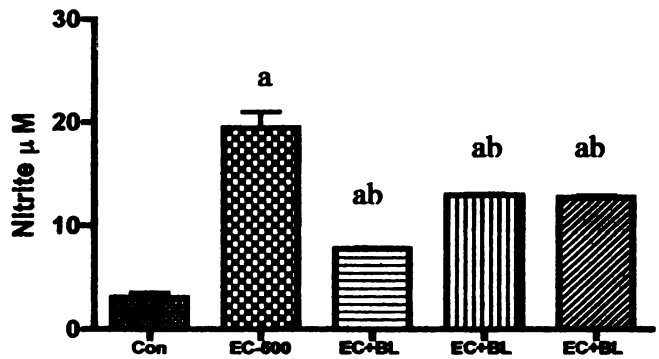


Figure 4.8 NO (Nitrite μM) production of IMCE cells treated with *O157: H7* (500 μg/ml) and cotreatments of *O157: H7* and *BL* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.01). b-Different compared to *O157: H7* (p-value <0.001)



EC 500	-	+	+	+	+
BL 500	-	-	+	-	-
BL 250	-	-	-	+	-
BL 25	-	-	-	-	+

Figure 4.9 NO (Nitrite μM) production of YAMC cells treated with *O157: H7* (500 μg/ml) and cotreatments of *O157: H7* and *LGG* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* (p-value <0.001)

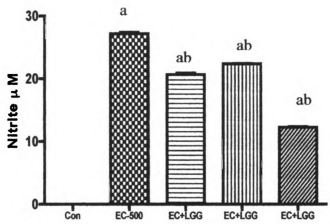
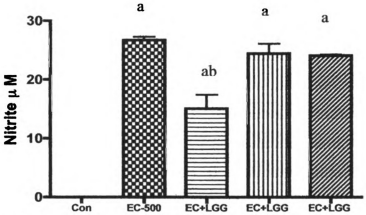


Figure 4.10 NO (Nitrite μM) production of IMCE cells treated with *O157: H7* (500 μg/ml) and cotreatments of *O157: H7* and *LGG* (μg/ml) for 48 hr. a-Different compared to control (p-value<0.001). b-Different compared to *O157: H7* (p-value <0.001)



EC 500	-	+	+	+	+
LGG 500	-	-	+	-	-
LGG 250	-	-	-	+	-
LGG 25	-	-	-	-	+

Figure 4.11 NO (Nitrite μM) production of YAMC cells treated with *O157: H7* (500 μg/ml) and cotreatments of *O157: H7* and *LS* (μg/ml) for 24 hr. a- Different compared to control (p-value <0.001). b-Different compared to *O157: H7* (p-value <0.001)

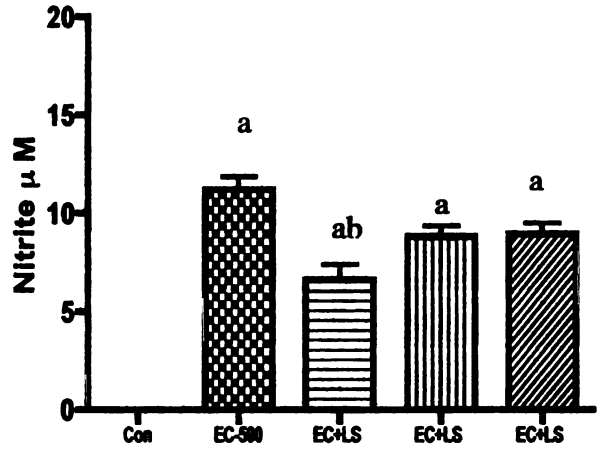
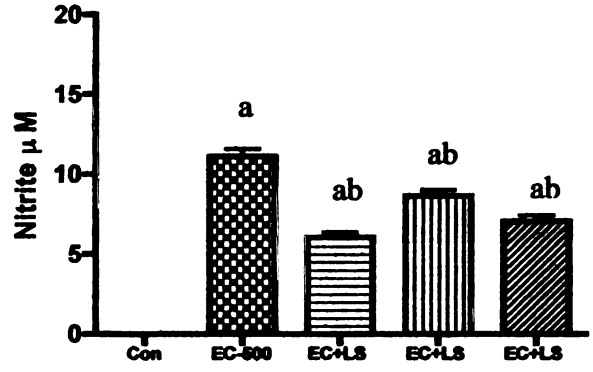


Figure 4.12 NO (Nitrite μM) production of IMCE cells treated with *O157: H7* (500 μg/ml) and cotreatments of *O157: H7* and *LS* (μg/ml) for 24 hr. a- Different compared to control (p-value <0.001). b- Different compared to *O157: H7* (p-value <0.01)



EC 500	-	+	+	+	+
LS 500	-	-	+	-	-
LS 250	-	-	-	+	-
LS 25	-	-	-	-	+

Figure 4.13 NO (Nitrite μM) production of YAMC cells treated with *O157: H7* (500 μg/ml) and cotreatments of *O157: H7* and *BB* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* (p-value <0.001)

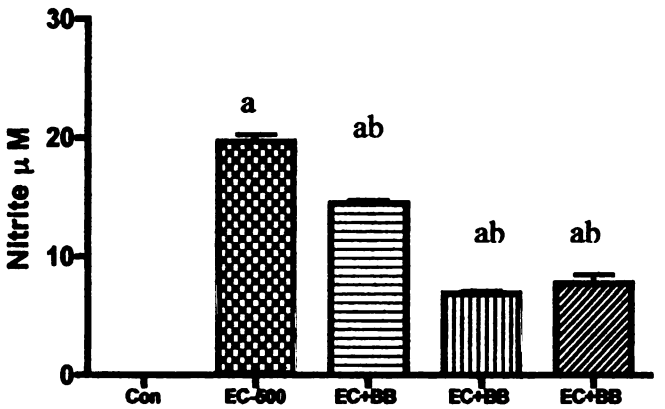
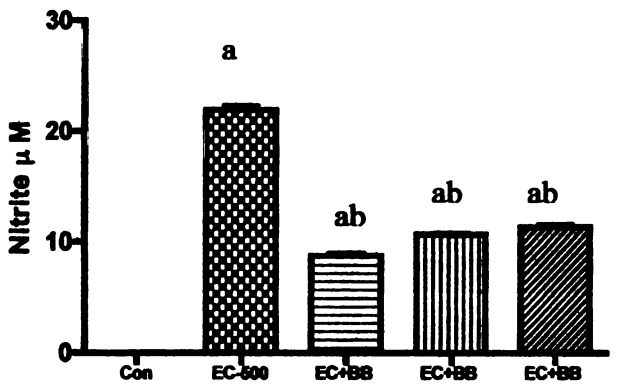


Figure 4.14 NO (Nitrite μM) production of IMCE cells treated with *O157: H7* (500 μg/ml) and cotreatments of *O157: H7* and *BB* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* (p-value <0.001)



EC 500	-	+	+	+	+
BB 500	-	-	+	-	-
BB 250	-	-	-	+	-
BB 25	-	-	-	-	+

Figure 4.15 Representative cell viability compared to control of YAMC cells cotreated with *O157: H7* and probiotic bacterial strains *LGG*, *BL*, *BB*, or *LS* (μg/ml) for 48 hr

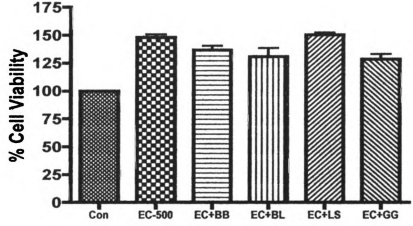
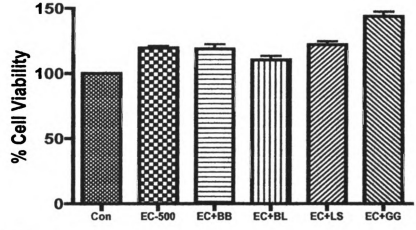


Figure 4.16 Representative cell viability compared to control of IMCE cells cotreated with *O157: H7* and probiotic bacterial strains *LGG*, *BL*, *BB*, or *LS* (μg/ml) for 48 hr



EC 500	-	+	+	+	+	+
BB 500	-	-	+	-	-	-
BL 500	-	-	-	+	-	-
LS 500	-	-	-	-	+	-
LGG 500	-	-	-	-	-	+

Figure 4.17 IL-6 (pg/ml) production of YAMC cells with *O157: H7* (500 µg/ml) and cotreatments of *O157: H7* and *BL* (µg/ml) for 48 hr. a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* (p-value <0.001)

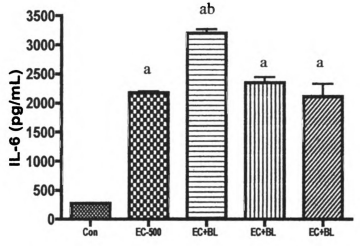
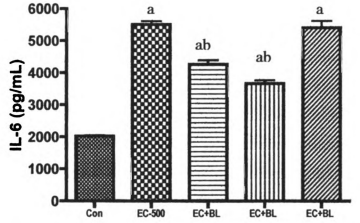


Figure 4.18 IL-6 (pg/ml) production of IMCE cells with *O157: H7* (500 µg/ml) and cotreatments of *O157: H7* and *BL* (µg/ml) for 48 hr. a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* (p-value <0.01)



EC 500	-	+	+	+	+
BL 500	-	-	+	-	-
BL 250	-	-	-	+	-
BL 25	-	-	-	-	+

Figure 4.19 IL-6 (pg/mL) production of YAMC cells with *O157: H7* (500 μg/ml) and cotreatments of *O157: H7* and *LGG* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* (p-value <0.001)

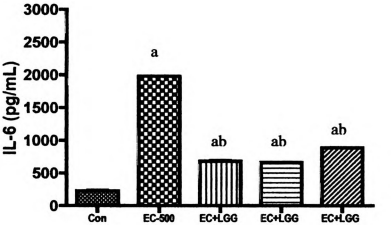
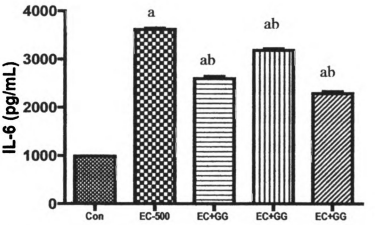


Figure 4.20 IL-6 (pg/mL) production of IMCE cells with *O157: H7* (500 μg/ml) and cotreatments of *O157: H7* and *LGG* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* (p-value <0.01)



EC 500	-	+	+	+	+
LGG 500	-	-	+	-	-
LGG 250	-	-	-	+	-
LGG 25	-	-	-	-	+

Figure 4.21 IL-6 (pg/mL) production of YAMC cells with *O157: H7* (500 μg/ml) and cotreatments of *O157: H7* and *LS* (μg/ml) for 24 hr. a-Different compared to control (p-value <0.001)

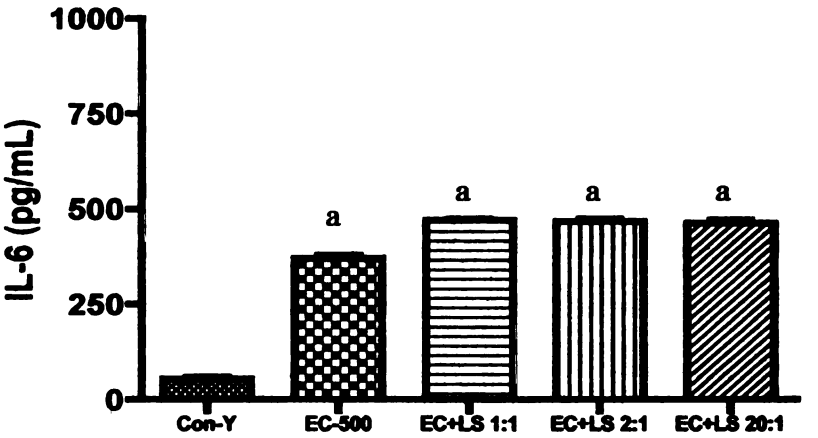
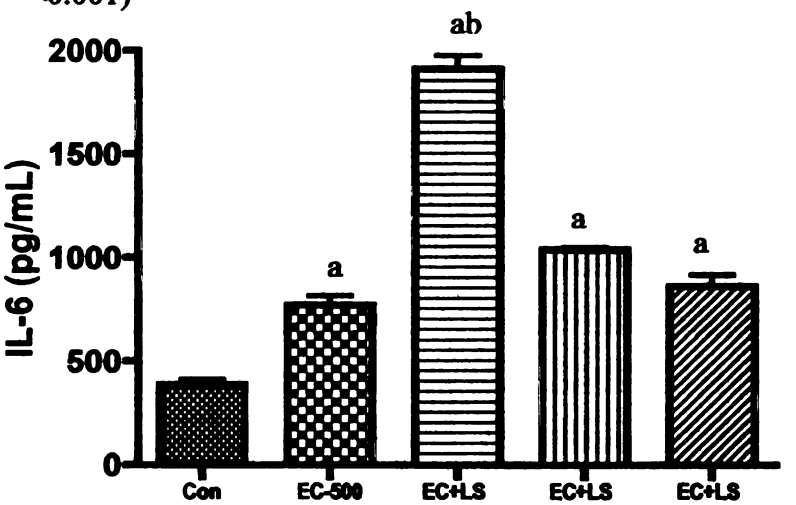


Figure 4.22 IL-6 (pg/ml) production of IMCE cells with *O157: H7* (500 μg/ml) and cotreatments of *O157: H7* and *LS* (μg/ml) for 24 hr. a-Different compared to control (p-value <0.01). b-Different compared to *O157: H7* (p-value <0.001)



EC 500	-	+	+	+	+
LS 500	-	-	+	-	-
LS 250	-	-	-	+	-
LS 25	-	-	-	-	+

Figure 4.23 IL-6 (pg/ml) production of YAMC cells with *O157: H7* (500 μg/ml) and cotreatments of *O157: H7* and *BB* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.01). b-Different compared to *O157: H7* (p-value <0.001)

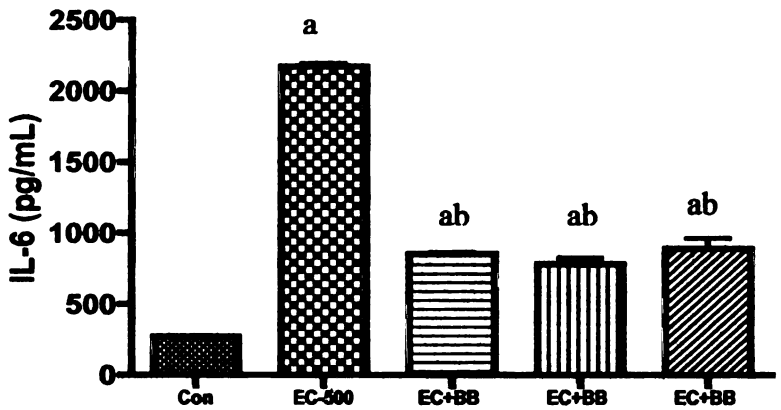
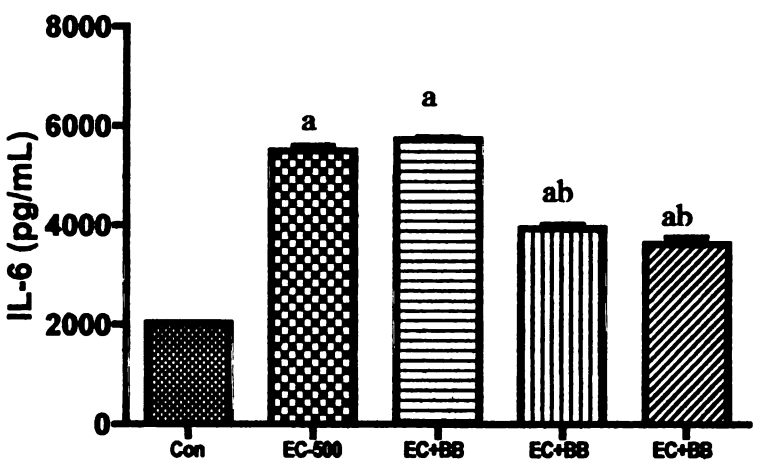


Figure 4.24 IL-6 (pg/ml) production of IMCE cells with *O157: H7* (500 μg/ml) and cotreatments of *O157: H7* and *BB* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* (p-value <0.001)



EC 500	-	+	+	+	+
BB 500	-	-	+	-	-
BB 250	-	-	-	+	-
BB 25	-	-	-	-	+

4.3 Effect of toll-like receptor 2, 4, 5, and 9 ligands on pro-inflammatory mediator production (NO and IL-6) in YAMC and IMCE cells

We hypothesized that bacterial treatments of YAMC and IMCE cells would result in activation of specific toll-like receptors. We exposed YAMC and IMCE cells to toll-like receptor 2 ligand (peptidoglycan), toll-like receptor 4 ligand (lipopolysaccharide), toll-like receptor 5 ligand (flagellin), and toll-like receptor 9 ligand (stimulatory and inhibitory CpG containing oligonucleotides). We had postulated that toll-like receptor 5 engagement by flagellin, a bacterial component identified as a TLR-ligand, may be responsible for the pro-inflammatory mediator production caused by bacteria. Since flagellin did not induce neither NO or IL-6 (data not shown), a western blot was performed to confirm the presence of toll-like receptor 2, 4, 5, and 9 protein in YAMC and IMCE cells (Figure 4.25). The western blot showed that both YAMC and IMCE cells had receptors to toll-like receptors 2, 4, and 9, but not to toll-like receptor 5. Ligands to TLR-2, TLR-4, and TLR-9 induced little NO production (Data shown in Appendix E); however, these ligands did induce IL-6 production, with a greater significance in IMCE cells compared to YAMC cells (p-value <0.001) (Figure 4.26 and 4.27).

Because literature has shown that probiotics do not block pro-inflammatory mediator production in TLR-9 knock out mice (Rachmilewitz, 2004), this led us to survey co-exposure of *O157: H7* with stimulatory and inhibitory ligands to TLR-9. Both stimulatory and inhibitory TLR-9 ligands blocked *O157: H7* induced IL-6 production (p-value <0.001) (Figure 4.28 and 4.29), but did not block NO production (Data shown in Appendix E) compared to *O157: H7* treatment. Upon exposing YAMC and IMCE cells

to stimulatory, but not inhibitory, TLR-9 ligands resulted in increased IL-6 production (p-value <0.001).

To gain further insight on how *O157: H7* is inducing NO and IL-6 production on YAMC and IMCE cells, monoclonal antibodies against TLR-2 and TLR-4 as well as polymyxin B (a chemical inhibitor of TLR-4 activity) were co-exposed with *O157: H7* and NO, MTT, and IL-6 were analyzed. Exposure of YAMC and IMCE cells to cotreatments of *O157: H7* and either monoclonal antibody against TLR-2 or TLR-4 resulted in a decreased production of NO in both cell types in a concentration-dependent manner (p-value < 0.001) (Figures 4.30 to 4.31). The cell viability was similar in both cell types and no treatment appeared to adversely affect cell viability (Figure 4.32 and 4.33). Exposure of IMCE cells to cotreatments of *O157: H7* and either TLR-2 or TLR-4 antibodies resulted in decreased production of IL-6 compared to *O157: H7* treatment (p-value <0.01), but not in YAMC cells (Figures 4.34 and 4.35).

Exposure of YAMC and IMCE cells to cotreatments of *O157: H7* and polymyxin B (inhibitor of TLR-4 signaling) for 48 hr resulted in decreased NO production (p-value <0.001) in both cell types (Figure 4.36 and 4.37). No treatment appeared to adversely affect cell viability (Figure 4.38 and 4.39). Exposure of YAMC cells and IMCE cells to cotreatments of *O157: H7* and polymyxin B for 48 hr resulted in decreased IL-6 production compared to *O157: H7* treatment (p-value <0.001) in both cell types (Figure 4.40 and 4.41).

We also co-exposed YAMC and IMCE cells to *O157: H7* and 10 μM of pyrrolidine dithiocarbamate (PDTC, an inhibitor of kappa kinase (IkK) that blocks *O157: H7*-dependent NF-kB activation) as NF-kB is activated by various toll-like receptor

ligands to produce NO and IL-6. Co-exposure of 10 μM PDTC with *O157: H7*, but not co-exposure of PDTC and cotreatment of *O157: H7* with BB to YAMC and IMCE cells resulted in a decreased production of NO (p-value <0.001) in both cell types (Data shown in Appendix F). Cell viability when compared to control was similar when comparing no PDTC cotreatment to 10 μM PDTC cotreatment. No treatment adversely affected cell viability except for those cotreated with 100 μM of PDTC.

Co-exposure of 10 μM PDTC and *O157: H7* to YAMC cells and IMCE cells did not result in a statistically significant decrease in IL-6 production in either cell type (p>0.05) when compared to *O157: H7* of 500 μg/ml. PDTC did not decrease IL-6 production when cotreating with BB, but rather increased IL-6 production in both cell types. PDTC reduced IL-6 production when cotreating with *O157: H7* and BB cotreatment in YAMC cells cell but not IMCE cells (p-value <0.05). All data for PDTC experiments can be found in Appendix F.

Figure 4.25 Western blot of total cell lysate of both YAMC and IMCE cells. Both cell types observed have receptors for TLR-2, TLR-4, TLR-9, but not TLR-5

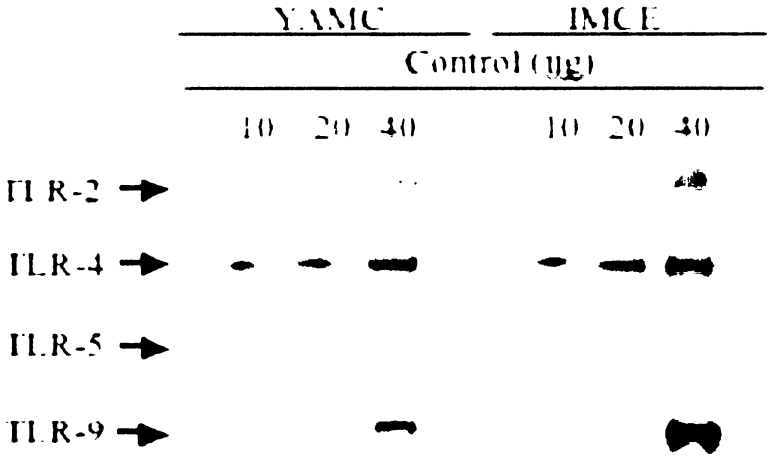


Figure 4.26 IL-6 (pg/ml) production of YAMC cells with peptidoglycan (TLR-2 ligand), lipopolysaccharide (TLR-4 ligand), and stimulatory CpG containing Oligonucleotide (TLR-9 ligand) for 24 hr. a-Different compared to control (p-value <0.05)

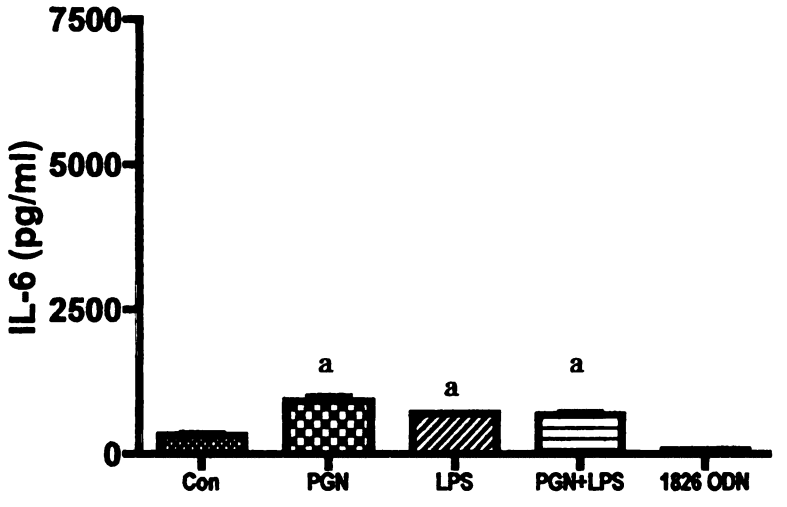


Figure 4.27 IL-6 (pg/ml) production of IMCE cells with peptidoglycan (TLR-2 ligand), lipopolysaccharide (TLR-4 ligand), and stimulatory CpG containing Oligonucleotide (TLR-9 ligand) for 24 hr. a-Different compared to control (p-value <0.01)

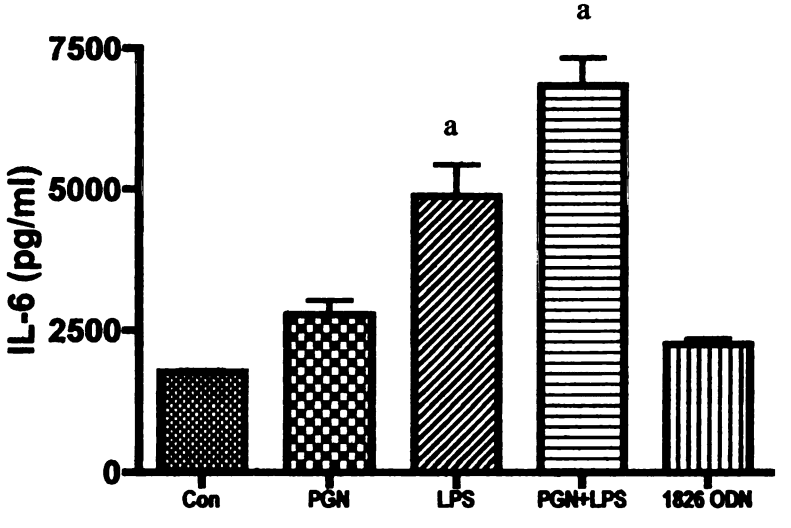


Figure 4.28 IL-6 (pg/ml) production of YAMC cells exposed to *O157: H7* (cotreated with stimulatory and inhibitory TLR-9 ligands for 24 hr. a-Different compared to control (p-value <0.01). b-Different compared to *O157: H7* at 500 μg/ml (p-value <0.001)

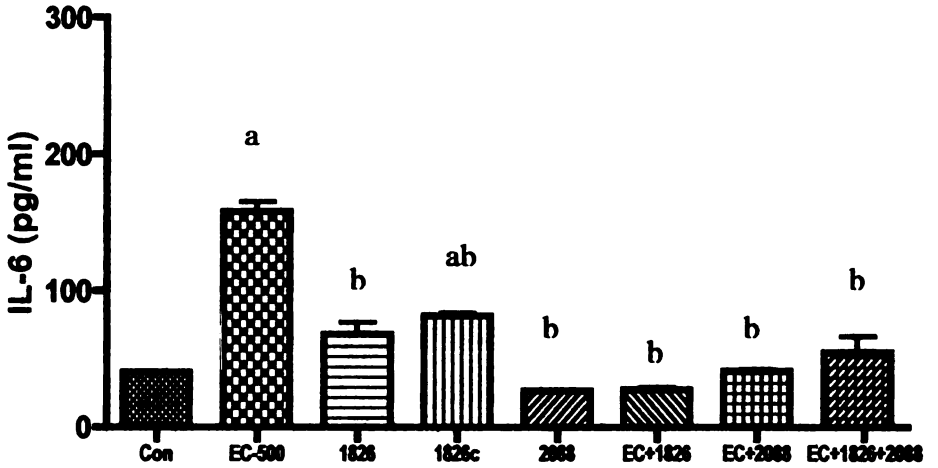


Figure 4.29 IL-6 (pg/ml) production of IMCE cells exposed to *O157: H7* cotreated with stimulatory and inhibitory TLR-9 ligands for 24 hr. a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* at 500 μg/ml (p-value <0.05)

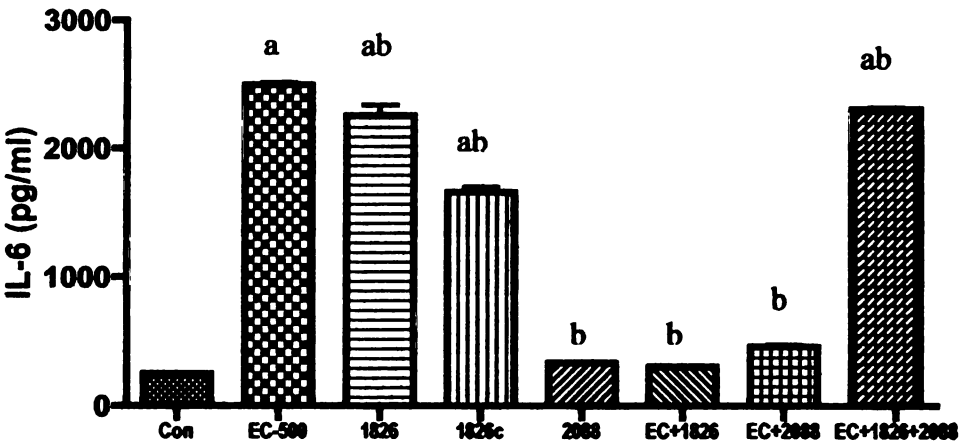


Figure 4.30 NO (Nitrite μM) production of YAMC cells cotreated with *O157: H7* and TLR-2 and TLR-4 monoclonal antibodies for 48 hr. a-Different compared to control (p-value <0.001) (*O157: H7*) or (p-value <0.05) (*O157: H7*+Anti-TLR 2 or 4). b-Different compared to *O157: H7* at 500 μg/ml (p-value <0.01). c-Different compared to *O157: H7* at 500 μg/ml (p-value <0.001)

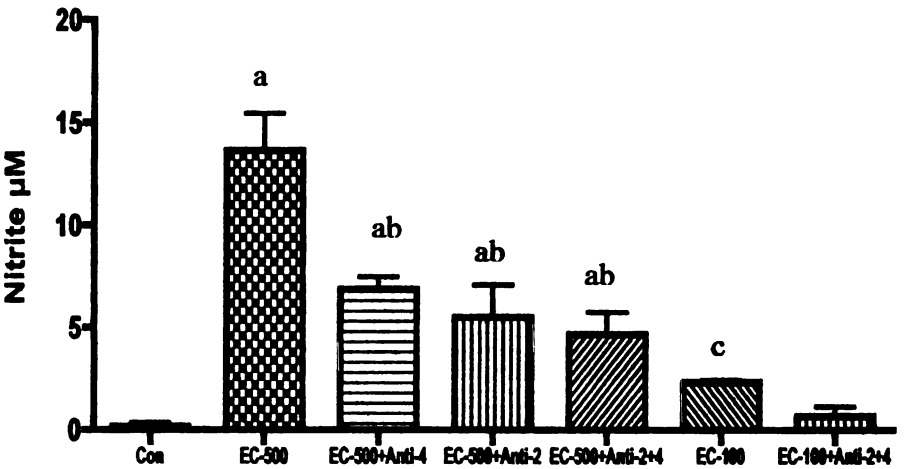


Figure 4.31 NO (Nitrite μM) production of IMCE cells cotreated with *O157: H7* and TLR-2 and TLR-4 monoclonal antibodies for 48 hr. a-Different compared to control (p-value <0.01). b-Different compared to *O157: H7* at 500 μg/ml (p-value <0.001)

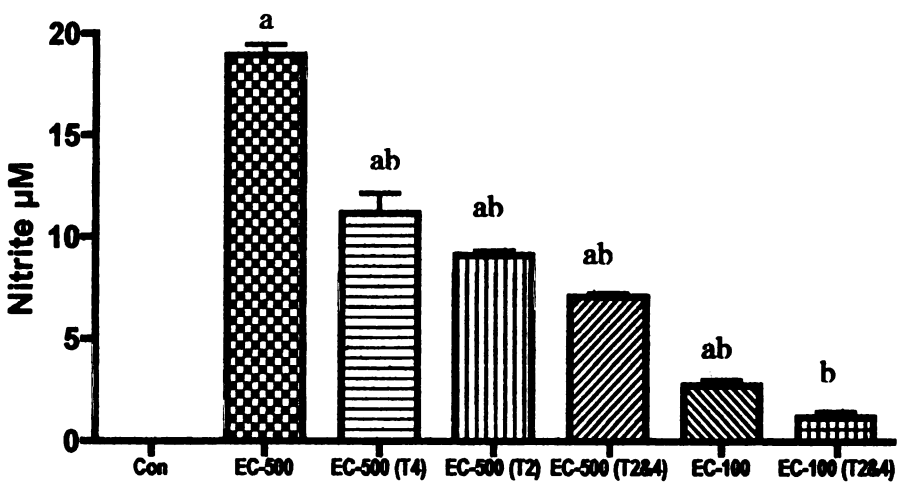


Figure 4.32 Cell viability compared to control of YAMC cells cotreated with 500 μg/ml compared to 100 μg/ml of *O157: H7* and TLR monoclonal antibodies of TLR2 and TLR4 ligands

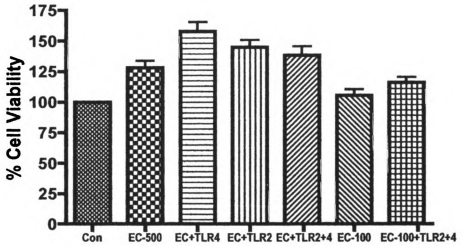


Figure 4.33 Cell viability compared to control of IMCE cells cotreated with 500 μg/ml compared to 100 μg/ml of *O157: H7* and TLR monoclonal antibodies of TLR2 and TLR4 ligands

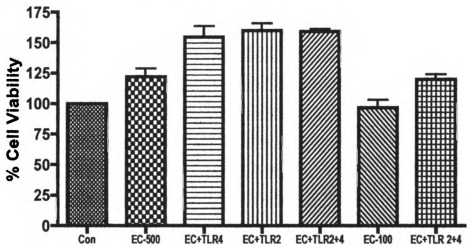


Figure 4.34 IL-6 (pg/ml) production of YAMC cells cotreated with 500 μg/ml or 100 μg/ml of *O157: H7* and TLR monoclonal antibodies of TLR2 and TLR4 for 48 hr. No statistical significance $p>0.05$

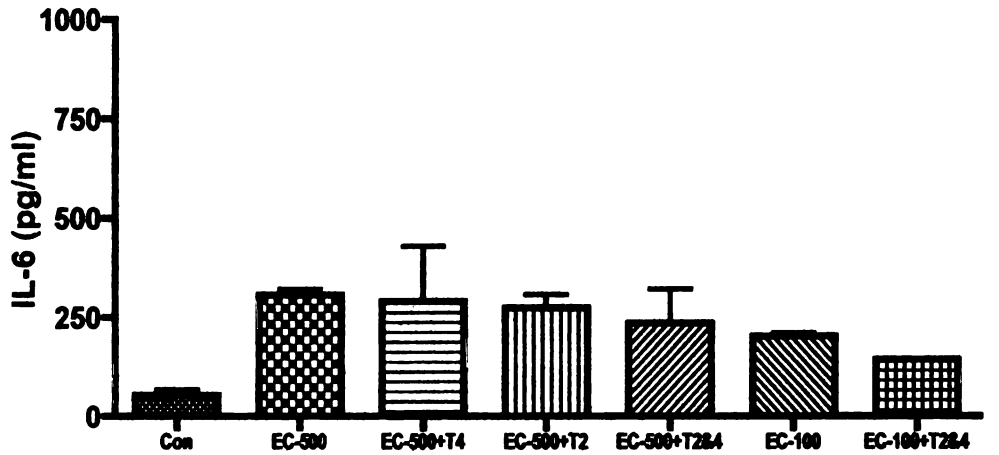


Figure 4.35 IL-6 (pg/ml) production of IMCE cells cotreated with 500 μg/ml or 100 μg/ml of *O157: H7* and TLR monoclonal antibodies of TLR2 and TLR4 for 48 hr. a-Different compared to control $p<0.01$. b-Different compared to *O157: H7* at 500 μg/ml $p<0.05$

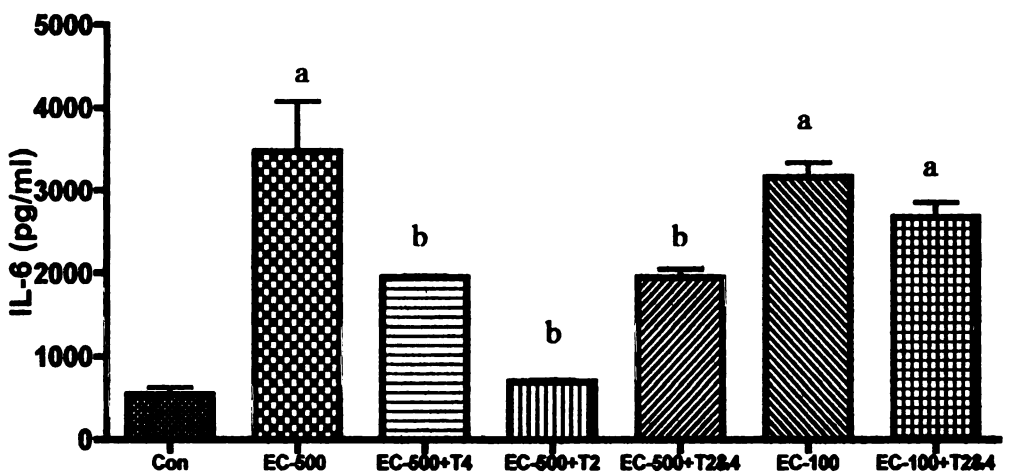


Figure 4.36 NO (Nitrite μM) production of YAMC cells cotreated with 500 μg/ml or 50 μg/ml of *O157: H7* and polymyxin B for 24 hr. a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* at 500 μg/ml (p-value <0.01)

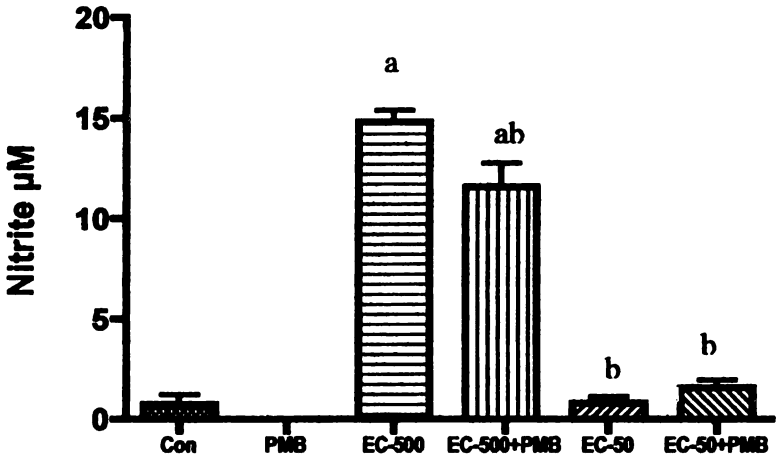


Figure 4.37 NO (Nitrite μM) production of IMCE cells cotreated with 500 μg/ml or 50 μg/ml of *O157: H7* and polymyxin B for 24 hr. a-Different compared to control (p-value <0.001) (50 μg/ml p-value <0.01). b-Different compared to *O157: H7* at 500 μg/ml (p-value <0.001)

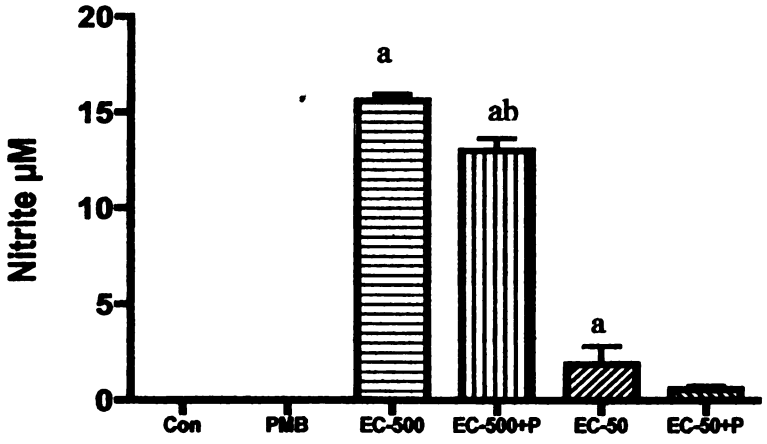


Figure 4.38 Cell viability compared to control of YAMC cells cotreated with *O157: H7* (μg/ml) and polymyxin B for 48 hr

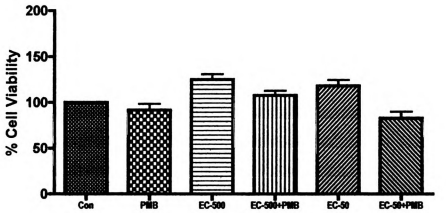


Figure 4.39 Cell viability compared to control of IMCE cells cotreated with *O157: H7* (μg/ml) and polymyxin B for 48 hr

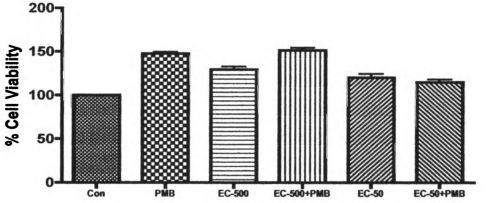


Figure 4.40 IL-6 (pg/ml) production of YAMC cells cotreated with *O157: H7* (μg/ml) and polymyxin B for 48 hr. a-Different compared to control (p-value <0.01). b-Different compared to *O157: H7* at 500 μg/ml (p-value <0.001)

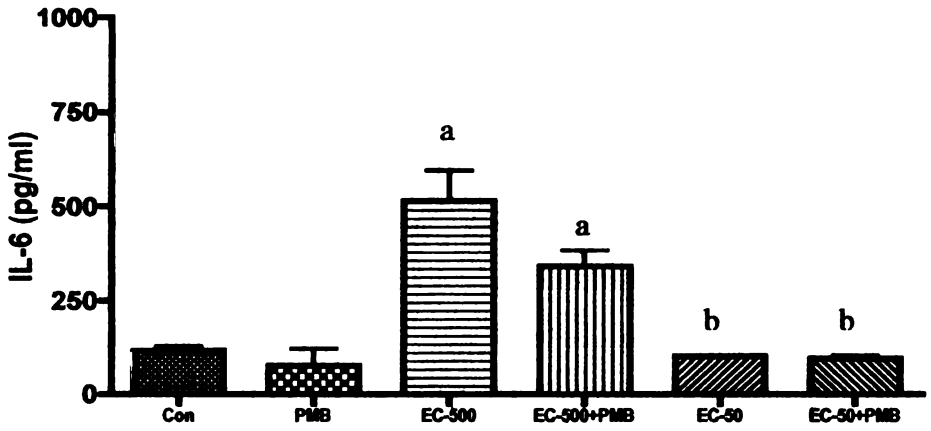
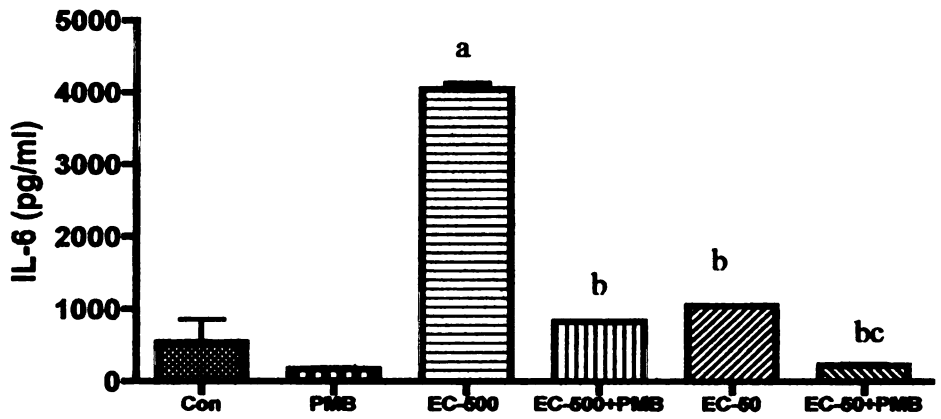


Figure 4.41 IL6 (pg/ml) production of IMCE cells cotreated with *O157: H7* (μg/ml) and polymyxin B for 48 hr. a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* at 500 μg/ml (p-value <0.001). c-Different compared to *O157: H7* at 50 μg/ml (p-value <0.05)



4.4 Effect of YAMC and IMCE treated supernatants exposed to *O157: H7* or co-exposed to *O157: H7* and probiotic bacteria on the production of pro-inflammatory mediators (NO and IL-6) on macrophages.

We hypothesized that upon exposing macrophages to conditioned media from *O157: H7* treated YAMC and IMCE cells, macrophage production of NO and IL-6 will be increased. We also hypothesized that upon exposing macrophages to conditioned media from *O157: H7* and probiotic bacteria cotreated YAMC and IMCE cells, macrophage production of NO and IL-6 will be decreased. Prior to exposing YAMC and IMCE conditioned media to macrophages to see the indirect effect that epithelial cells have on macrophage activation, we exposed directly to the macrophages either growth medium from *O157: H7* (Tryticase Soy-Yeast Extract) or from *O157: H7* cotreated with growth medium from each of the probiotic bacterial strains (MRS). Neither the *O157: H7* growth medium or the *O157: H7* cotreatment with growth medium from the probiotic bacteria induced NO or IL-6 (Data shown in Appendix G).

O157: H7 or cotreatments of *O157: H7* and probiotic bacterial strains BB, BL, LS, or LGG were exposed to macrophages directly to observe the effect that these bacteria have on macrophage NO and IL-6 production. We found that *O157: H7* induced NO and IL-6 production (p-value <0.01). Co-treatments of *O157: H7* and probiotic bacterial strains LS and LGG, but not BB and BL caused a decrease in macrophage NO production compared to *O157: H7* (p-value <0.01). Cotreatments of *O157: H7* and probiotic bacterial strains BB, BL, LS, and LGG caused an additive increase in macrophage IL-6 production compared to *O157: H7* (p-value <0.001) (Data shown in Appendix H).

Serum-starved macrophages were stimulated with supernatants from bacterial-treated YAMC and IMCE cells to determine whether these supernatants would cause macrophage activation. Exposure of macrophages to supernatants from YAMC and IMCE cells stimulated with *O157: H7* did not result in macrophage NO production as evidenced by the lack of increase compared to *O157: H7*-induced NO production in epithelial cells (Figures 4.42 and 4.43). When macrophages were coexposed to YAMC and IMCE cell supernatants stimulated with *O157: H7* and probiotic bacterial strains BB, LS, BL, or LGG, no change in NO was observed relative to NO production in epithelial cells.

Exposure of macrophages to YAMC and IMCE cell supernatants stimulated with *O157: H7* caused macrophages to produce IL-6. The increase in IL-6 production by the macrophages when treated with *O157: H7* was significantly higher in IMCE cell supernatants when compared to YAMC cell supernatants (p-value < 0.001). The probiotic bacteria reduced IL-6 production induced by O157: H7 in a genus- and species-dependent manner.

Figure 4.42 Macrophage activation treated with YAMC supernatants of *O157: H7* (500 μg/ml) or cotreatments of *O157: H7* and probiotic bacterial strains BB, BL, LS, or LGG (500 μg/ml each) for 48 hr. No statistical significance compared to YAMC NO production.

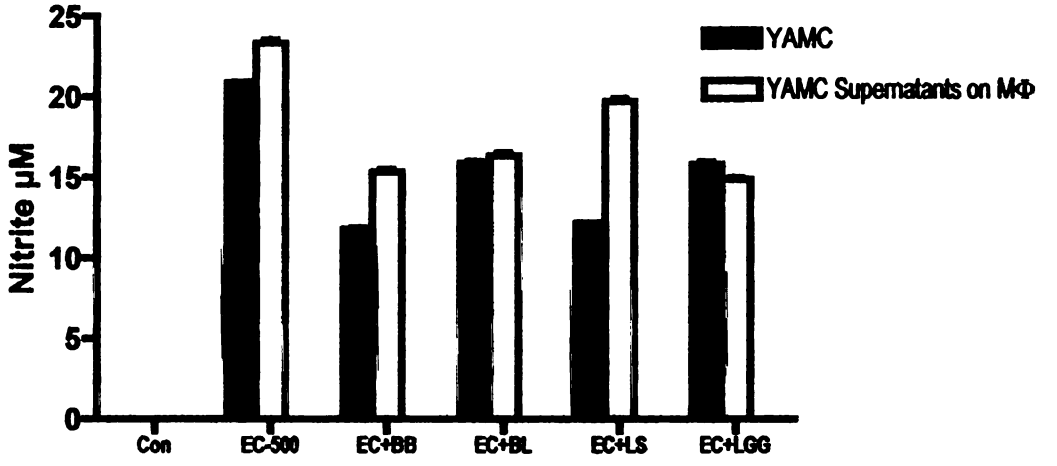


Figure 4.43 Macrophage activation treated with IMCE supernatants of *O157: H7* (500 μg/ml) or cotreatments of *O157: H7* and probiotic bacterial strains BB, BL, LS, and LGG (500 μg/ml each) for 48 hr. No statistical significance compared to IMCE NO production

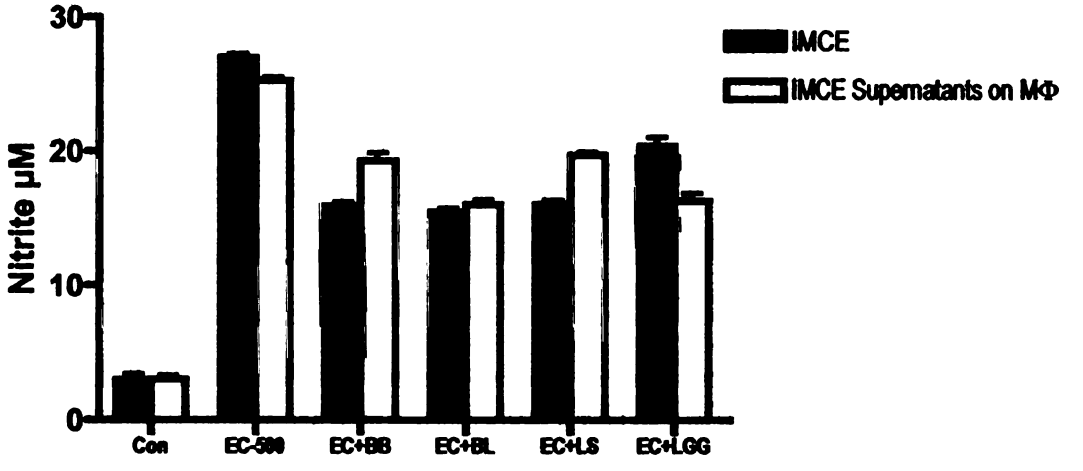


Figure 4.44 IL-6 (pg/ml) production of macrophages treated with YAMC supernatants from *O157: H7* (500 μg/ml) or cotreated with *O157: H7* and LS (500 μg/ml, 250 μg/ml, or 25 μg/ml). a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* exposed to macrophages (p-value <0.001)

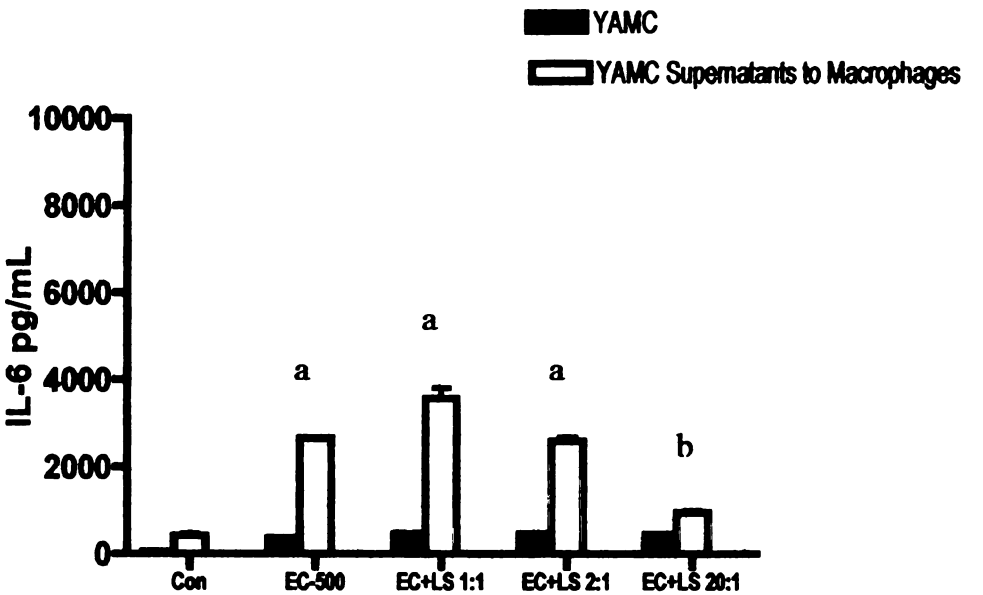


Figure 4.45 IL-6 (pg/ml) production of macrophages treated with IMCE supernatants from *O157: H7* (500μg/ml) or cotreated with *O157: H7* and LS (500 μg/ml, 250 μg/ml, or 25 μg/ml). a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* exposed to macrophages (p<0.05)

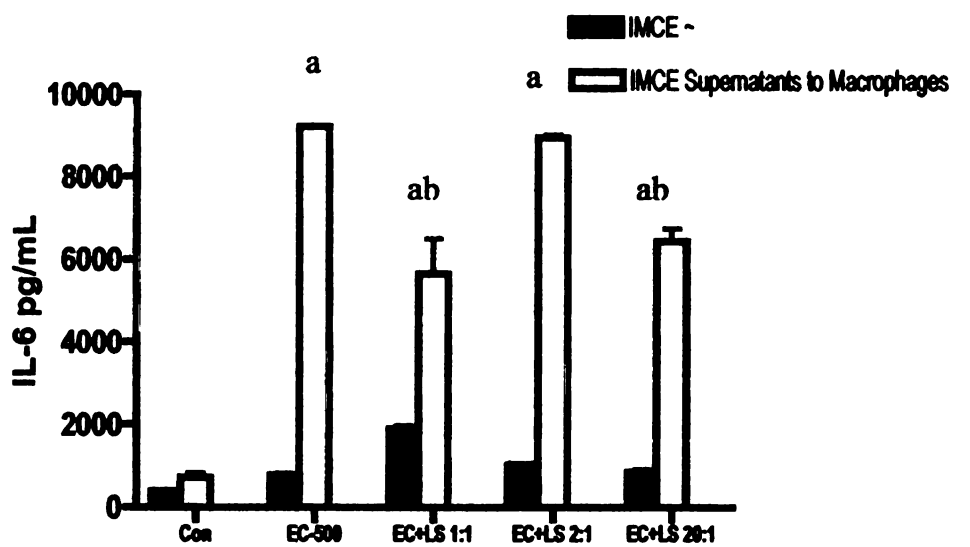


Figure 4.46 IL-6 (pg/ml) production of macrophages treated with YAMC supernatants from *O157: H7* (500 μg/ml) or cotreated with *O157: H7* and LGG (500 μg/ml, 250 μg/ml, or 25 μg/ml). a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* exposed to macrophages (p-value <0.01)

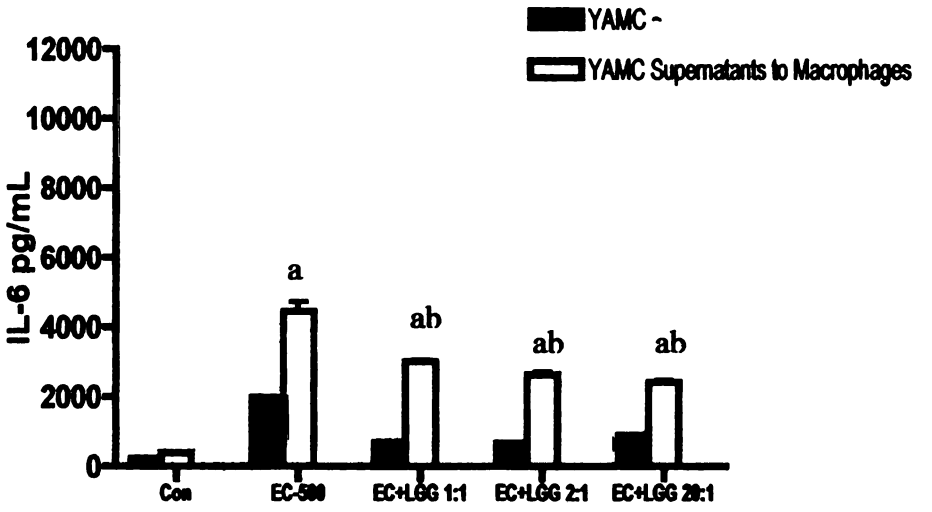


Figure 4.47 IL-6 (pg/ml) production of macrophages treated with IMCE supernatants from *O157: H7* (500 μg/ml) or cotreated with *O157: H7* and LGG (500 μg/ml, 250 μg/ml, or 25 μg/ml). a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* exposed to macrophages (p-value <0.001)

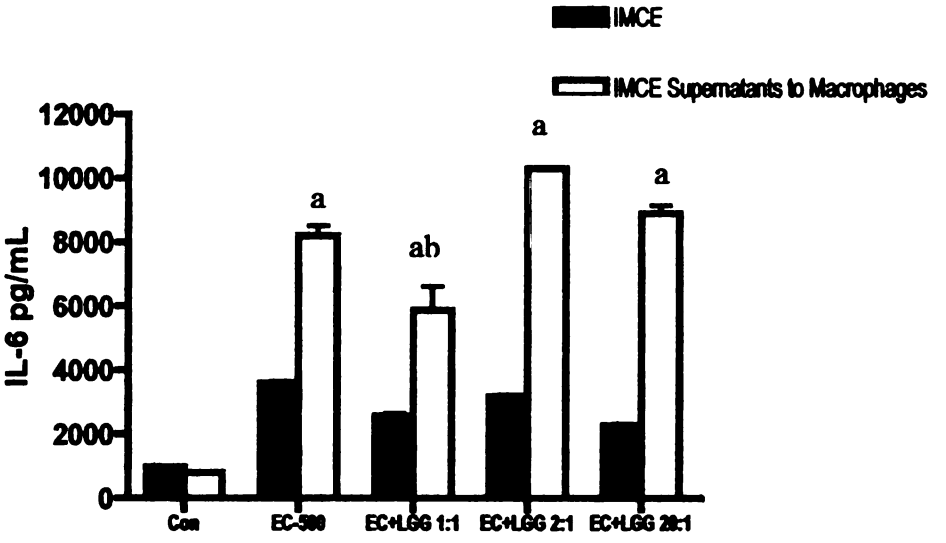


Figure 4.48 IL-6 (pg/ml) production of macrophages treated with YAMC supernatants from *O157: H7* (500 μg/ml) or cotreated with *O157: H7* and BB (500 μg/ml, 250 μg/ml, or 25 μg/ml). a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* exposed to macrophages (p-value <0.001)

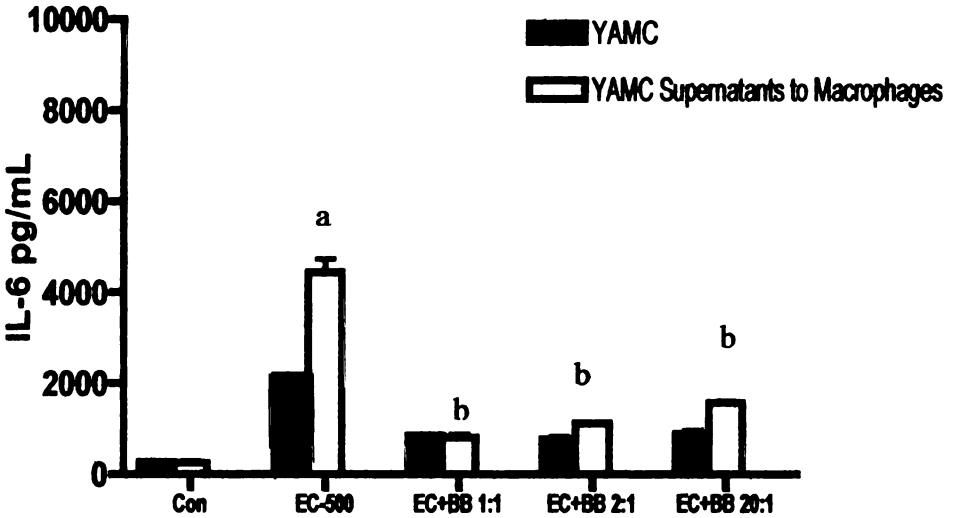
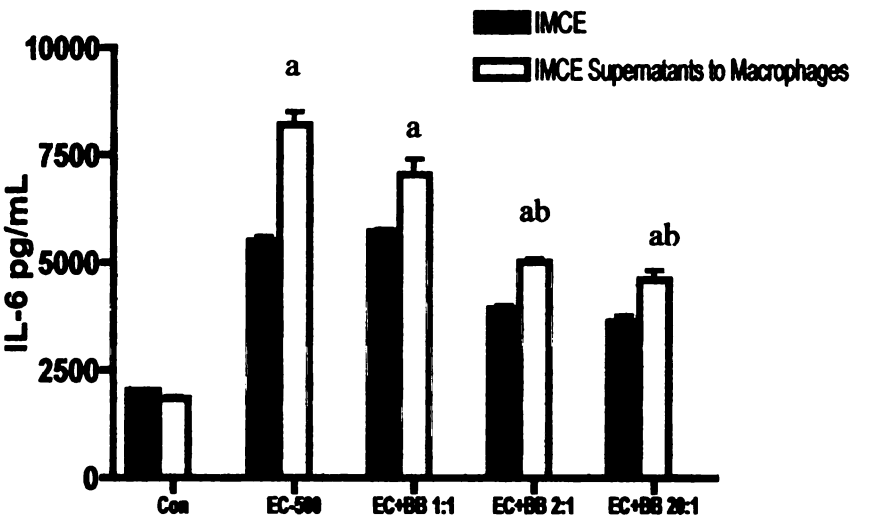


Figure 4.49 IL-6 (pg/ml) production of macrophages treated with IMCE supernatants from *O157: H7* (500 μg/ml) or cotreated with *O157: H7* and BB (500 μg/ml, 250 μg/ml, or 25 μg/ml). a-Different compared to control (p-value <0.001). b-Different compared to *O157: H7* exposed to macrophages (p-value <0.001)



4.5 Effect of IMCE and YAMC supernatants treated with *O157: H7* or cotreated with *O157: H7* and probiotic bacteria on macrophage chemotaxis

We hypothesized that exposure of macrophages to YAMC and IMCE cell supernatants stimulated with *O157: H7* would result in macrophage chemotaxis. We also hypothesized that exposure of macrophages to YAMC and IMCE cell supernatants co-stimulated with *O157: H7* and probiotic bacteria would result in decreased macrophage chemotaxis. Supernatants from the epithelial cell treatments were exposed to serum-starved macrophages to determine whether these supernatants would cause macrophage chemotaxis. Exposure of macrophages to supernatants from *O157: H7*-stimulated IMCE cells, but not those from *O157: H7*-stimulated YAMC cells, resulted in macrophage chemotaxis compared to control media (p-value <0.04) (Figure 4.50 through 4.53). No co-treatment of *O157: H7* and probiotic bacteria resulted in decreased macrophage chemotaxis; however, the co-treatment of *O157: H7* and LS resulted in increased macrophage chemotaxis compared to *O157: H7* (p-value <0.04).

Figure 4.50 Macrophage chemotaxis treated with YAMC supernatants of *O157: H7* or cotreated with *O157: H7* and *B. breve*. a-Different compared to control media (p-value <0.006)

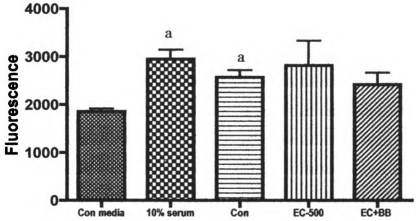


Figure 4.51 Macrophage chemotaxis treated with IMCE supernatants of *O157: H7* or cotreated with *O157: H7* and *B. breve*. a-Different compared to control media (p-value <0.01)

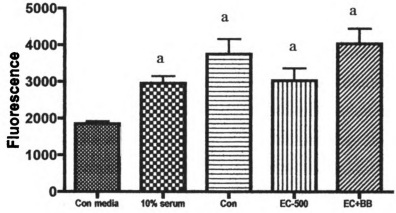


Figure 4.52 Macrophage chemotaxis treated with YAMC supernatants of *O157: H7*, or cotreated with *O157: H7* and *L. salivarius*. a-Different compared to control media (p-value <0.02)

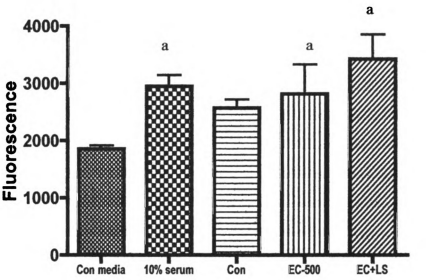
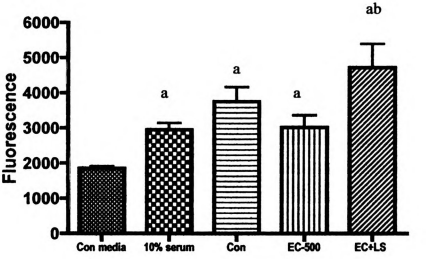


Figure 4.53 Macrophage chemotaxis treated with IMCE supernatants of *O157: H7* or cotreated with *O157: H7* and *L. salivarius*. a-Different compared to control media (p-value <0.01). b-Different compared to *O157: H7* (p-value <0.04)



CHAPTER 5

DISCUSSION

5.1 Effect of exposing YAMC and IMCE cells to *O157: H7* on pro-inflammatory mediator production (NO and IL-6)

In our study we observed that *O157: H7* treatment increased NO and IL-6 production in YAMC and IMCE cells and to a greater extent in IMCE cells. Epithelial cells, when exposed to enteropathogenic bacteria, like *O157: H7*, detect the presence of pathogens and produce signals that alert immune and inflammatory cells in the mucosa (Berin et al, 2002). This particular study investigated two pro-inflammatory mediators (NO and IL-6) produced by epithelial cells after exposure to *O157: H7*. Our *in vitro* model system reflects typical pro-inflammatory responses to this bacterial pathogen; however, it is likely that other bacterial pathogens could also induce increased production of NO and IL-6. It is also likely that epithelial cells exposed to bacterial pathogens produce additional cytokines, chemokines, and growth factors.

Epithelial cells can be affected by cytokines in their local environment (Panja et al, 1998), such as that of the colon. An altered cytokine environment can result from inflammation and have effects on epithelial cell growth, phenotype, and function (Panja et al, 1998); inflammatory conditions, such as that of IBD, are risk factors for colon carcinogenesis. Epithelial cells respond to invasive pathogens with an inflammatory phenotype including the production of cytokines, chemokines, and NO production (Berin et al, 2002). Similar to our study, human intestinal epithelial cells secreted elevated amounts of IL-8 when *O157: H7* was present. In response to *O157: H7* exposure, rat uterine epithelial cells produced elevated amounts of TNF-α (Crane-Godreau et al, 2004). Another study that further supports the effect of *O157: H7* on cytokine expression showed that *O157: H7* caused an up-regulation of IL-1ß and TNF-α, and a down-

regulation of TGF-ß1, which may have led to elevated cytokine production (Roselli et al, 2006). When the human colon carcinoma cell line, Caco-2, was exposed to *Bacilus subtilis*, increased levels of IL-6 and IL-8 were produced (Morita et al, 2002). These data show that production of IL-6 and IL-8 by enterocytes can occur as a result of *O157: H7* exposure. These findings are consistent with our observations that *O157: H7* increased pro-inflammatory mediator production in epithelial cells.

IL-6 production is increased during inflammation; however, IL-6 can have both pro- and anti-inflammatory properties (Hershko, 2002). IL-6, in addition to its role in inflammation and host defense, plays a direct role in protecting various epithelial cells, including the intestinal epithelium; processes mediated by IL-6 are tissue repair, barrier function, and angiogenesis (Rakoff-Nahoum et al, 2004). In the intestine, *in vivo* and *in vitro* studies show that IL-6 protects the intestine in response to injury through initiation of repair (Rakoff-Nahoum et al, 2004). IL-$6^{-/-}$ knockout mice exhibit a major impairment in acute-phase protein synthesis among having a reduction in antimicrobial resistance, impairment in T-cell growth and function, impairment in B-cell maturation, and deficiency in mucosal IgA production. The presence of these defects in the immune system of IL-6 knockout mice argues that IL-6 is required for normal immune functions (Papanicolaou et al, 1998). It is suggested that the inflammatory response is a balance, or trade-off, between the beneficial and detrimental effects of IL-6 production (Rakoff-Nahoum et al, 2004).

The targeted disruption of various genes encoding NF-kB in knockout mice revealed severe defects in immune function supporting a key regulatory role for NF-kB, or in particular IL-6, in the immune system (Neurath et al, 1998). We concentrated on

IL-6 production because it is a pleiotropic cytokine that plays an active role in the immune response and the development of the acute phase response in various epithelial tissues (de Haij et al, 2005). IL-6 is known to amplify local and systemic innate immune defenses against infection (Song et al, 2007). The IL-6 mediated signaling pathway is induced through activation of a number of transcriptional regulatory pathways, including NF-kB, STAT-3, MAPK, and TLR-4 activation of cyclic AMP pathway (Cho et al, 2007, Song et al, 2007, and Wang et al, 2003).

5.2 Effect of co-exposing YAMC and IMCE cells to *O157: H7* and probiotic bacteria on pro-inflammatory mediator production (NO and IL-6)

When we co-exposed YAMC and IMCE cells to *O157: H7* and probiotic bacteria BB, BL, LS, or LGG, the probiotic bacteria decreased *O157: H7*-induced NO and IL-6 production in a genus- and species-dependent manner. There are many potential mechanisms by which probiotic bacteria could exert their action. Some of which include, the secretion of biologically active components, competition with pathogenic bacteria, or acting through immunoregulatory effects mediated by their bacterial constituents (Marcinkiewicz et al, 2007). For example, lactic acid bacteria promote pro-inflammatory mediator production of TNF-α and NO, while others, such as LS, reduced TNF-α and NO in a rat colitis model (Peran et al 2005). These data, consistent with data presented here, show a genus- and species-dependent effect of probiotic bacteria that may result in either pro- or anti-inflammatory effects in epithelial cells.

Furthermore, two probiotic *Lactobacillus* strains inhibit *in vitro* adherence of *O157: H7* to the intestinal epithelial HT-29 tumorigenic cell line (Mack et al, 1999). This result may be due to probiotic bacteria, such as *L. plantarum* or *L. rhamnosus* GG,

binding to epithelial cells *in vitro* and stimulating epithelial cells to secrete antimicrobial substances that diminish enteric pathogen binding to epithelial cells and promoting local antibody responses in the gut wall (Mack et al, 1999 and Korhonen et al, 2001).

In addition, other probiotic strains were able to counteract the *O157: H7*-induced alterations of cytokine expression, and consequently block the pro-inflammatory mediators induced by *O157: H7* exposure (Roselli et al, 2006). These results are in agreement that probiotics may suppress the inflammatory response to infectious stimuli through regulation of pro-inflammatory mediator production (Roselli et al, 2006).

Cooperative as well as competitive interactions may occur between different bacterial ligands via TLRs and other components of the innate immune system leading to differential pro- and anti-inflammatory immune responses, thus maintaining gut homeostasis (Cario, 2005). The probiotic strain *L. casei Shirota* ameliorated murine chronic inflammatory bowel disease induced by dextran sodium sulfate and is associated with the down-regulation of pro-inflammatory cytokines such as IL-6 and IFN-γ production in lamina propria mononuclear cells. Therefore, this particular strain may be a useful probiotic for the treatment of human inflammatory bowel diseases (Matsumoto et al, 2005). While consistent with results of these animal studies, the *in vitro* nature of studies presented here regarding the anti-inflammatory roles of probiotic bacteria cannot be appropriately recommended for dietary applications.

Perhaps, the most intriguing activity of probiotics is their ability to balance the Th1/Th2 ratio due to altering cytokine patterns released by epithelial and other immune cells of the gut (Perdigon et al, 2002). Perdigon and colleagues observed that the anti-inflammatory cytokines IL-4 and IL-10 were produced by *lactobacilli* in response to a

pathogen. While induction of "anti-inflammatory" cytokines, like IL-10, in some model systems may explain the anti-inflammatory effect by probiotics, we have not detected IL-10 produced in epithelial cells exposed to probiotic bacteria (Block, 2004; unpublished observation). The ability of probiotic bacteria to attenuate pro-inflammatory mediator production (NO and IL-6) provides evidence that probiotics could be used as anti-inflammatory agents in protecting the host from inflammatory conditions, thus improving health (Block, 2004). These findings, along with our data, may suggest that the difference in cytokines released between pathogen and probiotic bacteria may be due to their cell wall structures (Perdigon et al, 2002). Since we did not measure IL-10 or other so-called 'anti-inflammatory" cytokines, we cannot speculate about the effects of probiotic bacteria on the production of these cytokines in our model system.

The production of NO and IL-6 by exposing probiotic bacteria alone to YAMC and IMCE cells was surveyed. We demonstrated that BB, BL, LS, or LGG alone induced low amounts of NO production in both YAMC and IMCE cells. These findings are supported by a similar study that demonstrated that LGG induced a low-level expression of iNOS protein and NO production by macrophages and human T84 intestinal epithelial cells (Korhonen et al, 2001). No strain of *bifidobacterium* exposure to HT-29 cells induced activation of NF-kB, suggesting that *bifidobacteria* themselves do not induce inflammatory events in IEC's (Riedel et al, 2006). When cytokines, such as TNF-α, are released in response to *O157: H7* but not *LGG* suggests a level of specificity in which epithelial cells distinguish between gram-negative pathogens and gram-positive probiotics (Crane-Godreau et al, 2004). In our model system, TNF-α production was not inducible under the conditions used in this study (Block, 2004; unpublished observation).

Unlike NO induction, probiotic bacteria induced IL-6 production in both YAMC and IMCE cell lines in a genus- and species-dependent manner. Our findings are bolstered by similar findings in colon tumor cell lines by investigators examining IL-6, IL-8 and TNF-α production. The probiotic bacterium strain *Bifidobacterium lactis BB12* triggered IL-6 gene expression in intestinal epithelial cell lines (Ruiz et al, 2005). These data provide evidence that the gram-positive strain induces IL-6 gene expression through TLR-mediated activation (Ruiz et al, 2005). IL-8 production from Caco-2 cells induced by TNF-α was modulated by the probiotic strain *LGG*; however, *LGG* without TNF-α cotreatment, caused a concentration-dependent increase in the production of IL-8 (Zhang et al, 2005). The absence of pro-inflammatory signals induced by adhesive lactic acid bacteria may also suggest a protective systemic immune response compared to bacterial invasion (Morita et al, 2002). These findings suggest that there may be a symbiotic interaction between enterocytes and lactic acid bacteria (Morita et al, 2002).

5.3 Effect of exposing YAMC and IMCE cells to toll-like receptor 2, 4, 5, and 9 ligands on the production of pro-inflammatory mediators (NO and IL-6)

In our study, exposure of toll-like receptor ligands to YAMC and IMCE cells resulted in increased levels of IL-6, but did not induce NO production. Gram-negative and gram-positive microbial products are believed to evoke different immune responses in which toll-like receptors play a large role (Tietze et al, 2006). Recognition of gram-negative bacteria is mediated by the cell wall constituent, LPS; while, gram-positive species are recognized through cell wall contact with PGN, lipoteichoic acid, or other extracellular toxins (Tietze et al, 2006). By interaction with different TLRs, the products of different gram-negative and gram-positive microbes may induce distinct patterns of cytokine

production (Tietze et al, 2006). Gram-negative species were found to induce higher amounts of TNF-α, while gram-positive species were found to induce higher amounts of IL-8. Similarly, the recognition of LPS by TLR-4 resulted in pro-inflammatory gene expression in diverse cell types (Abreu et al, 2001). While only minimally detected in colonic epithelial cells of normal, non-IBD mucosa, TLR-2 and TLR-4 was abundant in epithelial cells of active inflammation in IBD patients (Cario et al, 2000). Epithelial cells, relative to other cell types in the mucosa, express toll-like receptors in the gut mucosa (Cario et al, 2000). Since the cell types used in our model system (YAMC and IMCE) were found to express moderate levels of TLR-2 and TLR-4 without bacterial stimulation, we can conclude that these cells may serve as models of inflamed epithelium.

LPS, a key product of pathogenic gram-negative bacteria, activates signal transduction pathways in intestinal epithelial cells resulting in pro-inflammatory cytokine production. This observation suggests that TLR's participate in the innate immune response and signal the activation of adaptive immunity (Cario et al, 2000). It is biologically plausible that microbial compounds induce the production of pro-inflammatory cytokines since LPS and PGN induce the expression of pro-inflammatory cytokines and chemokines, such as IL-8 and TNF-α in epithelial cells, (Pivarcsi, et al, 2005). Furthermore, Tietze and colleagues observed that gram-negative and gram-positive bacteria induce a distinct pattern of cytokine production. Gram-negative bacteria induced higher amounts of epithelial cell TNF-_ while gram-positive bacteria induced higher amounts of IL-8. This differential pattern of cytokine induction might be a result of activation of different TLR's (Tietze et al, 2006). The differences in responses through

TLR-4 could be due to the differences in the chemical structure of lipid A moieties since LPS structures are a heterogeneous group of molecules with interspecies differences (Tietze et al, 2006).

We hypothesized that probiotic bacteria exerted an anti-inflammatory effect induced by *O157: H7* via a TLR-9 mediated pathway. To test this hypothesis, we used TLR-9 ligands to determine whether stimulation or inhibition of TLR-9 would augment or inhibit the effect of probiotic bacteria. Exposing IMCE cells to stimulatory or inhibitory TLR-9 ligands resulted in increased IL-6, but minimal NO production. YAMC cell exposure to TLR-9 ligands resulted in an absence of both NO and IL-6 production. Co-exposure of YAMC and IMCE cells to stimulatory or inhibitory TLR-9 ligands and *O157: H7* resulted in a decrease in IL-6, but did not decrease NO production.

Colonic epithelial cells are constantly exposed to bacterial DNA in the intestinal lumen. These cells must recognize and respond appropriately thereby distinguishing between pathogenic and non-pathogenic bacteria. In addition, bacterial DNA is recognized by TLR-9 (Ewaschuk et al, 2007). Therefore, one mechanism by which probiotic bacteria exert their effects is through activation of the innate immune system via TLR, specifically TLR-9 (Rachmilewitz et al, 2004). Rachmilewitz and colleagues demonstrated that by knocking out TLR-9 in a mouse colitis model, the course or severity of colitis was not affected. These data suggest that the amelioration of pro-inflammatory mediators by probiotic bacteria induced by *O157: H7* are mediated through TLR-9 versus some other metabolic activity performed by probiotic bacteria (Rachmilewitz et al, 2004). We found that any engagement of TLR-9 (stimulatory and inhibitory) decreased *O157: H7*-induced IL-6 production; therefore, these data allowed us to speculate that *O157: H7*

could be binding and/or acting through a TLR-9 mediated pathway. Therefore, we hypothesized probiotic bacteria may block *O157: H7*-induced IL-6 production through a TLR-9 mediated mechanism. Lack of availability of a neutralizing antibody against TLR-9 precluded blocking CpG oligonucleotide access to TLR-9.

Pedersen and colleagues observed that TLR-9 mRNA is variably expressed in normal human colonic mucosa as well as in mucosa of patients with inflammatory bowel disease. However, the average level of gene expression was reduced in the inflamed mucosa compared to that of the normal mucosa (Pedersen et al, 2005). Ewaschuk and colleagues observed that exposure of cells to DNA from pathogens, such as *O157: H7*, resulted in a significant increase in TLR-9 mRNA expression, whereas TLR-9 mRNA expression did not change as a result from *B. breve* exposure. (Ewaschuk et al, 2007). These results, along with our findings, suggest that the inflammatory response induced by pathogens is mediated in part by increased TLR-9 expression (Ewaschuk et al, 2007). Furthermore, intestinal epithelial cells do not respond equally to bacterial DNA and are thus capable of distinguishing between DNA from probiotic bacteria and that of pathogens (Ewaschuk et al, 2007). Previous studies, consistent with our observations, demonstrate that an inflammatory response does not always occur with the stimulation of TLR's. The interactions between TLRs' and TLR ligands are an integral component of intestinal homeostasis (Ewaschuk et al, 2007). Our data showing that either stimulatory or inhibitory ODN coexposure with *O157: H7* resulted in decreased IL-6 production indicates that TLR-9 occupancy effectively blocked *O157: H7*-induced IL-6 production. This finding is curious in light of our observation that stimulatory ODN, but not inhibitory ODN TLR-9 ligands alone increased IL-6 production. As irradiated probiotic

bacteria, it is unlikely that their metabolites or competitive inhibition with indigenous microflora were responsible for the protective effects on the colonic mucosa (Rachmilewitz et al, 2004).

5.3.1 Effect of toll-like receptor inhibitors 2 and 4 on pro-inflammatory mediator production (NO and IL-6)

Co-exposure of *O157: H7* with monoclonal antibodies against TLR-2 and TLR-4 and polymyxin B decreased the production of both NO and IL-6. This observation supports our hypothesis that *O157: H7* increases NO and IL-6 through TLR-2 and TLR-4 signaling. Both TLR-2 and TLR-4 are minimally expressed in normal tissue while being abundantly expressed by epithelial cells in IBD patients. This implies that TLR expression is altered in disease (Ewaschuk et al, 2007). Singh and colleagues support the finding that these changes in TLR expression may be the underlying factor in contributing to the hypersensitivity to bacterial antigens, a characteristic of IBD (Singh et al, 2005). Exposure of the human epithelium to CpG resulted in no inflammatory response, which assists to insure that there is not an inappropriate immune response to bacteria (Pederen et al, 2005). Inhibitors of TLR signaling provide another mechanism by which to limit TLR signaling in the intestine (Abreu et al, 2005). Vinderola and colleagues aimed at determining whether the non-pathogenic bacteria-intestinal epithelial cell interactions were taking place through TLR-2 and/or TLR-4 (Vinderola et al, 2005). They found that there was partial inhibition in anti-TLR4 treated IEC challenged with LPS; this could be due to the TLR-4 independent recognition of LPS (Vinderola et al 2005). Polymyxin B inhibited NO induced by LPS, but did not inhibit LGG induced NO production (Korhonen et al, 2002). These findings are consistent with our observation,

suggesting that the effect of polymyxin B acts through lipid A, which is a moiety of LPS within the cell wall of gram-negative bacteria (Daugelavicius et al, 2000). Therefore, we can conclude that polymyxin B is only active towards gram-negative pathogens rather than gram-positive probiotics.

5.3.2 Effect of PDTC (an inhibitor of NF-kB) on pro-inflammatory mediator production (NO and IL-6)

When PDTC was co-exposed to YAMC and IMCE cells with *O157: H7*, NO was decreased, but not IL-6 production. These findings are supported by the finding that PDTC and other dithiocarbamates inhibit the activation of NF-kB and possess antioxidative properties (Cuzzocrea et al, 2005). This particular study demonstrates that the production of pro-inflammatory cytokines plays an important role in the pathophysiology of inflammation. Further, NF-kB is the transcription factor that plays a pivotal role in the induction of genes involved in this pathophysiology (Block, 2004). Therefore, the inhibition of NF-kB by PDTC may be a useful therapy in ameliorating the inflammatory response (Cuzzocrea et al, 2005).

There is increasing evidence that formation of NO by iNOS also contributes to the inflammatory response and this study proves that PDTC decreases NO production *in vitro* (Cuzzocrea et al, 2005). The experiments of coexposing YAMC and IMCE cells to *O157: H7* or *O157: H7* and BB with PDTC did not decrease IL-6 production in either cell type except for cotreatment of *O157: H7* and BB for YAMC cells. This finding is supported by a study done by Németh and colleagues where mice were pretreated with PDTC, but this pretreatment did not alter LPS-induced production of IL-1α, IL-6, and IFN-gamma (Neméth et al, 1998). This same study showed that pretreatment of these

animals with PDTC prior to LPS challenge did result in a decrease in plasma levels of TNF-α and nitrite concentrations (Neméth et al, 1998). These results suggest that the production of NO via iNOS is regulated by the transcription factor NF-kB (Cavicchi et al, 1999). Furthermore, these findings also provide evidence that the production of IL-6 may be independent of NF-kB. The inability of PDTC to decrease IL-6 in IMCE cells may be due to the fact that PDTC increases MIP-2 production, which can further perpetuate IL-6 production (Block, 2004; unpublished observation).

5.4 Effect of exposing macrophages to YAMC and IMCE cell supernatants stimulated with *O157: H7* or co-stimulated with *O157: H7* and probiotic bacteria on macrophage NO and IL-6 production

Exposing serum-starved macrophages to YAMC and IMCE cell supernatants stimulated with *O157: H7* resulted in an increase in macrophage pro-inflammatory mediator IL-6 production, but did not result in macrophage NO production. Exposing serum-starved macrophages to YAMC and IMCE cell supernatants co-stimulated with *O157: H7* and probiotic bacterial strains BB, BL, LS, or LGG resulted in decreased macrophage IL-6 production, but did not result in decreased macrophage NO production. Hume and colleagues showed that macrophages exposed to LPS or other microbial agonists do not become refractory to stimulation; but rather, they have entered a new steady state requiring continued stimulation and in which other agonists can generate a further amplification of the response (Hume et al, 2001). These findings are also supported by the fact that not only macrophage activation, but also increase in the number of macrophages at the site of infection, may be important in augmenting infection against pathogens (Kim et al, 2006). These findings further suggest that specific

Lactobacillus strains can directly activate host immune component cells (Kim et al, 2006). Previous studies indicate that enteric inflammation increased activation of intestinal macrophages, releasing pro-inflammatory cytokines such as IL-6 (Zareie et al, 2001). Peran and colleagues observed that *L. salivarius* modified the cytokine profile in macrophages, reducing the amount of pro-inflammatory cytokines (TNF-α or IL-6), while increasing the amounts of anti-inflammatory cytokines, such as IL-10 (Peran et al, 2005).

Innate immune responses are induced upon detecting the conserved molecules produced by microorganisms (Kim et al, 2006). The stimulation of TLR's or other receptors of host cells by probiotics have important interactions with host immune cells of innate immunity that function to protect the host (Kim et al, 2006). Once an intestinal epithelial cell is exposed to a bacterial pathogen, it is stimulated to produce and release a number of signaling molecules that subsequently lead to the activation of immune cells during the inflammatory response (Mumy et al, 2005). In addition to chemoattraction, cytokines such as IL-6 stimulate macrophage activation and additional cytokine secretion, which perpetuates the inflammatory response (Mumy et al, 2005). These data implicate epithelial-macrophage paracrine signaling of pro-inflammatory mediator production. These findings also suggest that the probiotic bacteria may decrease the perpetuation of pro-inflammatory signaling of IL-6 via macrophages induced by bacterial-treated YAMC and IMCE cell supernatants. Because *O157: H7* treatment did not activate additional macrophage NO production compared to what YAMC and IMCE cells made, we can conclude that NO only acts locally versus systemically (Fenton et al, 2007). These findings, along with the data that we obtained, allow us to conclude that

epithelial cells transduce signals that discriminate between pathogens and probiotic bacteria (Haller et al, 2000).

5.5 Effect of macrophage chemotaxis exposed to YAMC or IMCE cell supernatants stimulated with *O157: H7* or co-stimulated with *O157: H7* and probiotic bacteria

Exposing serum-starved macrophages to YAMC and IMCE cell supernatants stimulated with *O157: H7* resulted in macrophage chemotaxis in IMCE, but not YAMC, cells. Exposing serum-starved macrophages to YAMC and IMCE cell supernatants co-stimulated with *O157: H7* and probiotic bacterial strains BB, BL, LS, or LGG did not result in decreased macrophage chemotaxis; however, *O157: H7* cotreated with LS resulted in an increase in macrophage chemotaxis. The recruitment of immune cells towards sites of infection or inflammation is the most fundamental process of innate immunity (Fillion et al, 2001). Host-derived chemoattractant factors are suggested to play pivotal roles in leukocyte recruitment elicited by inflammatory stimuli *in vitro* and *in vivo* (Fillion et al, 2001). Previous models of inflammation suggested that monocyte recruitment depends upon both the activation of endothelium and the generation of monocyte chemoattractants (Fillion et al, 2001). Veckman and colleagues demonstrated that both gram-positive *S. pyogenes* and *L. rhamnosus* stimulated macrophages to induce the migration of Th1 cells. Cell culture supernatants of the gram-positive pathogen stimulated macrophages induced cell migration almost three times more efficiently compared with supernatants from gram-positive probiotic-stimulated macrophages (Veckman et al, 2003). These findings, along with our observations, suggest that the ability for certain probiotic bacteria strains to enhance leukocyte chemotaxis could be considered an immunostimulatory mechanism (Veckman et al, 2003). Previous studies

showed that IMCE cells, but not YAMC cells possess the ability to facilitate the promotional influence of immune cells by promoting their activation and chemotaxis through the production of proinflammatory mediators induced by leptin treatment (Fenton et al, 2007). Fenton and colleagues also observed that normal YAMC cells do not make chemoattractant signals in response to leptin. These findings were consistent with our observations suggesting that YAMC cells do not make chemoattractant signals for macrophages in response to *O157: H7* treatment. Therefore, preneoplastic cells may respond differently to promotional influences that result in the elaboration of chemotactic cytokines.

CHAPTER 6

CONCLUSION AND FUTURE IMPLICATIONS

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Conclusions

Stimulation of YAMC and IMCE cells to *O157: H7*, induced both NO and IL-6 production. NO production was similar in both cell types; however, IL-6 production was greater in IMCE cells compared to YAMC cells. These findings allow us to conclude that epithelial cells were responsive to bacterial challenge through TLR-2 and TLR-4 mediated activation and in doing so initiate an appropriate immune response. Stimulation of YAMC and IMCE cells to BB, BL, LS, or LGG alone, induced IL-6 production, with a greater amount in *Bifidobacterium* compared to *lactobacillus* species; however, no probiotic bacterium induced NO production. These findings likely indicate a protective immunostimulatory role for probiotic bacteria (Trebichavsky et al, 2006).

Epithelial cells act as sensors to pathogen invasion that initiate defensive responses, releasing both chemokines and cytokines. Non-invasive as well as invasive microorganisms elicit production of pro-inflammatory mediators (Strober, 1998). Toll-like receptors (TLR's) play an important role in pathogen recognition and induction and regulation of the innate and adaptive immune response (Ritter et al, 2005). By targeting these TLR-mediated pathways, inflammatory conditions could be diminished.

Our data support the hypothesis that probiotic bacteria act through TLR-9 signaling to decrease *O157: H7*-induced IL-6 production, but not *O157: H7*-induced NO production. Occupancy of TLR-9 on YAMC or IMCE cells by either stimulatory or inhibitory ODN's effectively blocked *O157: H7*-induced IL-6 production. If probiotic

bacteria, such as LGG, LS, BL, or BB, are shown to act via TLR-9 dependent mechanisms *in vivo*, then they may be effective in modulating the immune response.

The NO-mediated pathway is primarily activated by TLR-2 and TLR-4 ligands, while the IL-6 mediated pathway is more complex. Thus, cumulative transcriptional pathways, including activation of NF-kB, MAPK, STAT-3, and TLR-4 activation of second messengers such as Ca^{2+} and cyclic AMP, regulate IL-6 production (Cho et al, 2007, Song et al, 2007, and Wang et al, 2003). The inability of the NF-kB inhibitor, PDTC, to decrease IL-6 in IMCE cells may be due to the fact that this inhibitor increases MIP-2 production, which may further increase IL-6 production. The use of enzymatic inhibitors of other cell signaling pathways (e.g., mitogen activated protein kinases) may be necessary with an NF-kB inhibitor to decrease IL-6 production.

We evaluated the effectiveness of four probiotic bacteria *LS, LGG, BB*, and *BL* to ameliorate *O157: H7*-induced production NO and IL-6 on YAMC and IMCE cells. All four probiotic bacterium reduced *O157: H7*-induced NO production; however *O157: H7*-induced IL-6 production was reduced by LGG, LS, and BB whereas BL increased IL-6 production in IMCE, but not YAMC, cells. Probiotic bacteria are microorganisms that are capable of ameliorating inflammatory conditions (Penner et al, 2005). Among the gram-positive probiotic bacteria examined, we observed a genus- and species-specific effect of these bacteria to decrease *O157: H7*-induced NO and IL-6 production. Because we used an *in vitro* model system of normal and preneoplastic murine colon epithelial cells, we are limited in the types of preventive inferences we can make to human nutrition. Even so, these cell culture models provided an opportunity to examine the effects of probiotic

bacteria on *O157: H7*-induced pro-inflammatory mediator production in an *Apc* genotype-dependent manner.

Consistent with our findings, Veckman and colleagues observed that gram-negative pathogenic and gram-positive probiotic bacteria act through direct and indirect cytokine-mediated mechanisms (Veckman et al, 2003). We have demonstrated a pivotal role for epithelial cells in the detection of signals originating from pathogenic bacteria as well as probiotic bacteria and the resulting ability of supernatants from bacteria-exposed epithelial cells to cause macrophage activation and chemotaxis. These data demonstrate the ability of epithelial cells to actively participate in immunomodulatory activities in response to bacterial challenge.

Our data suggest that IMCE cells, as models of preneoplastic epithelial cells, may indicate that preneoplastic cells may have the ability to elaborate paracrine signals in attracting macrophages that could enhance carcinogenesis. Our data, showing that probiotic bacteria decrease NO and IL-6 production in epithelial cells could have implications to decrease the promotional phase of carcinogenesis.

The ability of probiotic bacteria to reduce *O157: H7*-induced NO, but increase both *O157: H7*-induced IL-6 and macrophage chemotaxis, could lead us to conclude that some probiotic bacterium, such as BL or LS, provide immunostimulatory mechanisms that may act on adjacent epithelial cells and other local or systemic immune cells of the body. This may indicate that IL-6 may be protective rather than pathogenic under certain conditions and that receptor-mediated signaling pathways may be involved (Strober, 1998 and Giraud et al, 2006).

Research Strengths and Limitations

The strengths for using a cell culture model to analyze probiotic bacteria modulation on the immune response are that the cell culture model provides clear differences between the NO and IL-6 mediated pro-inflammatory pathways. It was also efficient in analyzing the differences between *O157: H7*-induced effects on normal epithelial cells and pre-neoplastic cells. With this cell culture model, we observed clear differences in probiotic modulation of the immune response induced by *O157: H7*, which may be useful in recommending LGG for human use or in an animal model system. The cell culture model that we used did not show real differences between cell types in macrophage activation, but clear differences were observed between cell types with macrophage chemotaxis.

The primary limitation of cell culture models is that they do not recapitulate the dynamic interactions between epithelial cells and numerous mucosal immune cells and extra cellular matrix components seen *in vivo*. Since extra cellular matrix components and other mucosal cell types may influence epithelial cell responses, care must be taken in drawing inferences from results using reductionist *in vitro* cell model systems.

The differential effect of probiotic bacteria used in this study on NO and IL-6 production in epithelial cells indicates that generalizations about probiotic bacteria are not possible. Some strains of probiotics, including BL and BB in our cell model produced large amounts of IL-6; and if IL-6 production is prolonged then the effects could be detrimental to the host. BL would be probably not be a good strain of probiotic bacteria to recommend for an inflammatory bowel disease patient, but would rather be a good strain to recommend for an infant with atopic Th2-mediated inflammation to shift the

cytokine profile towards Th1. Another limitation to our research is that we only observed the immune responses from individual probiotic bacteria and did not analyze the symbiotic nature by combining more than one bacterium.

Future Implications

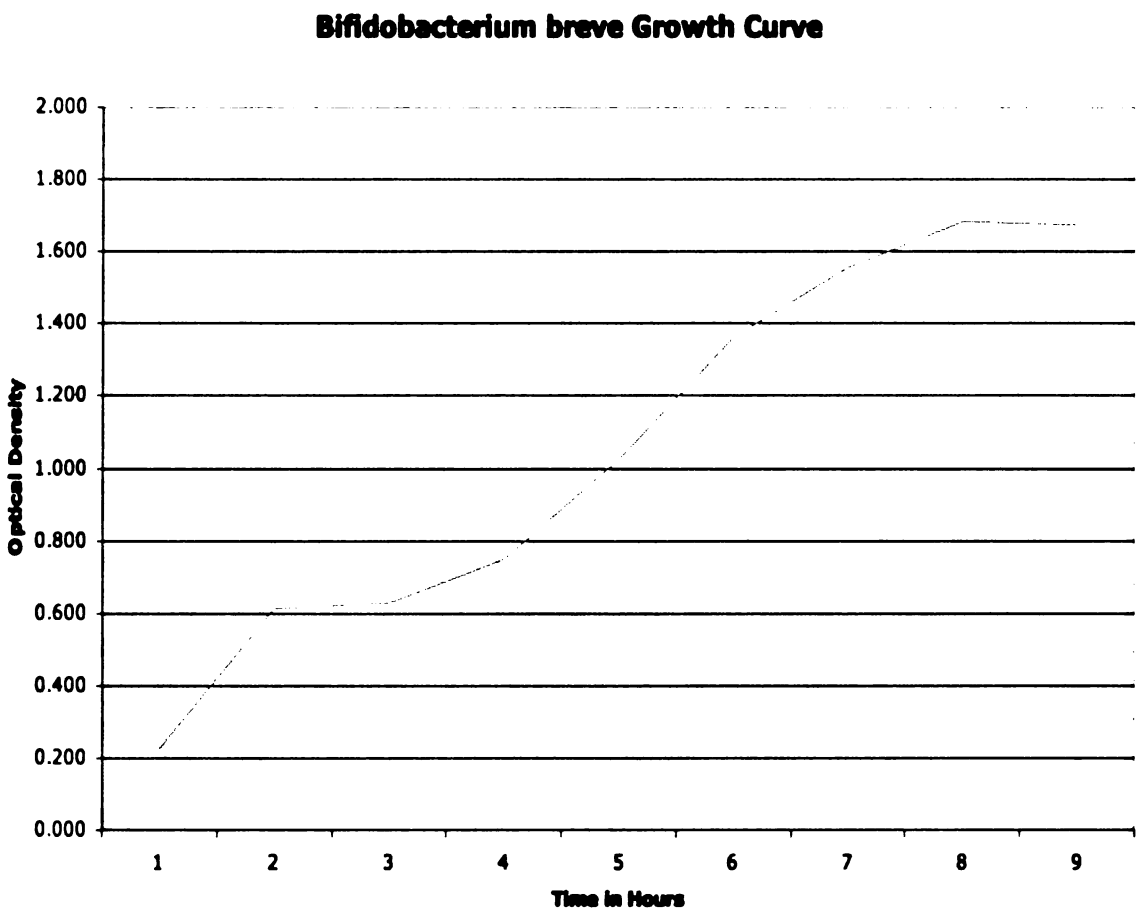
Gaining insight into host-pathogen interactions will help facilitate scientific investigations in the development of targeted treatment to minimize pathogen invasion that could otherwise lead to systemic disease (Acheson et al, 2004). The oral administration of probiotic therapies may be beneficial in ameliorating many diseases by either modulating the immune response locally (i.e., inside the GI tract) or systemically (i.e., atopic diseases) (Parvez et al, 2006 and Salminen et al, 2005). The use of multi-strain probiotic preparations (e.g., VSL #3, which is a mixture of four strains of *lactobacilli*, three strains of *bifidobacterium*, and one strain of *streptococcus*) (Corthesy et al, 2007) may have more synergistic potential than single strains in the mechanistic approach in improving illness (Penner et al, 2005). The consumption of fermented dairy products, containing probiotic bacteria, in healthy subjects, could be a key strategy in preventing colon carcinogenesis. Since the effects of probiotics are transient, future probiotic investigation for human use should be of an appropriate length of time and dose to see the totality of immunomodulatory effects being prolonged. The molecular mechanisms of activating signal transduction in intestinal epithelial cells by probiotic bacteria may be relevant for initiating and maintaining gut homeostasis (Ruiz et al, 2005). Probiotic strains that adapt to the colonic environment and possess anti-inflammatory properties may be good candidates in modulating host immunity and thereby have the potential to prevent disease (Corthésy et al, 2007).

Ongoing research will continue to identify and characterize existing strains of probiotic bacteria, identify strain-specific outcomes, and determine optimal concentrations of probiotics in eliciting consistent immunomodulatory responses. We provided data that illuminates one potential mechanism, such as TLR-9 mediated signaling, through which probiotic bacteria may exert their beneficial effects. However, there may be numerous other mechanisms through which probiotic bacteria may act. Ongoing research in our laboratory is investigating the role of probiotic bacteria in inducing the aforementioned negative regulators in TLR-signaling. In addition to these proteins, work is ongoing to examine other chemokines and cytokines induced by probiotic bacteria that may have immunomodulatory functions.

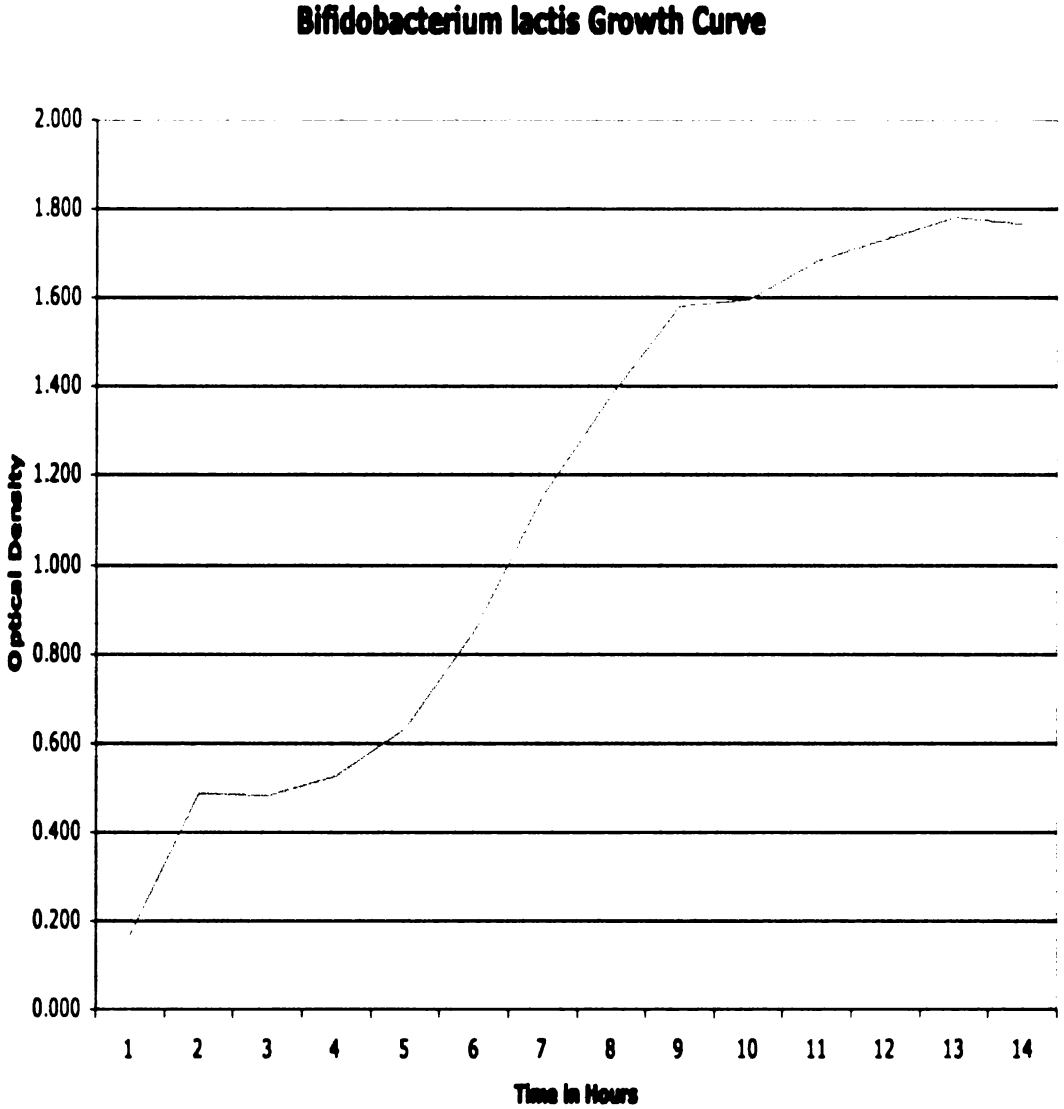
APPENDICES

APPENDIX A

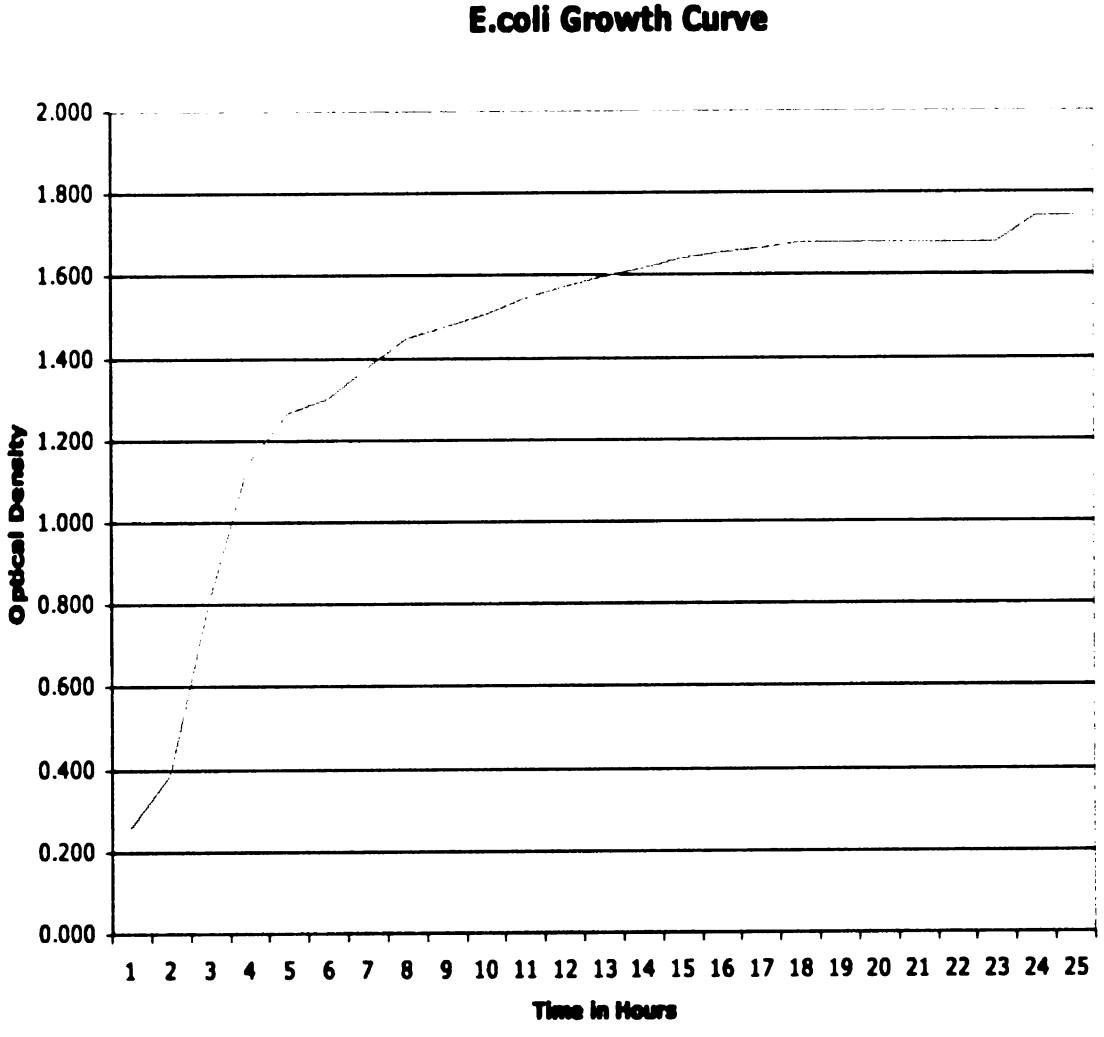
Growth Curves for Bacteria



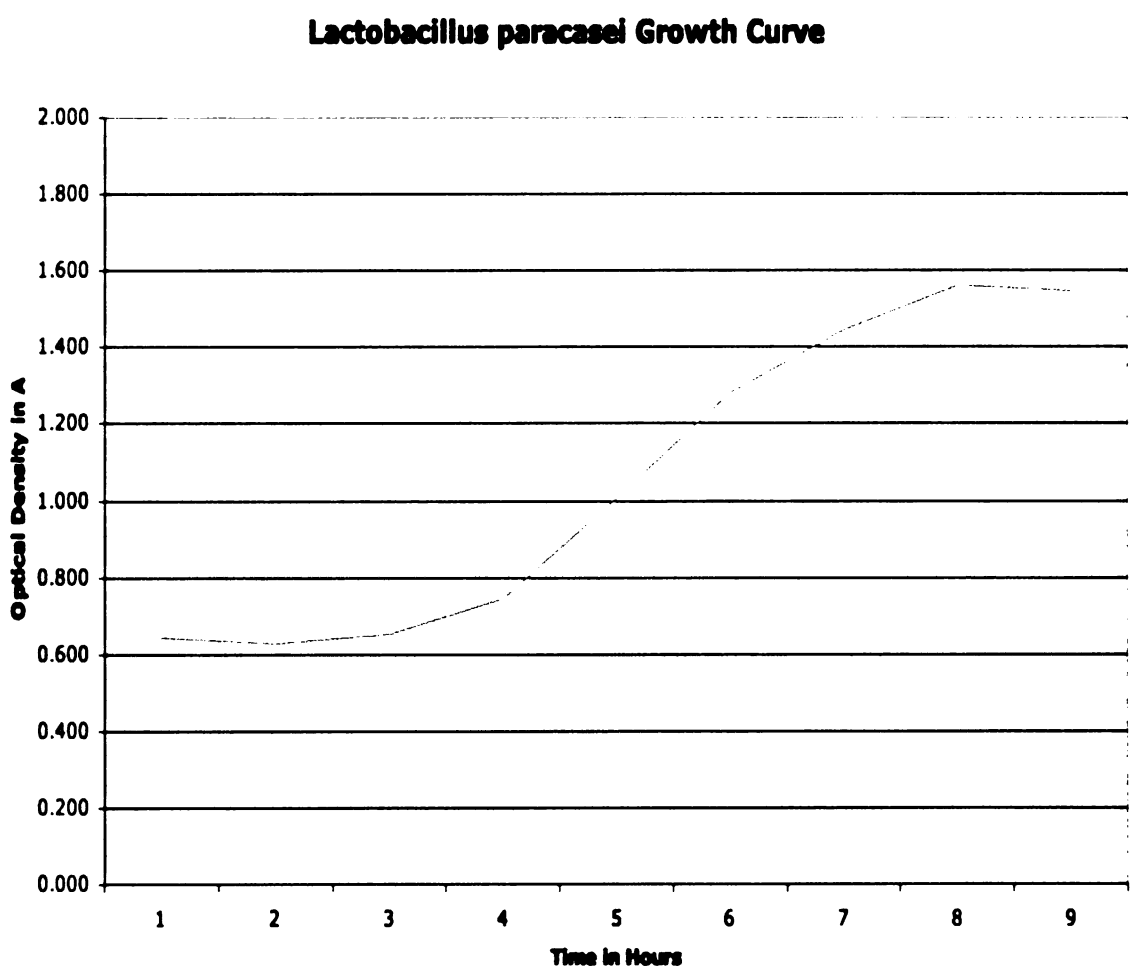
APPENDIX A


Bifidobacterium lactis Growth Curve
Optical Density
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time in Hours

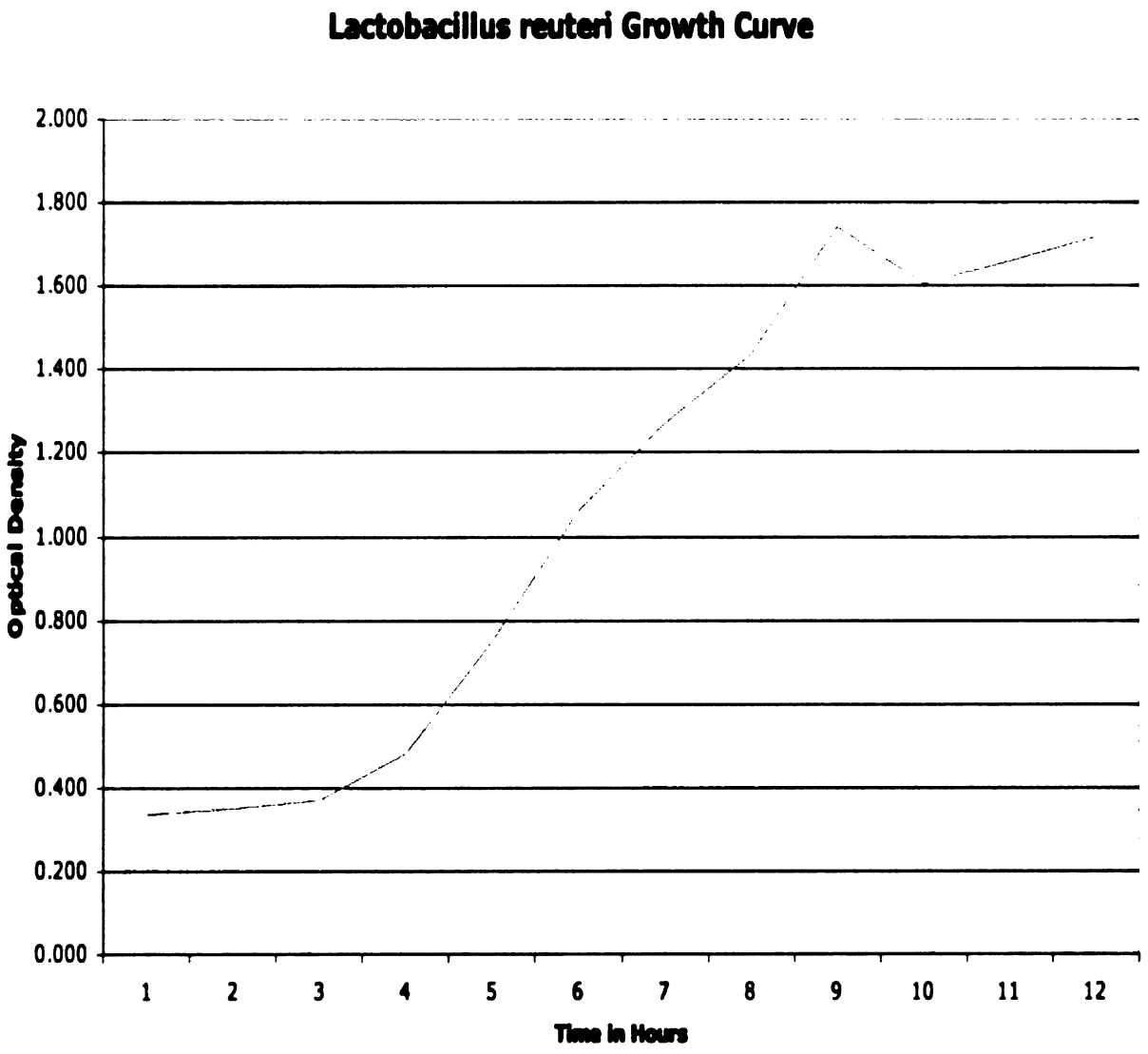
APPENDIX A


E.coli Growth Curve
Optical Density
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Time in Hours

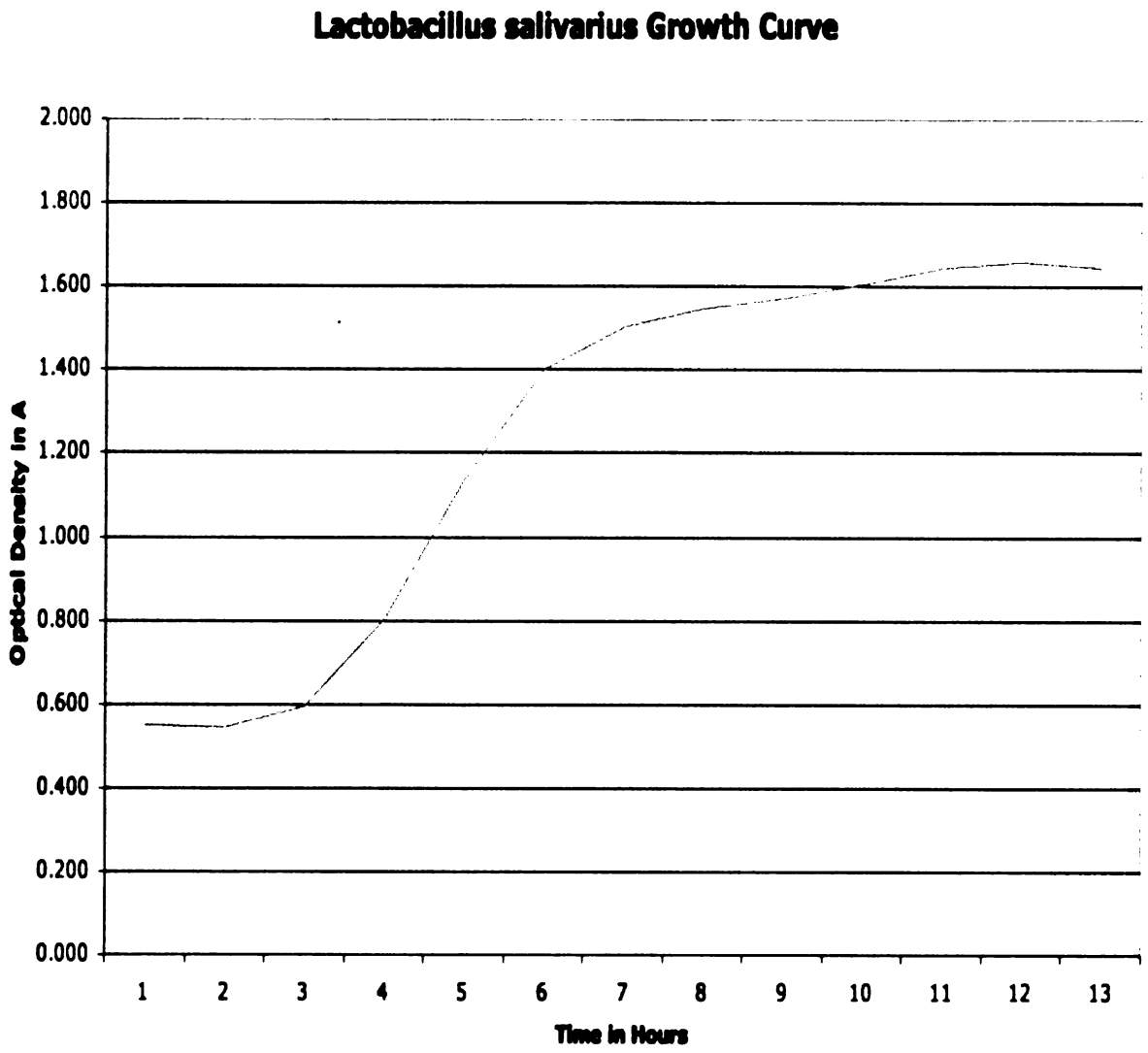
APPENDIX A


Lactobacillus paracasei Growth Curve
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Optical Density in A
1
2
3
4
5
6
7
8
9
Time in Hours

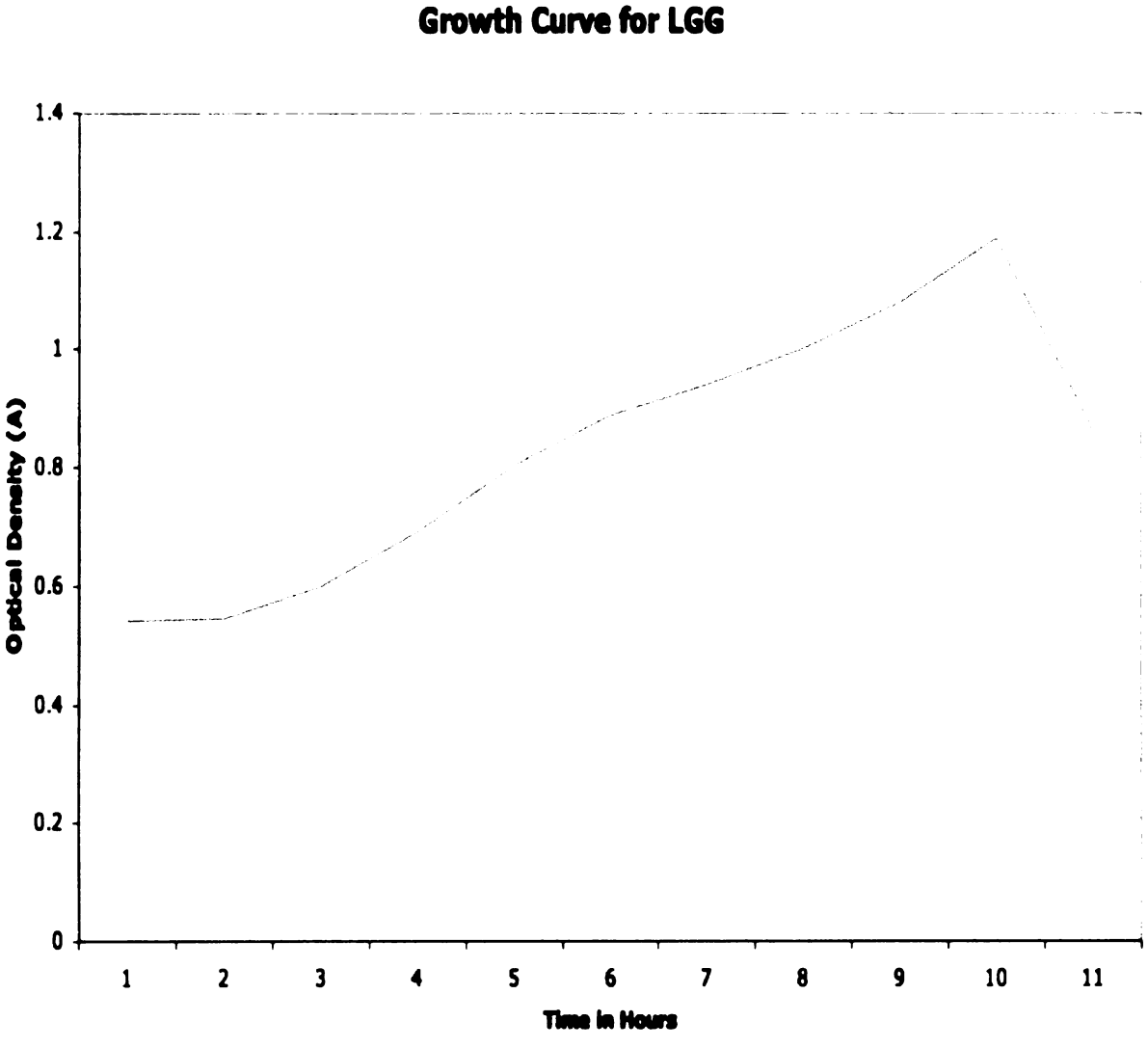
APPENDIX A


Lactobacillus reuteri Growth Curve
Optical Density
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
1
2
3
4
5
6
7
8
9
10
11
12
Time in Hours

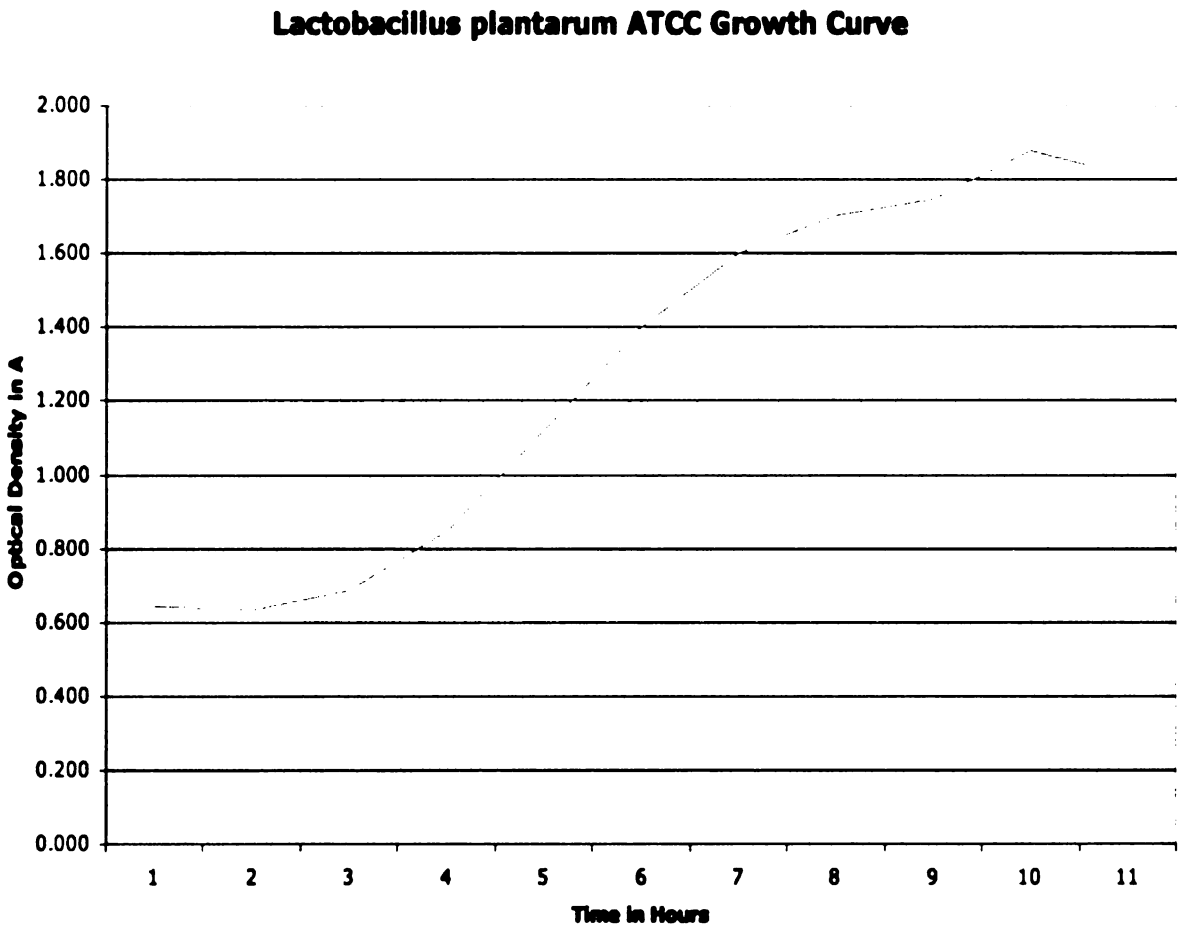
APPENDIX A


Lactobacillus salivarius Growth Curve
Optical Density in A
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
1
2
3
4
5
6
7
8
9
10
11
12
13
Time in Hours

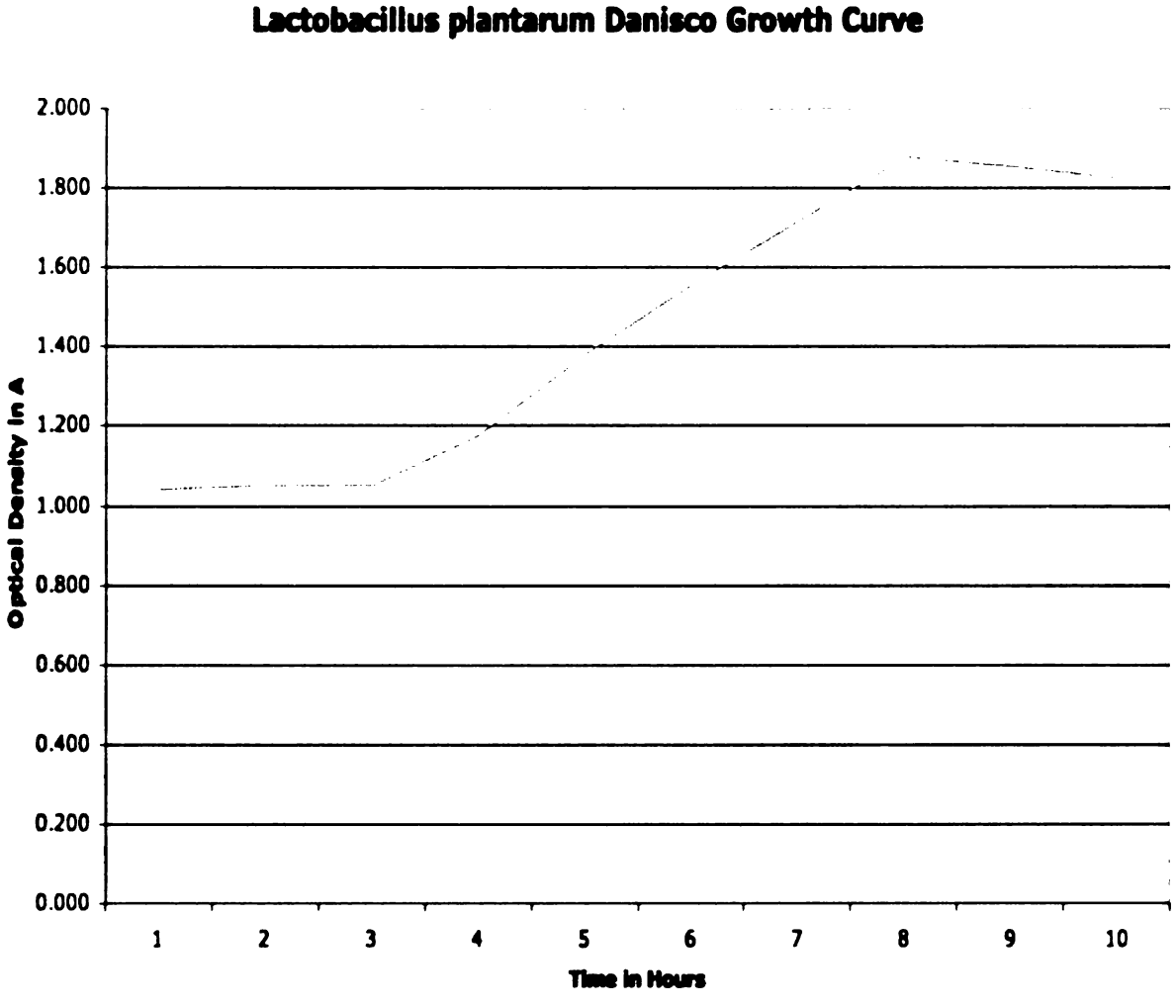
APPENDIX A


Growth Curve for LGG
Optical Density (A)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
9
10
11
Time in Hours

APPENDIX A


Lactobacillus plantarum ATCC Growth Curve
Optical Density in A
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
1
2
3
4
5
6
7
8
9
10
11
Time in Hours

APPENDIX A


Lactobacillus plantarum Danisco Growth Curve
Optical Density in A
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
1
2
3
4
5
6
7
8
9
10
Time in Hours

APPENDIX B

Algorithm for Lyophilization and Reconstitution of Bacteria

Weigh 1.5ml tubes to get pre-bacteria weight

↓

Aliquot 1ml into each tube

↓

Use speed vacuum to desiccate bacteria/PBS slurry

↓

Weigh tubes to get a post-weight and subtract out pre-weight to get net bacterial weights

↓

Calculate how much low serum RPMI media to add so bacteria concentration is 40 mg/ml

↓

Freeze all tubes in -80 C

↓

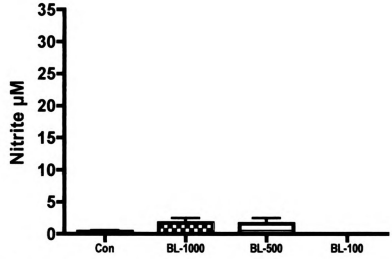
Repeat for other 7 strains of bacteria

Note that a test run with PBS alone through the speed vacuum and the average weight of residual salts was subtracted that from bacterial weight

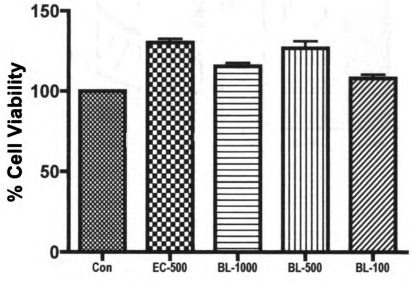
APPENDIX C

Effects of Probiotic Bacteria Alone on Epithelial Cell NO and IL-6 Production

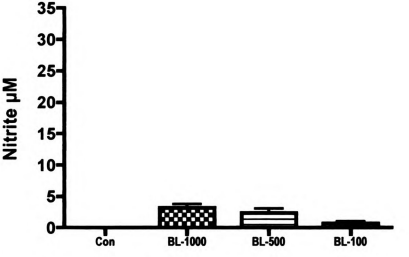
1. NO (Nitrite μM) production of YAMC cells treated with *Bifidobacterium lactis* (μg/ml) for 48 hr. No statistical significance $p>0.05$



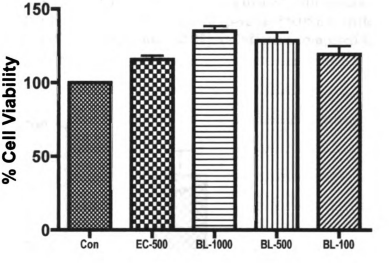
2. Representative cell viability compared to control of YAMC cells treated various concentrations of *Bifidobacterium lactis* (μg/ml) for 48 hr



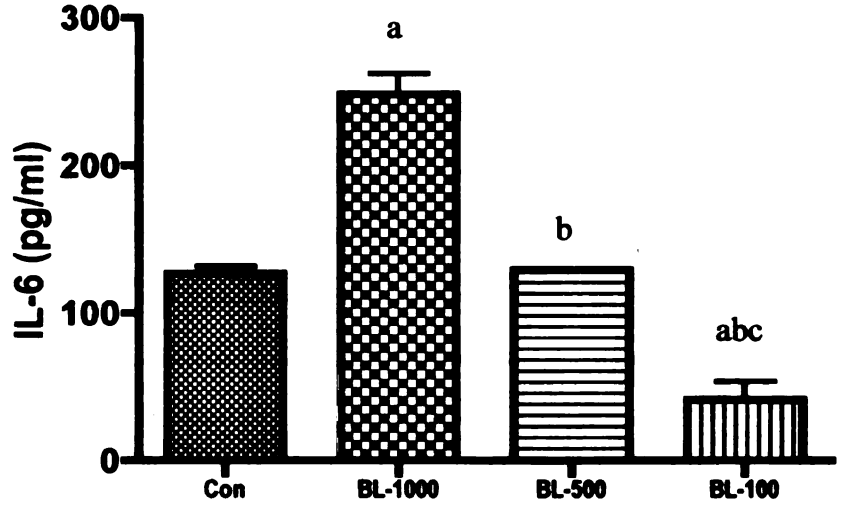
3. NO (Nitrite μM) production of IMCE cells treated with *Bifidobacterium lactis* (μg/ml) for 48 hr. No statistical significance p>0.05



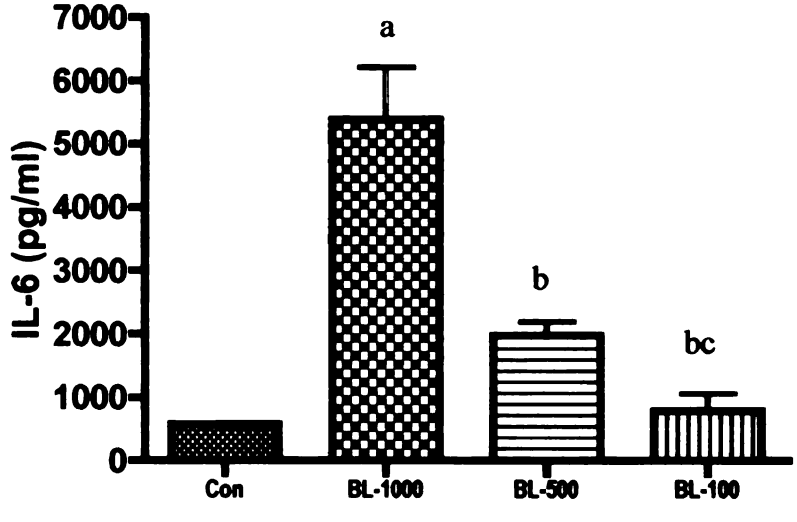
4. Cell viability compared to control of IMCE cells treated with *Bifidobacteirum lactis* (μg/ml) for 48 hr



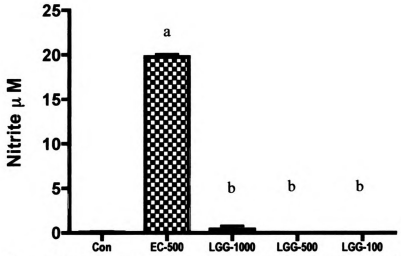
5. IL-6 production (pg/mL) production of YAMC cells treated with *Bifidobacteirum lactis* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.05). b-Different compared to *B. lactis* at 1000 μg/ml (p-value <0.01). c-Different compared to *B. lactis* at 500 μg/ml (p-value <0.05)



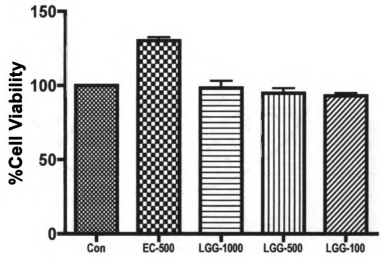
6. IL-6 production (pg/mL) production of IMCE cells treated with *Bifidobacterium lactis* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.05). b-Different compared *B. lactis* at 1000 μg/ml (p-value <0.01). c-Different compared to *B. lactis* at 500 μg/ml (p-value <0.05)



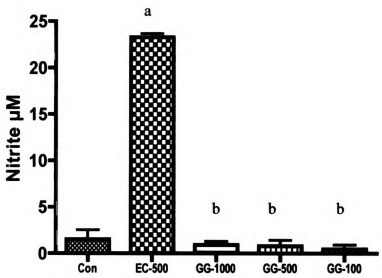
7. NO (Nitrite μM) production of YAMC cells treated with *Lactobacillus rhamnosus GG* (μg/ml) for 48 hr. a-Different compared to control p<0.001. b-Different compared to *O157: H7* at 500 μg/ml (p-value <0.001)



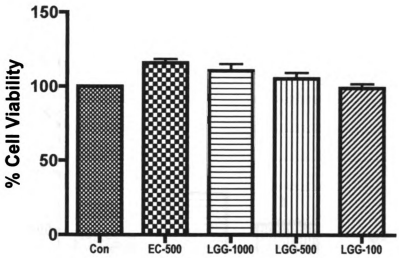
8. Representative cell viability compared to control of YAMC cells treated with varying doses of *Lactobacillus rhamnosus GG* (μg/ml) for 48 hr



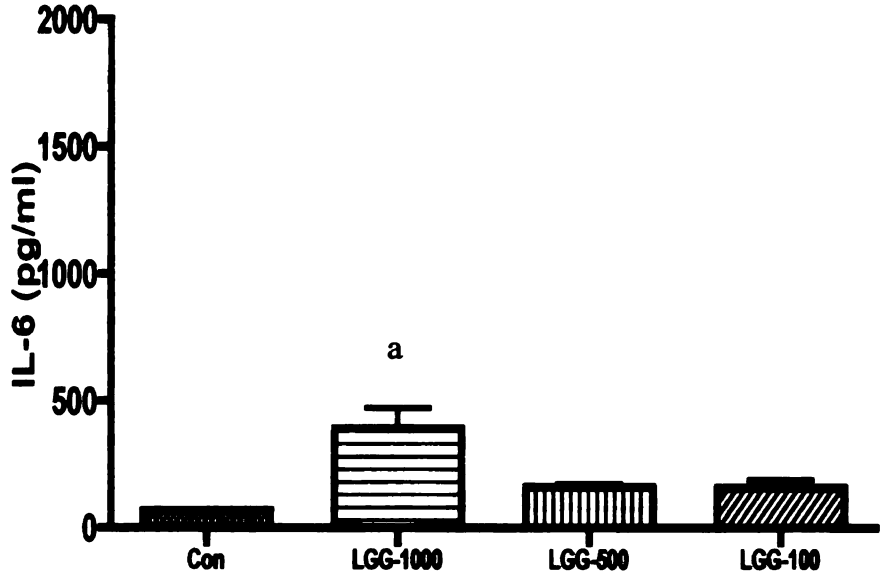
9. NO (Nitrite μM) production of IMCE cells treated with *Lactobacillus rhamnosus GG* (μg/ml) for 48 hr. a-Different compared to control p<0.001. b-Different compared to *O157: H7* at 500 μg/ml p<0.001



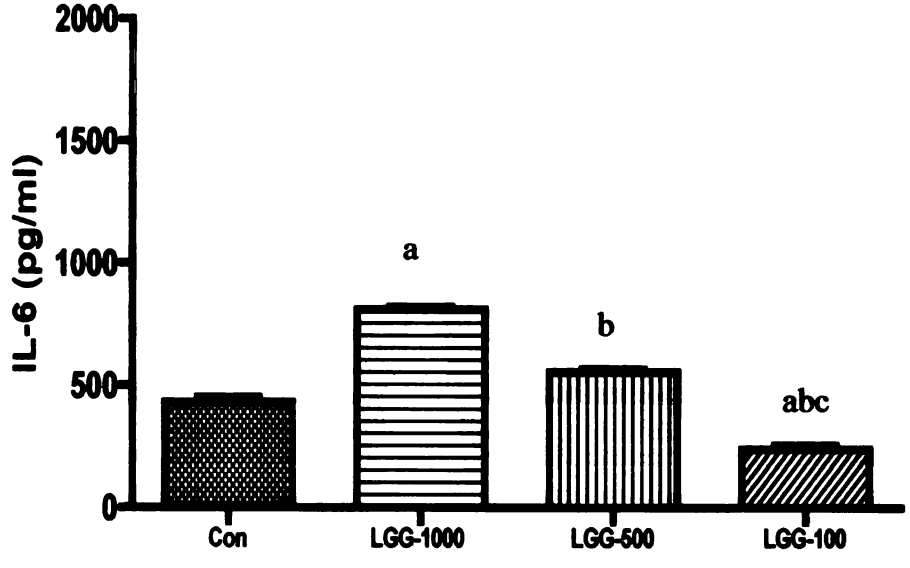
10. Cell viability compared to control of IMCE cells treated with varying doses of *Lactobacillus rhamnosus GG* (μg/ml) for 48 hr



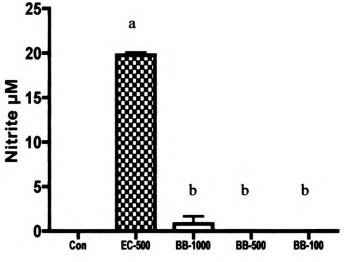
11. IL-6 production (pg/mL) production of YAMC cells treated with *Lactobacillus rhamnosus GG* (μg/ml) for 48 hr. a-Different compared to control ($p<0.05$)



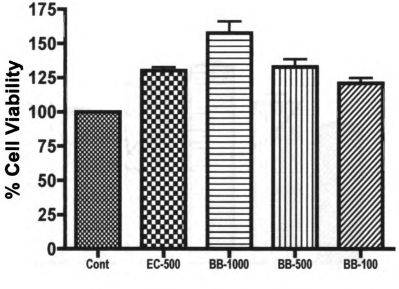
12. IL-6 production (pg/mL) production of IMCE cells treated with *Lactobacillus rhamnosus GG* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.05). b-Different compared to *L. rhamnosus GG* at 1000 μg/ml (p-value <0.01). c-Different compared to *L. rhamnosus GG* at 500 μg/ml (p-value <0.01)



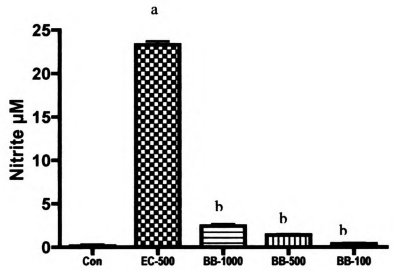
13. NO (Nitrite μM) production of YAMC cells treated with *Bifidobacterium breve* (μg/ml) for 48 hr. a-Different compared to control $p<0.001$. b-Different compared to *O157: H7* at 500 μg/ml $p<0.001$



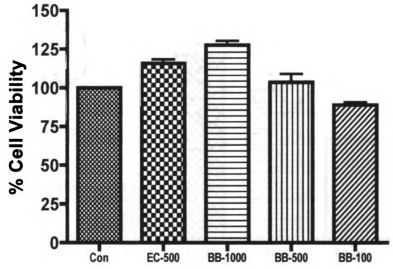
14. Cell viability compared to control of YAMC cells treated with varying doses of *Bifidobacterium breve* (μg/ml) for 48 hr



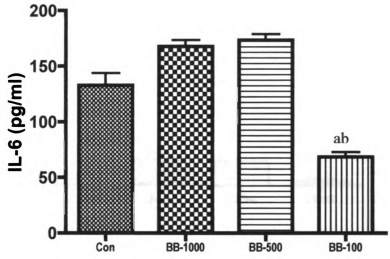
15. NO (Nitrite μM) production of IMCE cells treated with *Bifidobacterium breve* (μg/ml) for 48 hr. a-Different compared to control $p<0.001$. b-Different compared to O157: H7 at 500 μg/ml $p<0.001$



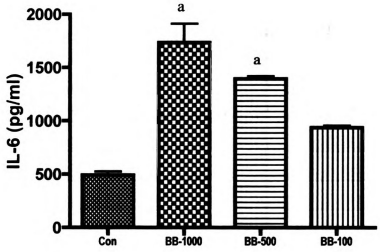
16. Cell viability compared to control of IMCE cells treated with varying doses of *Bifidobacterium breve* (μg/ml) for 48 hr



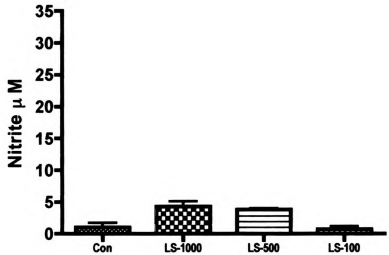
17. IL-6 production (pg/mL) production of YAMC cells treated with *Bifidobacterium breve* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.05). b-Different compared to *B. breve* at 1000 μg/ml or *B. breve* at 500 μg/ml (p-value <0.01)



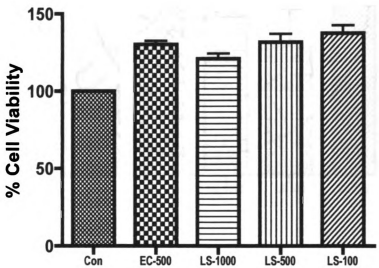
18. IL-6 production (pg/mL) production of IMCE cells treated with *Bifidobacterium breve* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.05)



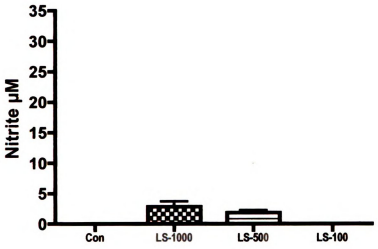
19. NO (Nitrite μM) production of YAMC cells treated with *Lactobacillus salivarius* (μg/ml) for 48 hr. No statistical significance $p>0.05$



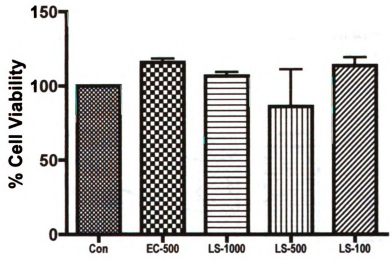
20. Cell viability compared to control of YAMC cells treated with varying doses of *Lactobacillus salivarius* (μg/ml) for 48 hr



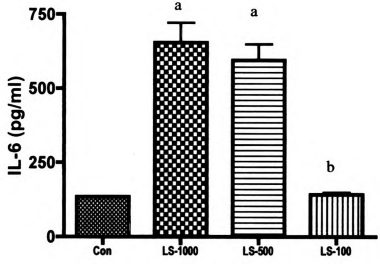
21. NO (Nitrite μM) production of IMCE cells treated with *Lactobacillus salivarius* (μg/ml) for 48 hr. No statistical significance p>0.05



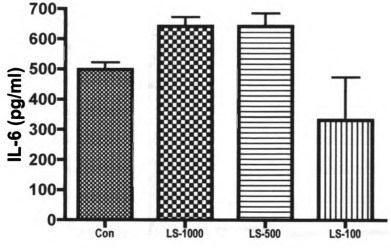
22. Cell viability compared to control of IMCE cells treated with varying doses of *Lactobacillus salivarius* (μg/ml) for 48 hr



23. IL-6 production (pg/mL) production of YAMC cells treated with *Lactobacillus salivarius* (μg/ml) for 48 hr. a-Different compared to control (p-value <0.01). b-Different compared to *L. salivarius* at 1000 μg/ml or *L. salivarius* at 500 μg/ml (p-value <0.05)



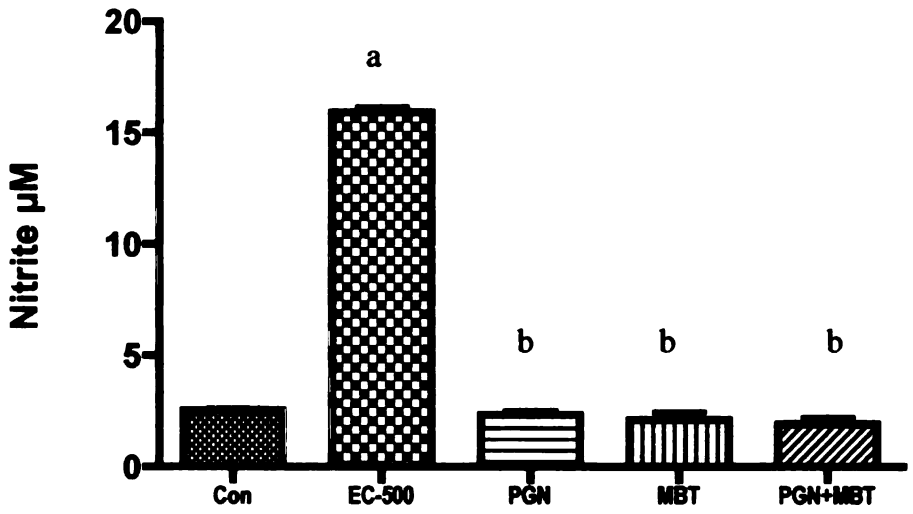
24. IL-6 production (pg/mL) production of IMCE cells treated with *Lactobacillus salivarius* (μg/ml) for 48 hr. No statistical significance (p-value >0.05)



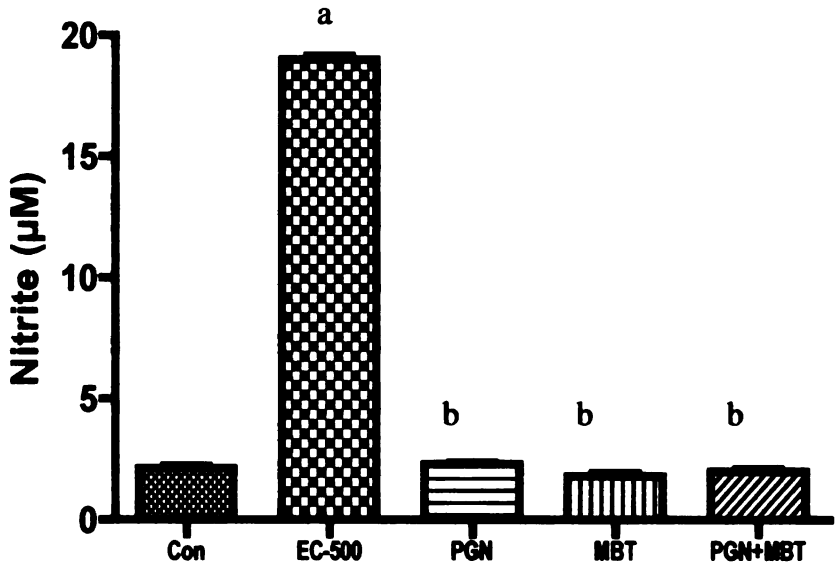
APPENDIX D

Effect of TLR Ligands 2, 4, and 9 on Epithelial Cell Production of NO

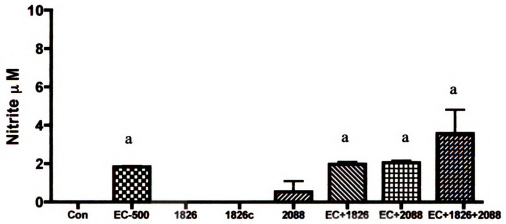
1. NO (Nitrite μM) production of YAMC cells with peptidoglycan (TLR-2 ligand) or murabutude (NOD-2 ligand) for 24 hr. a-Different compared to control $p<0.001$. b-Different compared to *O157: H7* at 500 μg/ml $p<0.01$



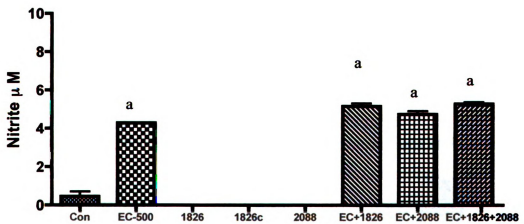
2. NO (Nitrite μM) production of IMCE cells with peptidoglycan (TLR-2 ligand) or murabutide (NOD-2 ligand) for 24 hr. a-Different compared to control $p<0.001$. b-Different compared to *O157: H7* at 500 μg/ml $p<0.001$



2. NO (Nitrite μM) production of YAMC cells with TLR-9 ligand for 24 hr. a- Different compared to control (p-value <0.05)



3. NO (Nitrite μM) production of IMCE cells with TLR-9 ligand for 24 hr. a- Different compared to control (p-value <0.001)

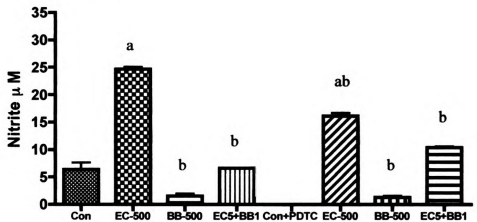


*****1826 is a stimulatory TLR-9 ligand and 2088 is an inhibitory TLR-9 ligand**

APPENDIX E

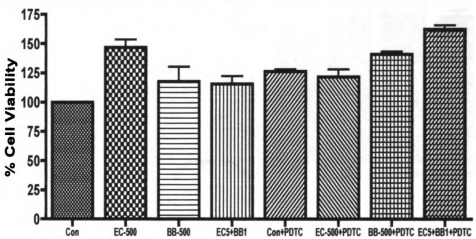
Effect of PDTC on Epithelial Cell Production of NO and IL-6

1. NO (Nitrite μM) production of YAMC cells cotreated with 500 μg/ml of *O157: H7*, 500 μg/ml of *B. breve* and cotreatments of 10μM PDTC (NF-kB inhibitor) for 48 hr. a-Different compared to control $p<0.001$. b-Different compared to *O157: H7* at 500 μg/ml $p<0.001$

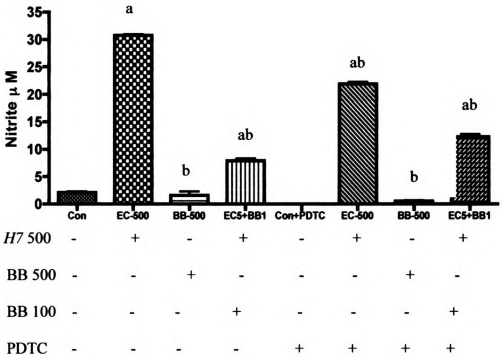


O157: H7 500	-	+	-	+	-	+	-	+
BB 500	-	-	+	-	-	-	+	-
BB 100	-	-	-	+	-	-	-	+
PDTC	-	-	-	-	+	+	+	+

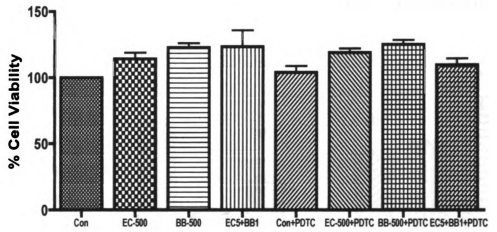
2. Cell viability compared to control of YAMC cells cotreated with *O157: H7* (500μg/ml) and *B. breve* (500μg/ml), and cotreatments with 10μM PDTC



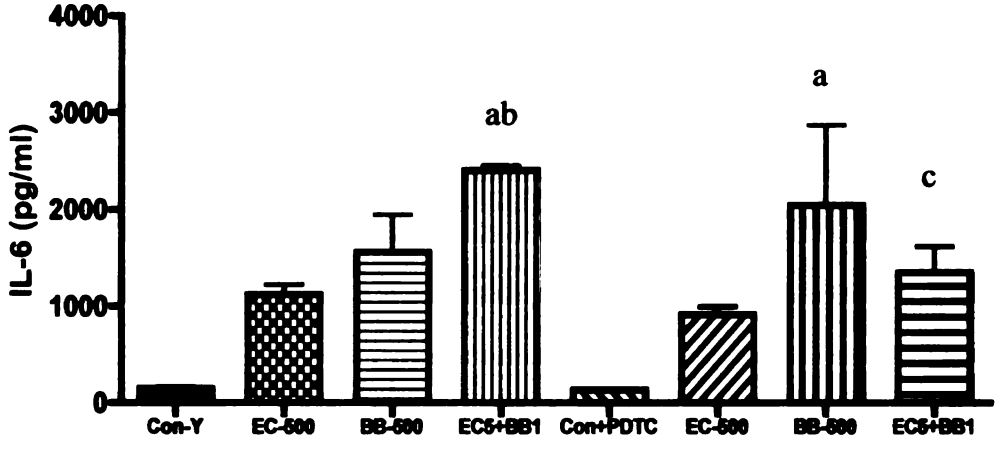
3. NO (Nitrite μM) production of IMCE cells cotreated with 500μg/ml of *O157: H7*, 500μg/ml of *B. breve*, and cotreatments of 10μM PDTC (NF-kB inhibitor) for 48 hr. a-Different compared to control p<0.001. b-Different compared to *O157: H7* at 500 μg/ml p<0.001



4. Cell viability compared to control of IMCE cells cotreated with *O157: H7* (500μg/ml) and *B. breve* (500μg/ml) and cotreatments with 10μM PDTC

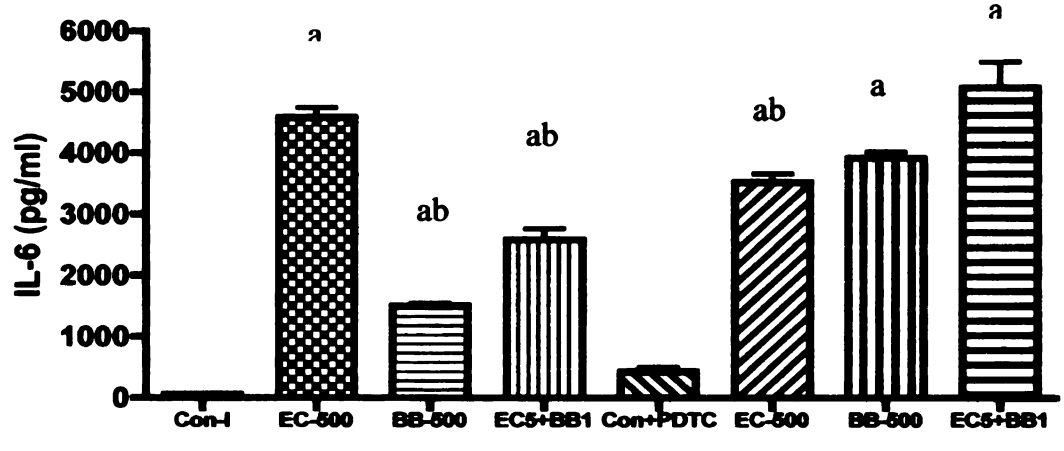


5. IL-6 (pg/mL) production of YAMC cells cotreated with 500μg/ml of *O157: H7*, 100μg/ml of *B. breve*, and cotreatments of 10μM PDTC for 48 hr. a-Different compared to control p<0.001. b-Different compared to *O157: H7* at 500 μg/ml (p-value <0.05). c-Different compared to *O157: H7*+BB (p-value <0.05)



O157: H7 500	-	+	-	+	-	+	-	+
BB 500	-	-	+	-	-	-	+	-
BB 100	-	-	-	+	-	-	-	+
PDTC	-	-	-	-	+	+	+	+

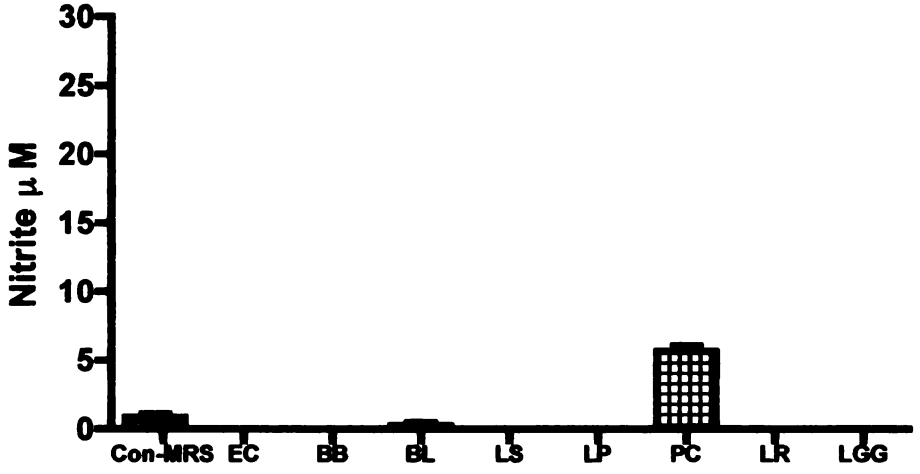
6. IL-6 (pg/mL) production of IMCE cells cotreated with 500μg/ml of *O157: H7*, 500μg/ml of *B. breve*, and cotreatments of 10μM PDTC for 48 hr. a-Different compared to control p<0.001. b-Different compared to *O157: H7* at 500 μg/ml (p-value <0.05)



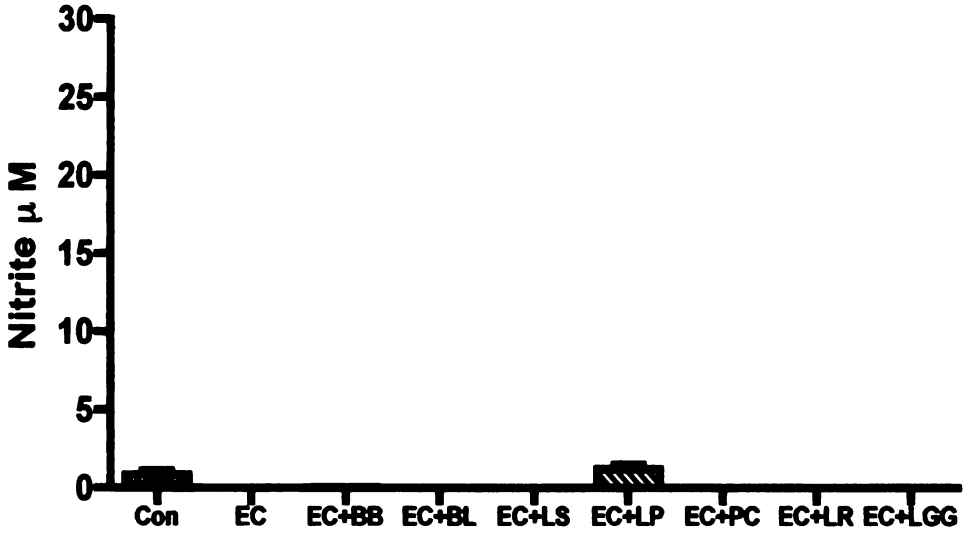
APPENDIX F

Effects of Bacterial Growth Medias on Macrophage NO and IL-6 Production

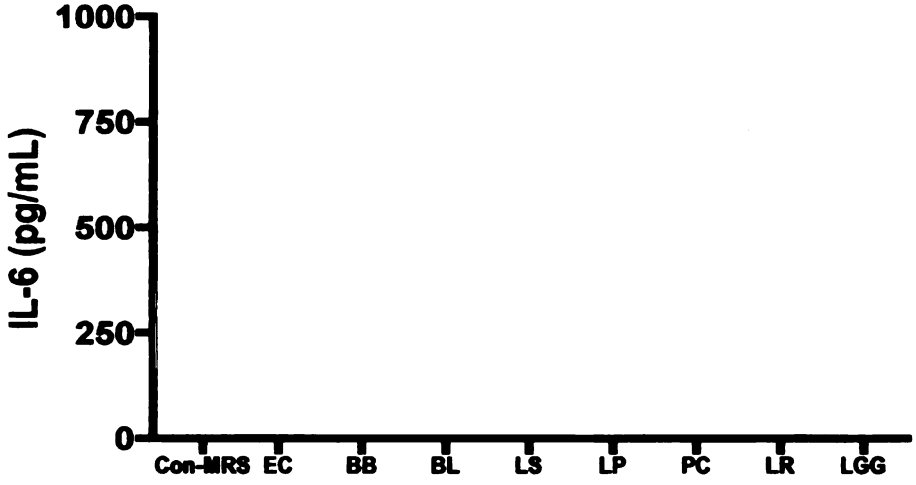
1. Macrophage activation treated with growth media bacterial supernatants (TSB-YE from growth of *O157: H7*; MRS from growth of *B. breve*, *B. lactis*, *L. salivarius*, *L. plantarum*, *L. paracasei*, *L. reuteri*, and *L. rhamnosus GG*) for 48 hr. No statistical significance $p>0.05$



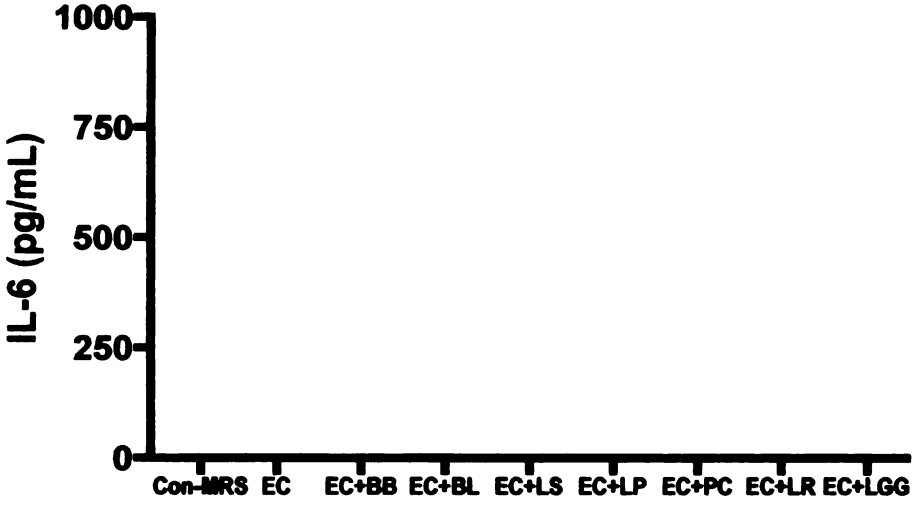
2. Macrophage activation treated with growth media bacterial supernatant cotreatments with (TSB-YE from growth of *O157: H7* cotreated with MRS from growth of *B. breve*, *B. lactis*, *L. salivarius*, *L. plantarum*, *L. paracasei*, *L. reuteri*, and *L. rhamnosus GG*) for 48 hr. No statistical significance $p>0.05$



3. IL-6 (pg/mL) production of macrophages treated with growth media bacterial supernatants (TSB-YE from growth of *O157: H7*; MRS from growth of *B. breve*, *B. lactis*, *L. salivarius*, *L. plantarum*, *L. paracasei*, *L. reuteri*, and *L. rhamnosus GG*) for 48 hr. No statistical significance $p>0.05$



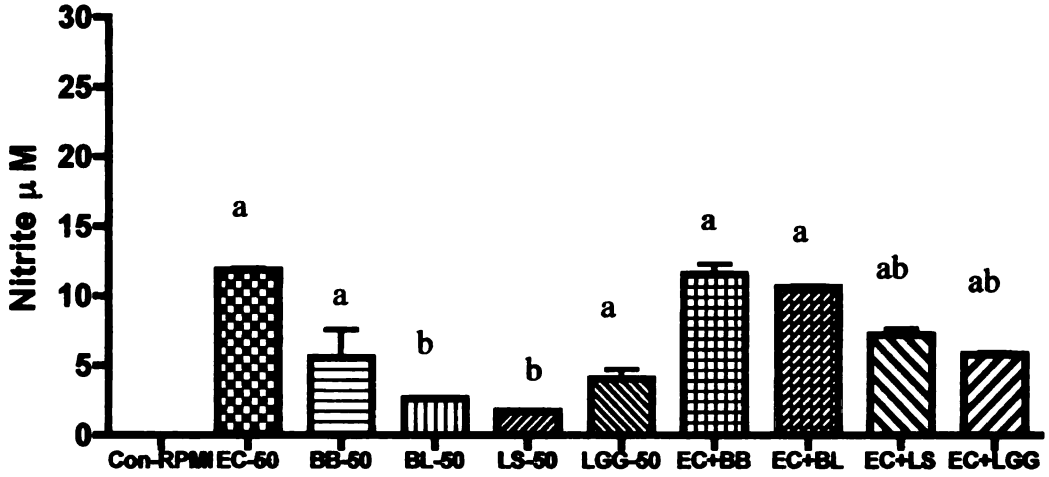
4. IL-6 (pg/mL) production of macrophages cotreated with growth media bacterial supernatants (TSB-YE from growth of *O157: H7* cotreated with MRS from growth of *B. breve*, *B. lactis*, *L. salivarius*, *L. plantarum*, *L. paracasei*, *L. reuteri*, and *L. rhamnosus GG*) for 48 hr. No statistical significance $p>0.05$



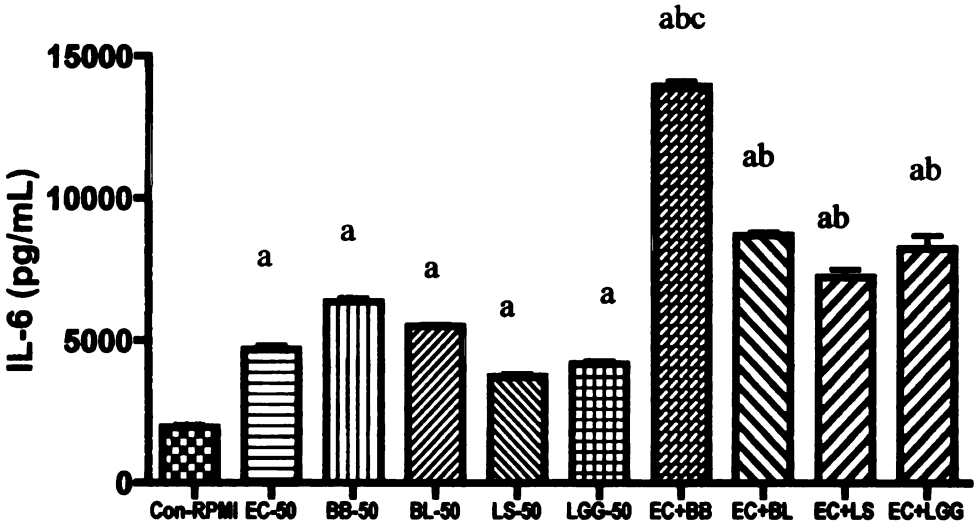
APPENDIX G

Effect of Exposing Bacteria Directly to Macrophages on NO and IL-6 Production

1. Macrophage activation treated directly with *O157: H7*, *B. breve*, B. lactis, *L. salivarius*, *L. rhamnosus GG*, or a combination thereof at 50μg/ml each for 48 hr. a-Different compared to control $p<0.01$. b-Different compared to *O157: H7* at 50 μg/ml $p<0.01$



2. IL-6 (pg/mL) production of macrophages treated with *O157: H7*, *B. breve*, *B. lactis*, *L. salivarius*, *L. rhamnosus GG*, or a combination thereof at 50μg/mL for 48 hr. a-Different compared to control $p<0.001$. b-Different compared to *O157: H7* at 50 μg/ml $p<0.001$. c-Different compared to other cotreatments (p-value <0.001)



APPENDIX H

Summary of Results from Chapter 4

Key		
	↑↑↑ p-value <0.001	↓↓↓ p-value <0.001
	↑↑ p-value <0.01	↓↓ p-value <0.01
	↑ p-value <0.05	↓ p-value <0.05

Bacterial and Bacterial Component Treatments

YAMC Treatment	NO	IL-6	Comments
EC	↑↑↑	↑↑↑	EC→TLR 2 or 4
BB	-	-	Differential IL-6 increases? Is it through TLR ligands or competing?
BL	-	↑	
LS	-	↑↑	
LGG	-	↑	
EC+BB (BB @ 500, 250, or 25 µg/ml)	↓↓↓ (500, 250, and 25)	↓↓↓ (500, 250, and 25)	NO: TLR access? (Block of turn on a negative signal) IL-6: Differential TLR? Or what other pathway might be playing a role
EC+BL (BL @ 500, 250, or 25 µg/ml)	↓↓↓ (500, 250, and 25)	↑↑↑ (500, 250, and 25)	
EC+LS (LS @ 500, 250, or 25 µg/ml)	↓↓↓ (500)	↑↑↑ (500, 250, and 25)	
EC+LGG (LGG @ 500, 250, or 25 µg/ml)	↓↓↓ (500, 250, and 25)	↓↓↓ (500, 250, and 25)	
PGN	-	-	
MBT	-	-	
LPS	-	↑	
1826	-	-	
1826c	-	-	
2088	-	-	
1826+EC	↑	↓↓	Negative or positive TLR-9 engagement to decrease IL-6 induced by EC, but not NO
2088+EC	↑	↓↓	
mAb TLR-2+EC	↓↓	-	No Effect on IL-6, Why?
mAb TLR-4+EC	↓↓	-	
Polymyxin B+EC	↓	-	TLR-4 via NO, not IL-6
PDTC+EC	↓↓↓	-	Decrease NO but not IL-6 in YAMC

IMCE Treatment	NO	IL-6	Comments
EC	↑↑↑	↑↑↑	**EC→TLR 2 or 4**
BB	-	↑	**Differential IL-6 increases? Is it through TLR ligands or competing?**
BL	-	↑	
LS	-	-	
LGG	-	↑	
EC+BB (BB @ 500, 250, or 25 μg/ml)	↓↓↓ **(500, 250, and 25)**	↓↓↓ **(250 and 25)**	**Genus/species-specific differences as well as cell type differences**
EC+BL (BL @ 500, 250, or 25 μg/ml)	↓↓↓ **(500, 250, and 25)**	↓↓ **(500 and 250)**	
EC+LS (LS @ 500, 250, or 25 μg/ml)	↓↓ **(500, 250, and 25)**	↑↑↑ **(500, 250, and 25)**	
EC+LGG (LGG @ 500, 250, or 25 μg/ml)	↓↓↓ **(500)**	↓↓ **(500, 250, and 25)**	
PGN	-	-	
MBT	-	-	
LPS	-	↑↑	**Cell type differences**
1826	-	↑↑↑	
1826c	-	↑↑↑	
2088	-	-	
1826+EC	↑↑	↓	**Cell type-direction same, but different magnitude**
2088+EC	↑↑	↓	
mAb TLR-2+EC	↓↓↓	↓	**NO is same, but with IL-6 there is a cell-type difference**
mAb TLR-4+EC	↓↓↓	↓	
Polymyxin B+EC	↓↓↓	↓↓↓	**Lipid A effect**
PDTC+EC	↓↓↓	↓	**NF-kB inhibition (IkK inhibition)**

Bacterial Growth Medias and Bacteria Directly Exposed to Macrophages

Treatments	NO	IL-6	Comments
EC (TSB-YE)	-	-	**Everything we observed is bacteria-dependent**
BB (MRS) or/+ EC (TSB-YE)	-	-	
BL (MRS) or/+ EC (TSB-YE)	-	-	
LS (MRS) or/+ EC (TSB-YE)	-	-	
LP (MRS) or/+ EC (TSB-YE)	-	-	
LPC (MRS) or/+ EC (TSB-YE)	-	-	

LR (MRS) or/+ EC (TSB-YE)	-	-	
LGG (MRS) or/+ EC (TSB-YE)	-	-	
EC 50	↑↑	↑↑↑	**All bacteria express TLR ligands that activate TLR's on macrophages**
BB 50	↑↑	↑↑↑	
BL 50	-	↑↑↑	
LS 50	-	↑↑↑	
LGG 50	↑↑	↑↑↑	
EC+BB (50µg/ml each)	↑↑	↑↑↑	**Why do Lactobacilli decrease NO, but not IL-6?**
EC+BL (50µg/ml each)	↑↑	↑↑↑	
EC+LS (50µg/ml each)	↓↓	↑↑↑	
EC+LGG (50µg/ml each)	↓↓	↑↑↑	

Macrophage Activation (YAMC and IMCE Supernatants Exposure)

YAMC Treatment	NO	IL-6	mAb IL-6 (NO)	mAb IL-6 (IL-6)	Comments
EC	-	↑↑↑	-	↓↓↓	**NO are all (-) and IL-6 has a dose-related effect as well as the mAb IL-6 showing that IL-6 in supernatant being a (+) regulator of IL-6 via macrophages**
EC+BB (500, 250, or 25 µg/ml)	-	↓↓↓ **(500, 250, and 25)**	-	↓↓↓	
EC+BL (500, 250, or 25 µg/ml)	-	**N/A**	-	**N/A**	
EC+LS (500, 250, or 25 µg/ml)	-	↓↓↓ **(25)**	-	↓↓↓	
EC+LGG (500, 250, or 25 µg/ml)	-	↓↓ **(500, 250, and 25)**	-	↓↓↓	

IMCE Treatment	NO	IL-6	mAb IL-6 (NO)	mAb IL-6 (IL-6)	Comments
EC	-	↑↑↑	-	↓↓↓	**NO are all (-) and IL-6 has a dose-related effect as well as the mAb IL-6 showing that IL-6 in supernatant being a (+) regulator of IL-6 via macrophages**
EC+BB (500, 250, or 25 µg/ml)	-	↓↓↓ **(250 and 25)**	-	↓↓↓	
EC+BL (500, 250, or 25 µg/ml)	-	**N/A**	-	↓↓↓	

EC+LS (500, 250, or 25 µg/ml)	-	↓ (500 and 25)	-	↓↓↓	
EC+LGG (500, 250, or 25 µg/ml)	-	↓↓↓ (500)	-	↓↓↓	

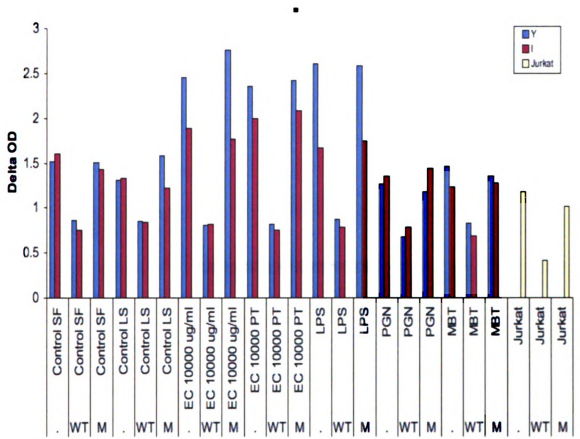
Macrophage Chemotaxis (YAMC and IMCE Supernatants Exposure)

YAMC Treatment	Migration	mAb IL-6	Comments
EC	-	-	**Normal cells-Bacterial treatment did not induce macrophage migration**
EC+BB	-	-	
EC+BL	-	-	
EC+LS	↑↑	-	

IMCE Treatment	Migration	mAb IL-6	Comments
EC	↑↑	-	**Preneoplastic cells-Bacterial treatments caused migration; and no probiotic strain decreased migration induced by EC**
EC+BB	↑↑	-	
EC+BL	↑↑	-	
EC+LS	↑↑	-	

APPENDIX I

NF-kappa B DNA Binding Assay (*JI Fenton and J Birmingham)



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