EFFECT OF LACTIC ACID BACTERIA AND BIFIDOBACTERIA ON INTERLEUKIN-6 AND INTERLEUKIN-8 PRODUCTION BY CACO-2 CELLS

By

Constance Wong

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ABSTRACT

EFFECT OF LACTIC ACID BACTERIA AND BIFIDOBACTERIA ON INTERLEUKIN-6 AND INTERLEUKIN-8 PRODUCTION BY CACO-2 CELLS

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The purpose of this study was to test the hypothesis that probiotics could enhance immune function by stimulating cytokine secretion by intestinal epithelial cells. To test this hypothesis, the effects of fermented and non-fermented reconstituted non-fat dry milk containing probiotic cultures (*Lactobacillus acidophilus, L. bulgaricus, L. casei, L. reuteri, Streptococcus thermophilus, Bifidobacterium, B. adolescentis*) on interleukin (IL)-6 and IL-8 production by Caco-2 cells were assessed. Three different concentrations (10^6, 10^7, 10^8 CFU/ml) of probiotic cultures were used to determine the optimum dose to elicit a maximal immune response. Probiotic cultures were inactivated by heat (95°C, 30 min) or irradiation (1 Mrad). In addition, milk components (lactose, α-lactalbumin, β-lactoglobulin) were evaluated for their ability to stimulate IL-6 and IL-8 production. In general, none of the cultures investigated significantly stimulated IL-6 or IL-8 production. There was a significant difference, however, between heat- and irradiation-inactivated samples. Heat-inactivated cultures caused more IL-6 and IL-8 production than their irradiated counterparts. These results suggest that the mode of inactivation may be important to immune stimulation. The milk components, α-lactalbumin and β-lactoglobulin elicited markedly high amounts of IL-6 and IL-8 production from Caco-2 cells. These results suggest that certain milk components have immunostimulating abilities in the gastrointestinal tract.

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I could not have made it this far without the support of my friends and family.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
α-la	α-Lactalbumin
ATCC	American Type Culture Collection
β-lg	β-Lactoglobulin
BSA	Bovine serum albumin
BGG	Bovine gamma globulin
CFU	Colony forming unit
CN	Casein
ConA	Concanavalin A
DMEM	Dulbecco's modified Eagle's media
ELISA	Enzyme-linked immunosorbent assay
ETEC	Enterotoxigenic *Escherichia coli*
F	Fermented
FBS	Fetal bovine serum
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HK	Heat-killed cells
I	Irradiated
IEL	Interepithelial lymphocyte
IFN	Interferon

Ig	Immunoglobulin
IL	Interleukin
LAB	Lactic acid bacteria
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein-1
MRS	De Man, Rogosa, Sharpe medium for lactobacilli
MRSL	De Man, Rogosa, Sharpe medium with 5% lactose
NF	Non-fermented
NFDM	Non-fat dry milk
NK	Natural killer
NO	Nitric oxide
NYA	National Yogurt Association
OA	Ovalbumin
OD	Optical density
OM	Ovomucoid
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.05% Tween-20
PMN	Polymorphonuclear
PP	Peyer's Patch
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha

CHAPTER 1

INTRODUCTION

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INTRODUCTION

The relationship between food, nutrition and health has long been known to exist. While early research focused on foods to prevent disease, current research is focused on foods to prolong and enhance health. Functional foods are generally defined as foods that provide a health benefit beyond inherent nutrition. This category of foods includes infant formulas, medical foods, dietary supplements, performance foods, probiotics and other foods designed to deliver specific nutrients or food components (i.e. fiber, vitamins, minerals, antioxidants, probiotics, dairy proteins, soy proteins and lipids).

Dairy products have been the focus of functional food research due to the bioactive properties of some milk components and their ability to serve as excellent carriers for probiotic organisms. Because of the perceived health benefits of probiotics, consumption and sales of yogurt in particular, rose to $2.2 million in 2001, which was a 6.6% increase from 2000 (Berry, 2002). This trend has been attributed to greater consumer interest in nutrition and health as well as the increase in published studies that indicate yogurt cultures may have additional health benefits.

The consumption of fermented milks has been associated with improved health for thousands of years. Hippocrates (circa 400 B.C.), the father of medicine, considered fermented milks to have medicinal qualities and prescribed them for stomach and intestinal ailments (Oberman, 1985). Eli Metchnikoff (1907) was the first to document the improved health of patients ingesting milk fermented by lactic cultures. According to his speculations, those who ingested fermented milk lived longer because the bacteria in

the milk helped to maintain a healthier intestine by decreasing toxic microbial activities. These health-promoting bacteria were identified as probiotic lactic acid bacteria (LAB).

Since Metchnikoff, many reports have described the benefits probiotics have on human health. Among them are the alleviation of lactose intolerance symptoms and diarrhea, anti-cancer effects, reduced serum cholesterol, and enhanced immune response (Fuller, 1991; Gilliland, 1990). Probiotics are thought to exert immune effects via the gastrointestinal (GI) tract where they interact with the gut-associated lymphoid tissue (GALT). It is believed that probiotic interaction leads various immune cells in the GI tract to mount an immunological response.

Several studies have demonstrated the ability of probiotics to enhance both non-specific and specific immune responses in humans (Gill and others, 2001; Donnet-Huges and others, 1999; Schiffrin and others, 1995). The efficacy of probiotics in humans was based on levels of immunoglobulins (Ig) and immune cells in blood rather than on stimulation of cytokines. An increase in Ig indicates that the body's adaptive immunity is responding to infection by a foreign substance (antigen). An increase in cytokine production, however, occurs via the innate immune response to recruit more phagocytic cells and effector molecules to the site of infection (Janeway and others, 1999). Most *in vitro* studies looked at cyokines but used mouse rather than human cell lines. We have chosen the Caco-2 cell line, which is considered a good model for human intestinal epithelial cells, in order to address issues concerning cytokine stimulation of cells by probiotic bacteria.

The hypothesis on which this research was based was that LAB and bifidobacteria, which are used in the production of fermented dairy foods, could enhance

immune function by stimulating cytokine secretion by intestinal epithelial cells. Therefore, the objectives of this research were as follows:

1) Examine the difference between fermented and non-fermented non-fat dry milk (NFDM) containing seven individual probiotic cultures on cytokine production by Caco-2 cells
2) Determine optimum levels of probiotic organisms to elicit a maximal immune response by Caco-2 cells
3) Investigate the effect of heat or irradiation inactivated cells on the stimulation of cytokine secretion by Caco-2 cells
4) Examine the effect of specific milk components on cytokine production by Caco-2 cells

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

2.1 Intestinal microflora

More than 400 species of bacteria are thought to inhabit the large intestine (Finegold and others, 1983) and make up to 40-55% of fecal solids for those on a western-type diet (Cabotaje and others, 1990). The dominant group of microflora is obligately anaerobic and includes the bacteria bacteroides, eubacteria, bifidobacteria, lactobacilli, anaerobic cocci and clostridia (Kleessen and others, 2000). Naturally occurring microflora serve as a protective barrier against invasion by pathogens (Tancrede, 1992). Most of these bacteria are found in the large intestine as the constant flow of gut contents keeps numbers in the small intestine relatively low (Pestka, 1993).

Commensal bacteria offer protection to the host from pathogens by blocking or attaching to receptors, competing for nutrients and by producing antimicrobial compounds (Vaughan and others, 1999). Though naturally occurring microflora exert these beneficial effects, their main role is to ferment carbohydrates (not digested earlier in the gut) to provide additional energy (Cummings and Macfarlane, 1991). The composition of commensal microflora varies between individuals, but the population is fairly stable in healthy adults (Kleessen and others, 2000). When the balance of microflora is disturbed due to advanced age, diet, illness or antibiotic treatment, the protective effect of the commensal bacteria is decreased and increases the chance of invasion by bacterial pathogens.

The most common disturbance of microflora results from the introduction of antimicrobial agents, antibiotics and medication, to the GI tract. Antibiotics reduce the types of bacteria in the GI tract, which allows for the growth of small populations of resistant bacteria (Wilson, 1997). When no longer kept in check by the predominant bacteria, these antibiotic resistant organisms can multiply and cause infection as they are typically more pathogenic than the bacteria which they are replacing (Wilson, 1997). Diarrhea is the most common symptom of GI infection. The ingestion of probiotic supplements has been demonstrated to lessen the duration of diarrhea (Isolauri and others, 1991; Kaila and others, 1992).

2.1.1 Lactic acid bacteria and bifidobacteria

Lactic acid bacteria are Gram-positive bacteria that produce lactic acid as the major product of lactose fermentation. The following genera are generally considered typical LAB: *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus and Streptococcus.* They are non-spore formers, highly acid tolerant, and grow best in a microaerophilic environment. LAB can be obligately homofermentative (producing only lactic acid as an end product) or facultatively heterofermentative (producing CO_2, acetic and lactic acids). LAB can change their metabolism depending on their growth conditions, but cannot compete with other bacteria in nutrient-poor conditions because they cannot produce all amino acids and vitamins necessary for their growth (Lucke, 1996).

Lactic acid bacteria are among the microorganisms that make up the indigenous gut microflora. Due to the acidic conditions in the stomach, few bacteria inhabit the beginning of the GI tract. Lactobacilli can be found in the stomach at $<10^3$/g stomach

contents (Salminen and others, 1998b) as some strains are more acid tolerant. Numbers of lactobacilli increase along the GI tract and typically reach between 10^4-10^9/g gut contents in the colon (Salminen and others, 1998b). Other genera of LAB found in the human large intestine are *Enterococcus*, and *Streptococcus* (Borriello, 1986).

Bifidobacteria, formerly grouped in the genus *Lactobacillus*, make up an important population of the gut microflora. The number of bifidobacteria increases along the GI tract and reaches 10^8-10^{11}/g gut content in the colon and can account for up to 10% of total flora and 25% of anaerobic strains (Mitsuoka, 1990; Salminen and others, 1998b). New molecular genetic techniques and chemotaxonomy developed in the 1960's allowed scientists to recognize bifidobacteria as a unique group of bacteria. It was determined that bifidobacteria were genetically different from *Lactobacillus*, *Corynebacterium* and *Propionibacterium* because they had >50% G+C in the DNA, whereas LAB had <50% G+C (Holzapfel and Wood, 1998).

Bifidobacteria are Gram-positive, strictly anaerobic, non-motile, non-spore forming bacteria. They are also unique in that they lack the enzymes aldolase and glucose-6-phosphate dehydrogenase needed for homo- and heterofementation. Instead, bifidobacteria degrade hexoses via the fructose-6-phosphate pathway, of which, fructose-6-phosphate phosphoketolase is the characteristic enzyme (Ballongue, 1993).

Mitsuoka (1990) reported that bacterial composition of gut microflora changes with age (Figure 2.1). Bifidobacteria populations decrease or disappear with an increase in age, whereas populations of streptococci, enterobacteria, clostridia and lactobacilli increase. *Clostridium perfringens*, which is associated with gastroenteritis, significantly

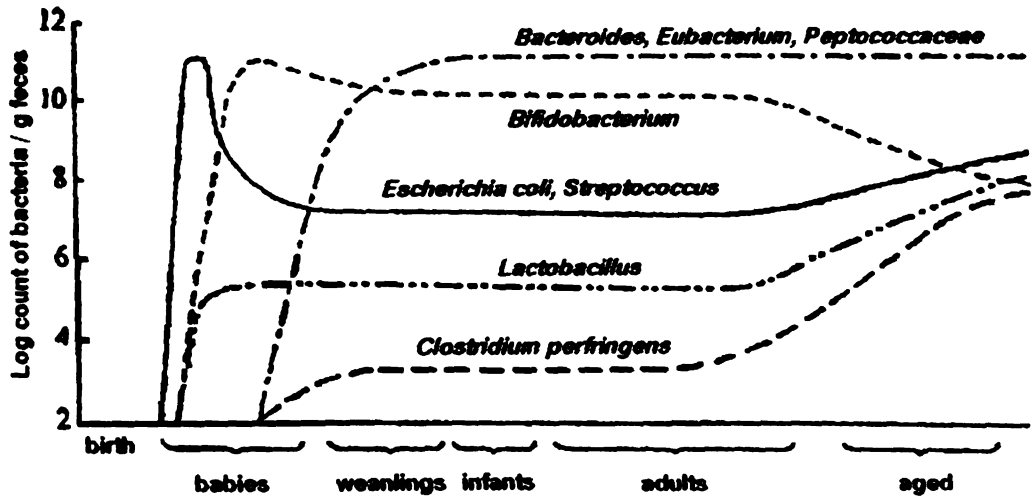

Figure 2.1 Changes in human fecal flora with increase in age. (Reproduced from Mitsuoka, 1990).

increases in the elderly. This alteration in microflora may make the elderly more susceptible to liver function disorders, pathogenic and toxic burdens, and cancer (Mallet and Roland, 1987). Bifidobacteria are able to maintain bacterial homeostasis in the gut with lactic and acetic acids produced during fermentation as well as production of other substances that are inhibitory to pathogens such as *C. perfringens* and *Escherichia coli* (Gibson and Wang, 1994).

2.1.2 Probiotics

Although earlier definitions describe probiotics as only having an affect in the gut, a definition that better fits recent studies was stated by Chandan (1999) as "strains of living microorganisms that on ingestion in certain doses exert health benefits beyond inherent basic nutrition." Bacteria from the genera *Lactobacillus* and *Bifidobacterium* are the most commonly studied probiotic bacteria (Sanders, 1999).

Probiotic cultures have been reported to have numerous health benefits, but only the alleviation of lactose intolerance symptoms and anti-diarrheal effects have been substantiated through scientific studies (Marteau and others, 2001; Sanders, 1999). LAB are thought to produce lactase when in the presence of bile and then aid the digestion of lactose in the gut lumen (de Vrese and others, 2001). The administration of probiotics has been demonstrated to lessen the duration of acute rotavirus diarrhea. Children fed *Lactobacillus casei* sp strain GG had significantly shorter (1.1 d) bouts of diarrhea compared to 2.5 d for the control group (Kaila and others, 1992). It has been hypothesized that probiotics could also prevent or lessen diarrhea by colonization

resistance, adhering to intestinal mucosa and by blocking adherence by pathogenic bacteria or by influencing gut flora populations (Sanders and Huis in't Veld, 1999).

Additional health benefits attributed to probiotics include anti-cancer effects, reduced serum cholesterol, antihypertensive effects, stomach health (prevention of infection by *Helicobacter pylori*) and enhanced immune response (Fuller, 1991, Gilliland, 1990; Sanders, 1999). Probiotics have been implicated in reduced cancer risk because they may affect intestinal epithelial cell kinetics and decrease cancer cell proliferation in the colon (Sanders, 1999). In a study by Aso and Akazan (1992), *L. casei* increased the time between incidences of bladder cancer in humans. Two possible mechanisms by which probiotics could reduce serum cholesterol have been proposed. Probiotics may assimilate the cholesterol molecule or enzymatically deconjugate bile acids (Sanders, 1999). If probiotics do deconjuate bile acids, however, some could by converted to secondary bile acids which are cancer promoters. Antihypertensive effects have been attributed to tripeptides created from fermentation of milk by probiotics. These tripeptides acted as angiotensin-I-converting enzyme inhibitors and reduced blood pressure (Sanders, 1999). Results from animal and human trials indicate that probiotics and their end products such as lactic acid can prevent colonization by *Helicobacter pylori*. Colonization of the stomach by *H. pylori* has been reported to result in peptic ulcers, chronic gastritis and increased risk of gastric cancer (Marshall, 1994). With respect to enhanced immune response, probiotics could reduce cancer risk as well as have anti-infective activity. Currently, the mechanism of how probiotics exert these effects is unclear.

2.2 GI immune system

The GI tract is made up of the stomach, small intestine and large intestine. Due to the exposure of these organs to foreign matter via ingested material, humans evolved with nonspecific and specific immune mechanisms for protection. The GI immune system plays an important role in the health of the individual. Nonspecific immunological defenses are intrinsic and include gastric acidity, small intestinal peristalsis, the indirect removal of bacteria by mucus and lysozymes and the gut microflora. To protect against antigens that survive these conditions, the host can launch a specific immune response that involves identification by lymphocytes, followed by proliferation and activation of additional immune cells (Pestka, 1993). Cells participating in the specific immune response include lymphoid follicles (Peyer's patches), isolated follicles, mesenteric lymph nodes, intraepithelial lymphocytes (IEL) and the lamina propria (Shanahan, 1994) (Figure 2.2). Collectively, these cells are called the GALT.

The intestinal epithelial cells give the first warning to underlying mucosa cells of bacterial invasion (Eckmann and others, 1993). More specifically, antigens from the gut lumen enter blood circulation via intestinal epithelial cells and Peyer's patches (PP), which are groups of lymphoid follicles (Pestka, 1993). After antigen uptake and presentation, an immune response is mounted that leads to the production of Ig and cell-mediated immune responses. Epithelial cells *in vitro* secrete cytokines such as interleukin (IL)-6 (Hedges and others, 1992) and IL-8 (Eckmann and others, 1993), which are believed to influence the development of an immune response from leukocytes in the intestinal mucosa. Stimulation of IL-6 and IL-8 has been the focus of this research.

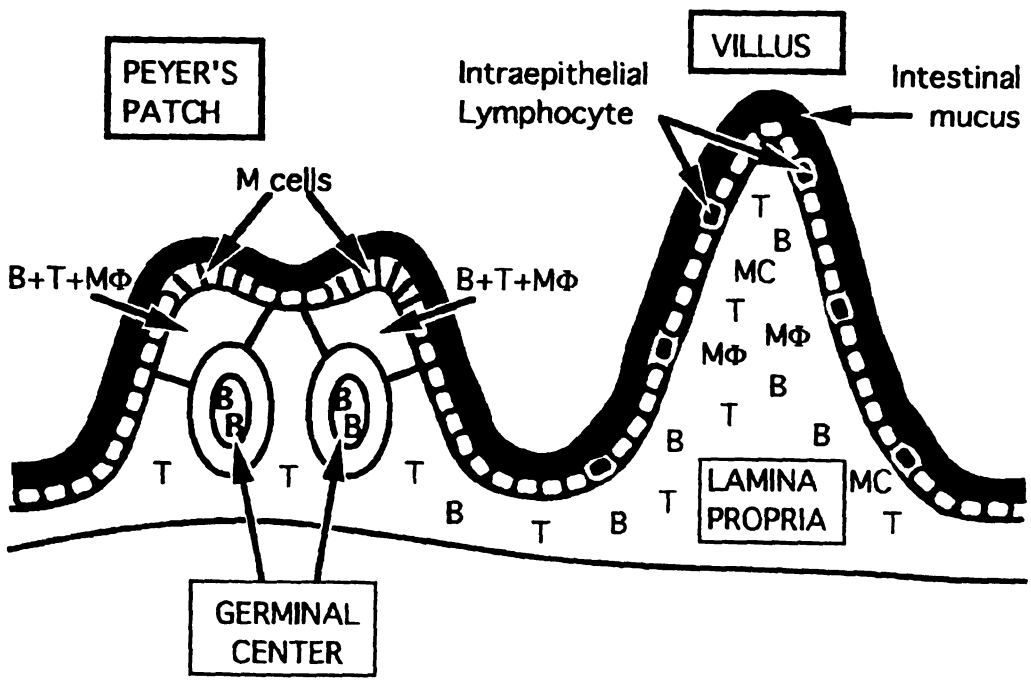

Figure 2.2 Gut-associated lymphoid tissue (GALT). Peyer's patches and lamina propria are important elements of the intrinsic GALT. Location of immune cells are shown by the following abbreviations: B = B lymphocytes, T = T lymphocytes, Mϕ = macrophage, MC = mast cells. (Reproduced from Pestka, 1993).

2.2.1 Cytokines

Cytokines are small non-antigen-specific protein molecules that cells use to influence each other. They work in a network where each cytokine may have multiple or overlapping functions (Playfair, 1996; Shanahan, 1994). Cytokines may work synergistically and stimulate the production of other cytokines. Depending on the effector cell type, they may have a harmful or a beneficial role in disease (Playfair, 1996)

Two cytokines will be examined in this project: IL-6 and IL-8. IL-6 is a B cell differentiation factor that is needed for antibody secretion. It also plays a role in acute phase response and enhances inflammatory response (Akira and others, 1993). IL-6 is produced by a number of cell types including: T cells, B cells, smooth muscle cells, endothelial cells and monocytes/macrophages (Akira and others, 1993). In the case of malaria, increased IL-6 levels over a period of time have been associated with organ damage. Although it is associated with the pathology of diseases such as rheumatoid arthritis, multiple myeloma and acquired immunodeficiency syndrome (AIDS), it also has antitumor activities. IL-6, therefore, can act in an inhibitory or stimulatory manner depending on cell type.

IL-8 is classified as a chemokine whose function is to attract T cells, monocytes and neutrophils to inflammatory sites (Playfair, 1996). Chemokines are a specific subset of cytokines characterized by a highly conserved sequence of four cysteines that influence their tertiary structures (Van Damme, 1994). IL-8 can protect blood vessel cells from neutrophil-mediated damage by inhibiting neutrophil adhesion to cytokine-activated endothelial cells (Gimbrone and others, 1989; Van Damme, 1994). High

amounts of IL-8, however, can stimulate adhesion of neutrophils to unactivated endothelial cells (Gimbrone and others, 1989). Increased IL-8 production also has been seen with infectious diseases of the central nervous system, gastric infection, ulcerative colitis and hemolytic uremic syndrome.

2.2.1 Effect of probiotics on immune responses in the GI tract

Probiotics may increase non-specific immunity against tumors and infection as mentioned earlier. They may achieve specific immune responses by activating macrophages, increasing levels of cytokines and IgA, and by increasing the activity of natural killer (NK) cells (Sanders, 1999). Probiotics can exert these positive effects, but do not cause a harmful inflammatory response like some enteric bacteria perhaps because of the lack of lipopolysaccharide (LPS) in the cell wall.

Although the exact mechanism by which probiotics exert their immune enhancing effects is not known, the ability to adhere to and colonize the intestine are thought to be important. Adherence allows the probiotics to be in close proximity to the GALT to have an effect and avoid 'washing-out' (Vaughan and others, 1999). Direct contact may be necessary for some immune effects such as enhanced leucocyte phagocyte activity against enterobacteria (Schiffrin and others, 1997). Colonization could ensure that the probiotics remained in the GI tract and continued to interact with the GALT. It is hypothesized that colonized probiotics exert protective effects by blocking attachment sites of pathogenic microorganisms and/or by steric hindrance (Tancrede, 1992). Probiotics also produce substances inhibitory towards other organisms. This capacity of

probiotics to produce bacteriocins and other antimicrobial peptides as well as the ability to alter pH, is defined as colonization resistance (Rolfe, 1996).

Although strains of lactobacilli and bifidobacteria have been shown to adhere to human intestinal cells *in vitro* (Bernet and others, 1994; Chauviere and others, 1992; Crociani and others, 1995), this has not been confirmed *in vivo*. In the body, probiotics are challenged with stomach acid, bile and peristaltic movement of the intestine before they can have the opportunity to colonize the gut. Colonization of the gut by probiotics may, therefore, be temporary. In clinical studies by Lidbeck and others (1987), after administration of *Lactobacillus* supplements ceased, the levels of *Lactobacillus* in feces returned to pre-experimental levels. Continual intake of the probiotic may be necessary to achieve and maintain maximum numbers of bacteria.

Other studies have shown, however, that probiotic bacteria may not need to be viable to exert an immunostimulatory response (Marin and others, 1997; Perdigon and others, 1986; Solis Pereyra and Lemonnier, 1993). It is possible that outer-membrane proteins of non-viable cells and/or their cell components may be all that is necessary to interact with receptors on GALT and provide an immune response. This would be similar to the immunostimulating effect of LPS, which is found on the outer membrane of Gram-negative bacteria. LPS can cause monocytes to secrete cytokines and also can potently activate B cells (Pestka, 1993). By contrast, some studies have compared live bacteria to nonviable cells and found that nonviable cells either did not produce an immune response or stimulated to a much lesser degree than the live cells (Haller and others, 1999; Miettinen and others, 1996). Due to the different models used for these

studies, further research is necessary to confirm whether viability is essential for probiotic function.

2.3 Immunostimulating effects of lactic acid bacteria, bifidobacteria and milk components

2.3.1 *In vitro* studies

One difficulty with intestinal studies is obtaining a reasonable model for the human GI tract. Due to the location of the intestinal tract in the body, human *in vivo* studies are not possible for most experiments. Therefore, most studies concerning probiotics and the immune system have used mouse models and various cell lines. Table 2.1 summarizes the recent *in vitro* studies on the immunostimulating effects of LAB, bifidobacteria and their cellular components.

Miettinen and others (1996) used both live and glutaraldehyde-fixed LAB to stimulate human peripheral blood mononuclear cells (PBMC). Glutaraldehyde is a cross-linking agent that denatures proteins. They reported that live bacteria were better able to stimulate PBMC to secrete the cytokines IL-6 and tumor necrosis factor (TNF)-α than glutaraldehyde-fixed bacteria. This effect, however, was strain specific. Although it is possible that the denaturation of proteins by glutaraldehyde altered their immune stimulating properties, Miettinen and others (1996) suggested that LAB should be viable to exert the optimal immunostimulating effect.

Several other experiments, however, demonstrate the immunostimulating effects of heat-killed LAB and bifidobacteria as well as their cellular components. Tejada-Simon and others (1999a) demonstrated that whole cells of bifidobacteria and LAB, their

Table 2.1 Recent *in vitro* studies of immune stimulation by lactic acid bacteria and bifidobacteria

Cell type	Experimental design	Results	Reference
Bone marrow-derived murine dendritic cells	6 irradiation-inactivated *Lactobacillus* strains	*L.casei* stimulated dose-dependent increases in IL-6, IL-10, IL-12 and TNF-α production. *L. reuteri* inhibited *L.casei*-induced IL-6, IL-12 and TNF-α production	Christensen and others, 2002
Murine splenocytes	LAB at 10^9 cfu/ml in RPMI 1640	Increased IL-10, IL-12 and inhibition of $CD4^+$ T cells *L. paracasei*	von der Weid and others, 2001
Caco-2 cell – human peripheral blood leucocyte co-culture	Non-pathogenic *Escherichia coli, Lactobacillus sakei, Lactobacillus johnsonii* at 1×10^6 or 1×10^7 cfu/ml	Co-culture necessary for *E. coli* and *L. sakei* induction of IL-8, MCP-1, IL-1β and TNF-α mRNA by Caco-2 cells	Haller and others, 2000
Human peripheral blood monocytes	*L. sakei, Lactobacillus paracasei, L. johnsonii.* Viable and heat-killed	Increased TNF-α response by live LAB	Haller and others, 1999
Murine splenocytes	Heat-killed *L. casei* strain Shirota	Induced IL-12 which stimulated IFN-γ	Kato and others, 1999
RAW 264.7 macrophage cells & murine peritoneal cells	Heat-killed LAB and their cell walls, and cytoplasmic extracts	LAB and their components stimulated RAW cells to produce TNF-α, IL-6 and nitric oxide (NO). Peritoneal cells stimulated to produce IL-6	Tejada-Simon and others, 1999a
RAW 264.7 cells	33 *Bifidobacterium* strains, heat-killed. With or without LPS	Increased H_2O_2, NO, IL-6 and TNF-α production	Park and others, 1999
RAW 264.7cells & & EL-4.IL-2 murine thymoma	4 *S. thermophilus* strains, *L. bulgaricus, B. adolescentis, B. bifidum*	RAW cells produced IL-6 and TNF-α; EL-4.IL-2 cells produced IL-2 and IL-5	Marin and others, 1998
RAW 264.7cells & & EL-4.IL-2 murine thymoma	14 heat-killed *Bifidobacterium* strains	IL-6 and TNF-α produced by RAW cells; EL-4.IL-2 cells secreted IL-2 and IL-5	Marin and others, 1997

Cell type	Experimental design	Results	Reference
Murine spleen macrophage cells	Phosphopolysaccharide from *Lactococcus lactis* ssp. *cremoris* at 1-200 ul/m	Increased IFN-γ and IL-1β produced	Kitazawa and others, 1996
Human peripheral blood mononuclear cells (PBMC)	10^5, 10^6, or 10^7 cfu/ml live or glutaraldehyde-fixed LAB	Strain specific increases in IL-6, IL-10 and TNF-α by live LAB	Miettinen and others, 1996
Murine spleen & Peyer's patch macrophage cells	Heat-killed *Lactobacillus gasseri*	Increased mRNA for IFNα by both types of macrophage cells	Kitazawa and others, 1994
Human PBMC	*L. bulgaricus* and *S. thermophilus* in different ratios, *L. casei, L. acidophilus, Bifidobacterium* and their cell walls	Bacteria and their cell walls increased IL-1β, TNF-α IFN-γ production	Solis Pereyra and Lemonnier, 1993

cell walls and cytoplasmic fractions could stimulate murine RAW 264.7 macrophage cells to produce TNF-α, IL-6 and nitric oxide (NO). Although all bacteria tested caused increased cytokine production, this effect was strain dependent.

Marin and others (1997) demonstrated that heat-killed bifidobacteria could enhance cytokine production by RAW 264.7 murine macrophage cells and EL-4.IL-2 thymoma cells (helper T-cell model). Incubation of fourteen different strains of bifidobacteria with RAW 264.7 cells significantly stimulated TNF-α and IL-6 production in a dose dependent manner. TNF-α and IL-6 production increased 21- to 872-fold and 9.3- to 204-fold, respectively, depending on strain. The addition of LPS tended to decrease the effect of bifidobacteria stimulation. Eight of the 14 strains of bifidobacteria significantly increased IL-2 production by EL-2.IL-4 cells at a concentration of 10^6 cells/ml. The effect of bifidobacteria on IL-5 production by EL-2.IL-4 cells was more inconsistent. Bifidobacteria were stimulatory or inhibitory depending on strain and concentration. Bifidobacteria Bf-6 and *B. adolescentis* M101-4 were among the most stimulatory strains for all cytokines tested and therefore, were chosen for this study. Bf-6 is used in commercial dairy products.

Park and others (1999) saw slightly varying results in RAW 264.7 cells stimulated with human and commercial isolates of bifidobacteria. Bifidobacteria with the addition of LPS increased IL-6 production synergistically. The same combination, however, reduced TNF-α production. While all strains of bifidobacteria stimulated IL-6 and TNF-α production without LPS, strain dependent differences were observed.

Compared to *L. bulgaricus, B. adolescentis* M101-4 and *Bifidobacterium* Bf-6, *S. thermophilus* was even more effective at cytokine stimulation of RAW 264.7 and

EL4.IL-2 cells (Marin and others, 1998). Generally, *S. thermophilus* St133 had the most stimulatory effect on RAW 264.7 cells. Solis-Pereyra and Lemonnier (1993) also found *S. thermophilus* to be among the most stimulatory to IL-1β, TNF-α and interferon (IFN)-γ production by human PBMC at a dose of 2×10^7 bacteria/2×10^6 PBMC.

Viability may not be necessary for probiotics to stimulate immune function. For example, Marin and others (1997, 1998) and Park and others (1999) used heat-killed LAB to stimulate cytokine production in mouse cell lines. However, Haller and others (1999) used *Lactobacillus sakei*, *Lactobacillus johnsonii* strain La1 and *Lactobacillus paracasei* strain Shirota at various stages of the cell cycle and observed a difference in cytokine production by human PBMC when stimulated with live and heat-killed bacteria. Live bacteria in the logarithmic growth phase were able to stimulate TNF-α production at a lower concentration than heat-killed bacteria from the same phase. However, heat-killed bacteria from the stationary growth phase were more effective at TNF-α stimulation than live bacteria also in stationary phase. Haller and others (1999) speculated that differences in bacterial growth phase were observed because the cell wall composition changes during growth and heat-inactivation may then affect these structures.

2.3.2 Animal studies

Feeding studies in animal models also have demonstrated an enhanced immune response to oral administration of LAB. Recent studies with animal models are summarized in Table 2.2. Perdigon and others (1986) orally administered a mixture of *Lactobacillus casei* and *Lactobacillus acidophilus* (a total of 2.4×10^9 cfu/d) in non-

Table 2.2 Recent animal studies of immune stimulation by lactic acid bacteria and bifidobacteria

Animal	Experimental design	Results	Reference
BALB/c mice	Heat-killed 10^7, 10^9, 10^{11} cfu *L. rhamnosus* HN001, 14 d	Dose-dependent enhancement on phagocytic activity of blood and peritoneal leukocytes in mice	Gill and Rutherfurd, 2001
Male BALB/c mice	10^9 cfu/d of *L. rhamnosus* HN001, *L. acidophilus* HN017, *B. lactis* HN019 in skim milk, 10 or 28d	Increased IFN-γ by spleen cells, increased serum antibodies	Gill., 2000b
Female BALB/c Mice	10^9cfu/d of LAB for 2, 5, or 7 d in non-fat milk	Dose dependent increase in IgA producing cells associated with the lamina propria of sm. intestine	Vitini and others, 2000
Male BALB/c mice	5 x 10^7, 10^9 or 5 x 10^{10}cfu/d *L. acidophilus* HN017, *L. rhamnosus* HN001 or *B. lactis* HN019 in skim milk	No adverse health effects: no abnormal clinical signs, no significant changes in feed intake, water intake, liverweight gain	Shu and others, 1999
Female $B6C3F_1$ mice	Yogurt produced using commercial starter cultures 21d, immunized with cholera toxin	Yogurt supplemented with *L. acidophilus* and *Bifidobacteria* caused increased IgA to anti-cholera toxin	Tejada-Simon and others, 1999b
Female $B6C3F_1$ mice	Oral gavage of 1 x 10^9 LAB in non fat dry milk	*L. acidophilus* and *L. casei* stimulated IL-6 and IL-12 production *ex vivo* in peritoneal cells. *L. acidophilus* also upregulated IFN-γ and nitric oxide	Tejada-Simon and others, 1999c
Male Sprague-Dawley rats	Saline solution with 10^{12} cells/L, 10ml/kg body wt of *L. bulgaricus* 191R *or S. thermophilus* CH3 in skim milk	*L. bulgaricus* prevented 1,2-dimethylhydrazine induced DNA breaks.	Wollowski and others, 1999
Male swiss mice	10^9 cfu/kg body wt *L. acidophilus, L. casei, Lactobacillus gasseri*, or *L. rhamnosus* for 7d	Strain specific effects on proliferation of splenic B- and T-lymphocytes	Kirjavainen and others, 1999
Male BALB/c Mice	1 x10^9 cfu LAB, 3wk after *Helicobacter pylori* administration	*Lactobacillus salivarius* suppressed *H. pylori* and reduced antibody response to *H. pylori*	Aiba and others, 1998

Animal	Experimental design	Results	Reference
Pathogen free, male BALB/c mice & Wistar rats	Biolactis powder with *L. casei* (heat-killed) Intraperitoneal injection of ovalbumin (OA)	Increased IFN-γ, IL-2, IL-12 production by spleen cells; Decreased IL-4, 5, 6, 10 production; IgE production in response to OA reduced by L. casei *in vivo* and *in vitro*	Matsuzaki and others, 1998
BALB/c mice	Yogurt fermented with *L. bulgaricus* and *S. thermophilus* at 2 x 10^8 cfu/ml	Increased IgA secreting cells; tumor growth inhibited possibly due to reduced inflammatory immune response	Perdigon and others, 1998
BALB/c mice	Infection with *Candida albicans*. 1.2 x 10^9 LAB/ Day, 2d	Increased phagocytosis by *L. casei* and *L. bulgaricus* as well as protection from *C. albicans*	de Petrino and others, 1995
Albino swiss mice	1.2 x 10^9 cfu *L. casei*/d with or without lipopoly-Saccharide (LPS), 2 d boost.	*L. casei* with LPS increased secretory IgA; protected against infection with *S.* Thyphimurium	Perdigon and others 1995
Piglets	Oral administration of *L. casei, L. bulgaricus, S. thermophilus, Streptococcus faecium* M74	*S. faecium* & *L.casei* combination increased IL-2 production, increased lysosomic elements in ileal epithelium	Tortuero and others, 1995
Male BALB/c mice	Biolactis powder containing *L. casei*	Suppression of tumor development	Kato and others, 1994
Female BALB/c mice	*L. bulgaricus* and *S. thermophilus* at 8 x 10^8 cfu/d, 7 or 15d	Increased IFN-γ and IFN-α production by spleen cells	Muscettola and others, 1994
Mice	Cell wall, cell wall skeleton and whole cell of *Bifidobacterium infantis*	Cell wall exhibited antitumor effects and activated polymorphonuclear cells and macrophages	Sekine and others, 1994
Female BALB/c & C57BL/6 mice	Mice were immunized with hen's egg ovomucoid (OM). *Ex vivo* incubation of lymph node cells & LAB	*Bifidobacterium adolescentis* M101-4 increased production of anti-OM antibody by lymph node cells	Lee and others, 1993

Animal	Experimental design	Results	Reference
BALB/c mice	Yogurt with *L. bulgaricus* & *S. thermophilus*, heat-treated. Yogurt with *L. casei*	Live and heat-treated yogurts increased specific antibody responses to *Vibrio cholerae* challenge	Portier and others, 1993
Female BALB/c mice	Pellet of *B. longum* or *L. acidophilus* at 4 x 10^{11} cfu/ml, non-viable	Increased antibody response to *B. longum* cytoplasm after 8 wks of feeding	Takahashi, and others, 1993

fermented milk to mice which resulted in a synergistic increase in macrophage and lymphocyte activation (up to 3.5 times greater than control). They concluded that using a mixture of lactobacilli was more effective than using individual strains.

Ingestion of LAB also protected against infection by pathogenic bacteria such as *H. pylori, Salmonella* Typhimurium and *E. coli* $O_{111}K_{58}$ (Aiba and others, 1998; Perdigon and others, 1990, 1991, 1995). Perdigon and others (1995) administered *L. casei*, LPS and a combination of *L. casei* and LPS to mice. They found that a combination of *L. casei* and LPS caused the greatest number of IgA-producing cells and T-lymphocytes. *L. casei* with or without LPS was effective at preventing *S.* Typhimurium infection which corresponded to increased IgA production. When these assays were conducted with non-viable *L. casei*, the protective effect was not observed.

Muscettola and others (1994) compared the effect of feeding live and heat-killed LAB to mice. They found that spleen cells from mice that had consumed heat-killed LAB did not produce a significant amount of IFN-γ when stimulated with concanavalin A (Con A). Con A is a mitogen which induces mitosis, and as a result, proliferation, in lymphocytes (Janeway and others, 1999). Con A-stimulated spleen cells from mice fed live LAB produced approximately 27 IU IFN-γ/10^6 cells compared to 7 IU IFN-γ/10^6 cells for the controls. Muscettola and others (1994) also demonstrated that if aged mice consume live LAB, they could restore levels of IFN- γ to those of young control mice. Cytokine production decreased with age and as a result, the immune system was not as active as it was in youth. This study supplies additional evidence that LAB can help boost the immune system by supplementing the indigenous microflora, which is also altered by age.

2.3.3 Human/clinical studies

Whether or not enhanced immune effects *in vitro* and in animal models can be extrapolated to humans remains to be seen. There are a limited number of properly conducted clinical trials with humans that investigated the effects of LAB on the immune system (Marteau and Rambaud, 1993). In short, studies report conflicting results on the effect of probiotics on the immune system in humans leaving a need for more research in this area. Table 2.3 summarizes recent studies that investigated the use of probiotics on immune enhancement in humans.

Clinical studies by Wheeler and others (1997), and Spanhaak and others (1998) indicated that consumption of yogurt with live LAB had no effect on most immune parameters measured. These studies, however, used small number of subjects (20 or fewer adults) that were only given LAB for four weeks. Both studies administered yogurt which contained approximately 6 x 10^8 CFU/ml of *L. bulgaricus* and *S. thermophilus* (Wheeler and others, 1997) and 10^9 CFU/ml *L. casei* (Spanhaak and others, 1998). These levels of cultures in yogurt are greater than the 10^8 CFU culture/g yogurt which is required by the National Yogurt Association (NYA) for the Live and Active Cultures seal (NYA website, 2002). Further research is required to determine whether 10^8 CFU culture/g yogurt is an effective dose in humans to mediate health benefits.

Other clinical trials demonstrated that ingestion of LAB resulted in an increase in phagocytic activity of monocytes and polymorphonuclear cells as well as increase in NK cell activity (Gill and others, 2001; Chiang and others, 2000). Future clinical trials should increase the subject size and duration of the study to examine the

Table 2.3 Recent human studies of immune stimulation by lactic acid bacteria and bifidobacteria

Subject	Experimental design	Results	Reference
5 elderly males 8 elderly females	2 x 200ml low fat milk with $5x10^9$ *Lacobacillus rhamnosus*/d, 3 wks	Increased phagocytic activity of monocytes and polymorphonuclear (PMN), natural killer (NK) cells	Gill and others, 2001
50 adults (no exact numbers on gender)	low fat milk with 10^9 cfu/g *Bifidobacterium lactis*, 3 wks	Increased PMN cell phagocytosis, NK cell tumor killing activity	Chiang and others, 2000
7 elderly males 18 elderly females	2 x 1.5 x 10^{11}cfu *B. lactis*/d, 6 wks	Increased IFN-α, PMN cell phagocytosis	Arunachalam and others, 2000
15 males 27 females	150ml milk, 3wks fermented by combinations of *Steptococcus thermophilus*, *Lactobacilli johnsonii*	Increased phagocytosis by peripheral blood leukocytes	Donnet-Hughes and others, 1999
4 boys 3 girls	200ml formula with 10^9 *B. lactis*, 20 d	Increased fecal IgA and anti-polovirus IgA	Fukushima and others, 1998a
4 males 13 females	2 x 200ml milk/d – 2.6 x 10^8 cfu *Lactobacillus* GG per day	Receptor expression on neutrophils and monocytes was increased in control, non-milk-hypersensitive, but not milk-hypersensitive subjects	Pelto and others, 1998
20 males	3 x 100ml fermented milk with 10^9 cfu *Lacto-bacillus casei*/ml, 4 wks	No change in NK cell activity, phagocytosis or cytokine production	Spanhaak and others, 1998
7 males 13 females	16 oz. Yogurt/d with 2.5-3.0 x 10^8/g *Lactobacillus bulgaricus* and 3.5-4.1 x 10^8/g *S. thermophilus*	No significant differences in IgG, M, E, IFN-γ, IL-2 IL-4	Wheeler and others, 1997
16 males 12 females	Milk with 1 x 10^{10} cfu *Bifidobacterium bifidum* or 7 x 10^{10} *L. acidophilus* LA1, 3 wks	Increased phagocytosis by blood lymphocytes. No change in lymphocyte subpopulation	Schiffrin and others, 1995

Subject	Experimental design	Results	Reference
16 males 14 females	3 x 125 g fermented milk/d with *L. acidophilus* & *B. bifidum* at >5 x 10^9 cfu, attenuated *Salmonella typhi*	Increased sIgA anti *S. typhi* & total serum IgA	Link-Amster and others, 1994
29 boys 15 girls	2 x milk fermented with 10^{10}-10^{11} *Lactobacillus* GG/d, 5 d	Increased IgA. Decreased duration of diarrhea	Kaila and others, 1992

possible long-term and dose effects of LAB in humans. It should be insured that these trials are randomized and placebo-controlled, as many studies in the past have not been.

2.3.4 Effect of milk components

Several milk components as well as bioactive milk peptides derived from milk proteins are thought to have immunomodulating effects. Bioactive milk peptides are amino acid sequences, which are released from the native milk protein upon digestion and can have "hormone-like properties" (Clare and Swaisgood, 2000). Digestive enzymes such as pepsin, trypsin and chymosin have often been used to synthesize bioactive milk peptides. Several studies have demonstrated that fermentation of milk by LAB can also release milk peptides with immunomodulating activities (Laffineur and others, 1996; McDonald and others, 1994). Table 2.4 describes recent studies of immunostimulation by milk components.

Since bioactive milk peptides are formed *in vivo* after the digestion of milk, Gill and others (2000a) suggested that they may help protect the neonatal bovine whose GI immune system has not yet fully developed. Several studies have examined the effects of bioactive milk peptides *in vitro* and *in vivo*, though most studies have used *in vitro* methods. More research on the immunomodulating effects of milk peptides remains to be done as well as clinical trials to determine their effects on human health.

Milk proteins are generally separated into two categories: caseins and soluble milk proteins. Table 2.5 lists bioactive milk peptides obtained from both types of milk proteins as well as their immune effects. Caseins are made up of α, β, and κ-caseins

Table 2.4 Recent studies of immunostimulation by milk components

Experimental design	Results	Reference
Peripheral blood mononuclear cells (PBMC) from children with and without atopic symptoms were stimulated with birch and β-lg	PBMC from atopic children had high levels of IL-5, 6, and 10 after stimulation with birch and β-lg	Jenmalm and others, 1999
Casein and whey proteins incubated with murine spleen cells	β-lg stimulated the most IgM production	Wong and others, 1998
Whey proteins incubated with ovine blood lymphocytes	Lactoferrin and lactoperoxidase inhibited proliferation and interferon-γ (IFN-γ) production by lymphocytes. α-la increased IL-1β production	Wong and others, 1997
Ultrafiltration permeate of milk with β-casein cultured with LAB incubated with human PBMC	Only *Lactobacillus helveticus* supernatant increased proliferation, increased IFN-γ, and decreased IL-2 production of PBMC stimulated with concanavalin A (ConA)	Laffineur and others, 1996
Caseins hydrolyzed by pepsin, trypsin and *Lactobacillus casei* strain GG was incubated with human PBMC	β- and α_{s1} - caseins hydrolyzed with pepsin and trypsin suppressed proliferation of PBMC, as did α_{s1}-, β- and κ-casein hydrolyzed by *L. casei* GG.	Sutas and others, 1996
Cell culture media with casein was inoculated with dairy starter culture and incubated with IEC-6 and Caco-2 cells.	IEC-6 cells had reduced rates of cell division, whereas Caco-2 had increased rates	MacDonald and others, 1994

Table 2.5 Immune effects of bioactive milk peptides

Milk protein precursor	Bioactive peptide	Immune effect
BSA	Serophin	Opioid agonist
α_{s1}-CN	Casecidin	Antimicrobial activity
	Isracidin	Antimicrobial activity
	α_{s1}-Casokinin-5	ACE inhibitor
	Caseinophosphopeptide	Calcium binding and Transport
β-CN	β-Casokinin-7	ACE inhibitor
	Antihypertensive peptide	Antihypertensive peptide
	Caseinophosphopeptide	Immunostimulatory
K-CN	Casoplatelin	Antithrombotic
	Casoxin C	Opioid antagonist
α-Lactalbumin	α-Lactorphin	Opioid agonist
		ACE inhibitor
β-Lactoglobulin	β-Lactorphin	Opioid agonist
		ACE inhibitor
Lactoferrin	Lactoferricin B	Immunostimulatory
		Antimicrobial activity
	Lactoferroxin A	Opioid antagonist
Lactotransferrin	Lactoferroxins A, B, C	Opioid agonist

Adapted from Clare and Swaisgood (2000) and Schlimme and Meisel (1995)

(CN). Bioactive components of CN have demonstrated antihypertensive, antithrombotic, opioid, and immunostimulating effects. The immunostimulating effects include increased phagocytosis of human macrophages and protection against *Klebsiella pneumoniae* (Clare and Swaisgood, 2000).

MacDonald and others (1994) used media containing CN digested by commercial yogurt cultures to determine if any of the end products of CN fermentation had an effect on colon cell kinetics. Their model included two intestinal cell lines: IEC-6 cells (from normal rat intestine) and Caco-2 cells. Rates of [^{3}H]thymidine incorporation and cell kinetics by flow cytometry were used to determine the effects of the bacteria-conditioned media on both cell lines. In general, IEC-6 cells had decreased rates of cell division, while Caco-2 cells demonstrated increased rates. Differences in cell divison were also observed between different starter cultures. Because a link has been observed between fermented milks and reduced risk of developing some cancers, a reduced rate of cell division could indicate a decrease in tumor development. The results from the two cell lines may differ because IEC-6 cells are normal, whereas Caco-2 cells are adenocarcinoma cells.

Laffineur and others (1996) also found that the effect of LAB fermented casein was strain dependent. Ten LAB were grown in ultrafiltration permeate of bovine milk supplemented with β-CN as the protein source. Supernatant from this digest was examined for its immunologic effects on human PBMC. Only supernatant from *Lactobacillus helveticus* 5089 caused lymphocyte proliferation from all blood donors. When PBMC were stimulated with the mitogen ConA, *L. helveticus* supernatant inhibited cytokine IL-2 production compared to the control sample which was not

fermented. Conversely, IFN-γ production was increased by ConA-stimulated PBMC. Laffineur and others (1996) suggest that some peptide formed from β-CN digestion by LAB can interact and stimulate proliferation of lymphocytes by increasing cytokine secretion.

Some soluble milk proteins and bioactive peptides obtained by their digestion have demonstrated immunomodulating activity. Soluble milk proteins include α-lactalbumin (α-la), β-lactobglobulin (β-lg), bovine serum albumin (BSA), Ig, lactoferrin and lactoperoxidase (Horton, 1995). Alpha-la and β-lg are currently used to supplement speciality foods such as infant formulas and sports and dietetic beverages (Horton, 1995). The bioactive peptides, α-lactorphin and β-lactorphin, obtained from digestion of α-la and β-lg, respectively, are agonist peptides with morphine-like activity (Clare and Swaisgood, 2000; Schlimme and Meisel, 1995).

Wong and others (1998) examined the effects of purified bovine milk proteins on murine spleen cells. They found that β-lg alone significantly increased cell proliferation and production of IgM compared to CN, and mixtures of α-la, β-lg, BSA, and bovine gamma globulin (BGG). Alpha-la, BSA and BGG alone did not stimulate IgM production, but the data were not shown. When β-lg was treated with alkaline or digested with trypsin, stimulation of IgM was greatly diminished. Wong and others (1998) found their results to conflict with other studies which determined that BSA was the most immunostimulatory bovine whey protein (Bounous and others, 1989; Bounous and Kongshavn, 1985). They suggested that their experimental model may account for the difference in results, but they also could not rule out the possibility of a copurifying substance in the β-lg.

2.4 Caco-2 cells

More recently, Caco-2 cells have been used as an *in vitro* model for human intestinal epithelial cells because of their physical and functional similarities. Caco-2 cells are enterocyte-like colonic adenocarcinoma cells, which are hypertetraploid. They are able to spontaneously differentiate in culture and form tight junctions, and thus resemble normal intestinal epithelial cells. Caco-2 cells also exhibit structures resembling brush border microvilli. As to why Caco-2 cells, isolated from the colon, are able to differentiate into enterocytes is still not well understood. Pinto and others (1983) speculated that Caco-2 cells have several chemical characteristics similar to fetal cells, which undergo differentiation.

Caco-2 cells are thought to be good models for immune studies because they secrete cytokines. Jung and others (1995) compared cytokine production from freshly isolated normal colon epithelial cells and colon epithelial cells lines. Freshly isolated epithelial cell production of monocyte chemotactic protein-1 (MCP-1, a chemokine) and IL-8 was upregulated by bacterial invasion or by IL-1β stimulation in the same order of magnitude as Caco-2 cells. Caco-2 cells produced mRNA for the cytokines TNF-α, IL-8, MCP-1 and granulocyte-macrophage colony-stimulating factor (GM-CSF) as a result of infection with invasive bacteria such as *Salmonella dublin*, *Shigella dysenteriae* and *L. monocytogenes* (Jung and others, 1995). In the case of IL-6, freshly isolated epithelial cells produced increased amounts of this cytokine from bacterial stimulation, whereas Caco-2 cells did not. From this study, it appeared that the Caco-2 cell line did not have the same response to stimuli as fresh epithelial cells. This comparison is important because cell lines are typically homogeneous and easier to grow in culture than fresh

cells. Several contrasting studies have shown that Caco-2 cells can produce IL-6 and IL-8 after exposure to bacterial pathogens (Michalsky and others, 1997; Eckmann and others, 1993; Hedges and others, 1992). Table 2.6 gives a summary of these and other studies of cytokine production by Caco-2 cells.

Vitkus and others (1998) found that Caco-2 cells were capable of producing IL-6 when stimulated with cytokines IL-1β and TNF-α. Caco-2 cells grown in 5% CO_2 produced significantly lower ($p<0.05$) amounts of IL-6 when stimulated with either IL-1β or TNF-α, as compared to those grown in 10% CO_2. Caco-2 production of IL-6 by independent stimulation with IL-1β and TNF-α was dose dependent; but co-stimulation resulted in a synergistic effect. Vitkus and others (1998) suggested that unstimulated Caco-2 cells were previously thought to be incapable of producing IL-6 because they were incubated in 5% CO_2. Caco-2 cells can therefore produce cytokines as a result of stimulation by other cytokines as well as bacterial pathogens. Based on the ability of Caco-2 cells to secrete IL-6, IL-8, and TNF-α, Vitkus and others (1998) concluded that Caco-2 cells make an excellent model for normal intestinal epithelial cell cytokine stimulation.

The use of Caco-2 cells as a model to investigate the immunostimulating effects of probiotics has been limited. Studies have primarily focused on adhesion of LAB to Caco-2 cells (Tuomola and Salminen, 1998; Bernet and others, 1994; Elo and others, 1991). Adhesion then allows LAB to exert other effects on Caco-2 cells such as

Table 2.6 Studies on stimulation of cytokine production by Caco-2 cells

Experimental design	Results	Reference
Co-culture of Caco-2 cells with human blood leukocytes. Incubation with *E. coli*[1] *L. sakei, L. johnsonii*	Co-culture necessary for *E. coli* and *L. sakei* induced expression of IL-8, MCP-1, IL-1β and TNF-α mRNA. *L. johnsonii* increased TGF-β mRNA	Haller and others, 2000
Stimulation with IL-1β, TNF-α in 5 or 10% CO_2	IL-6 production was greatest at 10% CO_2. Both cytokines stimulated IL-6 production; together they had a synergisitic effect	Vitkus and others, 1998
Apical or basal incubation with *E. coli*[2]	IL-6 and TNF-α production increased in a dose-dependent manner greatest on side of Caco-2 cell exposed to bacteria	Michalsky and others, 1997
Incubation with *S. dublin, Shigella dysenteriae, Yersinia entercolitica, L. monocytogenes, E. coli*[3]	All invasive bacteria upregulated MCP-1 and IL-8 secretion,	Jung and others, 1995
Stimulated with TNF-α, IL-1β, IFN-γ, LPS	Caco-2 cells only produced IL-8 in response to stimulation with IL-1β	Schuerer-Maly and others, 1994
Stimulated with TNF-α, IL-1β, IFN-γ, LPS	Caco-2 cells found to constitutively express mRNA for cytokines IL-1α, IL-1β, IL-8, IL-10 and TGF-β. Only IL-1β significantly Increased IL-8 production	Eckmann and others, 1993
Incubation with *E. coli*[4], *Staphylococcus saprophyticus*, LPS, lipid A	Caco-2 cells did not produce IL-6 after incubation with any of the stimuli	Hedges and others, 1992

[1] *E. coli* LTH 634 (non-pathogenic)
[2] *E.coli* C25 (non-pathogenic)
[3] *E. coli* serotype O111:B4 (enteroinvasive), *E. coli* DH5α (noninvasive), *E. coli* O157 (noninvasive)
[4] *E. coli* Hu734, *E. coli* GR12 (serotype O75:K5:H^-, Hly^-, $ColV^+$), *E. coli* C1212 (serotype O6:K2:H1), *E. coli* 536 (serotype O6:K15:H31)

inhibition of pathogen binding (Bernet and others, 1994; Coconnier and others, 1993; Chauviere and others, 1992). Haller and others (2000) examined the effect of non-pathogenic *E. coli* LTH 634, and *L. sakei* and *L. johnsonii* on cytokine production by Caco-2 cells and human blood leucocytes in co-culture. Using a transwell cell culture system, Caco-2 cells were incubated in separate but adjoining compartments which contained human PBMC (Figure 2.3). Non-pathogenic bacteria were not able to induce chemokine IL-8 or MCP-1 mRNA from Caco-2 cells alone. When Caco-2 cells and PBMC were cultured together, however, expression of mRNA for IL-8 and MCP-1 was observed. *E. coli* and *L. sakei* also had a stimulatory effect on PBMC-sensitized Caco-2 cells to produce the cytokines TNF-α and IL-1β which followed the same trend as mRNA expression. *L. johnsonii* did not stimulate production of TNF-α or IL-1β and also did not induce as much mRNA of IL-8 or MCP-1 as *L. sakei*. *L. johnsonii* did, however, stimulate transforming growth factor-β in Caco-2 cells. Haller and others (2000) concluded that immunocompetent cells were necessary for Caco-2 cells to recognize non-pathogenic bacteria. Their communication was thought to be through soluble factors since Caco-2 cells and PBMC were not in direct contact in the transwell culture plates.

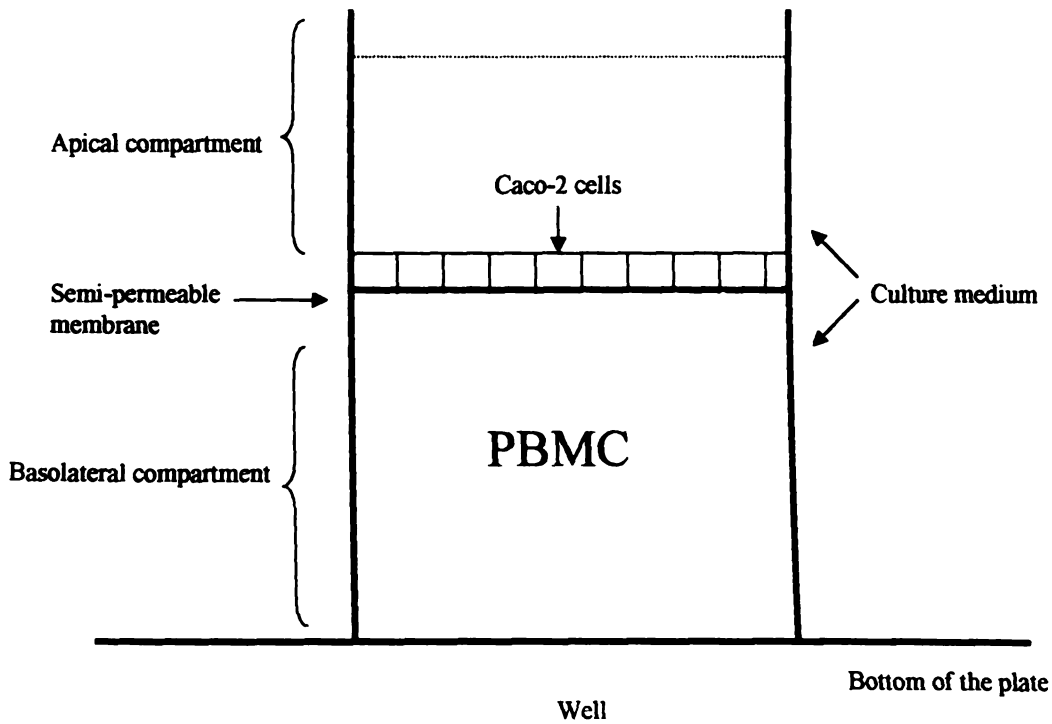

Figure 2.3 Schematic diagram of the transwell co-culture system used by Haller and others (2000). Caco-2 cells were grown on a cell culture insert and then placed in the apical compartment. They were separated from the peripheral blood mononuclear cells (PBMC) (2 x 10^6 cells/ml) in the basolateral compartment by a semi-permeable membrane. Both wells were filled with culture media.

2.5 Rationale for this research

Many questions concerning the immunomodulating effects of probiotics on the human immune system remain to be answered. Research needs to address if and how probiotics exert their effects. The limited number of studies investigating the interaction of probiotics and the immune system is due in part to the difficulty in finding an appropriate model system. To look at this interaction, the Caco-2 cell line was chosen because it is a well-established human cell line with similarity to epithelial cells.

The working hypothesis for this research was that LAB and bifidobacteria, which are used in the production of fermented dairy foods, can enhance immune function by stimulating cytokine secretion by intestinal epithelial cells. The probiotic bacterial strains were chosen based on their ability to stimulate cytokine secretion in murine macrophage and T-cells. Because nutritional composition of milk changes during fermentation, probiotic bacteria were allowed to ferment reconstituted non-fat dry milk. Prior to use in the cell culture system, bacterial cultures were also inactivated by heat or by irradiation to compare if the immune effects seen by heat-treated cells were due to changes in cellular proteins. Further studies were conducted to determine the effects of milk components on cytokine secretion.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

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3.1 Culture preparation

Seven probiotic organisms were selected based on their ability to stimulate cytokine production in previous experiments with murine macrophage and thymoma cell lines (Table 3.1) (Marin and others, 1997; Marin and others, 1998; Tejada-Simon and others, 1999). *Lactobacillus acidophilus* LA2 (LA), *Lactobacillus delbrückii* subsp. *bulgaricus* (hereafter referred to as *Lactobacillus bulgaricus*) NCK 231 (LB), *Lactobacillus casei* ATCC 39539 (LC), and *Lactobacillus reuteri* ATCC 23272 (LR) were grown in De Man, Rogosa, Sharpe (MRS) broth (Difco Laboratories, Detroit, MI). *Streptococcus thermophilus* St 133 (ST133) was grown in M17 broth (Difco). *Bifidobacterium* Bf-6 (BF6) and *Bifidobacterium adolescentis* M101-4 (M1014) were grown in MRS broth with 5% lactose under anaerobic conditions (GasPak®, BBL Microbiology Systems, Cockeysville, MD).

In preliminary experiments, standard curves of optical density (OD) vs CFU/ml were generated for each bacterium based on spectrophotometric and plate count methods. ODs of cultures in their respective broths were measured on a Spectronic 1001 Plus (Milton Roy, Rochester, NY) at 650 nm using uninoculated broth as a blank. Culture samples were then diluted and plated to correlate cell numbers with OD. Bacteria in broth were diluted using 0.1% bacto-peptone dilution buffer (Difco) to obtain ten-fold dilutions of 10^{-1} to 10^{-8} CFU/ml. One ml samples were plated using the pour plate method. Lactobacilli, streptococci and bifidobacteria were enumerated using MRS, M17 broth, and MRSL containing 1.5% agar, respectively. Lactobacilli and streptococci

Table 3.1 Sources of bifidobacteria and lactic acid bacteria cultures used in this study

Bacteria	Strain	Source
Bifidobacterium	Bf-6	Sanofi Bio-Industries, Waukesha, WI
Bifidobacterium adolescentis	M101-4	Japan Bifidus Foundation, Tokyo, Japan
Lactobacillus acidophilus	La-2	Sanofi Bio-Industries, Waukesha, WI
Lactobacillus bulgaricus	NCK 231	North Carolina State University, Raleigh, NC
Lactobacillus casei	ATCC 39539	American Type Culture Collection, Rockville, MD
Lactobacillus reuteri	ATCC 23272	American Type Culture Collection, Rockville, MD
Streptococcus thermophilus	St 133	Sanofi Bio-Industries, Waukesha, WI

plates were incubated at 37°C for 48 h and then counted using a Darkfield Quebec Colony Counter (American Optical Company, Buffalo, NY). Bifidobacteria were incubated anaerobically using the GasPak® system under the same conditions before being counted.

Figure 3.1 provides a schematic diagram of sample preparation. One ml of each of the stock cultures (stored at –80°C) was thawed before being added to 25 ml of their respective broths as mentioned above. The cultures were then incubated for 12 h at 37°C at which point they had reached late log phase of growth. After incubation, 5 ml of inoculum was transfered to 25 ml of fresh broth and incubated for 12 h at 37°C. This transfer was repeated 2 more times before cultures were prepared for use in cell culture.

After the third transfer, all probiotic cultures were harvested at late log phage (12 h). ODs were taken of the cultures to determine cell concentrations using the standard curves. The cultures were then centrifuged at 3000 x g for 15 min. The supernatant (broth) was discarded and cultures were washed with phosphate buffered saline (PBS) by centrifugation (3000 x g, 15 min) and decanted. The cultures were then resuspended in 10% reconstituted non-fat dry milk (NFDM) (Difco) to obtain final concentrations of 10^6, 10^7, and 10^8 CFU/ml according to calculations using the standard curves. Plate counts of bacterial samples were performed before inactivation to confirm cell numbers using the pour plate method as described previously.

Heat or irradiation was used to inactivate the prepared cultures. For heat-inactivated samples, bacteria were either heated (95°C, 30 min) immediately after preparation or after fermentation (37°C, 4 h), where cell numbers increased one log. The cultures at 10^8 CFU/ml were not fermented because the acid produced lowered the pH

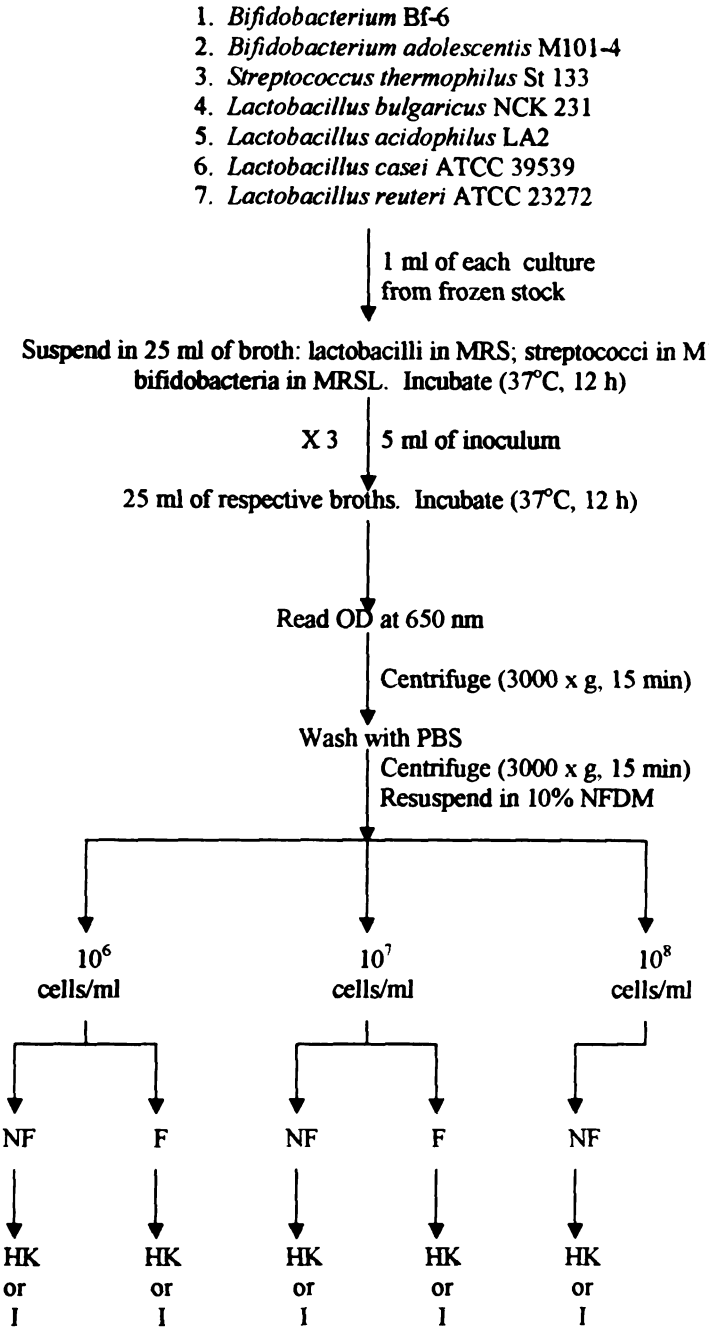

Figure 3.1 Schematic diagram of probiotic culture preparation in non-fat dry milk (NFDM).
MRS = DeMan, Rogosa, Sharpe; MRSL = MRS + 5% lactose; OD = optical density; NF = non-fermented; F = fermented (37°C, 4 h); HK = heat-killed (95°C, 30 min); I = irradiated (1 Mrad).

and caused the NFDM to coagulate. This made administering a uniform to the cell culture difficult. Samples were then frozen at –80°C until further use. For irradiated samples, bacteria were either frozen (-80°C) immediately after preparation, or after fermentation (37°C, 4 h). Frozen samples were exposed to 1 Mrad of cobalt-60 irradiation at the University of Michigan Memorial Phoenix Project (Ann Arbor, MI). Irradiated samples were stored frozen at –80°C until further use. Appendix I contains a certificate of compliance for irradiation of LAB and bifidobacteria. Plate counts of probiotic cultures were performed before their addition to cell culture to verify cell numbers using the pour plate method as described above.

3.2 Caco-2 cell culture

Caco-2 cells (ATCC HTB-37) were obtained from American Type Culture Collection (Rockville, USA) and grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA), 0.01% (v/v) antibiotic-antimycotic solution (10.0 units/ml penicillin G sodium, 10.0 μg/ml streptomycin sulfate, and 25.0 μg/ml amphotericin B in 0.85% saline) (Gibco), 0.01% (v/v) Fungizone reagent (Gibco), and 0.004% (w/v) sodium bicarbonate. Caco-2 cells were first grown in 25 cm^2 tissue culture flasks at 37°C and 6% CO_2. Cells were loosened from the flask using Trypsin-EDTA (Sigma, St. Louis, MO) and harvested by centrifugation at 1200 x g for 7 min. Cells were transferred to 48-well tissue culture plates (Costar, Cambridge, MA) at 5 x 10^5 cells/well. Cell numbers were determined using a Bright-line hemocytometer (American

Optical Co., Buffalo, NY). Monolayers of Caco-2 cells were incubated for 72 h until confluent before use in experiments.

3.3 Stimulation of cytokine production of Caco-2 cells by probiotic bacteria

Heat-inactivated or irradiated probiotic samples in NFDM as described in section 3.1 were added to a monolayer of Caco-2 cells at final concentrations of 10^6, 10^7, and 10^8 cells/ml in a well. Uninoculated NFDM and the Caco-2 supernatant alone were used as negative controls. The cytokine IL-1β (1 ng/ml) was used as a positive control for IL-6 and IL-8 induction. Supernatant was collected at 24 and 48 h and frozen at –80°C until analyzed for the cytokines IL-6 and IL-8 by enzyme-linked immunosorbant assays (ELISA).

3.4 IL-6 and IL-8 quantitation

Procedures included in the OptEIA™ Set (BD PharMingen, San Diego, CA) were followed for the ELISA. Briefly, 100 µl of anti-human IL-6 or IL-8 monoclonal antibodies diluted in 0.1 M sodium carbonate buffer (pH 9.5) was added to each well of microtiter strips (Immunolon II Removawell; Dynatech Technologies, Chantilly, VA) set in a Removawell holder (Dynatech Technologies). The plates were incubated overnight at 4°C. Wells were then washed 3x with 0.01 M PBS with 0.05% Tween-20 (v/v) (PBST) using the Ultrawash Plus ELISA washer (Dynatech Technologies) to remove unbound capture antibody. The plates were then incubated for 1 h with 200 µl of PBS buffer supplemented with 10% FBS (v/v) (pH 7.0) to reduce nonspecific binding. Next, the wells were washed 3 x with PBST before 100 µl of standards of recombinant human

IL-6 or IL-8 diluted with DMEM with 10% NFDM or sample were added to the wells. Plates were covered with aluminum foil and incubated at room temperature (~24°C) for 2 h. The wells were next washed 5 x with PBST to remove non-adhering antigens. One hundred µl of biotinylated anti-human IL-6 or IL-8 streptavidin-horseradish peroxidase conjugate (BD PharMingen) was added to each well and incubated at room temperature (~24°C) for 1 h. The wells were then washed 7x with PBST before 100 µl of tetramethylbenzidine substrate reagent (BD Pharmingen) was added to the wells. The plates were incubated at room temperature (~24°C, 30 min) in the dark. To stop the enzyme reaction, 50 µl of 2 N H_2SO_4 stopping solution was added to each well. Absorbance was read at 450 nm using a Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA). Cytokines were quantitated using a standard curve generated from the Softmax curve-fitting program (Molecular Devices).

Higher absorbance readings indicated greater amounts of cytokine present in the supernatant sample. Cytokine values were expressed as percent change from Caco-2 supernatant which was designated as 100%. Values were calculated as follows: 100-(100*((cytokine in Caco-2 supernatant-cytokine in sample)/cytokine in supernatant)).

3.5 Stimulation of cytokine production of Caco-2 cells by milk components

Lactose (Sigma-Aldrich, St. Louis, MO), α-la (Sigma-Aldrich), β-lg (Sigma-Aldrich) and NFDM were suspended in DMEM to obtain a 4% final concentration in cell culture. All solutions were filter sterilized using a 0.45µm Millex®HA syringe driven filter unit (Millipore Corporation, Bedford, MA). Solutions containing the milk

components were added to a monolayer of Caco-2 cells. After 2 h of incubation, IL-1β (1 ng/ml) was added to half of the samples. All samples were incubated (37°C, 6% CO_2) for 24 h. Supernatants were collected and frozen at –80°C until analyzed for the cytokines IL-6 and IL-8 by ELISA as described in section 3.4. The IL-6 and IL-8 standards, however, were suspended in DMEM for these experiments.

3.6 Statisitical analysis

Experiments were replicated three times in a randomized design. Percent change of IL-6 and IL-8 production relative to the Caco-2 cell supernatant (calculation described in section 3.4) was transformed by square root to correct for non-normality and heterogenous variances among samples. The data was analyzed using 'PROC MIXED' in The SAS system version 8.2 (SAS Institute Inc., 2001, Cary, NC). The model accounted for interaction between replication, probiotic organism, treatment, inactivation, concentration and plate. The main effects were probiotic organism (listed in Table 3.1), treatment (fermented or non-fermented), inactivation (heat or irradiation) and concentration (10^6, 10^7, 10^8 CFU/ml). Two-way interactions of these main effects were also examined (i.e. organism*treatment, organism*inactivation, etc.) Replication and plate were considered random effects. Significance of the main effects was tested using Type 3 sums of squares. The Satterthwaite degrees of freedom method was used with the Tukey-Kramer adjustment was conducted for multiple comparisons. A $p \leq 0.05$ was used as the level of significance.

CHAPTER 4

RESULTS AND DISCUSSION

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Effect of lactic acid bacteria and bifidobacteria on IL-6 production by Caco-2 cells

Heat-killed and irradiated lactic acid bacteria and bifidobacteria were incubated with a monolayer of Caco-2 cells for 24 and 48 h. The supernatants were collected and then analyzed for the cytokines IL-6 and IL-8 using ELISA. Due to the differences in baseline cytokine production between replications, percent change in cytokine production was calculated where the amount of cytokine in the Caco-2 DMEM supernatant alone was 100 percent. When stimulated by IL-1β, the positive control, Caco-2 cells were capable of producing 2 to 11 times more IL-6 than untreated controls. Uninoculated, reconstituted NFDM alone served as a negative control and did not significantly affect IL-6 and IL-8 production by Caco-2 cells during the 24 and 48 h incubations.

The 'PROC MIXED' statistical procedure was used to determine the significance of each main effect interaction (i.e. culture by fermentation, culture by method of inactivation). The data were organized so that two sets of comparisons could be made. The first set accounted for all non-fermented samples at 10^6, 10^7, and 10^8 CFU/ml, with the exception of ST133 which could not be grown to 10^8 CFU/ml. A separate analysis that did include ST133 indicated that it was not significantly different ($p>0.05$) from the other probiotic organisms at concentrations of 10^6 and 10^7 CFU/ml for any of the effects tested. The second set of data compared all fermented and non-fermented samples at 10^6 and10^7 CFU/ml. Standard errors of the means were not included in the figures because

they were generated based on the square root of the data. This was done to normalize the data.

4.1.1 Effect of culture

When comparing the non-fermented samples only, the type of culture was not significant ($p>0.05$) to IL-6 production at 24 h (Table 4.1), but became significant ($p<0.05$) at 48 h (Table 4.2). The pooled concentrations of BF6 stimulated significantly more IL-6 (112.6%) than LB (82%), LC (90.7%), and M1014 (89.2%) (Figure 4.1) at 48 h. LA stimulated significantly greater IL-6 (99.2%) than LB (82%). Stimulation of IL-6 by NFDM was only significantly different (110.5%) from LB. Levels of IL-6 stimulated by LR and ST133 were not significantly different from any other the other cultures. Although it was not significant ($p>0.05$), NFDM and BF6 stimulated more IL-6 compared to the Caco-2 supernatant. With the exception of BF6, the addition of culture to NFDM suppressed IL-6 production. LB was the only culture which significantly suppressed ($p<0.05$) IL-6 production compared to the supernatant from naïve Caco-2 culture.

Comparing the fermented and non-fermented samples, the type of culture was not significant ($p>0.05$) to IL-6 production at 24 (Table 4.3) and 48 h (Table 4.4).

4.1.2 Effect of dose

Dose was not significant to IL-6 production at either incubation time point. None of the concentrations were significantly different from one another. It cannot be concluded however, that there was no dose effect on IL-6 production by Caco-2 cells

Table 4.1 ANOVA table comparing non-fermented cultures at concentrations of 10^6, 10^7, and 10^8 CFU/ml on IL-6 production by Caco-2 cells after 24 hr incubation

Effect	Degrees of Freedom	F Value	Pr > F
Culture[1]	715	1.55	0.1486
Concentration[2]	711	1.91	0.1485
Inactivation[3]	715	60.9	**<0.0001**
Culture*Concentration	711	0.43	0.9589
Culture*Inactivation	710	4.99	**<0.0001**
Inactivation*Concentration	711	0.45	0.6387

[1] Probiotic cultures
[2] 10^6, 10^7, 10^8 CFU/ml
[3] Heat-killed/Irradiated

Table 4.2 ANOVA table comparing non-fermented cultures at concentrations of 10^6, 10^7, and 10^8 CFU/ml on IL-6 production by Caco-2 cells after 48 hr incubation

Effect	Degrees of Freedom	F Value	Pr > F
Culture[1]	708	4.67	**<0.0001**
Concentration[2]	710	0.53	0.5861
Inactivation[3]	716	148.1	**<0.0001**
Culture*Concentration	710	2.51	**0.0023**
Culture*Inactivation	693	3.46	**0.0012**
Inactivation*Concentration	710	0.91	0.4036

[1] Probiotic cultures
[2] 10^6, 10^7, 10^8 CFU/ml
[3] Heat-killed/Irradiated

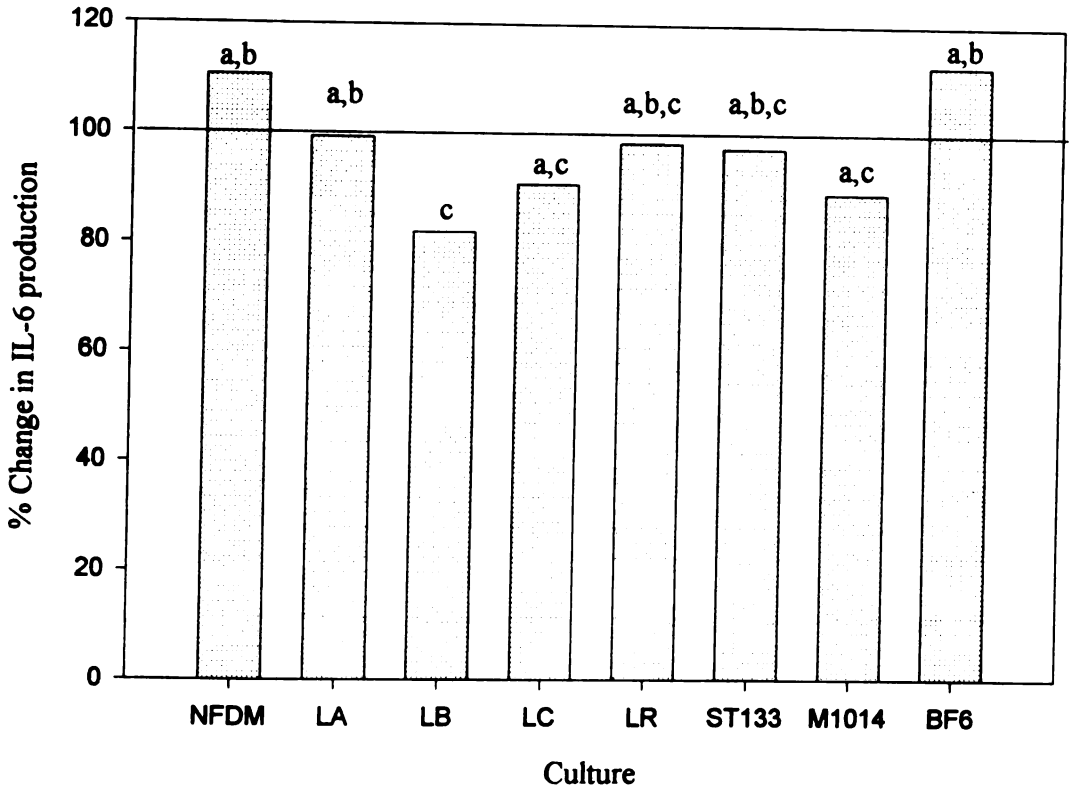

Figure 4.1 Effect of non-fermented probiotic cultures at concentrations of 10^6, 10^7, and 10^8 CFU/ml on IL-6 production by Caco- 2 cells (48 h incubation). Unstimulated IL-6 in Caco-2 supernatant was considered 100 percent

[a-c] Indicates that samples with different letters are significantly different from each other ($p<0.05$). NFDM = non-fat dry milk; LA = *L. acidophilus* LA2; LB = *L. bulgaricus* NCK 231; LC = *L. casei* ATCC 39539; LR = *L. reuteri* ATCC 23272; ST133 = *S. thermophilus*; M1014 = *B. adolescentis* M101-4; BF6 = *Bifidobacterium* Bf-6

Table 4.3 ANOVA table comparing fermented and non-fermented cultures at concentrations of 10^6 and 10^7 CFU/ml on IL-6 production by Caco-2 cells after 24 hr incubation

Effect	Degrees of Freedom	F Value	Pr > F
Culture[1]	1009	1.85	0.0748
Fermentation[2]	1010	5.71	**0.0171**
Concentration[3]	1003	1.01	0.3151
Inactivation[4]	1007	48.5	**<0.0001**
Culture*Fermentation	1005	2.50	**0.0152**
Culture*Concentration	1003	0.93	0.4808
Culture*Inactivation	1010	2.53	**0.0140**
Fermentation*Concentration	1004	0.27	0.6030
Fermentation*Inactivation	1014	2.09	0.1483
Inactivation*Concentration	1004	0.94	0.3315

[1] Probiotic cultures
[2] Fermented/Non-fermented
[3] 10^6, 10^7 CFU/ml
[4] Heat-killed/Irradiated

Table 4.4 ANOVA table comparing fermented and non-fermented cultures at concentrations of 10^6 and 10^7 CFU/ml on IL-6 production by Caco-2 cells after 48 hr incubation

Effect	Degrees of Freedom	F Value	Pr > F
Culture[1]	1011	1.04	0.4001
Fermentation[2]	1015	0.26	0.6118
Concentration[3]	1008	0.56	0.4537
Inactivation[4]	1013	172.84	**<0.0001**
Culture*Fermentation	1010	1.82	0.0799
Culture*Concentration	1008	0.98	0.4422
Culture*Inactivation	1013	1.96	0.0572
Fermentation*Concentration	1008	0.19	0.6615
Fermentation*Inactivation	1016	0.00	0.9506
Inactivation*Concentration	1008	0.15	0.7026

[1] Probiotic cultures
[2] Fermented/Non-fermented
[3] 10^6, 10^7 CFU/ml
[4] Heat-killed/Irradiated

because no single concentration stimulated or suppressed significantly different ($p>0.05$) amounts of IL-6 compared to the Caco-2 supernatant based on the confidence intervals.

4.1.3 Effect of fermentation

The effect of fermentation by probiotic bacteria on IL-6 production by Caco-2 cells was examined at doses of 10^6 and 10^7 CFU/ml. After 4 h incubation, cell numbers increased by one log. Fermentation of NFDM by probiotic bacteria had a significant effect ($p<0.05$) on IL-6 production by Caco-2 cells after 24 h incubation (Table 4.3), but this was no longer seen at 48 h (Table 4.4). Compared to the Caco-2 supernatant control, non-fermented samples suppressed IL-6 production to 96.5%. This was statistically higher than 90.9% IL-6 stimulated by fermented samples. Suppression of IL-6 production compared to the naïve Caco-2 supernatant, however, was not significant ($p>0.05$). These results indicate that fermentation yielded end products which were slightly inhibitory to IL-6 production by Caco-2 cells.

4.1.4 Effect of inactivation

The difference in IL-6 production between heat-killed and irradiated culture samples was significant ($p<0.05$) upon comparison of all non-fermented samples at 24 and 48 h (Tables 4.1, 4.2). More specifically, at 24 h, heat-killed samples stimulated IL-6 production to 106.9% control, whereas irradiated samples suppressed IL-6 production by 15.2%. None of the samples produced significantly ($p<0.05$) different levels of IL-6 compared to the Caco-2 supernatant. At 48 h, the same trend was seen where heat-killed

samples stimulated IL-6 production (114.6%), but in irradiated samples IL-6 production was suppressed (81%).

The comparison of fermented and non-fermented culture samples also showed that method of inactivation resulted in significantly different ($p<0.05$) levels of IL-6 production. At 24 h, irradiated cultures suppressed IL-6 production to 86%, which was significantly lower ($p<0.05$) than the heat-killed cultures (101.7%). At 48 h, heat-killed cultures stimulated more IL-6 (116.1%) than the irradiated cultures (82.4%).

These results suggest that the mode of inactivation is significant to the amount of cytokine produced. It is possible that the exposure to heat denatured proteins on the bacterial surface or in the cytoplasm which stimulated IL-6 production. Future studies may want to use irradiated cells to ensure more accurate results.

4.1.5 Effect of the interaction between culture and dose

The interaction between culture and dose was statistically significant ($p<0.05$) when comparing IL-6 production in the non-fermented cultures at 48 h (Table 4.2). Upon closer examination, however, the individual interactions were between different cultures, not within a culture. We were only interested in comparison between different doses of the same strain to determine if there was a dose effect on IL-6 production.

4.1.6 Effect of the interaction between culture and inactivation

The interaction between culture and inactivation was significant at both time points for the non-fermented samples (Tables 4.1, 4.2). Figure 4.2 illustrates the effect of culture and inactivation on IL-6 production by Caco-2 cells at 24 h. With the

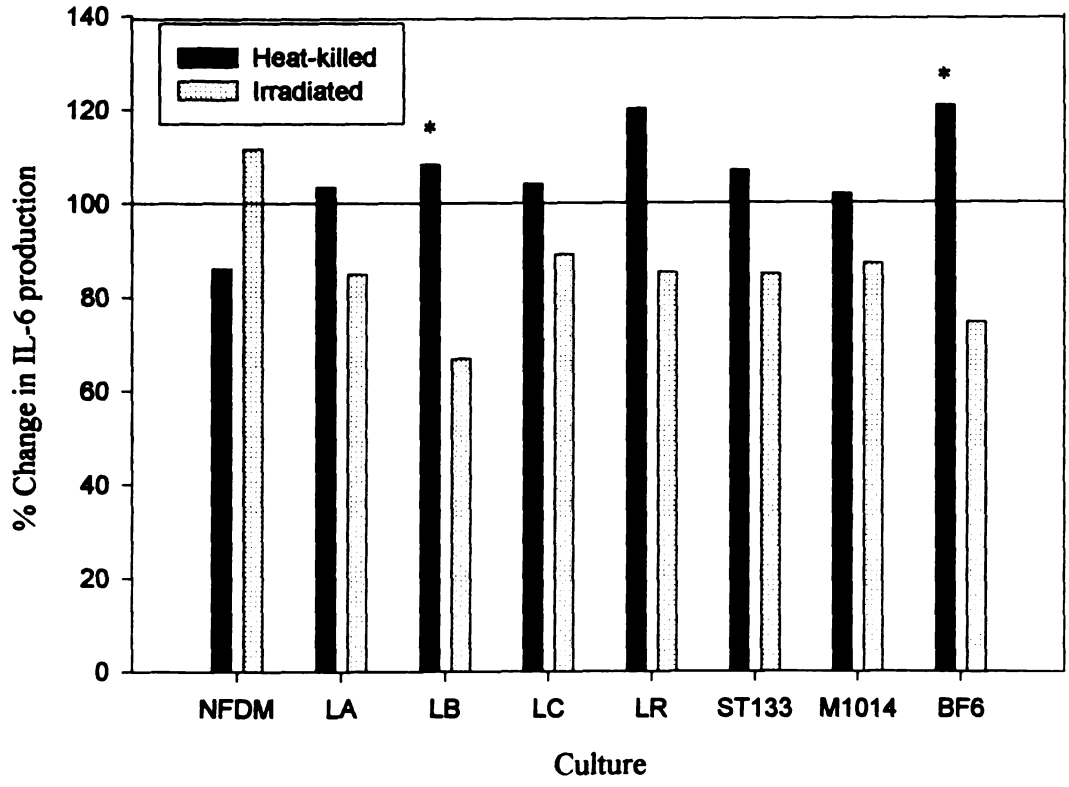

Figure 4.2 Effect of heat and irradiation inactivation of non-fermented lactic acid bacteria and bifidobacteria at concentrations of 10^6, 10^7, and 10^8 CFU/ml on IL-6 production by Caco-2 cells (24 h incubation). Unstimulated IL-6 in Caco-2 supernatant was considered 100 percent.
* Indicates that heat-killed are significantly different than the irradiated counterpart ($p<0.05$).
NFDM = non-fat dry milk; LA = *L. acidophilus* LA2; LB = *L. bulgaricus* NCK 231; LC = *L. casei* ATCC 39539; LR = *L. reuteri* ATCC 23272; ST133 = *S. thermophilus* St133; M1014 = *B. adolescentis* M101-4; BF6 = *Bifidobacterium* Bf-6

exception of NFDM, all heat-killed cultures caused Caco-2 cells to produce approximately the same amount of IL-6 compared to the Caco-2 supernatant. Irradiated cultures however, with the exception of NFDM, suppressed IL-6 production although it was not a significant amount ($p>0.05$) from the naïve Caco-2 supernatant. The heat-killed samples of BF6 and LB stimulated significantly higher levels of IL-6 ($p<0.05$) than their irradiated counterparts. None of the cultures when incubated with the Caco-2 cells resulted in IL-6 production that was significantly different from the naive Caco-2 cells.

Figure 4.3 demonstrates that at 48 h, the heat-killed and irradiated cultures showed the same trends on IL-6 production by non-fermented cultures as at 24 h (Figure 4.2). At this later time point, however, there was a significant difference ($p<0.05$) between the heat-killed and irradiated cultures of LA, LB, LC, LR and BF6. The interaction of LB, LC, LR, and M1014 and irradiation resulted in suppression of IL-6 production that was significantly lower than the IL-6 induction by the naïve Caco-2 cells.

Comparing the interaction between culture and inactivation for fermented and non-fermented cultures indicated that this interaction was only significant at 24 h. Figure 4.4 illustrates that irradiated cultures suppressed IL-6 production by Caco-2 cells to a much greater extent than heat-killed cultures. There was a significant difference ($p<0.05$) between the irradiated and heat-killed cultures of LC, M1014 and BF6. None of the cultures resulted in IL-6 production that was significantly different from the Caco-2 supernatant.

The difference between heat- and irradiation-inactivation was significant for several of the cultures, however this was not consistent between time points or comparisons. Heat-inactivation resulted in substances that were not as inhibitory to IL-6

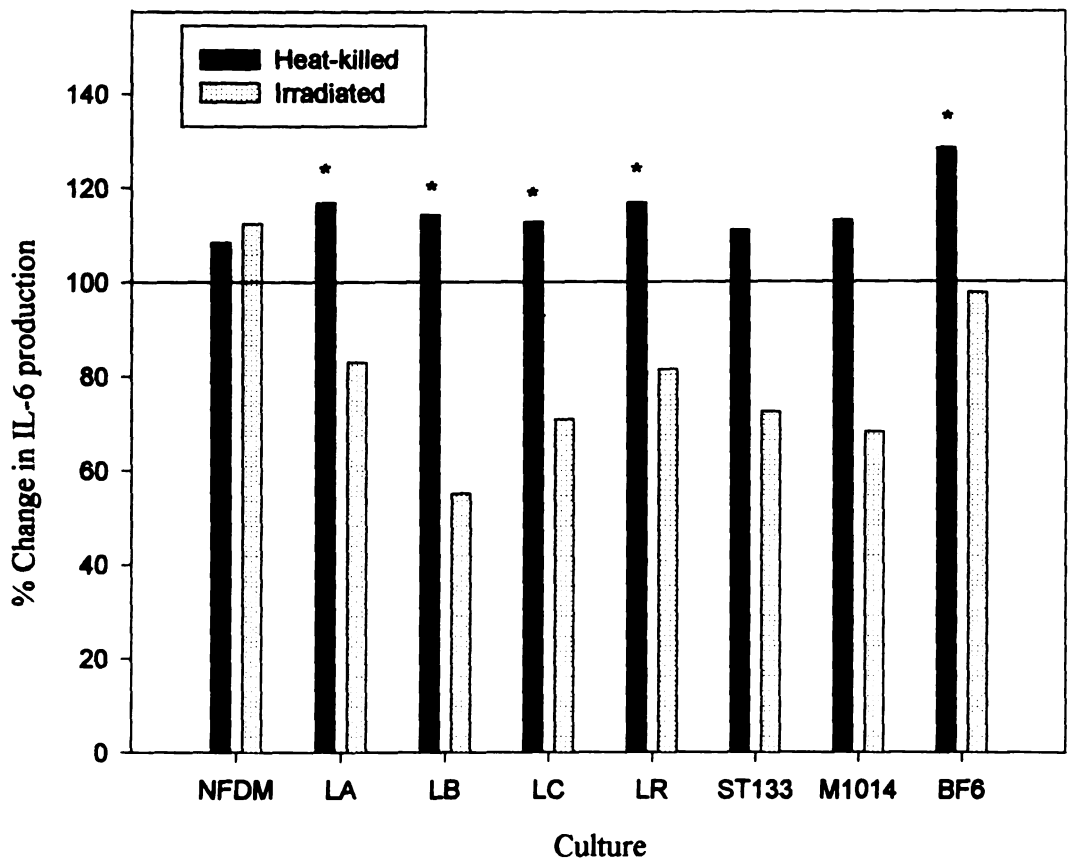

Figure 4.3 Effect heat and irradiation inactivation on non-fermented lactic acid bacteria and bifidobacteria at concentrations of 10^6, 10^7, and 10^8 CFU/ml on IL-6 production by Caco-2 cells (48 h incubation). Unstimulated IL-6 in Caco-2 cell supernatant was considered 100 percent.
* Indicates that heat-killed were significantly different than irradiated counterpart ($p<0.05$). NFDM = non-fat dry milk; LA = *L. acidophilus* LA2; LB = *L. bulgaricus* NCK 231; LC = *L. casei* ATCC 35935; LR = *L. reuteri* ATCC 23272; ST133 = *S. thermophilus* St133; M1014 = *B. adolescentis* M101-4; BF6 = *Bifidobacterium* Bf-6

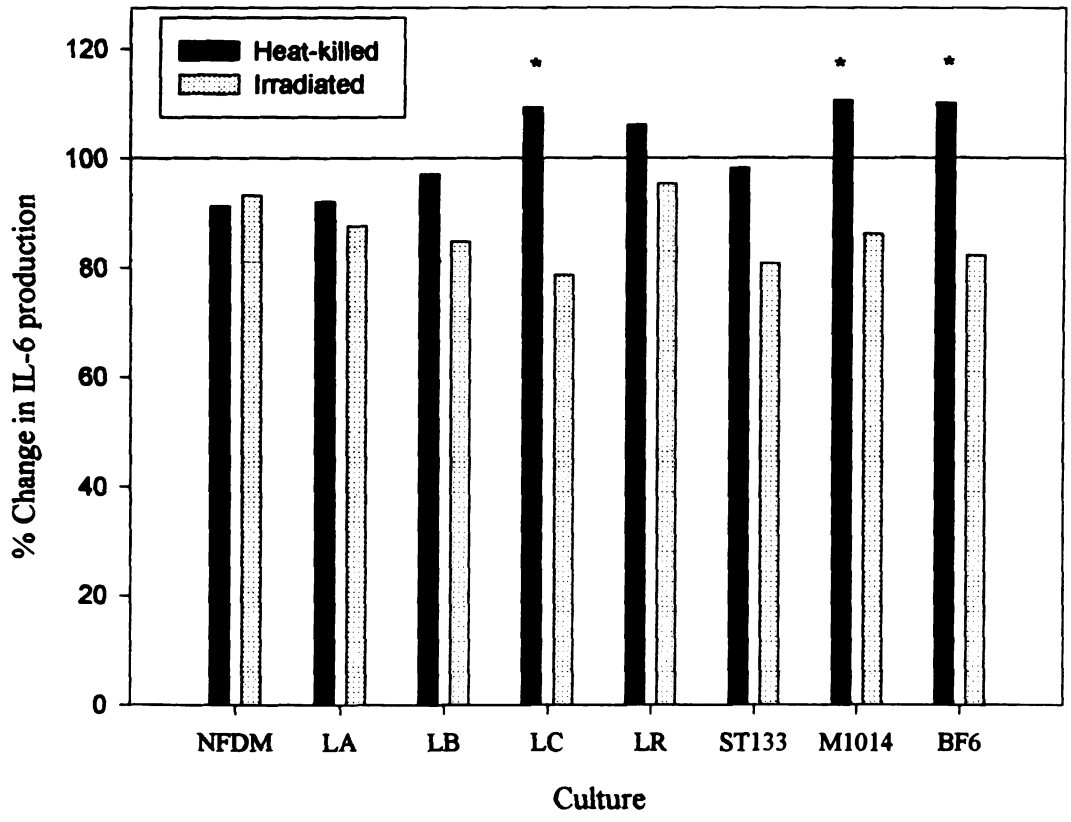

Figure 4.4 Effect of heat and irradiation inactivation of fermented and non-fermented lactic acid bacteria and bifidobacteria at concentrations of 10^6 and 10^7 CFU/ml on IL-6 production by Caco-2 cells (24 h incubation). Unstimulated IL-6 in Caco-2 supernatantwas considered 100 percent.
* Indicates that heat-killed were significantly different from irradiated counterpart ($p<0.05$). NFDM = non-fat dry milk; LA = *L. acidophilus* LA2; LB = *L. bulgaricus* NCK 231; LC = *L. casei* ATCC 39539; LR = *L. reuteri* ATCC 23272; ST133 = *S. thermophilus* St133; M1014 = *B. adolescentis* M101-4; BF6 = *Bifidobacterium* Bf-6

production by Caco-2 cells than irradiated cultures.

4.1.7 Effect of the interaction between culture and fermentation

The interaction between culture and fermentation was significant ($p<0.05$) at 24, but not at 48 h. Upon closer examination, however, the specific interactions that were significant were not between the same culture and therefore not a valid comparison for this study. It can be concluded that fermentation did not make a significant difference in IL-6 production for any of the cultures studied.

4.1.8 Discussion on the effect of lactic acid bacteria and bifidobacteria on IL-6 production by Caco-2 cells

In general there were no consistent culture- or dose-dependent observations relative to IL-6 production by Caco-2 cells. None of the cultures significantly stimulated ($p>0.05$) IL-6 production at any dose which is contrary to the findings of Marin and others (1998), who examined the effect of LAB and bifidobacteria on cytokine production by mouse RAW 264.7 macrophage cells and mouse EL4.IL-2 thymoma cells. In the latter study, all bacteria were heat-killed and incubated with cell lines at concentrations of 10^6, 10^7, and 10^8 bacteria/ml. Probiotic strain- and dose-dependent increases were observed with respect to IL-6 and TNF-α production by RAW 264.7 cells as well as in IL-2 and IL-5 production by EL4.IL-2 cells. Compared to other bacteria studied, *S. thermophilus* ST133 (also used in our experiments) had the greatest enhancing effects on cytokine production. In general, they observed that as the concentration of all bacteria increased, so did the amount of cytokine produced by the respective cell lines.

There are other reports of strain and concentration effects of probiotics and their components on cytokine production (Park and others, 1999; Tejada-Simon and Pestka, 1999; Miettinen and others, 1996). Our results also differed from these previous studies, possibly because the cell models differed. It is also possible that Caco-2 cells may require communication with underlying immune cells in order to respond to Gram-positive bacteria. Haller and others (2000), as mentioned in section 2.7, used Caco-2 cells in a co-culture system with human blood leukocytes where the two types of cells were separated by a membrane in transwell culture plates. Without the leukocytes, Caco-2 cells could not be stimulated by *L. sakei* to produce cytokines TNF-α and IL-1β. Transfer experiments were performed using leukocyte-sensitized Caco-2 cells to determine if it was the Caco-2 cells or leukocytes responsible for cytokine production. After a 12 h initial incubation with leukocytes, Caco-2 cells were transferred to another plate. Leukocyte-sensitized Caco-2 cells continued to produce a high level of TNF-α and to a lesser extent, IL-1β. They concluded that cross talk between Caco-2 cells and underlying immune cells is necessary for Caco-2 cells to recognize and respond to non-pathogenic bacteria.

It was hypothesized that differences between fermented and non-fermented milk could stimulate different amounts of IL-6 from Caco-2 cells. Fermented cultures induced significantly more IL-6 than non-fermented cultures at 24, but not at 48 h. In the process of yogurt production, fermentation by LAB and bifidobacteria changes the composition of milk. Yogurt has increased folic acid, lactic acid and decreased lactose and vitamin B_6 compared to non-fermented milk (Meydani and Ha, 2000; Shahani and Chandan, 1979). Calcium is also more bioavailable from yogurt. Bacterial enzymes can

break down proteins and lipids in milk. It is possible that the digestion of certain milk proteins could result in the production of bioactive milk peptides that may have immunomodulatory activity (McDonald and others, 1994; Laffineur and others, 1996). The inactivation carried out in this research may have created compounds with suppressive effects from the bioactive milk peptides or substances associated with the fermentation process.

The mode of bacterial inactivation did have a significant effect on IL-6 production. The main difference between these two modes of inactivation is that heat denatures proteins in the milk as well as on the surface and cytoplasm of the probiotic bacteria. In contrast, irradiation causes molecular changes in the DNA leaving the protein structure intact. These changes eventually lead to alterations in metabolism that can result in cell death if the irradiation damage is sufficiently extensive (Olson, 1998).

Heat treatment of the cultures resulted in greater amounts of IL-6 compared to the NFDM control (Figures 4.2 and 4.3). The irradiated culture, however, resulted in lower amounts of IL-6 compared to the irradiated NFDM control. Many previous *in vitro* studies used heat-killed probiotic cultures and demonstrated immunostimulatory capabilities of the cultures. Comparison between the two modes of inactivation, heat vs irradiation, sought to determine if heat-denatured proteins from the cultures were responsible for cytokine stimulation as seen in previous studies (Marin and others, 1998; Park and others, 1999). Lysis of the bacterial cell is also possible with heat inactivation. The release of proteases, DNA, cytoplasmic proteins and exposure of new epitopes could also be responsible for cytokine stimulation.

Although the stimulation and suppression of IL-6 production by heat-killed and irradiated cultures, respectively, were not significantly different from the naïve Caco-2 supernatant, our results suggest that heat inactivation leads to the generation of stimulatory factors on the cultures. These stimulatory factors may be recognized by membrane bound Toll-like receptors (TLR) on the Caco-2 cells, that can recognize microbial components (Matzinger, 2002). TLRs can induce an immune response, including the production of cytokines. Perhaps the differences between heat and irradiation inactivation would be more pronounced using the co-culture system of Haller and others (2000).

The current definition for probiotics stipulates that they should be ingested live to have immunostimulating effects in the body. While live bacteria were not investigated here, this research suggests that inactivated probiotics, specifically by irradiation, can suppress IL-6 production by gut epithelial cells. Because IL-6 plays a role in many immune functions, as well as inflammatory responses, its suppression may or may not be desirable.

4.2 Effect of lactic acid bacteria and bifidobacteria on IL-8 production by Caco-2 cells

Heat-killed and irradiated lactic acid bacteria and bifidobacteria were incubated with a monolayer of Caco-2 cells for 24 and 48 h. The supernatants were collected and then analyzed for the cytokines IL-6 and IL-8 using ELISA. Due to the differences in baseline cytokine production between replications, percent change in cytokine production was calculated where the amount of cytokine in the Caco-2 DMEM supernatant alone

was 100%. When stimulated by IL-1β, the positive control, Caco-2 cells produced 11.6 to 90 times more IL-8. Uninoculated, reconstituted NFDM alone served as a negative control and did not significantly affect IL-6 and IL-8 production by Caco-2 cells during the 24 and 48 h incubations.

The 'PROC MIXED' statistical procedure was used to determine the significance of each main effect interaction (i.e. culture by fermentation, culture by method of inactivation). The data were organized so that two sets of comparisons could be made. The first set accounted for all non-fermented samples at 10^6, 10^7, and 10^8 CFU/ml, with the exception of ST133 which could not be grown to 10^8 CFU/ml. A separate analysis that did include ST133 indicated that it was not significantly different ($p>0.05$) from the other probiotic organisms at concentrations of 10^6 and 10^7 CFU/ml for any of the effects tested. The second set of data compared all fermented and non-fermented samples at 10^6 and10^7 CFU/ml. Standard errors of the means were not included in the figures because they were generated based on the square root of the data. This was done to normalize the data.

4.2.1 Effect of culture

The type of culture was only significant ($p<0.05$) to IL-8 production when comparing the non-fermented cultures at 24 h (Table 4.5). LC stimulated significantly greater IL-8 (116.9%) ($p<0.05$) than NFDM (68%). None of the cultures, however, caused IL-8 production which was significantly different than levels of IL-8 in the Caco-2 supernatant. At 48 h, the type of culture was no longer significant ($p>0.05$) (Table 4.6).

Table 4.5 ANOVA table comparing non-fermented cultures at concentrations of 10^6, 10^7, and 10^8 CFU/ml on IL-8 production by Caco-2 cells after 24 hr incubation

Effect	Degrees of Freedom	F Value	Pr > F
Culture[1]	250	2.26	**0.0299**
Concentration[2]	713	1.15	0.3176
Inactivation[3]	620	12.6	**0.0004**
Culture*Concentration	707	1.91	**0.0266**
Culture*Inactivation	226	2.66	**0.0116**
Inactivation*Concentration	713	0.12	0.8878

[1] Probiotic cultures
[2] 10^6, 10^7, 10^8 CFU/ml
[3] Heat-killed/Irradiated

Table 4.6 ANOVA table comparing non-fermented cultures at concentrations of 10^6, 10^7, and 10^8 CFU/ml on IL-8 production by Caco-2 cells after 48 hr incubation

Effect	Degrees of Freedom	F Value	Pr > F
Culture[1]	621	1.62	0.1267
Concentration[2]	708	2.04	0.1302
Inactivation[3]	712	2.85	0.0920
Culture*Concentration	708	6.21	**<0.0001**
Culture*Inactivation	610	7.90	**<0.0001**
Inactivation*Concentration	708	2.35	0.0959

[1] Probiotic cultures
[2] 10^6, 10^7, 10^8 CFU/ml
[3] Heat-killed/Irradiated

Table 4.7 ANOVA table comparing fermented and non-fermented cultures at concentrations of 10^6 and 10^7 CFU/ml on IL-8 production by Caco-2 cells after a 24 hr incubation

Effect	Degrees of Freedom	F Value	Pr > F
Culture	901	1.04	0.3988
Fermentation	992	0.13	0.7211
Concentration	1009	0.45	0.5013
Inactivation	1016	39.11	**<0.0001**
Culture*Fermentation	884	0.64	0.7247
Culture*Concentration	1010	1.38	0.2088
Culture*Inactivation	934	3.36	**0.0015**
Fermentation*Concentration	1009	7.99	**0.0048**
Fermentation*Inactivation	910	0.47	0.4921
Inactivation*Concentration	1009	0.44	0.5081

[1] Probiotic cultures
[2] Fermented/Non-fermented
[3] 10^6, 10^7 CFU/ml
[4] Heat-killed/Irradiated

Table 4.8 ANOVA table comparing fermented and non-fermented cultures at concentrations of 10^6 and 10^7 CFU/ml on IL-8 production by Caco-2 cells after a 48 hr incubation

Effect	Degrees of Freedom	F Value	Pr > F
Culture	961	0.70	0.6748
Fermentation	1004	0.23	0.6314
Concentration	1003	8.85	**0.0030**
Inactivation	1010	18.33	**<0.0001**
Culture*Fermentation	948	3.41	**0.0013**
Culture*Concentration	1004	2.94	**0.0047**
Culture*Inactivation	977	2.77	**0.0074**
Fermentation*Concentration	1003	0.16	0.6847
Fermentation*Inactivation	971	1.00	0.3185
Inactivation*Concentration	1003	1.57	0.2104

[1] Probiotic cultures
[2] Fermented/Non-fermented
[3] 10^6, 10^7 CFU/ml
[4] Heat-killed/Irradiated

In a comparison of fermented and non-fermented samples, the effect of culture was not significant ($p>0.05$) to IL-8 production at either time point (Tables 4.7, 4.8).

With the exception of the difference between NFDM and LC at 24 h in the comparison of the unfermented cultures, the results indicate that culture does not have a significant effect on IL-8 production by Caco-2 cells.

4.2.2 Effect of dose

Dose was significant when the IL-8 production by fermented and non-fermented cultures was evaluated at 48 h (Table 4.8). Cultures at a concentration of 10^6 CFU/ml significantly suppressed ($p<0.05$) IL-8 (75.4%) compared to cultures at 10^7 CFU/ml (83.4%). This comparison indicates that as the concentration of the culture increased, the amount of IL-8 induced by Caco-2 cells also increased.

4.2.3 Effect of fermentation

There were no significant differences ($p>0.05$) between fermented and non-fermented cultures after 24 or 48 h of incubation (Tables 4.7 and 4.8).

4.2.4 Effect of inactivation

A comparison of the non-fermented cultures determined that the mode of inactivation was significant at 24 h ($p<0.05$) (Table 4.5), but not at 48 h (Table 4.6). At 24 h, heat-killed cultures stimulated IL-8 (106.9%), whereas irradiated cultures suppressed IL-8 production (84.8%).

When fermented and non-fermented cultures were evaluated together, the mode of inactivation was significant at 24 and 48 h ($p<0.05$). After 24 h of incubation, heat-killed samples stimulated IL-8 production 108.7%, whereas irradiated samples suppressed IL-8 production 78.2%. At 48 h, the amounts of IL-8 decreased where heat-killed samples resulted in 85.3% IL-8 and irradiated cultures suppressed IL-8 even further to 73.6%. The amount of IL-8 may have decreased at 48 h because the cytokine degraded or denatured over time. These results indicate that the mode of inactivation is an important factor of experimental design and should be carefully considered in future experiments.

4.2.5 Effect of the interaction between culture and dose

The interaction of culture and dose was significant when comparing the non-fermented cultures at 24 (Table 4.5) and 48 h (Table 4.6). The individual comparisons that were significant ($p<0.05$) at 24 h, however, were between different cultures. We were only interested in different doses of the same culture and therefore these comparisons were not valid for this study. At 48 h, LB at a concentration of 10^8 CFU/ml significantly suppressed IL-8 production compared to LB at concentrations of 10^6 and 10^7 CFU/ml (Figure 4.5). This trend is opposite from several other studies, where it was observed that the higher the dose, the greater the cytokine response (Marin and others, 1998).

When comparing the fermented and non-fermented cultures, the interaction between culture and dose was significant at 48 (Table 4.8) but not 24 h (Table 4.7). Caco-2 cells may have needed the longer incubation with the cultures in NFDM to

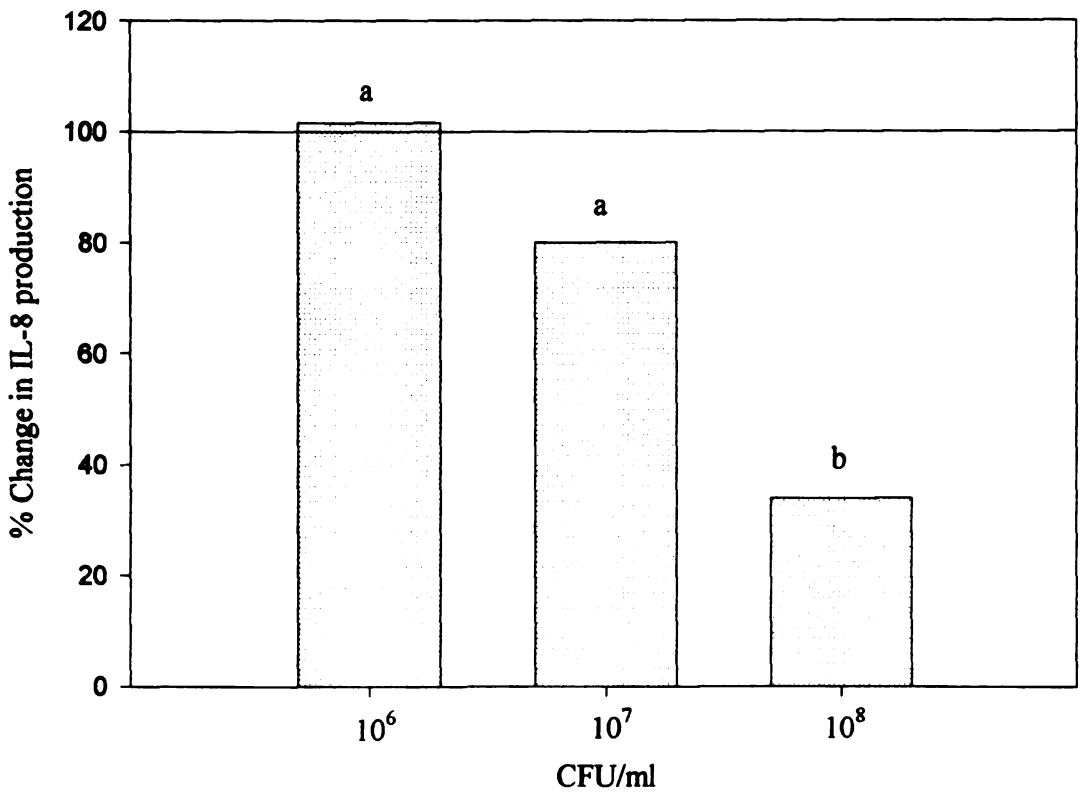

Figure 4.5 Effect of dose of non-fermented heat- and irradiation-inactivated LB on IL-8 production by Caco-2 cells (48 h incubation). Unstimulated IL-8 in Caco-2 supernatant was considered 100%.
[a-b] Concentrations with different letters are significantly different from each other ($p<0.05$).

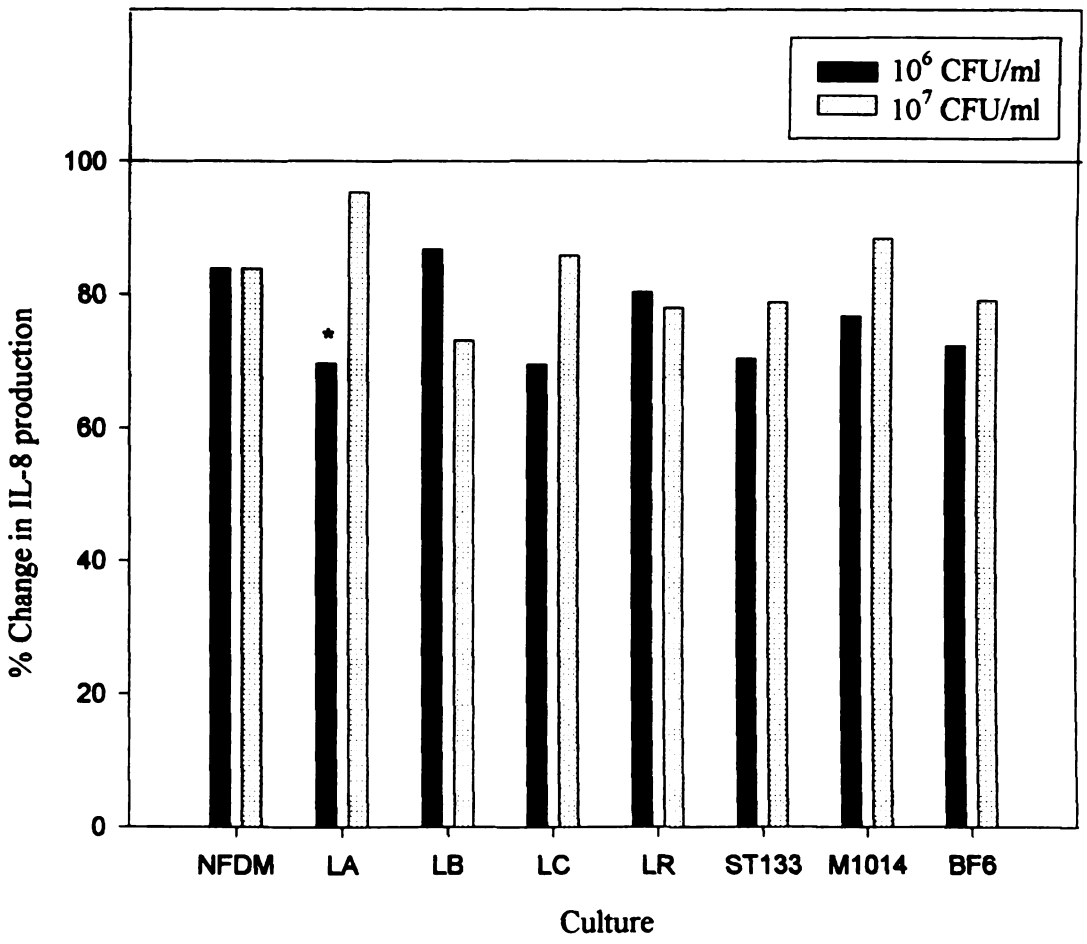

Figure 4.6 Effect of concentration (10^6 and 10^7 CFU/ml) of fermented and non-fermented lactic acid bacteria and bifidobacteria on IL-8 production by Caco-2 cells (48 h incubation). Unstimulated IL-8 in Caco-2 supernatant was considered 100 percent.

* Indicates significant difference between concentrations 10^6 and 10^7 CFU/ml for that culture ($p<0.05$). NFDM = non-fat dry milk; LA = *L. acidophilus* LA2; LB = *L. bulgaricus* NCK 231; LC = *L. casei* ATCC 35935; ST133 = *S. thermophilus* St133; M1014 = *B. adolescentis* M101-4; BF6 = *Bifidobacterium* Bf-6.

produce IL-8 which demonstrated this interaction. With the exception of LB and LR, cultures at 10^7 CFU/ml resulted in greater IL-8 production than cultures at 10^6 CFU/ml (Figure 4.6). Only LA, however, demonstrated a significant difference ($p<0.05$) between the two concentrations. All of the cultures, including NFDM, suppressed IL-8 production relative to the Caco-2 supernatant. There may be a substance in the NFDM that caused this effect on IL-8 production.

4.2.6 Effect of the interaction between culture and inactivation

The interaction of culture and mode of inactivation was significant ($p<0.05$) for comparisons of non-fermented cultures alone at both time points. At 24 h (Table 4.5), however, the specific effects of non-fermented cultures and mode of inactivation that were significant were between different cultures, not the same culture. Therefore, these results were disregarded. At 48 h (Table 4.5), heat-killed LB and LC cultures stimulated significantly higher amounts of IL-8 from Caco-2 cells than their irradiated counterparts (Figure 4.7). The irradiated cultures of BF6, LB, and LC significantly suppressed IL-8 production compared to the Caco-2 supernatant.

Interaction of culture and inactivation was also significant ($p<0.05$) for comparisons of fermented and non-fermented cultures at 24 (Table 4.7) and 48 h (Table 4.8). At 24 h, the heat-killed samples of NFDM and BF6 stimulated significantly greater ($p<0.05$) amounts of IL-8 than their irradiated counterparts (Figure 4.8). Although not significant ($p>0.05$), the trend of heat-killed culture resulting in greater levels of IL-8 than the irradiated culture was seen for all cultures tested. It could be that irradiation resulted in some kind of change in the NFDM that was inhibitory to IL-8 production.

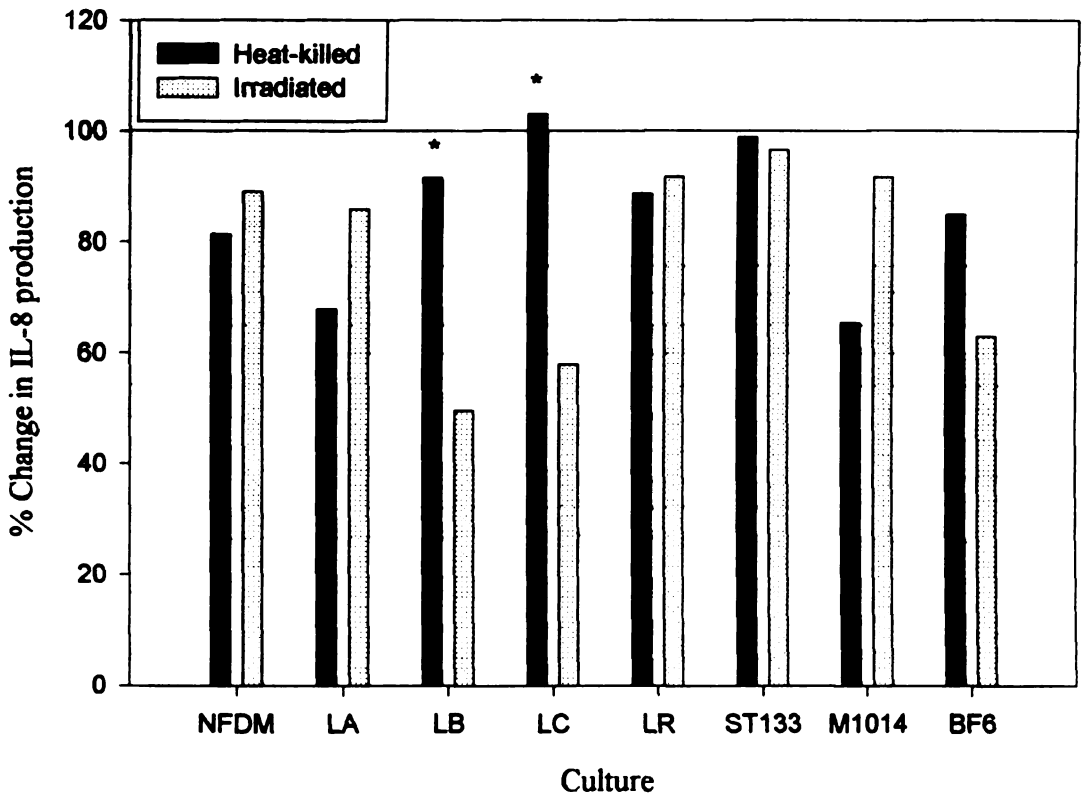

Figure 4.7 Effect heat and irradiation inactivated non-fermented lactic acid bacteria and bifidobacteria at concentrations of 10^6, 10^7, and 10^8 CFU/ml on IL-8 production by Caco-2 cells (48 h incubation). Unstimulated IL-8 in Caco-2 supernatant was considered 100%.
* Indicates that heat-killed were significantly different than irradiated counterpart ($p<0.05$). NFDM = non-fat dry milk; LA = *L. acidophilus* LA2; LB = *L. bulgaricus* NCK 231; LC = *L. casei* ATCC 39539; LR = *L. reuteri* ATCC 23272; ST133 = *S. thermophilus* St133; M1014 = *B. adolescentis* M101-4; BF6 = *Bifidobacterium* Bf-6

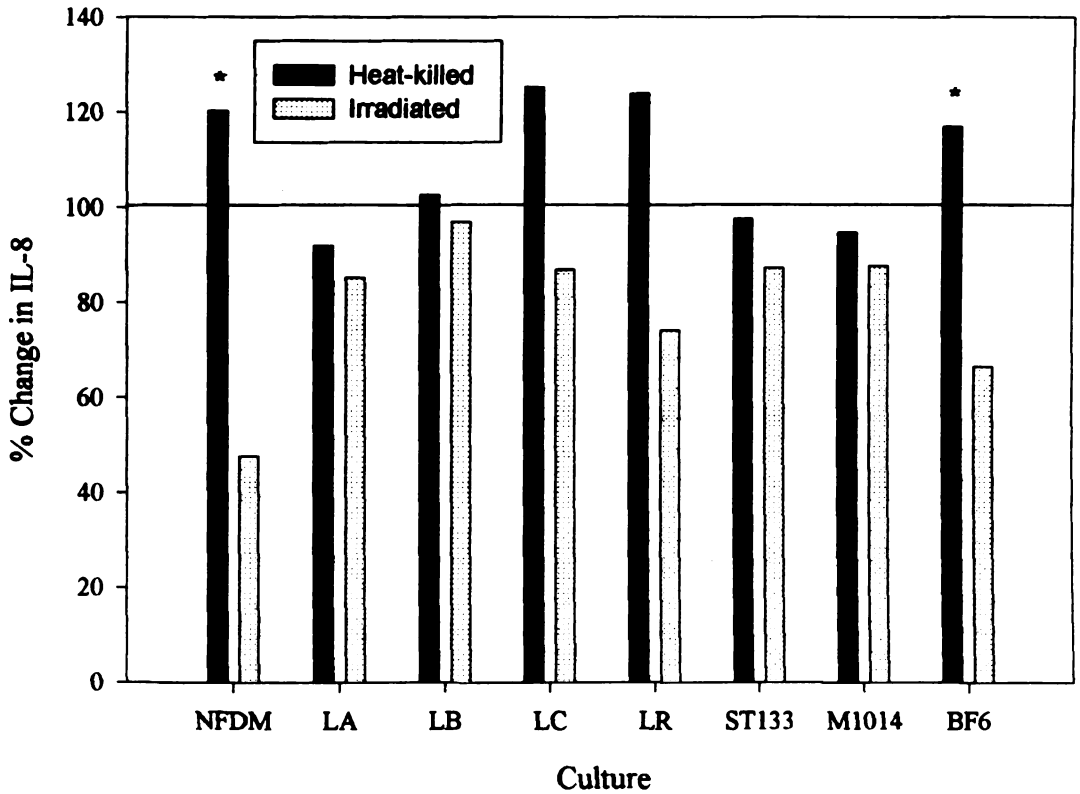

Figure 4.8 Effect of heat and irradiation inactivation on fermented and non-fermented lactic acid bacteria and bifidobacteria at concentrations of 10^6 and 10^7 CFU/ml on IL-8 production by Caco-2 cells (24 h incubation). Unstimulated IL-8 in Caco-2 supernatant was considered 100 percent.
* Indicates that heat-killed was significantly different than irradiated counterpart ($p<0.05$). NFDM = non-fat dry milk; LA = *L. acidophilus* LA2; LB = *L. bulgaricus* NCK 231; LC = *L. casei* ATCC 39539; LR = *L. reuteri* ATCC 23272; ST133 = *S. thermophilus* St133; *B. adolescentis* M101-4; BF6 = *Bifidobacterium* Bf-6.

The irradiated sample of NFDM alone suppressed IL-8 production the most and was significantly different than the level of IL-8 in the Caco-2 supernatant.

After the 48 h incubation, the interaction of culture and mode of inactivation for fermented and non-fermented cultures was still significant ($p<0.05$) (Table 4.8). More specifically, heat-killed LB cultures suppressed IL-8 (95.5%), which was significantly more IL-8 than the suppression by irradiated LB cultures (65.4%) (Figure 4.9). As with the 24 h cultures, most irradiated cultures at 48 h suppressed IL-8 production more than the heat-killed cultures.

4.2.7 Effect of the interaction between culture and fermentation

Although the interaction of culture and fermentation was found to be statistically significant at 48 h (Table 4.8), the specific interactions were between different cultures not among the same culture. These interactions were not valid for this study.

4.2.8 Effect of the interaction between fermentation and concentration

The interaction between fermentation and concentration was found to be significant at 24 h ($p<0.05$). Upon closer examination, however, there were no individual differences. This can occur in multiple comparison statistical analysis when the sample sizes are not balanced (Smith, 2002)

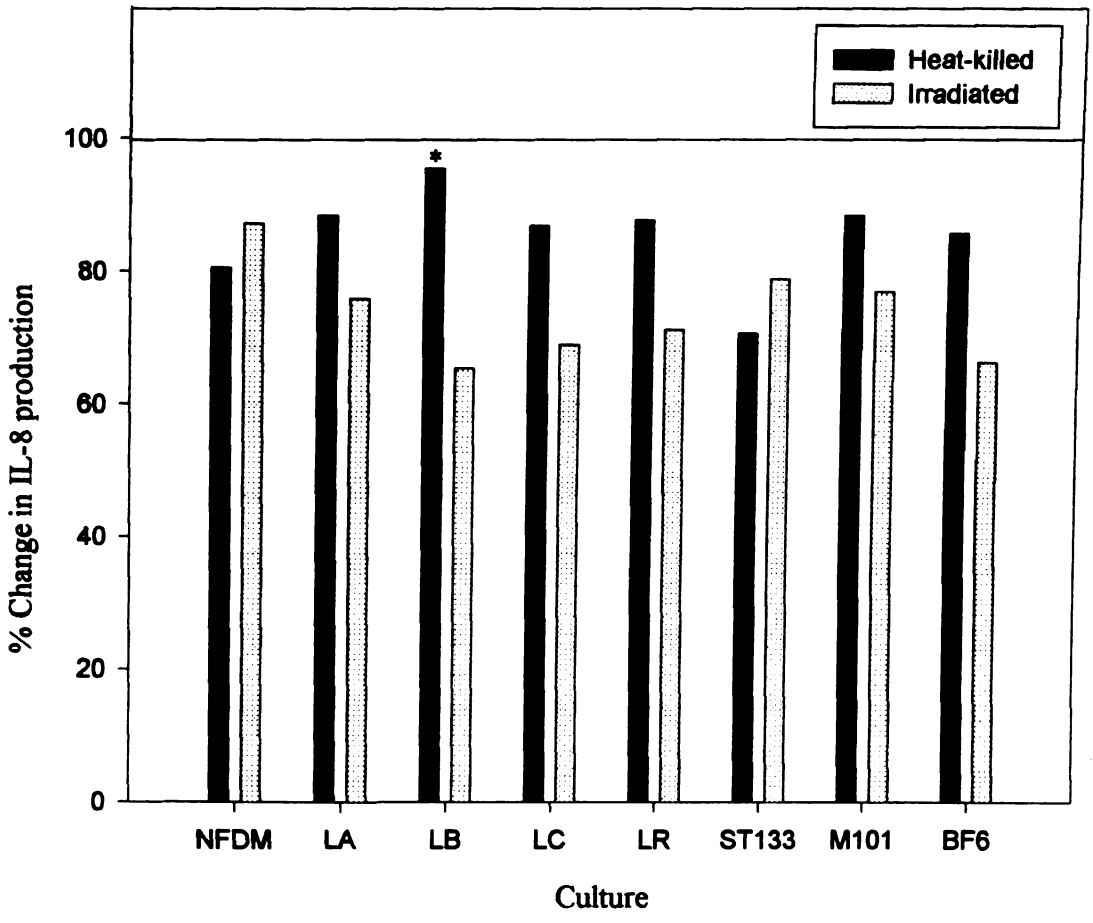

Figure 4.9 Comparison of heat and irradiation inactivation of fermented and non-fermented lactic acid bacteria and bifidobacteria at concentrations of 10^6 and 10^7 CFU/ml on IL-8 production by Caco-2 cells (48 h incubation). Unstimulated IL-8 in Caco-2 cell supernatant was considered 100%.
* Indicates heat-killed were significantly different from irradiated counterpart ($p<0.05$). NFDM = non-fat dry milk; LA = *L. acidophilus* LA2; LB = *L. bulgaricus* NCK; LC = *L. casei* ATCC 39539; LR = *L. reuteri* ATCC 23272; ST133 = S. thermophilus St133; M1014 = B. adolscentis M101-4; BF6 = *Bifidobacterium* Bf-6

4.2.9 Discussion on the effect of lactic acid bacteria and bifidobacteria on IL-8 production by Caco-2 cells

While some significant differences were noted between LC and NFDM, there were no consistent patterns to the effect of culture on IL-8 production. As was true for IL-6, none of the cultures significantly stimulated ($p>0.05$) IL-8 production at any dose which was contrary to experiments conducted by Marin and others (1998) who examined the effect of LAB and bifidobacteria on cytokine production by mouse RAW 264.7 macrophage cells and mouse EL4.IL-2 thymoma cells. All bacteria were heat-killed and incubated with cell lines at concentrations of 10^6, 10^7, and 10^8 bacteria/ml. Probiotic strain- and dose-dependent increases were observed with respect to IL-6 and TNF-α production by RAW 264.7 cells as well as in IL-2 and IL-5 production by EL4.IL-2 cells. Compared to other bacteria studied, *S. thermophilus* ST133 (also used in our study) had the greatest enhancing effects on cytokine production. In general, they observed that as the concentration of all bacteria increased, so did the amount of cytokine produced by the respective cell lines.

The effect of dose was only significant when comparing fermented and non-fermented cultures, but not for non-fermented cultures alone. Although the effect of fermentation alone was not significant, these results suggest that fermentation enhances the effect of dose. It was hypothesized that differences between fermented and non-fermented milk could stimulate different amounts of IL-8 from Caco-2 cells. During yogurt production, fermentation by LAB and bifidobacteria changes the composition of milk. Yogurt has increased folic acid, lactic acid and decreased lactose and vitamin B_6 compared to non-fermented milk (Meydani and Ha, 2000; Shahani and Chandan, 1979).

Calcium is also more bioavailable from yogurt. Bacterial enzymes can break down proteins and lipids in milk. It is possible that the digestion of certain milk proteins could result in the production of bioactive milk peptides which may have immunomodulatory activity (McDonald and others, 1994; Laffineur and others, 1996). The inactivation carried out in this research may have created compounds with suppressive effects from the bioactive milk peptides or substances associated with the fermentation process.

There are other reports of strain and concentration effects of probiotics and their components on cytokine production (Park and others, 1999; Tejada-Simon and Pestka, 1999; Miettinen and others, 1996). Our results were contrary to these previous studies, possibly because the cell models differed. It is also possible that Caco-2 cells require communication with underlying immune cells in order to respond to Gram-positive bacteria. Haller and others (2000), as mentioned in section 2.7, used Caco-2 cells in a co-culture system with human blood leukocytes where the two types of cells were separated by a membrane in transwell culture plates. Without the leukocytes, Caco-2 cells could not be stimulated by *L. sakei* to produce cytokines TNF-α and IL-1β. After the initial incubation the two wells were separated. Leukocyte-sensitized Caco-2 cells continued to produce a high level of TNF-α and to a lesser extent, IL-1β. They concluded that cross talk between Caco-2 cells and underlying immune cells is necessary for Caco-2 cells to recognize and respond to non-pathogenic bacteria.

The mode of bacterial inactivation did have a significant effect on IL-8 production. The main difference between these two modes of inactivation is that heat denatures proteins in the milk as well as on the surface of the probiotic bacteria. In contrast, irradiation leaves the protein structure intact, but causes molecular changes in

the DNA. These changes eventually lead to alterations in metabolism which can result in cell death if the irradiation damage is sufficiently extensive (Olson, 1998).

Heat-treated cultures generally resulted in greater amounts of IL-8 compared to the irradiated cultures. These amounts of IL-8, however, were lower than the Caco-2 supernatant and were unexpected based on previous experiments which used heat-inactivated cultures (Marin and others, 1998; Park and others, 1999). The comparison between the two modes of inactivation, heat vs irradiation, in our experiments sought to determine if heat-denatured proteins on the cell surface of the cultures were responsible for cytokine stimulation as seen in previous studies.

Although the stimulation or suppression of IL-8 production by heat-killed and irradiated cultures, respectively, in our experiment were not significantly different from the naïve Caco-2 supernatant, these results suggest that heat inactivation leads to the generation of stimulatory factors on the cultures. These stimulatory factors may be recognized by membrane bound TLR on the Caco-2 cells, that can recognize microbial components (Matzinger, 2002). Perhaps the differences between heat and irradiation inactivation would be more pronounced using the co-culture system of Haller and others (2000).

The current definition for probiotics stipulates that they should be ingested live to have immunostimulating effects in the body. While live bacteria were not investigated here, this research suggests that inactivated probiotics, specifically by irradiation, can suppress IL-8 production by gut epithelial cells. Because IL-8 plays a role in many immune functions, particularly in the inflammatory response, its suppression may or may not be desirable.

4.3 Future research for lactic acid bacteria and bifidobacteria in NFDM on cytokine production by Caco-2 cells

Future research could utilize the co-culture system previously described to determine if the probiotic cultures used in these experiments are capable of stimulating cytokine production in Caco-2 cells. Experiments with the co-culture system should investigate a dose effect of probiotic bacteria in NFDM on cytokine stimulation by human Caco-2 cells. Based on the previous *in vitro* studies on probiotic dose, the probiotic organisms used in these experiments would likely cause cytokine production by Caco-2 cells with the aid of underlying immune cells.

4.4 Effect of milk components on cytokine production by Caco-2 cells

Although not statistically significant, a trend was observed from the previous experiments where NFDM suppressed IL-6 and IL-8 production by Caco-2 cells. Thus, the intent of the next experiment was to investigate the effect of individual milk components on IL-6 and IL-8. The milk components lactose, α-la, β-lg as well as NFDM were incubated with Caco-2 cells with or without IL-1β for 24 h. IL-6 and IL-8 production were calculated using the amount of cytokine in the Caco-2 DMEM supernatant alone as 100%.

The milk component effect on IL-6 production by Caco-2 cells was statistically significantly ($p<0.0001$). Figure 4.10 illustrates IL-6 production by the specific milk components with and without IL-1β. The error bars represent standard error based on raw data. All milk components and NFDM stimulated significantly greater ($p<0.05$) IL-6

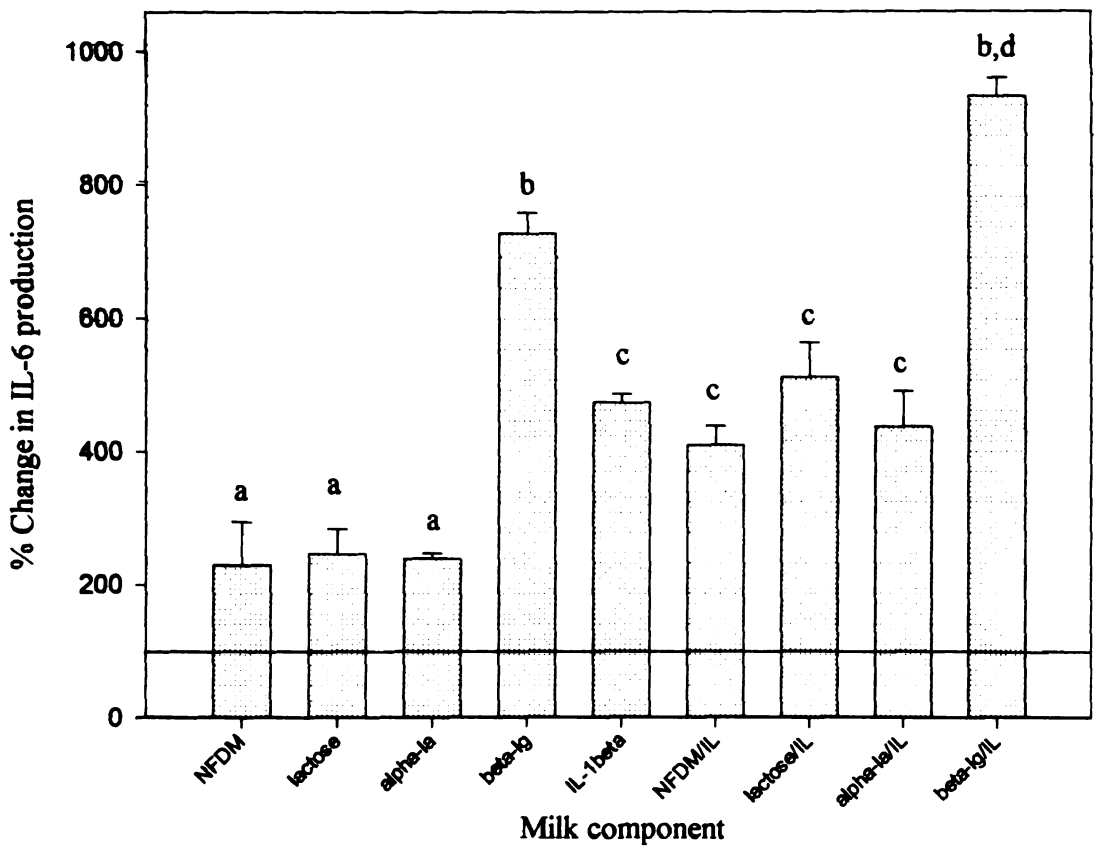

Figure 4.10 Effect of various milk components on IL-6 production by Caco-2 cells with or without stimulation by IL-1beta.
NFDM = non-fat dry milk; IL = IL-1beta; alpha-LA = alpha-lactalbumin; beta-LG = beta-lactoglobulin. IL-6 produced by Caco-2 supernatant alone was considered 100 percent IL-6 production.
[a-d] Indicates that means with different letters are significantly different from each other ($p<0.05$).

than was present in the naïve Caco-2 cell supernatant. IL-6 production stimulated by lactose and α-la were not significantly different from NFDM or each other. Beta-lg alone, however, stimulated significantly greater IL-6 production than NFDM and caused Caco-2 cells to produce 726% more IL-6 than the Caco-2 supernatant. This was significantly more ($p<0.05$) IL-6 than the samples with IL-1β alone, NFDM with IL-1β, lactose with IL-1β and α-la with IL-1β. Although β-lg with IL-1β stimulated the most IL-6 (931.4%) of all milk component samples, it was not significantly greater than the β-lg alone.

The effect of milk components on IL-8 production by Caco-2 cells was also significant ($p<0.0001$), but did not follow the same trends as for IL-6 production. Figure 4.11 shows the effect of the specific milk components on IL-8 production by Caco-2 cells. The error bars represent standard error based on raw data. All milk components, with the exception of NFDM and lactose, stimulated significantly greater ($p<0.05$) IL-8 than the naïve Caco-2 cell supernatant. Alpha-la and β-lg stimulated significantly ($p<0.05$) more IL-8 than NFDM and lactose. Although not significant, α-la caused Caco-2 cells to produce more (596.2%) IL-8 than IL-1β (468.1%) ($p>0.05$). The combination of milk components and NFDM with IL-1β had an additive effect on IL-8 production ($p<0.05$).

Several milk components have been reported to have immunostimulating effects (Gill and others, 2000). In our study, IL-6 stimulation by all milk components was at least 200% greater than the Caco-2 supernatant. Beta-lg is considered a potent milk allergen (Tsuji and others, 2001). Jenmalm and others (1999) found that β-lg could

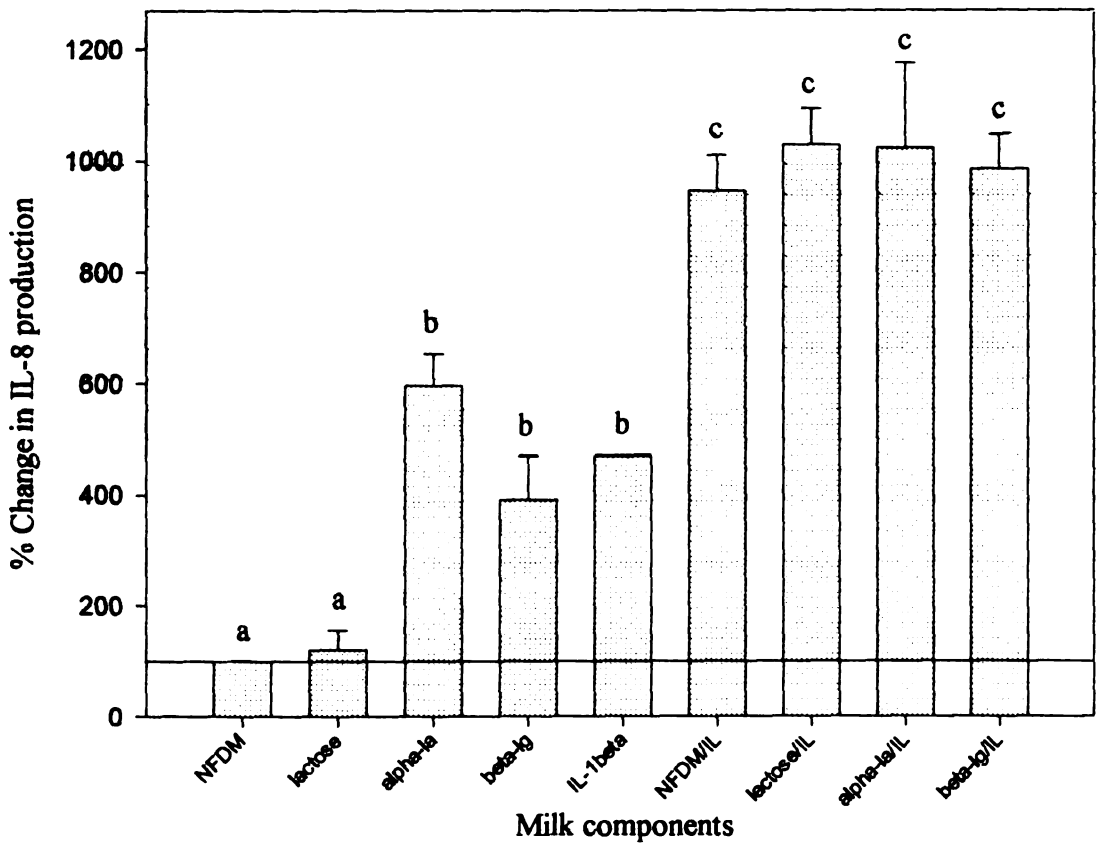

Figure 4.11 Effect of various milk components on IL-8 production by Caco-2 cells with or without stimulation by IL-1beta.
NFDM = non-fat dry milk; alpha-LA = alpha-lactalbumin; beta-LG = beta-lactoglobulin; IL = IL-1beta. IL-8 in Caco-2 supernatant was considered 100 percent IL-8 production.
[a-c] Indicates that means with different letters are significantly different from each other ($p<0.05$).

induce increased IL-6 by PBMCs from eight-year old children with and without atopic symptoms. After 96 h of incubation with β-lg, PBMCs from atopic children produced as much as 100 ng/ml IL-6, which was significantly greater ($p<0.05$) IL-6 than those from children without atopic symptoms at 60 ng/ml. While it is a different cell system, their results may be an indicator of the stimulatory effect of β-lg in the body.

Comparing the milk components and NFDM without IL-1β, α-la was a more potent stimulator of IL-8 than even IL-1β alone. Wong and others (1997) found that α-LA (400 μg/ml) increased IL-1β production by ovine blood lymphocytes 56.9 percent. Although the cell models are different, it is possible that α-la could increase IL-1β production by Caco-2 cells and as a result, increase IL-8 production.

These experiments investigating cytokine production by milk components gave conflicting results compared to the previous two experiments. Previously, NFDM suppressed cytokine production. In these experiments, NFDM and the milk components stimulated IL-6 production and α-la and β-lg stimulated IL-8 production. This could be due to the fact that the milk components and NFDM solutions were more concentrated (4% solution in culture) than in experiments with the probiotic organisms (2% NFDM solution in culture). The purity of the milk components also could have played a role in the results. According to Wong and others (1997), the more pure bovine whey fractions were, the more clear-cut the immune response observed. Further experiments with dose of NFDM and milk components should be conducted to determine the effects on cytokine production by Caco-2 cells.

4.4.1 Future research

Because α-la and β-lb in 4% solutions stimulated IL-6 and IL-8 production, experiments with dose of these and other milk components should be performed to understand their role in cytokine production by Caco-2 cells. Irradiation of the milk component solutions would also help to understand the differences seen between heat- and irradiation-inactivated samples in the previous experiments with probiotic cultures. Perhaps a co-culture system would give further insight as to the function of milk components in the gut.

CHAPTER 5

SUMMARY

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SUMMARY

In this research, we conducted experiments to determine the immunomodulatory effects of dose and inactivation of seven different probiotic cultures on the human Caco-2 cell culture system. Further experiments with milk components and the Caco-2 cell line were also conducted to determine if any of the components had an inhibitory effect on immune responses.

The results of the investigation with probiotics suggest that Caco-2 cells were largely unresponsive to incubation with the cultures. The majority of the cultures did not significantly suppress or stimulate cytokine production (IL-6 and IL-8) ($p>0.05$). The dose of culture also did not have a significant effect. Although it was not significant for every culture, the mode of culture inactivation had an effect on cytokine production. In general, IL-6 and IL-8 production by Caco-2 cells were suppressed by irradiated probiotic cultures more than their heat-killed counterparts.

We also discovered that the soluble whey proteins, α-la and β-lb, stimulated more cytokine production than the other milk components examined. Production of IL-8 was synergistically enhanced when the cytokine IL-1β was added to the Caco-2 cells in combination with the soluble whey proteins. These results may be due to the dose at which they were incubated with Caco-2 cells relative to the first set of experiments.

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APPENDIX I

University of Michigan

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

CERTIFICATE OF COMPLIANCE

Version 4

This is to certify that the following specimens were irradiated in the facility's cobalt-60 irradiator. Dose rates were measured with Reuter-Stokes ion chamber model RS-C4-1606-207, serial number Z-8943, which is calibrated annually by the manufacturer or Phoenix against a National Institute of Standards & Technology source.

Organization:	MSU - Food Science & Human Nutrition
Irradiation Date:	8/20/01
Specimen Type:	Lactic Acid Bacteria in 15cc tubes
Specimen Identification:	082001MSU-FS02
Distance from Irradiator (cm):	Center Well
Gamma Dose Rate (rad/hr):	1228850
Irradiation Time (hr):	0.817 (4 Irradiations)
Interrupt Time (min):	0
Gamma Dose (Mrad):	1.00

Aug 20, 2001
Date

Robert B. Blackburn
Asst. Manager of Laboratory Operations

APPENDIX II

MICHIGAN STATE
UNIVERSITY

March 9, 2000

TO: Zeynep USTUNOL
136 G.M. Trout Food Science Bldg

RE: **IRB# 00-120** CATEGORY:1-E
APPROVAL DATE: March 7, 2000
TITLE: EFFECT OF LACTIC ACID BACTERIA AZND BIFIDO-BACTERIA ON THE CYTOKINE PRODUCTION BY CACO-2 CELLS

The University Committee on Research Involving Human Subjects' (UCRIHS) review of this project is complete and I am pleased to advise that the rights and welfare of the human subjects appear to be adequately protected and methods to obtain informed consent are appropriate. Therefore, the **UCRIHS approved this project.**

RENEWALS: UCRIHS approval is valid for one calendar year, beginning with the approval date shown above. Projects continuing beyond one year must be renewed with the green renewal form. A maximum of four such expedited renewals possible. Investigators wishing to continue a project beyond that time need to submit it again for a complete review.
REVISIONS: UCRIHS must review any changes in procedures involving human subjects, prior to initiation of the change. If this is done at the time of renewal, please use the green renewal form. To revise an approved protocol at any other time during the year, send your written request to the UCRIHS Chair, requesting revised approval and referencing the project's IRB# and title. Include in your request a description of the change and any revised instruments, consent forms or advertisements that are applicable.
PROBLEMS/CHANGES: Should either of the following arise during the course of the work, notify UCRIHS promptly: 1) problems (unexpected side effects, complaints, etc.) involving human subjects or 2) changes in the research environment or new information indicating greater risk to the human subjects than existed when the protocol was previously reviewed and approved.

If we can be of further assistance, please contact us at 517 355-2180 or via email: UCRIHS@pilot.msu.edu. Please note that all UCRIHS forms are located on the web: http://www.msu.edu/user/ucrihs/

Sincerely,

David E. Wright, Ph.D.
UCRIHS Chair

DEW: bd

cc: Constance Wong
2125 S. Anthony

MICHIGAN STATE
UNIVERSITY

February 28, 2001

TO: Zeynep USTUNOL
2105 S. Anthony Hall

RE: **IRB # 00-120** CATEGORY: EXEMPT 1-E
RENEWAL APPROVAL DATE: February 27, 2001

TITLE: EFFECT OF LACTIC ACID BACTERIA AND BIFIDO-BACTERIA ON THE CYTOKINE PRODUCTION BY CACO-2 CELLS

The University Committee on Research Involving Human Subjects' (UCRIHS) review of this project is complete and I am pleased to advise that the rights and welfare of the human subjects appear to be adequately protected and methods to obtain informed consent are appropriate. Therefore, the **UCRIHS APPROVED THIS PROJECT'S RENEWAL.**

RENEWALS: UCRIHS approval is valid for one calendar year, beginning with the approval date shown above. Projects continuing beyond one year must be renewed with the green renewal form. A maximum of four such expedited renewal are possible. Investigators wishing to continue a project beyond that time need to submit it again for complete review.
REVISIONS: UCRIHS must review any changes in procedures involving human subjects, prior to initiation of the change. If this is done at the time of renewal, please use the green renewal form. To revise an approved protocol at any other time during the year, send your written request to the UCRIHS Chair, requesting revised approval and referencing the project's IRB# and title. Include in your request a description of the change and any revised instruments, consent forms or advertisements that are applicable.
PROBLEMS/CHANGES: Should either of the following arise during the course of the work, notify UCRIHS promptly: 1) problems (unexpected side effects, complaints, etc.) involving human subjects or 2) changes in the research environment or new information indicating greater risk to the human subjects than existed when the protocol was previously reviewed and approved.

If we can be of further assistance, please contact us at 517 355-2180 or via email: UCRIHS@pilot.msu.edu.

Sincerely,

Ashir Kumar, M.D.
Interim Chair, UCRIHS

AK: rj

cc: Constance Wong
2125 S. Anthony

MICHIGAN STATE
UNIVERSITY

January 31, 2002

TO: Zeynep USTUNOL
2105 S. Anthony Hall
MSU

RE: IRB # 00-120 CATEGORY: 1-E EXEMPT
RENEWAL APPROVAL DATE: January 30, 2002

TITLE: EFFECT OF LACTIC ACID BACTERIA AND BIFIDO-BACTERIA ON THE CYTOKINE PRODUCTION BY CACO-2 CELLS

The University Committee on Research Involving Human Subjects' (UCRIHS) review of this project is complete and I am pleased to advise that the rights and welfare of the human subjects appear to be adequately protected and methods to obtain informed consent are appropriate. Therefore, the UCRIHS APPROVED THIS PROJECT'S RENEWAL.

RENEWALS: UCRIHS approval is valid for one calendar year, beginning with the approval date shown above. Projects continuing beyond one year must be renewed with the green renewal form. A maximum of four such expedited renewal are possible. Investigators wishing to continue a project beyond that time need to submit it again for complete review.
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If we can be of further assistance, please contact us at 517 355-2180 or via email: UCRIHS@pilot.msu.edu.

Sincerely,

Ashir Kumar, M.D.
UCRIHS Chair

AK: kb

cc: Constance Wong
2125 S. Anthony
MSU