EFFECTS OF LACTIC ACID BACTERIA ON THE IMMUNE SYSTEM

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ABSTRACT

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Lactic acid bacteria are essential for the fermentation of products such as cheese, buttermilk and yogurt. An increasing number of functional foods and pharmaceutical preparations are being promoted with health claims based on the potential probiotic characteristics of some of these bacteria and on their capacity for stimulating the host immune system. A possible mechanism for these effects is direct stimulation of the gastrointestinal immune response. The specific objectives of these studies were to evaluate the effects of *in vivo*, *ex vivo*, and *in vitro* exposure to viable, non-viable strains, and cell extracts of lactic acid bacteria (*Lactobacillus acidophilus*, *L. bulgaricus, L. casei*, *L. gasseri*, *L. helveticus*, *L. reuteri*, *Streptococcus thermophilus* and *Bifidobacterium*) on leukocyte function. *In vivo* studies showed that growth rate of mice as well as immunoglobulin levels were not affected by direct oral administration of lactic acid bacteria. Although basal cytokine mRNA expression in spleen and Peyer's patches was not affected by repeated oral lactic acid bacteria administration (*in vivo*), single exposures to certain bacteria altered subsequent mitogen induced cytokine and nitric oxide

production by peritoneal cells (*ex vivo*). When mice were fed a fermented milk manufactured with starter cultures containing different species/strains of lactic acid bacteria for three weeks and after immunizing twice with 10 μg cholera toxin, those mice responded by producing specific intestinal and serum IgA-anti cholera toxin, isotype that was significantly increased in mice fed yogurts made with starters containing *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (yogurt bacteria) supplemented with *L. acidophilus* and *Bifidobacterium* spp. These results suggested that lactic acid bacteria may alter immune function in a strain dependent manner. The effects of *in vitro* exposure to heat-killed cells, their cell walls, and their cytoplasmic extracts on proliferation, cytokine and intermediate production were examined in the RAW 264.7 macrophage cell line, spleen and Peyer's patch cells as well as in peritoneal cells from mice. Lactic acid bacteria as well as their cytoplasmic and cell wall fractions were able to stimulate cloned macrophages to produce very significant amounts of TNF-α, IL-6 and nitric oxide. While similar effects were not noted in spleen and Peyer's patch cell cultures from mice, a pronounced enhancement in IL-6 production by peritoneal cells was observed when cultured with those extracts. The results suggested that as a group, the lactic acid bacteria are capable of stimulating macrophages and/or other immune cells to produce cytokines and nitric oxide.

To Klaus, your love and support kept me motivated

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ABBREVIATIONS

ABTS	2, 2'-azino-bis (3-ethylbenz-thiazoline-6 sulfonic acid
AC	Adenylate cyclase
AIN	American Institute of Nutrition
ANOVA	Analysis of variance
APS	Antigen presenting cells
ATCC	American Type Culture Collection
ATP	Adenosine tri-phosphate
BMC	Blood mononuclear cells
β2-MG	β2-microglobulin
BSA	Bovine serum albumin
$[Ca^{2+}]$	Intracellular calcium concentration
CaM	Calmodulin
cAMP	Cyclic adenosine mono-phosphate
CF	Cytoplasmic fraction
CFU	Colony forming unit
CPM	Counts per minute
CT	Cholera toxin
CW	Cell wall
DAG	Diacylglycerol
DMEM	Dubelcco's modified Eagle's medium
DMSO	Dimethylsulfoxide

DPA	Diphenilamine
ELISA	Enzyme-linked immunosorbent assay
EMP	Embden-Meyerhoff-Parnas pathway
FBS	Fetal bovine serum
GALT	gut-associated lymphoid tssue
GI	Gastrointestine
HBSS	Hank's buffer saline solution
I	Ionomycin
IEL	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ip	Intraperitoneal
IP_3	inositol tri-phosphate
iv	Intravenous
LAB	Lactic Acid Bacteria
LP	Lamina propria
LPS	Lipopolysaccharide
LTA	Lipoteichoic acids
MRS	De Man-Rogosa-Sharpe medium for lactobacilli
MRSL	MRS + 5% lactose (w/w)
MTT	(3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide)

NED	N-(1-naphthyl) ethylenediamine dihydrochloride
NFDM	non fat dry milk
NK	Natural killer cells
NO	Nitric oxide
NOS	Nitric oxide synthase
NPNL	Neomycin sulfate, paromomycin sulfate, nalidixic acid, lithium chloride medium for bifidobacteria
PBS	Phosphate buffered saline
PBS-T	PBS + 0.2% Tween 20
PCR	Polymerase chain reaction
PIP_2	Phosphoinositol bi-phosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate-13 acetate
PP	Peyer's patches
RT	Reverse transcriptase
SC	Secretory component
S.E.M	Standard error of the mean
spp.	Species
ssp.	Subspecies
TA	Teichoic acids

TCA	Trichloroacetic acid
TdT	Terminal deoxyribonucleotide transferase
Th	T helper cells
TNF	Tumor necrosis factor
TTE	0.01M Tris-Cl with 1mM EDTA
WC	Whole cell

INTRODUCTION

For thousands of years lactic acid bacteria have been used to produce fermentation of a variety of foods and improve the shelf-life, flavor and texture of these products. The properties attributed to lactic acid bacteria such as anticholesterol activity, improvements in intestinal motility, metabolism of drugs, vaginitis, immunological status, and the decreased gastrointestinal disorders and tumors seem to be due to the metabolic activity of these bacteria and their interaction with other microflora and/or different cells present in the gut (Fuller, 1991). There is an extensive body of literature addressing the possible health benefits associated with the consumption of lactic acid bacteria. The putative mechanisms involved in the production of these favorable effects include changes in viable counts of microorganisms in the intestinal flora after ingestion, competition for adhesion sites and nutrients between the ingested bacteria and potential pathogens, production of antibacterial substances and the action of these bacteria through stimulation of the immune system. However, convincing scientific experiments which validate these mechanisms are limited (Gasson, 1993).

The effect of oral administration of lactic acid bacteria on immunity has not been well defined yet but it is believed that this group of bacteria target the intestine, resist the digestion process and establish themselves in the gut. Several cell types present in the intestinal wall such as macrophages, could be stimulated (Perdigon et al., 1986a, 1987, 1988, 1990, 1992; Saito et al., 1987). Cytokines might be produced as a response (Halpern et al., 1991; Kishi et al., 1996; Kitazawa et al., 1992, 1994; De Simone et al., 1986; Miettinen et al., 1996) thereby driving a cascade of events which could eventually lead to activation of other immune cells, further production of cytokines and mediators as well as

immunoglobulin secretion.

Cells of a large number of microbial species as well as bacterial products have been shown to possess mitogenic and polyclonal activation properties, and are fully capable of inducing DNA synthesis, blast formation and ultimately division of lymphocytes. One of the most widely studied bacterial products with this mitogenic activity is lipopolysaccharide (LPS), which is present in the cell wall of Gram negative bacteria (Gammage et al., 1996). Some bacterial species that represent major human pathogens are Gram positive and possess a basic Gram positive cell wall structure. Gram positive purified cell walls containing teichoic acid and peptidoglycan also seem to be mitogenic and to induce production of certain cytokines by human monocytes (Heumann et al., 1994).

Several fundamental questions need to be answered regarding the use and activity of lactic acid bacteria to better understand their putative probiotic effects in order to ensure the rational development of new healthy foods. These include precise knowledge of the specific strains producing beneficial effects, the active constituent of lactic acid bacteria responsible for each potential effect, the target site in the body, the ecological conditions necessary for the activity of the active constituent, the pharmacokinetics of ingested lactic acid bacteria, the percentage of survival at the target site, the concentrations of bacteria, the duration of passage and its effects, and the type of cells being altered by these bacteria (Fuller, 1991). All these studies are relevant to the food industry and could be translated into novel commercially viable foods.

The underlying hypothesis in this dissertation is that lactic acid bacteria have immunomodulatory effects in cells of the mucosal and systemic compartment and that the immunopotentiating activity resides in the cell walls of lactobacilli, streptococci, and

bifidobacteria.

The objectives of this research were as follows:

1. To assess the effects of *in vivo* oral exposure to viable and non-viable strains of lactic acid bacteria using a murine model.
2. To assess the effects of *ex vivo* exposure of primary leukocytes to lactic acid bacteria.
3. To determine the *in vitro* effects of lactic acid bacteria and their extracts obtained by fractionation on proliferation, cytokine and intermediate production by exposing a macrophage cell line.
4. To determine the *in vitro* effects of lactic acid bacteria and their extracts on cytokine and intermediate production by exposing primary leukocytes from mice.
5. To compare the effects of ingestion of yogurts manufactured with different starter cultures on the gastrointestinal immune system of the mouse.

The aforementioned issues were addressed in this research. The dissertation presented here is composed of five chapters. Chapter 1 reviews recent and classical literature on lactic acid bacteria, characteristics and structure, use in industry and probiotic research. Chapter 2 describes the effects of lactic acid bacteria ingestion on mucosal and systemic cytokine gene expression and secretion in a murine model. Chapter 3 investigates the *in vitro* effects of lactic acid bacteria and their extracts on cytokine production in a macrophage cell line, and in primary leukocytes from mice. Chapter 4 determines the effects of feeding a fermented milk manufactured with different strains of lactic acid bacteria on the immunoglobulin response to bacterial toxin. Finally, Chapter 5 summarizes these inter-related studies.

CHAPTER 1

LITERATURE REVIEW

1.1 Lactic acid bacteria

1.1.1 Historical background.- For thousands of years, fermentation with lactic acid bacteria has been used to produce a variety of foods with improved shelf-life, different flavors and textures. These fermented products may contain a variety of strains belonging to different genera and species but each having the major characteristic of producing lactic acid. The special metabolism of these microorganisms may contribute to possible nutritional and health benefits. Selection and development of new strains of lactic acid bacteria is currently research of the highest priority.

Humans have used fermentation as one of the more effective and oldest techniques for the preservation of foods. Fermented products were an important aspect of survival in mountainous or desertic areas particularly during seasons where the ability to obtain fresh food was impossible. Old European civilizations believed that these foods were produced by their gods and the technology delivered to humans by them. Such a belief motivated man to name these products with words such as "life", "long life" and "health" (Benoît, 1981). Lactic acid bacteria have an essential role in the majority of these food fermentation processes. The traditional elaboration of these products consisted of leaving raw whole milk at room temperature, therefore allowing development of the natural bacteria to initiate fermentation. It is believed that when husbandry practice was initiated, men utilized animal products such as milk and when this milk was not consumed fast enough, it would become acidified thereby originating the first fermented milks. Often, a small amount of the old product was added as inoculum.

Western Europe and Mediterranean countries have classically been the biggest consumers of fermented milk products. This fact is mentioned in the Bible where Moses classified them among the foods that Jehovah procured (Deuteronomy, XXXII, 14). Also Abraham 's life is linked to these type of foods (Genesis, XVIII, 8). Marco Polo, a Venetian traveler who explored Asia from 1271 to 1295, wrote in his book "Travels of Marco Polo": "...they are prepared with mare's milk and a pleasure to drink, being similar to white wine...". Therapeutical characteristics were recognized and reported by Arab writers such as Abumahomet Abdullah who said that "leben" (a fermented milk with its origin in Egypt) fortified the stomach, cured diarrhea, stabilize the body temperature, purified the blood and improved the color of the skin, mucosa surfaces and lips (Mateos Garcia, 1984).

Elie Metchnikoff (1907) wrote in his book "The Prolongation of Life" that the long life span attributed to eastern European populations was due in part to the consumption of great amounts of fermented milks. He believed that overgrowth of some organisms in the gastrointestinal tract produced damaging substances resulting in aging due to the stimulation of autoimmune reactions. It was then when he established the controversial theory about antagonism between lactic acid bacteria and intestinal pathogens, a theory that today is still accepted with some modifications. He evidenced *Lactobacillus bulgaricus* as the main agent causing milk fermentation. It was due to these first investigations that lactic acid bacteria became very popular. As a result of his writings, acidophilus milk and koumiss were introduced in the United States and Soviet Union, respectively, as beneficial products for the treatment of tuberculosis and some other diseases of the time. Kopeloff (1926) and Rettger et al. (1935) conducted probably the first studies describing the use of these microorganisms

in humans to cure intestinal illnesses. The study of the intestinal microflora flourished after World War II and along with it the theory of possible beneficial effects on health by these microorganisms and their metabolic functions, notably in infants and the elderly (Speck, 1980).

Today, there is an extensive body of literature addressing the possible health benefits associated with the consumption of lactic acid bacteria. One of the theories to explain these effects include stimulation of the immune system, but convincing scientific experiments which prove this are limited. Modern molecular and immunological techniques do, however, provide a powerful strategy with which to address some of these issues (Gasson, 1993).

1.1.2 Description and classification.- The lactic acid bacteria produce lactic acid as the major product from the energy-yielding fermentation of sugars. They are gram positive rods or cocci, anaerobic, micro-aerophilic or aero-tolerant, catalase negative, without cytochromes. They do not form spores (except for *Sporolactobacillus*) and are non-motile (except for *Vagococcus*) (Wood and Holzapfel, 1995). Much has happened regarding classification of lactic acid bacteria. Recently, several species have been stripped of their dairy and enteric species (i.e. *Streptococcus* of *Lactococcus* and *Enterococcus*), newly identified (i.e. *Sporolactobacillus*) or characterized (i.e. *Vagococcus*).

Traditionally, the formation of lactic acid as a endproduct of carbohydrate metabolism defined the classification of lactic acid bacteria. But some other microorganisms (such as *Actinomyces*) under appropriate conditions are also able to convert carbohydrates to lactic acid. The development of new molecular biological techniques made possible to

more accurately study taxonomy and phylogeny of lactic acid bacteria (Lortal et al., 1997). The current classification is mainly based on comparative sequence analysis of 16S rRNA, DNA:DNA homology, DNA:RNA and G+C content. Gram positive bacteria are divided in two groups, one with a low G+C content ranging between 33-50 G+C mol % (*Clostridium* branch) and another with a high G+C content ranging between 55-67 G+C mol % . Typical lactic acid bacteria have a G+C < 50 mol%. The genera used as starters by the food industry consist of *Lactobacillus, Streptococcus, Lactococcus*, *Leuconostoc* and *Pediococcus*. *Lactobacillus delbrueckii*, *L. acidophilus*, *L. gasseri*, *L. helveticus* and some other species are mainly homofermenters (only lactic acid is produced). *Lactobacillus casei* and *Pediococcus* could be included in another group where facultative heterofermenters (lactate, acetate, ethanol and CO_2 are produced), obligate heterofermenters or homofermenters are found. *Leuconostoc* is an obligate heterofermenter. The genus *Streptococcus* can be divided into oral, pyogenic and viridans groups and also it can be differentiated by the composition of the cell wall, classifying it into the so-called Lancefield groups (groups A, B, C, E, F,). *S. thermophilus* belong to the oral streptococci (*S. salivarius* spp. *thermophilus*). It is closely related to the genus *Lactococcus* (group N Lancefield group), and *Enterococcus* (group D Lancefield group).

Other genera of lactic acid bacteria include *Enterococcus*, *Carnobacterium*, *Vagococcus* and *Tetragenococcus*. *Enterococcus* appears to be also closer to *Carnobacterium* (unique because of its ability to grow at pH between 8.5-9.0) and *Vagococcus* (the only motile group N streptococci) when 16S rRNAs are compared. *Pediococcus halophilus* was also reclassified as a new genus called *Tetragenococcus*, and

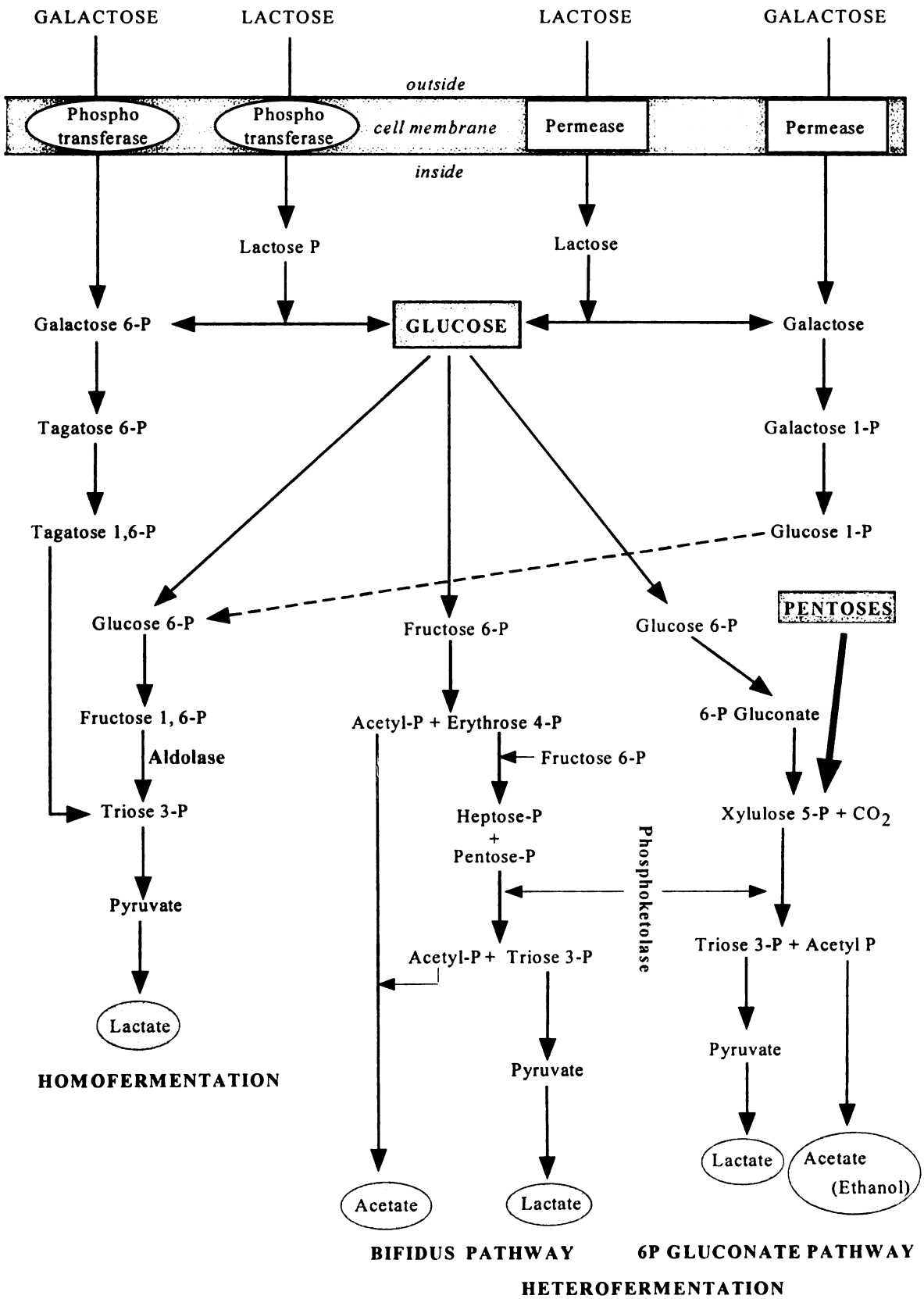
it is capable of growing at very high salt concentrations.

Originally, *Bifidobacterium* was considered to be a typical lactic acid bacteria, but its G+C content (>55 mol%) and 16S rRNA showed to be a distinct group (Woods and Holzapfel, 1995b). Nevertheless, members of the genus *Bifidobacterium* are still considered to as genuine lactic acid bacteria with saccharoclastic fermentation producing lactate and acetate, but the pathway used for hexose fermentation is different or special as compared to typical lactic acid bacteria (Woods and Holzapfel, 1995).

1.1.3 Metabolism.- Lactic acid bacteria use carbohydrates as a primary energy source. Hexoses are degraded to lactate mainly and possibly to additional products such acetate, ethanol, formate, succinate or CO_2 (Gilliland, 1985). Lactic acid bacteria can be classified as homolactic or homofermenters and heterolactics or heterofermenters, depending on the pathway followed for carbohydrate fermentation and the end-products obtained. Homofermenters follow the Embden-Meyerhoff-Parnas (EMP) pathway for glycolysis producing lactate. Heterofermenters produce lactate as well as acetate, ethanol and CO_2. Figure 1.1 shows a general schematic representation of lactose and galactose uptake and conversion to lactate and other products. Pentoses also can be used, incorporating them to this pathway as xylulose 5P.

The organic acids fermented most frequently by lactic acid bacteria are malate and citrate. Citrate is metabolized by some lactic acid bacteria to acetate, acetoin, diacetyl, 2,3-butylene glycol and CO_2, when there is a fermentable carbohydrate in the media (Gilliland, 1985). Malic acid is actively fermented by most strains of *Leuconostoc* producing lactate and

Figure 1.1. Carbohydrate fermentation pathways in lactic acid bacteria.


GALACTOSE
LACTOSE
LACTOSE
GALACTOSE
outside
Phospho transferase
Phospho transferase
cell membrane
Permease
Permease
inside
Lactose P
Lactose
Galactose 6-P
GLUCOSE
Galactose
Tagatose 6-P
Galactose 1-P
Tagatose 1,6-P
Glucose 1-P
Glucose 6-P
Fructose 6-P
Glucose 6-P
PENTOSES
Fructose 1, 6-P
Aldolase
Acetyl-P + Erythrose 4-P
Fructose 6-P
6-P Gluconate
Triose 3-P
Heptose-P
+
Pentose-P
Xylulose 5-P + CO_2
Phosphoketolase
Pyruvate
Acetyl-P + Triose 3-P
Triose 3-P + Acetyl P
Pyruvate
Pyruvate
Lactate
Lactate
Acetate
(Ethanol)
Acetate
Lactate
HOMOFERMENTATION
BIFIDUS PATHWAY
6P GLUCONATE PATHWAY
HETEROFERMENTATION

CO_2, besides acetate, acetoin, diacetyl and 2,3-butyleneglycol (Figure 1.2). Citrate is present in many foods such as fruit, vegetables and milk. Its degradation results in the formation of aroma compounds that have a different effect depending on the type of fermented food. For instance, the presence of diacetyl is desirable for butter, buttermilk, cottage cheese, but it is not wanted for products such as beer, wine or sausages (Hugenholtz, 1993).

Lactic acid bacteria cannot synthesize several amino acids, and this is one of the reasons for these microorganisms being fastidious regarding their nutritional requirements. Lysis of proteins from the substrate where they are growing is necessary for these bacteria to proliferate. Lactic acid bacteria possess a limited proteolytic capability, having some proteolytic enzymes which are located in the cell wall, cell membrane and cytoplasm (Figure 1.3). Lactic acid bacteria are able to degrade proteins and use them as a nitrogen source. This potential is exploited by using this bacteria in dairy technology as starters or as ripening agents to contribute to the development of flavor and texture of the product (Pritchard and Coolbear, 1993).

The properties attributed to lactic acid bacteria such as anticholesterol activity, improvements in intestinal motility, metabolism of drugs, vaginitis, immunological status, and the decreased gastrointestinal disorders and tumors might be due, in part, to the metabolic activities of these bacteria. Specifically they produce a great amount of acid very rapidly and also some other metabolic products such as H_2O_2, CO_2, diacetyl and the so-called bacteriocins (Davidson and Hoover, 1993). Acetic and lactic acids can inhibit other bacteria and act in foods as a preservative extending the shelf-life of these products. Lactic acid bacteria produce large amounts of H_2O_2 through pyruvate metabolism. They do not produce

Figure 1.2. Citrate and malate fermentation pathway in lactic acid bacteria.

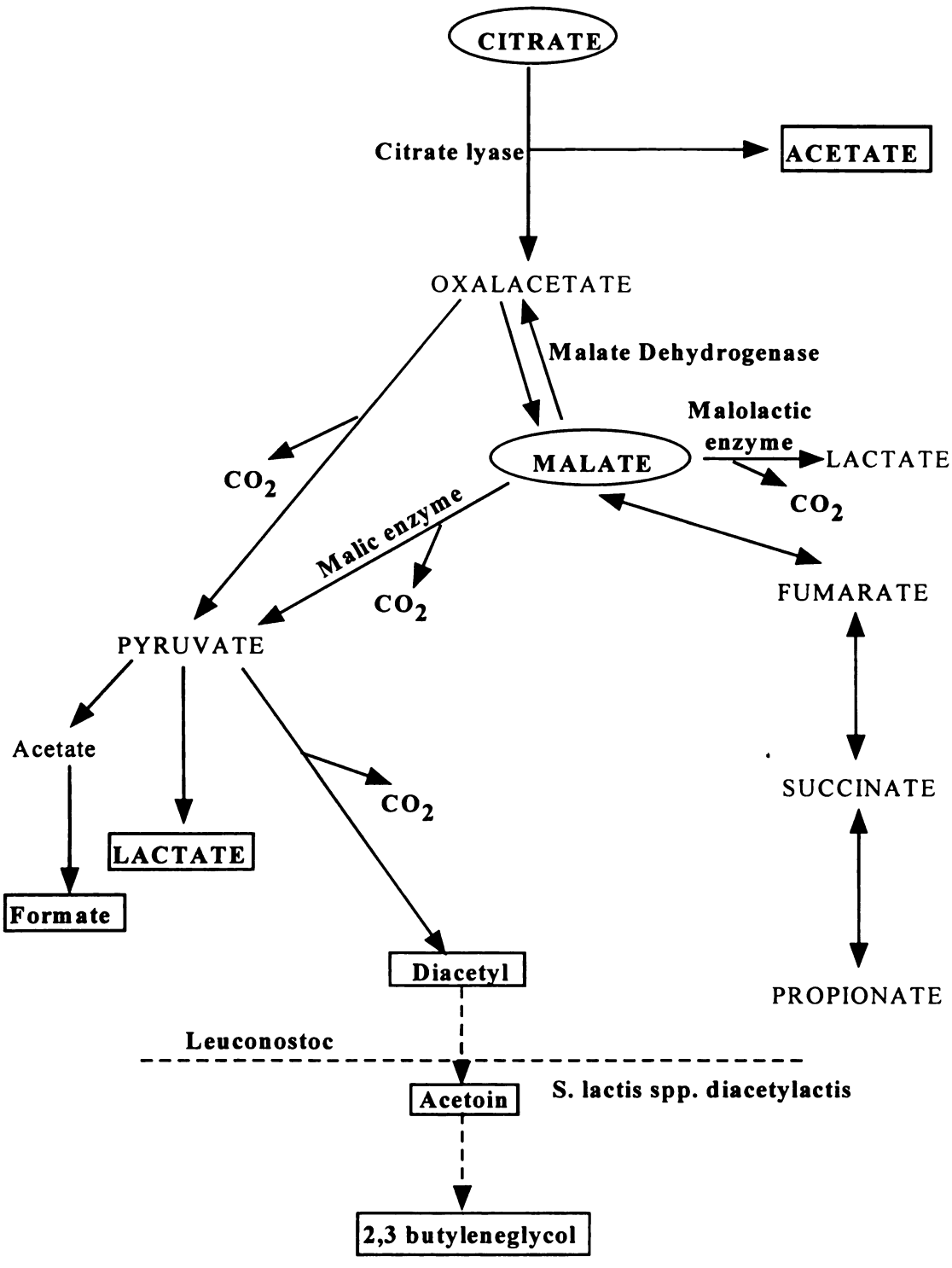

CITRATE
Citrate lyase
ACETATE
OXALACETATE
Malate Dehydrogenase
Malolactic enzyme
MALATE
LACTATE
CO_2
CO_2
Malic enzyme
CO_2
FUMARATE
PYRUVATE
Acetate
CO_2
SUCCINATE
LACTATE
Formate
Diacetyl
PROPIONATE
Leuconostoc
S. lactis spp. diacetylactis
Acetoin
2,3 butyleneglycol

Figure 1.3. Proteolysis in lactic acid bacteria.

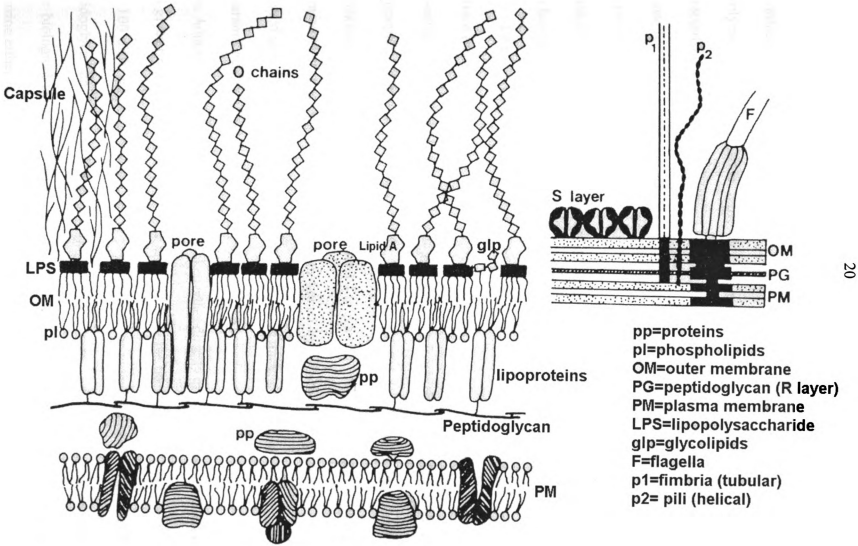
Casein
Large peptides
Small peptides
Amino acids
PROTEASE
PEPTIDASE
Cell Wall
Cell Membrane
Cytoplasm
Oligopeptide transport system
Amino acid transport system
Small peptides
Endopeptidases
Amino acids

catalase but they are resistant to H_2O_2, while other microorganisms are very sensitive and die in its presence. Diacetyl is also synthesized from pyruvate by fermentation of citrate. It is known to be able to inhibit yeast and gram negative bacteria but at very high concentrations (Salminen and von Wright, 1993). There are some other antimicrobial substances produced by lactic acid bacteria which have a peptide/protein nature known as bacteriocins. Klaenhammer (1988) defined bacteriocins as "proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producer microorganism". Bacteriocins are heterogeneous compounds which vary in molecular weight and many other biochemical characteristics. An all comprehensive coverage on bacteriocins can be found in the reviews cited here (Davidson and Hoover, 1993; Lindgren and Dobrogosz, 1990; Klaenhammer, 1993; Nes et al., 1996).

1.1.4 Structure of bacteria cell wall.- The cell wall of bacteria is a rigid structure that confers the characteristic shape. This structure prevents the cell from expanding, bursting because of water uptake, and in general it protects the cell from adverse physical conditions (Pelczar et al., 1993). Usually this structure accounts for 10 - 40 % of the dry weight of the entire cell. Cell walls from different bacteria vary in thickness as well as composition. Among bacteria, the Gram negative species possess a thinner wall than those of Gram positive bacteria.

For Gram negative bacteria (Figure 1.4) the main distinctive characteristic is the presence of lipopolysaccharide (LPS). The basic molecule of LPS consists of a lipid component, the lipid A, covalently bound to a heteropolysaccharide of two distinct regions:

Figure 1.4. Gram negative bacterial cell envelope.

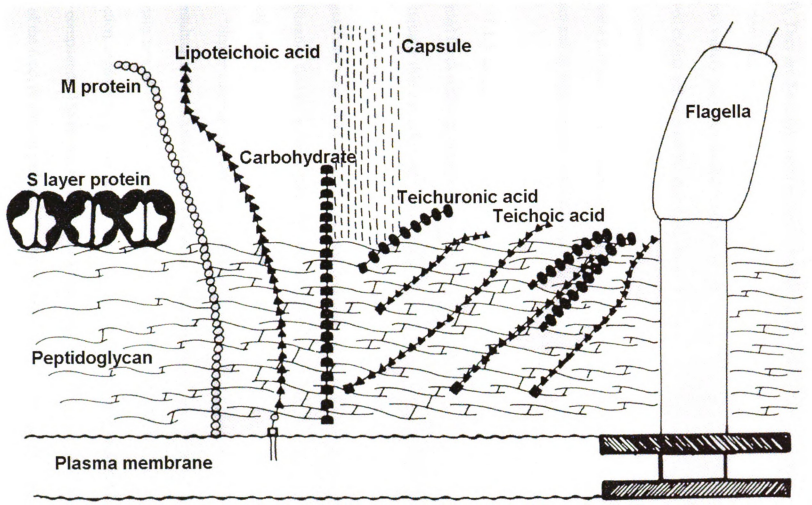


pp=proteins
pl=phospholipids
OM=outer membrane
PG=peptidoglycan (R layer)
PM=plasma membrane
LPS=lipopolysaccharide
glp=glycolipids
F=flagella
p1=fimbria (tubular)
p2= pili (helical)

the core oligosaccharide and the O-specific chain (Stewart-Tull et al. 1985). It is also liable to contain polysaccharide chains of variable lengths. The O-specific chain is composed of a polymer of oligosaccharide molecules in repeating units, the nature of which is characteristic and unique for a given LPS. The O-specific chain is linked to a subterminal glucose residue in the core which in turn is covalently linked through a 2-keto-3-deoxy-D-manno-octonate to an unusual lipid region, lipid A. Lipid A consists of a phosphorylated β(1-6)-linked D-glucosamine disaccharide backbone to which long-chain fatty acids are attached. LPS has been recognized as a macrophage stimulator.

Gram positive purified cell walls (Figure 1.5) contain teichoic acid and peptidoglycan. Peptidoglycan (40-90% of the cell wall) is an essential cell wall polymer consisting of a polysaccharide backbone (glycan strand) which is cross-linked through oligopeptides (Stewart-Tull et al. 1985). It consist of β(1-4)-glycosidically linked N-acetylglucosamine residues (which are believed to play an immunodominant role). Each alternate N-acetylglucosamine residue is substituted by a D-lactic acid ether in its C-3 hydroxyl group. This derivative of glucosamine is called muramic acid. The carboxyl group of muramic acid is substituted by an oligopeptide which contains alternating L- and D- amino acids. Adjacent peptide subunits are cross-linked either directly or via an interpeptide bridge. This gives rise to a huge macromolecule encompassing the bacterial cell. There are more than 100 different primary structures of peptidoglycan or murein types. It is believed that peptidoglycan has an immunoadjuvant effect and it is capable of B-cell activation among other biological activities. The teichoic acids are found in most gram positive bacteria, occurring either in the cell wall or associated with the cell membrane (Stewart-Tull et al.

Figure 1.5. Gram positive bacterial cell envelope.


Lipoteichoic acid
Capsule
M protein
Flagella
Carbohydrate
S layer protein
Teichuronic acid
Teichoic acid
Peptidoglycan
Plasma membrane

1985). They are basically stable polymers which usually consist of a glycerophosphate or ribitol phosphate backbone upon which glycosyl or D-alanyl groups may be substituted. The glycerol variety may contain fatty acids. It is not known if fatty acids or other esters are removed during cell wall purification procedures. Chain lengths of those polymers may vary upon extraction procedures. Lipoteichoic acid (LTA) appears to be associated with the membrane surface. Teichoic acids (TA) dissociate readily from bacterial cells in the culture medium and by washing cells with saline solutions.

1.1.5 Industrial applications.- Lactic acid bacteria have been used to produce fermented foods with improved preservation, flavors and textures as compared to the original food. Initially this was achieved without understanding the process and the scientific basis for the fermentation. Now, a broad variety of foods contain lactic acid bacteria such a sausages, ham, wine, cider, beer, pickles, milk products, olives and bakery products (Table 1.1). Notably, fermentation of milk with lactic acid bacteria yields more than a thousand products with specific organoleptic characteristics of taste, aroma and texture.

"Starter cultures" are the specific strains of lactic acid bacteria used to inoculate a food resulting in fermentation of that product by the metabolic activity of bacteria. There are two main sets of criteria for selection of starters. One set is based on the rate of acid production, capacity for polysaccharide production, ability of proteolysis and production of flavor compounds. The second set of criteria is based on their ability to increase nutritional value of the food, producing perhaps a beneficial health effect (Gilliland, 1985). In addition new methods being considered for strain selection include adhesion, potential for immune

TABLE 1.1. Lactic acid bacteria used in the fermentation of foods (McKay et al., 1990)

Foods	Microorganisms
Vegetable fermentations	*Leuconostoc mesenteroides*
	Pediococcus pentosaceous
	Lactobacillus plantarum
Meat and fish fermentation	*Lactobacillus plantarum*
	Pediococcus acidilactici
Alcoholic beverages	*Leuconostoc oenos*
	Lactobacillus delbrueckii
Coffee and cocoa	Various LAB
Soy sauce	*Lactobacillus delbrueckii*
	Pediococcus soyae
Silage	*Lactobacillus plantarum*
Bakery products	
Sourdough	*Lactobacillus sanfrancisco*
	Lactobacillus brevis
	Lactobacillus plantarum
	Lactobacillus fermentum
Soda crackers	*Lactobacillus plantarum*
	Lactobacillus delbrueckii
	Lactobacillus leichmanii
	Lactobacillus casei
	Lactobacillus brevis
Fermented dairy products	*Lactococcus lactis* spp. *lactis*
	Lactococcus lactis spp. *cremoris*
	Lactococcus lactis spp. *lactis* var. *diacetylactis*
	Leuconostoc mesenteroides spp. *cremoris*
	Leuconostoc lactis
	Streptococcus salivarius spp. *thermophilus*
	Lactobacillus delbrueckii spp. *bulgaricus*
	Lactobacillus helveticus
	Lactobacillus acidophilus
	Lactobacillus casei

effects, and gastrointestinal colonization (Salminen et al., 1996a, 1996b, 1996c).

The dairy industry is interested in mesophilic starters capable of forming acid and flavor compounds (cheese, fermented milks, cream butter, [Table 1.2, 1.3]) as well as in thermophilic starters capable of growing at high cooking temperatures (yogurt, a large variety of cheeses such as Grana, Gruyere, Emmental) (Sharpe, 1979; Auclair and Accolas, 1983). Their main function for manufacturing these products is fermentation of sugars, protein hydrolysis, synthesis of texturizing agents and flavor compounds and production of inhibitory components which avoid cross-contaminations. Basically, when temperature, water activity, pH conditions are optimal and allow the growth of these bacteria in these products, lactic acid bacteria develop faster than competiting microflora, the pH decreases by acid production leading to a microbiologically stable fermented product (Steele and Ünlü, 1992).

The same principle is applicable in meat and vegetable products, except that in some cases the meat industry has to add fermentable sugars, salt, and spices to adjust the product to a more favorable conditions for lactic acid bacteria to grow (Salminen and von Wright, 1993; Egan, 1983). Vegetable products can be fermented using pure cultures of lactic acid bacteria (cucumbers, cabbage, olives). Nevertheless, these products undergo natural fermentation if the product is handled adequately and by establishing a designated salt concentration, which is typical of that particular product.

Lactic acid bacteria starters continue to be used in the baking industry in manufacture of breads such as natural sour rye bread, San Francisco sourdough French bread, Pumpernickel, Italian Panettone and Pandoro cakes, and soda crackers (Gilliland, 1985).

TABLE 1.2. Types of fermented milks (Tejada-Simon and Seisdedos, 1986).

Name	Country	Microorganisms	Type of milk
Acidophilus milk	U.S.A.	*Lactobacillus acidophilus*	cow
Buttermilk	U.K. and U.S.A.	*Streptococcus lactis* *Streptococcus cremoris* *Leuconostoc cremoris* *Leuconostoc dextranicum* *Streptococcus diacetylactis* *Leuconostoc citrovorum*	cow
Sour cream	U.S.A.	same as buttermilk	cow
Mazum	Armenia	Various	goat, sheep, buffalo
Tarho	Hungary	Various	cow, sheep
Kos	Albania	Various	sheep
Grusavin	Balkan Peninsula (Montenegro)	Various	cow, sheep
Skorup	Balkan peninsula	Various	cow, sheep
Kaimak	former Yugoslavia	Various	cow, sheep
Biogurt		*Lactobacillus acidophilus* *Streptococcus lactis*	cow
Bulgarian milk	Bulgaria	*Lactobacillus bulgaricus*	cow
Yogurt	Various countries	*Lactobacillus bulgaricus* *Streptococcus thermophilus*	cow

TABLE 1.3. Types of alcoholic fermented milks (Tejada-Simon and Seisdedos, 1986)

Name	Country	Microorganisms	Type of milk
Kefir	Russia	*Saccharomyces kefir* *Torula kefir* *Streptococcus cremoris* *Streptococcus lactis* *Lactobacillus caucasicus* *Acetobacter* (polution) *Klebsiella* (polution)	cow,sheep,goat 0.6% alcohol
Koumiss	Russia	*Streptococcus thermophilus* *Lactobacillus bulgaricus* *Lactobacillus acidophilus* *Saccharomyces lactis* *Torula* *Mycoderma*	mare, camel, goat 0.5% alcohol
Leben	Egypt and Africa	Various	cow, sheep, buffalo
Gioddu	Sardinia (Italy)	Various	goat, sheep, cow
Taette	Scandinavia	*Streptococcus lactis* *Lactobacillus spp.* *yeast*	cow some alcohol
Araka	Turkey	Various	sheep
Fuli	Finland	Various	cow
Puma	Finland	Various	cow skim milk
Tatte mjoelk	Norway	Various	cow
Lang mjoelk	Sweden	Various	cow

Lactic acid bacteria can be both useful or detrimental in brewing and wine making. In the first case, products contain large quantities of organic acids which lactic acid bacteria can metabolize thus affecting the final product favorably or unfavorably. The predominant organic acids in grape wine, cider and other fruits are malic acid and tartaric acid. Malic acid is transformed to lactic acid and CO_2 (malo-lactic fermentation) producing not only the reduction on the acid content but also a characteristic flavor. In contrast, the transformation of tartaric acid by these bacteria leads to a spoiled wine (Radler, 1975).

Lactic acid bacteria are also of interest as biopreservation or biocontrol cultures. The purpose is to extend the storage life and safety of certain foods by using the natural flora and the products of their metabolism. In the United States lactic acid bacteria are generally considered as harmless and they are afforded GRAS (Generally Recognized as Safe) status. They are capable of preserving the food by lowering pH and by producing lactic acid as well as bacteriocins (Stiles, 1996). This application of lactic acid bacteria has been studied in dairy and meat products, and recently is being examined for use in minimally processed fruits and vegetables, creating the so-called minimally processed refrigerated foods (MPR) (Breidt and Fleming, 1997).

1.1.6 Lactic acid bacteria as probiotics and animal supplements.- The term probiotic derives from the greek meaning "for life". The first definition of probiotics was as "organisms and substances which contribute to intestinal microbial balance", where antibiotics and organic acids could be included. Fuller (1991) redefined the term as " a live microbial feed supplement which beneficially affects the host animal by improving its

microbial balance". There are numerous reports of potential health and nutritional benefits afforded by lactic acid bacteria (Gilliland, 1990; Marteau and Rambaud, 1993; Lee and Salminen, 1995; Hughes and Hoover, 1991; Tanaka, 1995; Teuber, 1991; Sanders, 1993; Wood and Holzapfel, 1995). These include studies of lactose digestion, cholesterol metabolism, diarrheal disorders (Gonzalez et al., 1995; Salminen et al., 1996a, 1996b), prophylaxis of intestinal or urogenital infections (McGroarty, 1993), immunomodulation (Perdigon et al., 1986a, 1986b, 1987, 1988, 1990, 1991a, 1991b, 1992, 1995) and oral vaccination (Wells et al., 1996).

To better understand previous results and to rationally develop new healthy foods or even drugs using lactic acid bacteria, the pharmacology of these microorganisms must be determined. This includes precise knowledge of the active constituents of lactic acid bacteria responsible for each potential effect, the target site in the body, the ecological conditions necessary for the activity of the active constituent, the requirement for viability, the pharmacokinetics of ingested lactic acid bacteria as percentage of survival at the target site, the concentrations of bacteria, the duration of the passage (Fuller, 1991; McCann et al., 1995). Also research must consider possible negative side effects of lactic acid bacteria.

Factors required to maintain high viability include the proper ratio of strains and inoculum levels, suitable growth factors, and control of pH and redox potential (Shin, 1997). Once bacteria are ingested, they can be destroyed by the acid in the stomach and bile in the intestine. Their survival depends on their intrinsic resistance but also on the host and the product in which they are ingested. Yogurt bacteria (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) have a very poor intrinsic resistance to acid and bile.

Lactobacillus acidophilus and *Bifidobacterium* are more resistant but there are numerous strain differences. Most information concerning the pharmacokinetics of lactic acid bacteria relates to the human gut. After ingestion, *L. acidophilus*, *L. casei* and *Bifidobacterium* can colonize the small bowel and colon and reach high concentrations (10^6 - 10^8/ml or more) (Marteu and Rambaud, 1993). Lactic acid bacteria administered exogenously can persist in the colon for at least 3 weeks or more.

Some of the lactic acid bacteria used as probiotics may offer an alternative to the use of antibiotics to improve growth and performance of livestock. Much of this interest has been due to the concern over the use of banned antibiotics for this purpose (Gilliland et al., 1990). Beneficial effects have been observed for some strains of lactic acid bacteria administered orally in pigs (Tortuero et al., 1995; Abe et al., 1996), calves (Nousiainen and Setälä, 1993) and poultry (Jin et al., 1996a, 1996b).

1.1.7 Immunomodulation.- Relative to immunity, the effects of lactic acid bacteria are intrinsically related to the diet and nutritional status of the individual. There is a strong relationship between nutrition and immunity created by the presence of mechanisms responsible for decreasing the resistance to infection. Specific nutrient deficiencies can interfere with nonspecific defense mechanisms that include flora, anatomical barriers (skin, mucosa, and epithelium); secretory substances such as lysozymes, mucus, and gastric acid; the febrile response; endocrine changes; and binding of serum and tissue iron (Solis-Pereyra et al., 1997). Protein deficiency produces impaired antibody formation, decreased serum immunoglobulin, decreased secretory immunoglobulin A, decreased thymic function and

splenic lymphocytes, delayed cutaneous hypersensitivity, decreased complement formation, decreased interferon, and effects on nonspecific mechanisms that include anatomic barriers and secretory substances such as lysozymes and mucus (Scrimshaw and San Giovanni, 1997). Deficiency in vitamin A increases susceptibility to infection, decreases thymus and spleen sizes, reduces natural killer cell activity, lowers production of interferon, impairs delayed cutaneous hypersensitivity and lowers lymphocyte response to stimulation by mitogens. β-carotene can stimulate mitogenesis in lymphocytes and increase human natural killer cell and T helper cell numbers. Vitamin B-12 and folic acid interfere with cellular replication, antibody formation. Deficiency of these vitamins can produce anemia and cell-mediated immunity depression. Vitamin C deficiency also decreases immune function, besides decreasing iron absorption, but there are claims of a favorable effect on infection with massive doses of vitamin C, studies which have not been scientifically confirmed. The killing power of lymphocytes is reduced if the dose of vitamin E is not appropriate. Clearly impaired phagocytic killing power has been reported with iron and zinc deficiency, as well as cytokine function or production, immunoglobulin and B cell function (Schrimshaw and San Giovanni, 1997). All these effects are more severe in the elderly (Lesourd, 1997; Paavonen, 1994; Schmucker, 1996). Thus, when studying immune function, it is very important to design experiments in which the nutritional status of the population under study is controlled and equilibrated.

Several studies have tried to decipher the events which occur after the first contact of ingested lactic acid bacteria with the immune system in the gut-associated lymphoid tissue (GALT). Perdigon et al. (1990) showed an increase in levels of immunoglobulins against

Salmonella in the intestinal fluid of mice after oral administration of *L. casei*, *L. acidophilus*, *L. bulgaricus* or *S. thermophilus* and challenge with *Salmonella typhimurium*. De Simone et al. (1987a, 1988b) reported that yogurt or heated yogurt, when administered to mice, increased the percentage of B lymphocytes in the Peyer's patches. The antibacterial activity of the Peyer's patch cells is also increased by viable yogurt (De Simone et al., 1987a). These effects might be due to the activation of the macrophages and non-specific immunity by cell wall components of lactic acid bacteria.

In human studies, De Simone et al. (1986, 1987b) showed stimulation of interferon-γ (IFN-γ) production in supernatants of blood lymphocyte cultures when lactic acid bacteria were administered as a yogurt filtrate to the cultures. At very large doses (10^{11} - 10^{12}/ day) of yogurt, serum IFN-γ is elevated (Halpern et al., 1991). While lower doses of yogurt did cause a significant increase of serum IFN-γ, blood lymphocytes did have an increased capacity to produce IFN-γ *in vitro*. Because such a stimulation of IFN-γ might exert both beneficial and detrimental effects, more experiments and trials are needed to reach a conclusion (dose-response studies, clinical end-points, etc.). Tables 1.4 through 1.6 summarize the recent researches on lactic acid bacteria modulation of the immune system which is relevant to this dissertation.

1.1.8 Side effects and toxicity of lactic acid bacteria.- Lactic acid bacteria have been observed to produce side effects after intraperitoneal injection, causing septicemia, endocarditis in mice (Okitsu-Negishi et al., 1996), systemic infections, fever, and arthritis in rats (Wilson et al., 1993; Blancuzzi et al., 1993). However, during oral administration no

complications have been shown in mice (Perdigon et al., 1991a). In humans, cases of clinical infections are extremely rare, but the fact that they appear in several publications suggest that lactic acid bacteria might act, rarely, as opportunistic pathogens. Nevertheless, all severe cases were reported in immunocompromised patients (Abgrall et al., 1997), and only a few cases describing local infections were produced in immunocompetent individuals (Gasser, 1994; Donohue et al., 1993).

TABLE 1.4.Recent human studies on immunostimulation by lactic acid bacteria.

Subject	Experimental design	Results	Reference
27 patients > 10 yr	oral antibiotics + Lactinex (*L. acidophilus* + *L .bulgaricus*) 1.2 x 10^8/day	Fewer yeast infections and gastrointestinal disorders	Witsell et al., 1995
33 women	8 oz. yogurt/d with *L. acidophilus* (10^8cfu)	Fewer candidal infection in yogurt group	Hilton et al., 1992
20 adults	4 x 10^9 *L. acidophilus* & *Bifidobacterium bifidum* for 7 d, 3 times /d	Microflora restoration	Black et al., 1991
Adults	2 x 150 g/d for 28 d of *L. acidophilus* 10^8cfu	Increased Ig secretion not altered	Marteau et al., 1996
28 adults	3 x 129 ml milk (3wks) + *B. bifidum* or *L. acidophilus* (3wks) 7 x 10^{10}cfu + milk (6 wks)	Increased phagocytic acitivity, no change lymphocyte population	Schiffrin et al., 1995
30 adults	3 wks,3 times /d, 125g FM *L. acidophilus* & *B. bifidum* (>5x10^9cfu each) + attenuated *S. typhi*	Increased sIgA anti *S. typhi* & total serum IgA	Link-Amster et al., 1994
25 elderly	*L .acidophilus* 10^6cfu & *B. bifidum* 8 capsules/d, 28 d	Increased B cell & TNF-α in peripheral blood	De Simone et al., 1992
Humans	*L. casei* GG, oral, 10^{10}-10^{11} cfu	Increased IgA, IgG, IgM secreting cells, IgA to rotavirus, recovery from diarrhea	Kaila et al., 1992 Isolauri et al., 1991
Humans	Lyophilized lactobacilli, oral, 3 times10^{10} cfu + 200 g yogur, 28 d	Increased NK cells and IFN-γ	De Simone et al., 1988d
Human blood cells	*L. acidophilus* La1 7 x 10^{10} + *Bifidobacterium bifidum* 1 x 10^{10}, 3-6 wks	Increased phagocytosis, no change lymphocyte subpopulation.	Schiffrin et al ., 1995
Infants (vaccination)	*L. casei* GG, 10^{10} cfu/d, 5 d, oral, rotavirus	Increased IgM and IgA-anti-rotavirus	Isolauri et al., 1995

TABLE 1.4. (cont'd)

Subject	Experimental design	Results	Reference
Human blood cells	*L. brevis* ssp.*coagulans*, heat-killed, oral, 4 wk, 3 x 10^8 cfu/d	Increased IFN-γ	Kishi et al., 1996
Human blood cells	*L. casei* GG, 10 d, 10^{10}cfu, 2x/d	Increased gut IgA	Malin et al., 1996
Adults	Yogurt (Dannon), 16 oz., 1 month, 2.5-3 x 10^8/g *L. bulgaricus* + 3.5-4.1 x 10^8 *S. thermophilus/g*	No improvement in total blood count, Ig M,A,E, allergy, IFN-γ, IL-2, IL-4	Wheeler et al., 1997
Blood mononuclear cells (BMC)	*S. thermophilus, L. acidophilus, S. lactis, Bifido-bacterium*, yogurt, 7 d with + 7 d without	Increased IFN production, IL-1β, IL-6	Aattori and Lemonnier 1997

Table 1.5. Recent animal studies on immunostimulation by lactic acid bacteria

Subject	Experimental design	Results	Reference
Female BALB/c mice	*L. bulgaricus* & *L. casei*, i.p, 8 d, 1.2 x 10^9cfu	Increased IgM, peritoneal macrophage function	Perdigon et al., 1986b
Female BALB/c mice	*L. bulgaricus* & *S. thermophilus*, oral and ip, 8 d, 1.2 x 10^9 cfu	Increased IgM, peritoneal macrophage function	Perdigon et al., 1987
Mice	*L. bulgaricus* LB51 , gavage, lysate, 150 mg/kg body weight	Increased serum TNF-α and IL-1	Davidhova et al., 1992
Female BALB/c mice	*L. casei* or *L. acidophilus*, oral, 2-8 d, 1. 2 x 10^9 cfu/mouse	Increased peritoneal macrophage function	Perdigon et al., 1992
Female BALB/c mice	*L. acidophilus*, oral, 1.2 x 10^9 cfu/mouse	Increased Ig secreting cells in lamina propria	Smeyanov et al., 1992
Female BALB/c mice	*L. bulgaricus, L. acidophilus*, *L.casei*, oral, 7 d, 1. 2 x 10^9 cfu/mouse	Increased intestinal IgA to *Salmonella*	Perdigon et al., 1990
Mice	*L. casei*, iv, 4-40 mg/Kg body weight	Increased colony-stimulating factor	Nanno et al., 1988
Mice	*L. casei*, ip, 0.5 mg/mouse	Increased cytostatic factor by macrophage	Hashimoto et al., 1987
Mice	*L. casei*, subcutaneous, 0.1-1 mg/mouse	Increased hematopoiesis	Nomoto et al., 1991 Yokokura et al., 1986
Mice	*L. casei*, iv, 0.5 mg/mouse	Increased phagocytosis and intracellular killing	Nomoto et al., 1985
Mice	*L. casei*, heat killed, im, 1 mg/mouse/wk	Increased macrophage function	Saito et al., 1987 Tomioka et al., 1990
Mice	*S. thermophilus*, 20 mg/kg, injection	Increased T cell-mediated antitumor action	Kaklij et al., 1991

TABLE 1.5. (cont'd)

Subject	Experimental design	Results	Reference
Female BALB/c mice	*S .thermophilus*, oral, 7 d, 1.2 x 10^9 cfu	Increased resistance to *Salmonella*	Perdigon et al., 1990
Female BALB/c mice	*B. longum*, *L. acidophilus*, oral, 2-8 wk heat-killed, 8 x 10^{10}/d, cell wall & cytoplasm	Proliferative response of PP, increased antibody cell wall and cytoplasm of *L.acidophilus*	Takahashi et al., 1993
Mice	Yogurt, oral	Increased Ig, stimulation lymph follicles	Bourlioux, 1986
Mice	7 fermented milks: *B. longum*, *L. delbrueck* ii ssp. *bulgaricus*, *L. casei*, *L. helveticus*, *L .lactis* ssp *cremoris*, *L. lactis*, *S. salivarius* ssp .*thermophilus*.	Increased activation of macrophages	Moineau and Goulet 1991
Male BALB/c mice	*B. breve*, gavage, 7 d, 2 x 10^{11} cfu	Increased antibody production	Yasui et al., 1989
Male BALB/c mice	*B. longum*, *B. animalis*, ip, collection of peritoneal macrophages after 3 h, 5.7 x 10^5 cfu	Increased IL-1β, IL-6, IL-10, TNF-α mRNA, by RT-PCR	Sekine et al., 1994
Female BALB/c mice	*Lactobacillus* + TNP-*lactobacillus*, oral, 3 times, 500 μg bacteria, 1 x 10^{10} cfu/ml TNP-L	Increased systemic memory, IgG	Gerritse et al., 1990
BALB/c mice	*L. casei*, *L. bulgaricus*, *L. acidophilus*, *S. thermophilus*, oral, 2 d, 1.2 x 10^9 cfu/d	Increased non-specific & specific immunity	De Petrino et al., 1995
Male BALB/c mice	*B. infantis* cell wall, 4 wk, sc, 100 μg, spleen	T cell mediated immunity increased	Sekine et al., 1994, 1995a
Swiss mice	*L. bulgaricus*, *S. thermophilus*, ip, 72 h, peritoneal macrophages, 0.2-25 x 10^7cfu	Increased TNF and IFN-α/β	Solis-Pereyra et al.,1991
Female BALB/c mice	*L. casei*, oral, 2 d, 2 x 10^9cfu/d, viable-non viable	Secretory IgA-anti-*S. typhimurium* increased plasma cells and T-lymphocytes increased	Perdigon et al., 1995
Mice	*L. bulgaricus* lysate, oral, 10 d, 150 mg/kg/d	Increased IL-1 in peritoneal macrophages	Popova et al., 1993

TABLE 1.5. (cont'd)

Subject	Experimental design	Results	Reference
Swiss albino mice	1.5×10^8 cfu/d, *L. casei* & *L. acidophilus* 8 d, challenged *L. monocytogenes* or *E. coli*	Increased anti-pathogen antibodies in sera	Nader de Macias et al., 1993
Swiss albino mice	1.5×10^8 cfu/d, *L. casei* & *L. acidophilus* 8 d, challenged *Shigella sonnei*	Increased anti-shigella antibodies in sera	Nader de Macias et al., 1992
Swiss albino mice	*L. acidophilus, L. casei* or yogurt, 1.2×10^9cfu 2, 5, 7 d	Increased plasma cells, IgA, IgG producing cells, alteration in mucosa	Perdigon et al., 1993
Female BALB/c mice	*L. acidophilus* or *B. longum*, 8×10^{10} cfu/d, 12 wks, cytoplasm and cell wall fraction	Increased *L. acidophilus* cell wall, cytoplasm proliferative response but weak immunity	Takahashi et al., 1993
Mice	*B. infantis,* injection cell wall	Tumor regression	Sekine et al., 1985
Mice	Lactic acid bacteria, 8 strains, oral, 3×10^7cfu of each strain, 13 d	Increased serum IgG and resistance *Klebsiella pneumoniae*	Saucier et al., 1992
Female BALB/c mice	*L. casei & acidophilus,* oral, 8 d, 2×10^9 cfu	Increased peritoneal macrophages function, IgM	Perdigon et al.,1986a, b 1987

TABLE 1.5. (cont'd)

Subject	Experimental design	Results	Reference
BALB/c	*L bulgaricus* $5x10^7$/d + *S.thermophilus* $5.7x10^7$/d heated-yogurt ($<10^{2-3}$cfu/ml), *L. casei* $1.1x10^8$/d, 20d, oral, 2 x 200 µl/d yogurt, 200 µg CT ip + 100 µg CT oral, 7wks total experiment	Increased antibody anti-cholera in serum	Portier et al., 1993
DBA/2J	Yogurt + *Salmonella* challenge at 8 d, 4 wk	Increased proliferation spleen, serum IgA anti-*Salmonella*	Puri et al., 1996
Rats	*L. reuteri* R2LC, *L. plantarum* DSM 9843 6 d, intragastric infusion, 4 ml	sIgA, CD4, CD8 increased	Mao et al., 1996
Rabbits	*L. bulgaricus* LB51, im and iv, 10^6 cfu	Increased IL-1, TNF-α, IL-6	Guencheva et al., 1992
Murine spleen cells	*L. bulgaricus, S. thermophilus*, oral, 7-15 d $8 x 10^8$ cfu/d/mouse	Increased IFN-γ, and NK activity	Muscettola et al., 1994

TABLE 1.6. Recent *in vitro* studies on immunostimulation by lactic acid bacteria

Subject	Experimental design	Results	Reference
Murine splenocytes, Peyer's patch cells	*B. adolescentis*, whole cell (WC), sonicated cells (SC), cell wall (CW), cytoplasm (CF).	Proliferation of spleen cells	Hosono et al., 1997
Murine splenocytes Peyer's patch cells lymph node cells	*B. adolescentis,* CW, SC, WC, CF	Antibody production anti-egg ovomucoid	Lee et al., 1993
Whole blood cells	*S. mitis* cell wall, 160 μl cell wall material	Increased TNF	Le Roy et al., 1996
Peritoneal macrophages	*L. acidophilus*, heat-killed, 100 mg/ml, 3-72 h	Induced IFN-α/β	Kitazawa et al., 1992
Murine splenocytes, Peyer's patch, T and/or B cells	*L.acidophilus, L. gasseri*, WC, CW, 50 μg, mitogens	Increased mitogenic response with CW	Takeda et al., 1997
J774 macrophage	*L. acidophilus, B. longum*, + LPS, 1 g (20 μg protein/ml), *S. typhimurium* challenge.	Macrophage alteration strain-dependent	Hatcher et al., 1993
RAW 264.7 macrophage	*Lactobacillus* spp.,10^6-10^8 cfu/ml, 2 d culture	Enhanced cytokine production w/ concentration	Marin et al., 1997a
	S. thermophilus,10^6-10^8 cfu/ml, 2 d culture	Increased TNF-α, IL-6	Marin et al., 1997c
	Bifidobacterium spp.,10^6-10^8 cfu/ml, 2 d culture	Enhanced cytokine production w/ concentration	Marin et al., 1997b
EL-4 Thymoma cell	*Lactobacillus* spp.,10^6-10^8 cfu/ml, 2 d culture	Increased cytokine production, strain dependent	Marin et al., 1997a
	S. thermophilus,10^6-10^8 cfu/ml, 2 d culture	Increased IL-2, IL-5	Marin et al., 1997c
	Bifidobacterium spp.,10^6-10^8 cfu/ml, 2 d culture	Enhanced cytokine production w/ concentration	Marin et al., 1997b

TABLE 1.6. (cont'd)

Subject	Experimental design	Results	Reference
Blood mononuclear cells			
BMC 2 x 10^6/ml	*L. bulgaricus* lysate, 3 μg/ml	Increased IL-1, TNF-α	Popova et al., 1993
BMC 106 /ml	*Streptococcus* fractions, 10 μg/ml	Increased TNF-α	Vallejo et al., 1996
BMC 2 x 10^6/ml	7 strains of lactic acid bacteria, 2 x 10^7cfu	Increase IFN-γ, IL-1β, TNF–α	Solis-Pereyra et al., 1993
BMC	10 strains of lactic acid bacteria, 10^5-10^7/ml	Increased TNF-α, IL-6, IL-10	Miettinen et al., 1996
Spleen macrophages	13 strains of heat-killed *L. gasseri*	1 strain, IFN-α mRNA increased	Kitazawa et al., 1994
mouse lymphocytes	*B. breve*	Increased cytokine production by macrophages	Yasui & Ohwaki, 1991
mouse lymphocytes	*L. bulgaricus* + *S. thermophilus*, 1-3 μg/ml	Increase IFN-γ	DeSimone, 1986, 1987b 1988c

1.2 Gastrointestinal immune system

The gastrointestinal system consists of stomach, small intestine, and large intestine. Defense against infections can involve nonimmunologic mechanisms such as gastric acidity, digestive enzymes, peristalsis, mucus, enteric microflora, and the self-regenerative capacity of the epithelium (Pestka, 1993). Specific immunologic responses are mediated by the gut-associated lymphoid tissue (GALT) (McBurney, 1994). GALT consists of lymphoid follicles (Peyer's patches) located in the small intestine, appendix and large intestine, isolated follicles, the mesenteric lymph nodes, and a diffuse tissue comprising the intraepithelial lymphocytes (IELs) and lamina propria (LP) (Shanahan, 1994). Peyer's patches consist mainly of lymphocytes of type B, T, macrophage and accessory cells. Intraepithelial lymphocytes are essentially regulatory and cytotoxic T cells. Lamina propria possesses mainly plasma cells (Ig-secreting B cells) and memory T cells. The Peyer's patches are the central focus for the induction of T and B-cell responses following an oral immunization. These organs lie below a specialized layer of epithelial cells called M cells. The mucosal immune response is initiated by contact between the antigen and the lymphocytes and antigen presenting cells in the dome of the Peyer's patch. Here, once the antigen has traversed the M cells, antigen presenting cells within the Peyer's patch can take up the antigen and present it to adjuvant regulatory T cells. These B cells expressing IgM or IgD on their surface upon stimulation proliferate and differentiate into lymphoblasts expressing IgA on their surface. The response induced in Peyer's patches leads to activation, switching, proliferation and differentiation of B cells under the control of T cells and other

accessory cells (Ogra et al., 1994).

The mucosal immune system B lymphoblasts derived from the GALT can migrate to respiratory, genital tissues as well as other sites in the intestine. Lymphocytes migrate from Peyer's patches via the mesenteric lymph node into the efferent lymphatics, thoracic duct, and the systemic compartment to the lamina propria located beneath the intestinal epithelia. These lymphoid cells which originate at the inductive sites can enter distant effector sites as lamina propria of the intestine repopulating it. At these effector sites B cells proliferate and undergo terminal differentiation to IgA secreting plasma cells in response to antigen and further signaling by cytokines produced by T cells and macrophages. Lamina propria plasma cells synthesize monomeric IgA which eventually is joined by a J chain to form dimers or longer polymers. Polymeric IgA binds to the secretory component (SC) which is the epithelial receptor responsible for the transportation of this immunoglobulin, being then secreted across the epithelial cell into the lumen (Pestka, 1993). This complex IgA-SC binds to the antigen present in the intestinal mucus and lumen, avoiding antigen access to the mucosa and producing its degradation. IgA secreted by B cells in the lamina propria and released to the intestine follows later the bowel content or goes to the blood stream and raises the serum IgA levels. Although Peyer's patches are primarily inductive sites for IgA response, IgG+ cells are known to be also present in these organs. During this process cytokine production can be also stimulated, controlling first cytokine mRNA formation and resulting eventually in an elevated translation.

Of particular relevance to this dissertation are the recent reports indicating that B-cell activation, switching, proliferation and differentiation to IgA synthesis as well as

cytotoxic T-cell development are regulated in the mucosal and systemic immune compartments by several cell types and their cytokines (McGhee et al., 1989). Cytokines produced by activated macrophages and mucosal T cells also mediate the gastroimmmune response. There are two different types of T cells: T-helper and T-cytotoxic cells. T-helper cells can be classified as Th1 (producers of IL-2, IL-3, IFN-γ) and Th2 (producers of IL-6, IL-4, IL-5) (Pascual et al., 1996). T lymphocytes located in lamina propria and Peyer's patches are capable of cytokine production such as IL-2, IFN-γ, IL-4, and IL-5. T cells present in mesenteric lymph node have a potential for IL-4 and IL-5 production, and to some extent for IL-2 and IFN-γ. Activated macrophages are able to secrete IL-1, TNF-α, IL-12 and IL-6, as well as some other mediators that can participate in the immune response such as nitric oxide. Some of these cytokines mediate proliferation, switching and differentiation of B cells to become IgA plasma cells and up-regulation of the expression of secretory component (Shanahan, 1994).

The most likely site for interaction among immune cells, food antigens and lactic acid bacteria is the GALT, where the intestinal microflora is also a source of antigenic stimulation. Possible targets are the Peyer's patches, via specialized epithelial cells called M cells (Pestka, 1993). They are more permeable to luminal components and as such encourage interaction with underlying immune cells. Bacteria signals can also act directly on the proximal small intestine, increasing the proportion of intraepithelial cells (Muscettola et al., 1994). This lymphoid population can produce cytokines which can act locally or alter the systemic reactivity of the host.

Thus, numerous factors of host and of microbial origin participate in the

development of an individual gastrointestinal ecosystem and in the generation of gut immune responses. The composition and number of microorganisms in the gut are mostly influenced by bacterial physicochemical activities. There are specific substances excreted by some bacteria which inhibit the growth of others (bacteriocins, organic acids, short-chain fatty acids, H_2O_2), allowing them a better adherence to the gut epithelial cells, forming a local microbial community which will determine for instance gas concentration and composition, the pH and reduction-oxidation potential, and the mucosal secretions (lysozyme, cytokines, immunoglobulins) in the gut (Sanders, 1993). It is very difficult to have available data about interactions between different niches established along the gut in a particular host and obviously there is great difficulty in getting individuals with a similar or identical GI ecosystem. The microbial ecosystems are individually different inside the gastrointestinal system even in cases of animals with a similar origin or/and husbandry.

1.3 Rationale for this research

A number of published studies suggest that lactic acid bacteria and their fermented products have beneficial effects on the health of human beings due not only to the nutritional properties of such bacteria but also due to their action on the immune system. Much interest has been focused recently on the immunological effects of these bacteria. Several lines of evidence using the mouse model suggest that probiotic bacteria could enhance immunity both locally on the mucosal surfaces and at the systemic level.

To enhance immune function in severely immune-compromised individuals, high

doses of IFN or strong immunopotentiators such as *S. pyogenes* (OK432), Bacille bilie de Calmette-Guerin (BCG) have often been administered. However, repeated use of them has been reported to cause severe side effects such as fever, malaise, depilation or depression in some cases (Kishi et al., 1996). Oral administration of lactic acid bacteria could be an alternative way to stimulate host immunity and stimulate the systemic immune response. Immunological effects of dairy cultures reported previously in our laboratory and by others suggest that some strains of lactobacilli as well as bifidobacteria (Marin et al., 1997a, 1997b) or their cellular components can stimulate macrophage activation in animals. As of now, the biological significance of this enhancement in macrophage activity is not clear.

Researchers have made the assumption that lactic acid bacteria are beneficial to humans and most of them have designed experiments to prove this hypothesis. There is a lack of studies relating to a clear identification of most of the strains used and consequently the claims of positive effects are not well sustained. Furthermore, not all data reported in literature are based on well designed and controlled experiments and often lack statistical analysis. Research needs for specific target areas have been suggested by a panel of experts on the subject (Sanders, 1993), recommending that further research on immune system stimulation must use "focused animal studies prior to human studies until a working hypothesis is obtained". There is a clear need for critical study of the effects of strain selection on health, and also there is a lack of clear mechanistic information about how cultures could affect immunological function in leukocytes of gut origin (Peyer's patch, mesenteric lymphoid node, lamina propria, intraepithelial lymphocyte cells). Elucidation of specific effects after ingestion of lactic cultures on leukocytes found in the immune

system is becoming a critical need. Recent advances in the immunology field may now allow us to explain the reasons for immune enhancement by consumption of dairy cultures that have been observed previously. Thus, the dairy industry could be provided with a basis for choosing species and strains to optimize immune enhancement and this could be used in new or improved fermented dairy products.

In this research, I hypothesized that lactic acid bacteria have an immunomodulatory effect in the gastrointestinal system and that the immunopotentiating activity resides in the cell walls of lactobacilli, streptococci and bifidobacteria. To determine the effects of lactic acid bacteria on immunity, *in vivo* feeding studies were conducted using the mouse model. Secondly, I conducted *in vitro* studies, where the conditions were defined and kept constant. By exposing clones and primary leukocytes to the whole bacterial cell and to different fractions of lactic acid bacteria, I attempted to identify the cellular components that possess the immunopotentiating activity.

CHAPTER 2

EFFECTS OF LACTOBACILLI, STREPTOCOCCI AND BIFIDOBACTERIA INGESTION ON MUCOSAL AND SYSTEMIC CYTOKINE GENE EXPRESSION AND SECRETION IN A MURINE MODEL

2.1 ABSTRACT

Lactic acid bacteria are essential for the fermentation of products such as cheese, buttermilk and yogurt. An increasing number of functional foods and pharmaceutical preparations are being promoted with health claims based on the potential "probiotic" characteristics of some of these bacteria and on their capacity for stimulating the host immune system. However, the immune effects of oral administration of these microbes has not yet been well defined. In this study, I tested the hypothesis that cytokine gene expression and production in mice is affected by orally administered lactobacilli, streptococci or bifidobacteria. The specific objectives of this study were to evaluate the effects of *in vivo* exposure to viable and non-viable strains of lactic acid bacteria (*Lactobacillus acidophilus*, *L. bulgaricus, L. casei, L. gasseri, L. helveticus, L. reuteri, Streptococcus thermophilus* and *Bifidobacterium*) in mice on: (1) cytokine mRNA expression in systemic (spleen) and mucosal (Peyer's patches) lymphoid tissue, (2) cytokine and nitric oxide production by mitogen-stimulated Peyer's patch, spleen and peritoneal cell cultures and (3) immunoglobulin levels in serum and fecal pellets. Growth rate of mice as well as immunoglobulin levels were not affected by treatments. Although basal cytokine mRNA expression in spleen and Peyer's patches was not affected by repeated lactic acid bacteria administration, single exposures to certain bacteria altered subsequent mitogen-induced cytokine and nitric oxide production by peritoneal cells. The results suggest that lactic acid bacteria may alter leukocyte responsiveness to an activation signal in a strain dependent manner.

2.2 INTRODUCTION

Almost a century ago, Metchnikoff (1907) suggested in his book "The Prolongation of Life" that consumption of fermented dairy products resulted in improved health and a longer life. Lactic acid bacteria are mainly associated with fermented dairy products such as cheese, buttermilk and yogurt. Today, an increasing number of health foods and so-called functional foods as well as pharmaceutical preparations are promoted with health claims based on the "probiotic" characteristics of some of these bacteria. The most evident effects of probiotics involve changes in viable counts of microorganisms in the intestinal flora after ingestion, which can be caused by competition for adhesion sites and nutrients between the ingested microorganisms and potential pathogens (Rafter, 1995). Another known mode of action for probiotics is production of antibacterial substances. Some of the health effects attributed to lactic cultures are improved absorbability of certain nutrients, alleviation of lactose intolerance symptoms, improved metabolism of some drugs, serum cholesterol reduction, improvement of intestinal motility, anticancer effects, creation of an antagonistic environment for intestinal pathogens by production of inhibitors, inactivation of enterotoxins from pathogens, alleviation of constipation, relief from vaginitis and stimulation of the immune system (Gilliland, 1990; Fuller, 1991).

T cell regulation, IgA synthesis and cytotoxic T cell development are tightly regulated in the mucosal and systemic immune compartment by several cell types and the cytokines they produce (McGhee et al., 1989). The gastrointestinal immune system is a critical site of host defense (Hanson et al., 1983) formed by Peyer's patches, lamina propria and mesenteric

lymph node, which represent the mucosal compartment, while spleen and lymph nodes dispersed along the body represent the systemic immune compartment. The intestinal wall contains macrophages and over half of the lymphocytes (T and B-cells) present in the body. Differentiation of B cells to committed IgA producing cells requires T helper type 2 cells (Th2), which secrete IL-4, IL-5 and IL-6. In contrast, T helper type 1 cells (Th1) can secrete IL-2 and interferon-γ (IFN-γ) (Pestka, 1993). These matured B -cells produce mainly IgA which is located in the Peyer's patches and lamina propria in the small intestine, or travel to spleen using the systemic circulation. The secretory IgA system acting by binding microbes and toxins, is the biggest component of the gut immune defense system. A number of investigations suggest that lactic acid bacteria immunopotentiate this gut system (Perdigon et al., 1986a, 1987, 1988, 1990, 1992, 1995; Saito et al., 1987) via activation of the macrophage fraction. Macrophages can regulate T and B cell activity by activation and secretion of IL-1 and tumor necrosis factor-α (TNF-α) (Abbas et al., 1994). Increased secretion of these two cytokines by peritoneal macrophages occurs after intraperitoneal exposure to *Bifidobacterium longum*, or *B. animalis* (Sekine et al., 1994) and *Lactobacillus bulgaricus,* or *Streptococcus thermophilus* (Solis-Pereyra et al., 1991). Even though the peritoneal cavity surrounds the gastrointestinal system, the exact relationship between these two components of the immune system remain unclear.

The effect of oral administration of lactic acid bacteria on cytokine expression and production is not yet well defined. In general, cytokines are considered a diverse group of protein hormones produced through the effector phase of an immune response (Abbas et al., 1994). Their synthesis is initiated by transcriptional activation of DNA, mRNA production,

and translation of this mRNA to proteins (i.e. cytokines). Once synthesized, cytokines are usually secreted as needed, playing a very important function in host immunity. In this study, I hypothesized that cytokine expression and production in mice is affected by orally administered lactobacilli, streptococci or bifidobacteria. The objectives of this study were to evaluate the effects of *in vivo* oral exposure to viable and non-viable strains of lactic acid bacteria (*Lactobacillus acidophilus*, *L. bulgaricus, L. casei*, *L. gasseri*, *L. helveticus*, *L. reuteri*, *Streptococcus thermophilus* and *Bifidobacterium*) in mice on 1) cytokine mRNA gene expression in systemic and mucosal lymphoid tissue and 2) cytokine production by mitogen-stimulated Peyer's patch, spleen, and peritoneal cell cultures. These latter effects were further related to immunoglobulin and nitric oxide production.

2.3 MATERIAL AND METHODS

2.3.1 Microorganisms

Representative cultures of *L. acidophilus*, *L. bulgaricus*, *L. casei*, *L. gasseri, L. helveticus*, *L. reuteri*, *S. thermophilus* and *Bifidobacterium* were obtained from three different sources: American Type culture Collection (Raleigh, NC), Dr. T.R. Klaenhammer (North Carolina State University) and Sanofi Bio-Industries (Waukesha, WI) (Table 2.1). Initially, lactobacilli and streptococci were grown in MRS broth (De Man et al., 1960) while bifidobacteria were grown in MRS + 5% (w/w) lactose (MRSL) broth. All organisms were incubated at 37°C for a period of approximately 15 h. A 1% (v/v) inoculum was transferred to fresh broth, and this was incubated for 15 h at 37°C. Bacteria were recovered by centrifugation at 1100 x g for 15 min, resuspended in 10% (w/v) non fat dry milk (NFDM) plus 10% (v/v) glycerol (cryoprotective agent) and frozen at - 80^{0}C for long time storage.

Growth curves were calculated for each bacterial species, by measuring every 30 min the percentage of light absorption at 650 nm in a newly growing culture, which was kept at 37°C after each measurement until the turbidity no longer increased. These measurements are directly proportional to the cell concentration in the culture and can be correlated with total number of bacterial cells. Aliquots at each time point were plated in MRS agar for lactobacilli, modified ST agar (Lee's agar) (Lee et al., 1974) for streptococci and neomycin sulfate, paromomycin sulfate, nalidixic acid, lithium chloride (NPNL) agar (Teraguchi et al., 1978) for bifidobacteria in order to determine bacterial counts at these same points. Agar plates were incubated at 37°C for 48 h aerobically for lactobacilli and streptococci and

TABLE 2.1. List of cultures

Bacteria	Strain	Source
Bifidobacterium	Bf-1, Bf-6, Bf-12, Bf-13	Sanofi Bio-Industries, Waukesha, WI
Lactobacillus acidophilus	La-1, La-2, La-7	Sanofi Bio-Industries, Waukesha, WI
	ATCC 521, ATCC 53545	American Type Culture Collection, Rockville, MD
	BG2FO4 (NCK96), NCFM-N2 (NCK56)	North Carolina State University, Raleigh, NC
Lactobacillus bulgaricus	Lr-28, Lr-78, Lr-79	Sanofi Bio-Industries, Waukesha, WI
Lactobacillus bulgaricus 1489	NCK 231	North Carolina State University, Raleigh, NC
Lactobacillus casei	Lr-95	Sanofi Bio-Industries, Waukesha, WI
Lactobacillus casei ssp. *casei*	ATCC 39539, ATCC 334	American Type Culture Collection, Rockville, MD
Lactobacillus gasseri ADH	NCK 101	North Carolina State University, Raleigh, NC
Lactobacillus helveticus	Lr-92	Sanofi Bio-Industries, Waukesha, WI
Lactobacillus reuteri	ATCC 23272	American Type Culture Collection, Rockville, MD
Streptococcus thermophilus	St-133, St-52, St-113, St-134	Sanofi Bio-Industries, Waukesha, WI

anaerobically with Gas Pak® (Becton Dickinson Co., Cockesysville, MD) for bifidobacteria. Colonies were counted using a Quebec counter (Fisher Scientific, Pittsburgh, PA). The absorbance at 650 nm versus log number of bacterial cells per ml were plotted and used when an estimation of bacteria present in a suspension was needed.

Prior to *in vivo* experiments, frozen bacteria were thawed with moderate agitation in a 37°C water bath, inoculated (1% v/v) using broths described above and incubated at 37°C until entry into log phase (8 - 20 h, depending on the species). A 1% (v/v) inoculum was transferred to fresh broth and the culture was incubated at 37°C for an identical time period. Total cell numbers were estimated at this point by measuring the culture's absorbance at 650 nm and relating these data to previous quantitative plate counts.

Some bacteria cultures were heat killed at 100°C for 50 minutes in their own culture broth. Viable and non-viable cultures were centrifuged at 1100 x g for 15 min to recover bacteria, washed once with a physiological saline solution and resuspended in 10% (w/v) NFDM. Doses were aliquoted in single vials containing 0.3 ml 10% NFDM with 1.0×10^9 cells and frozen at - 80°C for no longer than 3 weeks. To determine the effects of freezing storage in lactic acid bacteria doses prepared, viability was checked by plating a single dose every 5 days during 15 consecutive days, using agar media and conditions described above.

2.3.2 Experimental Design

2.3.2.1 *In vivo* studies

Animal model. Eight week old female B6C3F1 mice (6-8 mice per experimental group) were used for *in vivo* experiments. All animal handling was conducted in strict accordance

with regulations established by the National Institutes for Health. Experiments were designed to minimize numbers of animals required to adequately test the proposed hypothesis and approved by Michigan State University Laboratory animal Research committee. Mice were housed 3-4 per cage in a 24 hr light/dark cycle. Water was provided *ad libitum*. The basic feed was nutritionally complete (AIN-93G) (Reeves et al., 1993).

Lactic acid bacteria administration. Bacteria were orally administered as suspensions in 10% (w/v) NFDM by gavaging doses of 1.0×10^9 cells per mouse per day. Matching controls were gavaged 10% (w/v) NFDM without organisms. At 1, 7, 14 days, and 2 h after last dose was administrated, blood was collected and mice were sacrificed by cervical dislocation after gentle anesthesia for extraction of Peyer's patches, mesenteric lymph node, and spleen.

Serum preparation. Blood was collected from anesthetized mice from the retroorbital plexus. Serum was obtained after overnight incubation at 4^0C and centrifugation at 1,000 x g for 15 min. Serum samples were aliquoted, stored at -80^0C prior to assay for immunoglobulins.

Semiquantitative RT-PCR for cytokines. Total RNA was extracted from Peyer's patches, spleen and mesenteric lymph node using the RNA STAT-60™ isolation reagent (TEL-TEST "B" Inc., Friendswood, TX) according to the manufacturer's instructions. Total RNA content was determined spectrophotometrically at 260 nm (Sambrook at al., 1989). A

reverse transcriptase (RT) reaction for first strand cDNA synthesis was carried out by the method of Kawasaki (1991). Cytokines were detected by the semiquantitative RT-PCR method of Svetic et al. (1991) using the PCR primers and probes for DNA hybridization synthesized at GIBCO-BRL which sequences are described in Table 2.2. PCR was performed in a final volume of 50 μl using a 9600 Perkin Elmer Cycler (Perkin-Elmer Corporation, Norwalk, CT) with a 5 min incubation step at 95°C for denaturation, followed by a three step temperature cycling (1 min denaturation at 95°C, 1 min primer annealing at 52°C, and 3 min extension at 72°C) terminated by a 72°C incubation for 10 min then cooled to 4°C. The cycles were repeated for an optimized number for each transcript (15, 20, 24, and 25 cycles for β2 microglobulin [β2-MG], TNF-α, IFN-γ, and IL-6, respectively). β2-MG was used as a "housekeeping gene" to verify initial equal quantities of RNA and the integrity of the RNA preparation.

Hybridization analysis were carried out to determine the relative abundance of PCR-amplified cDNAs using previously described protocols (Azcona-Olivera et al., 1995; Zhou et al., 1997). Briefly, PCR products (10 μl) were treated with 1N NaOH and 0.2M EDTA, boiled for 10 min and applied to a Nytron membrane (Nytran, Schleicher &Schuell Inc., Keene, NH) using a Bio-dot SF micro filtration apparatus (Bio-Rad Laboratories, Hercules, CA). Hybridizations employed ^{32}P-labeled probes prepared using a DNA 3'-End labeling system which uses terminal deoxyribonucleotide transferase (TdT) (Promega Co., Madison, WI) (Table 2.2). All oligoprobes had at least a specific activity of 1 x 10^9 cpm/μg and all hybridizations were performed with no less than 1 x 10^7cpm/ml. Blots were exposed to Kodak X-OMAT-AR film (Eastman Kodak, Rochester, NY) with an intensifying screen at

TABLE 2.2. Primer sequences for cytokine cDNA amplification and probe sequences for slot-blot hybridization to detect amplified cDNA product.

Cytokine	Sense and antisense primer 5' 3'	5' position	Probe 5' 3'	5' position
β-2 MG	TGACCGGCTTGTATGCTATC[a]	96	CTCACGCCACCCACCGGAGA[d]	142
	CAGTGTGAGCCAGGATATAG[a]	318		
IL-6	TTCCATCCAGTTGCCTTCTTGG[b]	56	ACTTCACAAGTCCGGAGA[b]	110
	CTTCATGTACTCCAGGTAG[b]	314		
IFN-γ	GCTCTGAGACAATGAACGCT[a]	59	GGAGGAACTGGCAAAAGGA[b]	230
	AAAGAGATAATCTGGCTCTGC[a]	287		
TNF-α	TCTCATCAGTTCTATGGCCC[b]	5195	CCTGTAGCCCACGTCGTAG[a]	5436
	GGGAGTAGACAAGGTACAAC[b]	5877		

Primers and probe sequences were provided by (a) Dr. David Shire (Sanofi Elf Bio Recherches), (b) Svetic et al. (1991), (c) Wesselingh et al. (1994) or (d) designed with OLIGO (national Biosciences, Hamel, MN) software.

-80^{0}C for variable length periods depending upon signal strength. For the purposes of comparison, exposure periods for each cytokine were identical among untreated and treated groups. Autoradiographic bands were quantified with an Epson ES-1000C scanner and Sigma-Scan program (Jandel Scientific, San Rafael, CA). Relative amounts of cytokines mRNA were estimated by dividing the densitometric area of the cytokine autoradiography band by the densitometric area of the housekeeping gene β2-MG autoradiography band.

2.3.2.2 *Ex vivo* studies

Animal model and Lactic acid bacteria administration. Eight weeks old female B6C3F1 mice (6-8 mice per experimental group) were also used for *ex vivo* experiments. Bacteria were orally administered to mice by gavaging a single dose of 1.0 x 10^9 cells per mouse in 10% (w/v) NFDM. Eight hours after administration of the bacteria, fecal pellets and blood were collected, and mice were killed by cervical dislocation after gentle anesthesia for peritoneal cells, Peyer's patches, and spleen extraction.

Serum and fecal pellet preparation. Blood was collected from anesthetized mice from the retroorbital plexus. Serum was obtained after overnight incubation at 4^{0}C and centrifugation at 1,000 x g for 15 min. Serum samples were aliquoted, stored at -80^{0}C prior to monitor for immunoglobulins.

Fecal samples were prepared as described by de Vos and Dick (1991). Briefly, feces were collected, aseptically weighted and placed into centrifuge tubes. Ten ml of 0.01 M phosphate buffered saline (PBS) per gram of feces (v/w) were added, and the mixture was

incubated for 15 min at room temperature. Samples were mixed by vortexing until suspended, left to settle for 15 min, mixed again, and centrifuged at 22,000 x g for 10 min in a Sorval RC 5C centrifuge (Du Pont Co., Wilmington, DE). The supernatant was removed and stored at -80^{0}C for immunoglobulin measurement.

Leukocyte preparation. Peritoneal cells were prepared by injecting 2-3 ml RPMI 1640 medium containing 10% (v/v) heat inactivated fetal bovine serum (FBS) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 5 x 10^{-5} M 2-mercaptoethanol, 25 mM Hepes buffer, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids (10% FBS-RPMI 1640) into the peritoneal cavity of euthanized mice with a 10 ml syringe fitted with a 20 gauge needle. After gentle massage of the abdomen and without withdrawing the needle, the remaining 7-8 ml of medium were injected. Abdomen was massaged again and the liquid was slowly drawn back inside the syringe. After all the fluid was evacuated with the syringe, a small hole was cut in the peritoneum and the remaining fluid was collected with a sterile Pasteur pipette. The lavage fluid was centrifuged 800 x g for 5-10 min and the supernatant discarded. Ten ml of same medium was added to resuspend the cells. Cells were counted with a Neubauer hemacytometer to determine number of cells per ml.

Lymphocyte preparation. Peyer's patches were removed, teased apart, and passed through 85-mesh stainless-steel screen. Cells were suspended in 5 ml 10% FBS-RPMI 1640 medium, washed by centrifugation at 450 x g for 10 min and resuspended in 2 ml of the same medium and counted. Spleen was removed aseptically, teased apart with tissue forceps

in 10 ml 10% FBS-RPMI 1640, and centrifuged at 450 x g for 10 min. Erythrocytes were lysed for 5 min at room temperature in 5 ml of a buffer containing 9 parts of 0.16 M ammonium chloride plus 1 part 0.17 M TRIS buffer (pH 7.2). Ten ml of fresh medium were added and cells were centrifuged at 450 x g for 10 min, and counted.

Leukocyte cultures. Spleen, Peyer's patch lymphocytes (5×10^5 cells/ml) and peritoneal cells (1.5×10^5 cells/ml) were cultured in 24-well tissue culture plates with 10% FBS-RPMI 1640 medium in the absence (unstimulated) and in the presence of inducing agents as lipopolysaccharide (LPS, 1 μg/ml) from *Salmonella typhimurium*, phorbol 12-myristate-13 acetate (PMA, 10 ng/ml) and ionomycin (I, 0.5 μg/ml) and incubated at 37^0C in 7% CO_2 atmosphere. The tissue culture supernatants were collected after 2 and 5 days and monitored for cytokine levels (IL-12, IL-6, IFN-γ, TNF-α) and IgA production by ELISA.

Cytokine quantification. Cytokines were quantitated in supernatants by enzyme-linked immunosorbent assay (ELISA). Immunolon 4 Removawell microtiter strips (Dynatech Laboratories Inc., Chantilly, VA) were coated overnight at 4^0C with 50 μl/well of a purified rat anti-mouse cytokine capture antibody (PharMingen, San Diego, CA) in 0.1M sodium bicarbonate buffer, pH 8.2 (1 μg/ml [IL-6, TNF-α], 2 μg/ml [IFN-γ] or 0.5 mg/ml [IL-12]). Plates were washed three times with 0.01M phosphate buffered (pH 7.2) saline (PBS) containing 0.2% Tween 20 (PBS-T). Plates were blocked with 300 μl of 3% (w/v) bovine serum albumin (BSA) in PBS (BSA-PBS) at 37^0C for 30 min and washed 3 times with the PBS-T. Standard murine cytokines (PharMingen) or samples were diluted in 10% (v/v)

FBS-RPMI 1640, and 50 µl aliquots were added to appropriate wells. Plates were incubated at 37^{0}C for 60 min, washed 4 times with PBS-T, and 50 µl of biotinylated rat anti-mouse cytokine detection monoclonal antibody (1.5 µg/well; PharMingen) diluted in BSA-PBS were added to each well. Plates were incubated at room temperature for 60 min, washed six times with PBS-T and 1 additional time with distilled water. Fifty µl of streptavidin-horseradish peroxidase conjugate (1.5 µg/well; Sigma Chemical Co., St. Louis, MO) diluted in BSA-PBS were added to each well and plates were incubated at room temperature for 60 min. Plates were then washed 8 times with PBS-T and two more times with distilled water, and 100 µl of substrate [10 mM citric phosphate buffer (pH 5.5), containing 0.4 mM tetramethylbenzidine (TMB; Fluka chemical Corp., Ronkonkoma, NY) and 1.2 mM H_2O_2] were added to each well. The reaction was stopped by adding an equal volume (100 µl) of 6N H_2SO_4. Absorbance was read at 450 nm on a Vmax kinetic Microplate reader (Molecular Devices Co.) and cytokine concentrations were quantitated by using Vmax Software. The sensitivity of this assay was 0.5 ng/ml for IL-6, 1.2 ng/ml for IFN-γ, 3 ng/ml for TNF-α and 75 pg/ml for IL-12.

Immunoglobulin quantification. IgA and IgG were quantitated in supernatants and sera by enzyme-linked immunosorbent assay (ELISA). Immunolon 4 Removawell microtiter strips (Dynatech Laboratories Inc., Chantilly, VA) were coated overnight at 4^{0}C with 50 µl/well of a heavy-chain specific goat anti-mouse IgA or IgG (Capel Worthington, Malvern, PA) (10 µg/ml) in 0.1M carbonate buffer, pH 9.6. Plates were washed three times with 0.01M phosphate buffered (pH 7.2) saline (PBS) containing 0.2% (v/v) Tween 20 (PBS-T).

To reduce nonspecific protein binding, plates were blocked with 300 µl of 1% (w/v) bovine serum albumin (BSA) in PBS (BSA-PBS) at 37^{0}C for 30 min and washed 3 times with the PBS-T. Standard mouse reference serum (ICN Immunobiologicals, Costa Mesa, CA) or samples were diluted in BSA-PBS (sera) or in 10% (v/v) FBS RPMI-1640 (supernatants), and 50 µl were added to appropriate wells. Plates were incubated at 37^{0}C for 60 min, washed 5 more times, and then 50 µl of goat anti-mouse immunoglobulin horseradish peroxidase (γ- or α- chain specific, Cappel Worthington, Malvern, PA) diluted 1:1000 for IgA and 1:500 for IgG in BSA-PBS was added to each well. Plates were incubated at 37^{0}C for 30 min and washed 6 times with PBS-T. Bound peroxidase was determined with 2,2'-azino-bis (3-ethylbenz-thiazoline-6 sulfonic acid) (ABTS) substrate [0.4 mM ABTS, 50 mM citrate buffer (pH 4.0), and 1.2 mM hydrogen peroxide] as described previously by Pestka et al., 1980. Absorbance was measured at 405 nm on a Vmax kinetic Microplate reader (Molecular Devices' SOFTmax, Molecular Devices Co., Menlo Park, CA) and immunoglobulins were quantified using the Vmax software. The sensitivity of this assay was 0.1 µg of IgA or IgG per ml.

NO production. The method of Dietert et al., (1995) was used to detect nitrite production by macrophages. Cells were cultured as described above for 2 and 5 days and nitric oxide (NO) production was assessed by measuring nitrite accumulation, a stable metabolic product of NO, in the culture supernatants. Nitrite concentrations were determined by the Griess reaction. Equal amounts of N-(1-naphthyl) ethylenediamine dihydrochloride (NED; Sigma) [100 mg dissolved in 100 ml of distilled water] and sufanilamide (Sigma) [1

g dissolved in 100 ml of a 5% phosphoric acid solution] solutions were mixed prior to each assay (Griess reagent or chromogenic reagent). Nitrite standards [2 mM stock solution; from 0 to 200 μM] were diluted in the same media in which the cells were suspended. Equal amounts of Griess reagent and $NaNO_2$ standards or samples (100 μl) were placed in a 96 well plate in duplicate and incubated for 5 min at room temperature to allow the chromophore to develop and stabilize. Absorbance was read at 550 nm using the Vmax kinetic Microplate reader (Molecular Devices Co.) and nitrite concentrations were quantitated by using Vmax Software. The sensitivity of the assay was 4 μM.

2.3.3 Statistical methods

Data summary was done with descriptive statistics such as the mean and standard error of the mean. Statistical comparisons of treatment and control groups were analyzed using a Student's *t* test for comparison between two groups or by Dunnett's test following one way analysis of variance (ANOVA) using the SigmaStat Statistical Analysis System (Jandel Scientific, San Rafael, CA). A *p* value of less than 0.05 was considered statistically significant.

2.4 RESULTS

2.4.1 Viability of lactic acid bacteria.

An initial study was performed to determine the ability of representative lactic acid bacteria to recover, remain viable and in high numbers after being frozen at -80^{0}C over 15 days. MRS agar was used to enumerate these cells after being frozen for 0, 2, 5, 10, and 15 days. Table 2.3 summarizes the survival of several lactic acid bacteria suspended in NFDM at this temperature. None of the tested lactobacilli exhibited a significant decrease in cell concentration, which verified that primarily viable organisms would be used in *in vivo* studies. Since all species and strains tested showed a similar behavior, I assumed a similar trend for the rest of the species used in my studies.

2.4.2 Study I.- *In vivo* effects of lactic acid bacteria on cytokine mRNA expression.

All animals appeared healthy during these experiments. For all *in vivo* studies, weight gain in mice was monitored to determine if any change occurred due to feeding lactic acid bacteria during 1 and 2 weeks (Figure 2.1). No significant effects on weight gain were observed in mice fed lactic acid bacteria as compared to NFDM controls.

To assess potential immune modulation of bacterial feeding, basal cytokine mRNA expression in spleen, Peyer's patches and mesenteric lymph node was evaluated after administration of lactic acid bacteria for 1, 7 and 14 days at 1.0 x 10^9 cells per dose per day. Figure 2.2 represents a typical result of exposure to viable and non viable lactic acid bacteria (*L. bulgaricus* 1489 [NCK 231] in this case) for basal expression of IFN-γ and TNF-α

TABLE 2.3. Effects of freezing (-80^{0}C) on survival of lactic acid bacteria

Days	0	2	5	10	15
Microorganisms	log (CFU/ml)[a]				
L.bulgaricus Lr-79	9	9.8	9.7	10	10
L.bulgaricus Lr-78	10.3	10.3	10.3	10.3	9.3
L.bulgaricus Lr-28	9.5	9.5	9.5	9.6	8.5
L.acidophilus La-7	10	11	11	10.8	10.9
L. acidophilus La-1	9.8	10.5	10.5	10.6	10.5
L.acidophilus ATCC 53545	9	9	9	9	9
L. acidophilus ATCC 521	9.3	8.7	8.9	8.6	9
L.casei ATCC 334	9	9	9	9	ND[b]
L.casei ATCC 39539	9.5	9.8	9.8	9.8	9.8

[a]Bacteria were plated in MRS agar, incubated for 48 h at 37^{0}C. The bacterial count is reported as the log of colony forming units per ml.

[b] ND = non determined

Figure 2.1. Weight gain of mice fed lactic acid bacteria for 7 and 14 days (1 x 10^9 cells/dose/day). Data are mean ± S.E.M (n = 8) and representative of three different experiments.

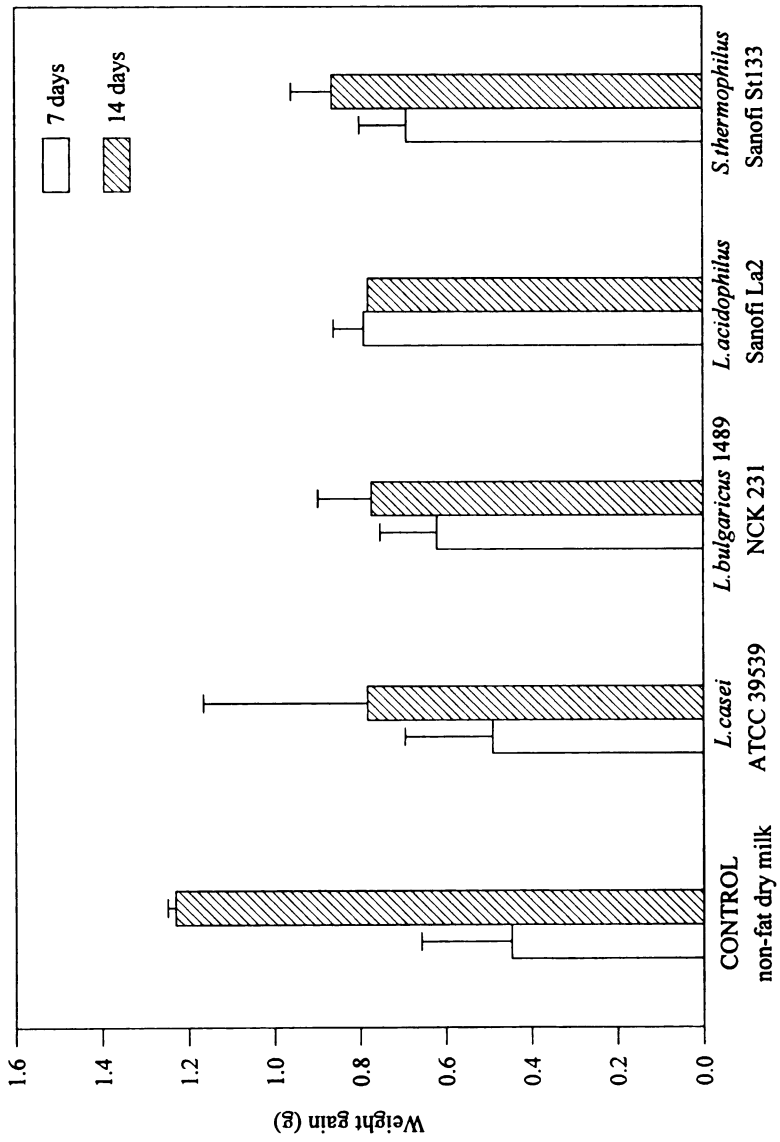
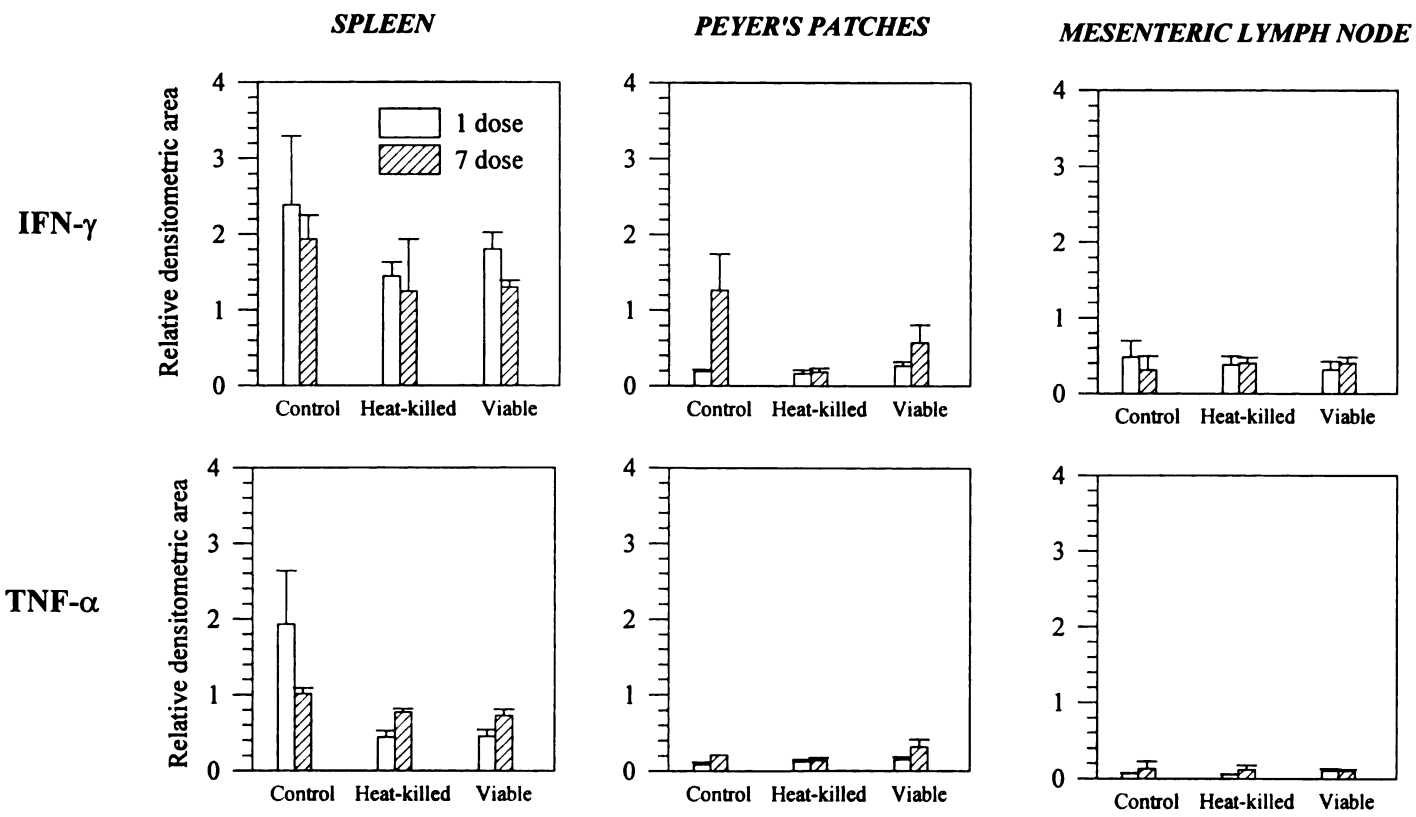

7 days
14 days
Weight gain (g)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CONTROL
non-fat dry milk
L.casei
ATCC 39539
L.bulgaricus 1489
NCK 231
L.acidophilus
Sanofi La2
S.thermophilus
Sanofi St133

Figure 2.2. Cytokine mRNA levels in mice after oral exposure to live and dead *Lactobacillus bulgaricus* 1489 (NCK 231) for 1 and 7 days. Data are mean ± S.E.M (n = 6) and are representative of two separate experiments.


SPLEEN
PEYER'S PATCHES
MESENTERIC LYMPH NODE
IFN-γ
TNF-α
Relative densitometric area
1 dose
7 dose
0
1
2
3
4
Control
Heat-killed
Viable

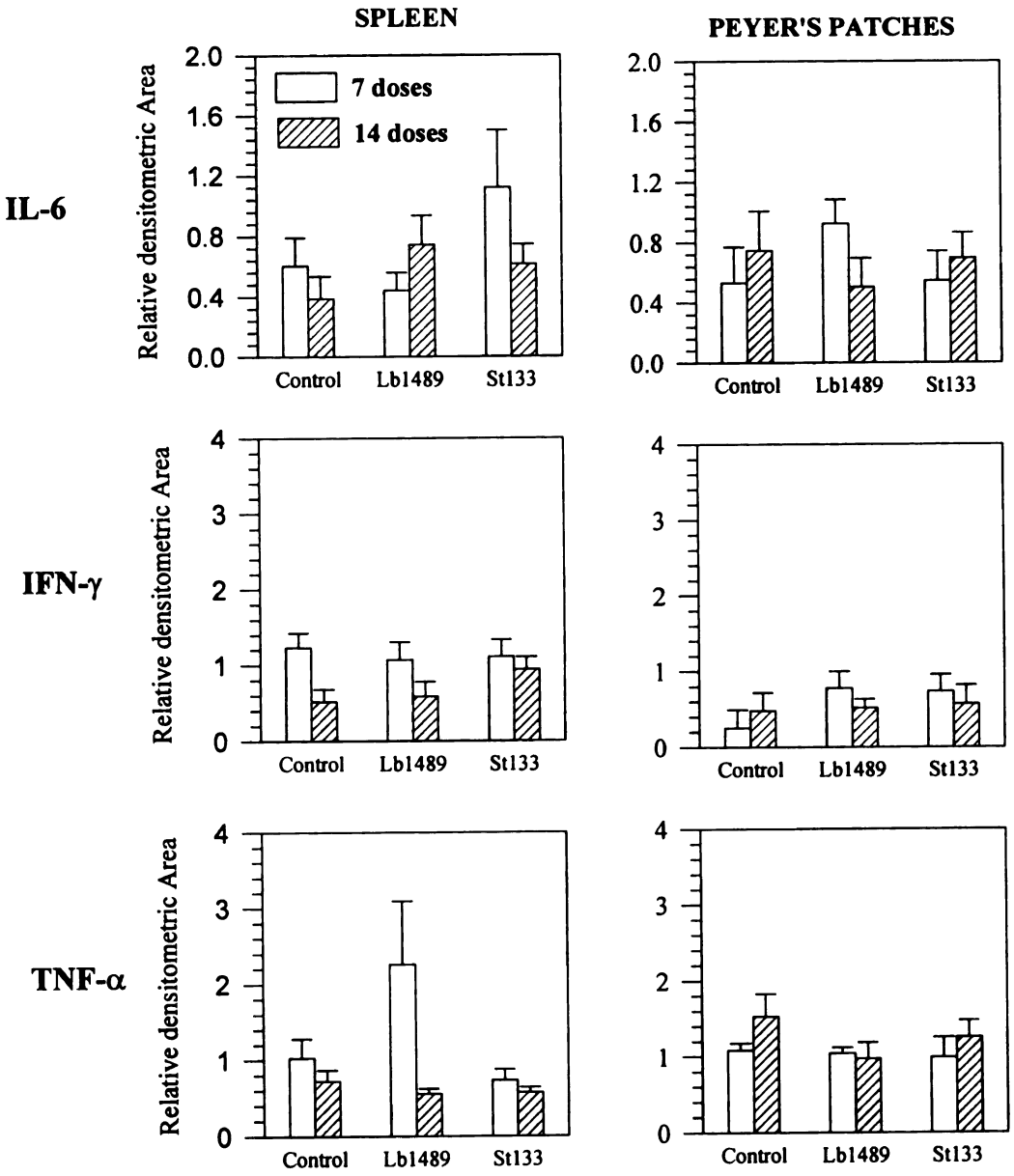
mRNA. In spleen, basal expression of IFN-γ and TNF-α mRNA was higher than in Peyer's patches and mesenteric lymph node for all groups. No difference was found between treatment and controls of the species tested (*Lactobacillus casei* ATCC 39539 and *L. bulgaricus* 1489 [NCK 231]) regardless of whether viable or non-viable bacteria were used.

When *L. bulgaricus* 1489 (NCK 231) and *Streptococcus thermophilus* St-133 were administered for 1 and 2 weeks (1 x 10^9 cells/mouse/day), significant induction or inhibition was not observed for the basal mRNA for any cytokine tested in either spleen or Peyer's patches (mesenteric lymph node was not tested) (Figure 2.3). Expression of IL-6, IFN-γ and TNF-α mRNA was similar for mice treated during 1 and 2 weeks with *L. bulgaricus* 1489 (NCK 231), *S. thermophilus* St-133 or NFDM used as a control. These results typify those obtained from 3 separate experiments not only for *L. bulgaricus* 1489 (NCK 231), *S. thermophilus* St-133, but also for *L. bulgaricus* 1489 (NCK 231), *S. thermophilus* St-133, *L. casei* ATCC 39539 and *L. acidophilus* La-2 (data not shown).

2.4.3 Study II.- *Ex vivo* effects of lactic acid bacteria on cytokine, nitric oxide and immunoglobulin production.

The effects of exposure to different strains of *L. acidophilus*, *L. bulgaricus*, *L. casei*, *L. helveticus*, *L. gasseri*, *L. reuteri* and *S. thermophilus* in mice on cytokine production were assessed in mitogen stimulated and unstimulated leukocyte cultures. Mice were fed a single dose of a microbe suspension and sacrificed after 8 hours. Peyer's patch and spleen cells were isolated, peritoneal cells were extracted, and cultured with or without mitogens (LPS or PMA + ionomycin [PMA+I]) for 2 and 5 days. LPS induces activation of B cells, antigen

Figure 2.3. Cytokine mRNA levels in mice after oral exposure to *Lactobacillus bulgaricus* 1489 (NCK 231) and *Streptococcus thermophilus* St- 133 for 7 and 14 days. Data are mean ± S.E.M (n = 6) and are representative of three separate experiments.


SPLEEN
PEYER'S PATCHES
IL-6
IFN-γ
TNF-α
Relative densitometric Area
7 doses
14 doses
Control
Lb1489
St133
0.0
0.4
0.8
1.2
1.6
2.0
0
1
2
3
4

presenting cells and macrophages (Beutler and Kruys, 1995). PMA is an activator of protein kinase C while I is a calcium ionophore which increases the levels of intracellular calcium (Truneh et al., 1985). Together they are strong enhancers of T cell activation.

Lactic acid bacteria did not affect cytokine production by Peyer's patch and spleen cell supernatants (TNF-α, IL-6, IL-12, and IFN-γ, or NO). Furthermore, TNF-α was undetectable in spleen, Peyer's patch or peritoneal cultures. However, IL-6 was produced in considerable amounts by peritoneal cells from mice fed NFDM, *L. bulgaricus* 1489 (NCK 231), *S. thermophilus* St-133, *L. casei* ATCC 39539 and *L. acidophilus* La-2, when stimulated with LPS or with PMA + I, with LPS treated cells showing the highest IL-6 levels (Figure 2.4). Effects on cytokine production by peritoneal cells were strain dependent. IL-6 was also produced in appreciable amounts by peritoneal cells from mice fed *L. helveticus* Lr-92, *L. gasseri* ADH (NCK 101), *L. reuteri* ATCC 23272, and *Bifidobacterium* Bf-1 when cells were stimulated with LPS, but synthesis of this cytokine seemed to be inhibited when these same cells were stimulated with PMA + I (Figure 2.4).

Peritoneal cells stimulated with PMA + I but not LPS showed a high capability for IFN-γ production (Figure 2.5). Those cells coming from mice fed just one dose of *L. bulgaricus* 1489 (NCK 231), *S. thermophilus* St-133, *L. casei* ATCC 39539 and *L. acidophilus* La-2 produced a equivalent or greater IFN-γ level when compared to the control group fed NFDM. In contrast, those cells coming from mice treated with *L. helveticus* Lr-92, *L. gasseri* ADH (NCK 101), *L. reuteri* ATCC 23272, and *Bifidobacterium* Bf-1, exhibited markedly depressed IFN-γ production as it was observed for IL-6.

Figure 2.4. IL-6 levels in peritoneal cell (1.5 x 10^5 cells/ml) cultures from mice fed a single dose of lactic acid bacteria. Peritoneal cells were obtained 8 h after administration of lactic acid bacteria as described in methods and cultured in the absence and in the presence of inducing agents (LPS, 1 μg/ml; PMA, 10 ng/ml + Ionomycin, 0.5μg/ml). Culture supernatants were collected after 2 and 5 days and assayed for IL-6 by ELISA. **Lb**= *L. bulgaricus*, **St**= *S. thermophilus*, **Lc**= *L. casei*, **La**= *L. acidophilus*, **Lh**= *L. helveticus*, **Lg**= *L. gasseri*, **Lr**= *L. reuteri*, **Bf**= *Bifidobacterium*. Data are mean ± S.E.M of duplicate cultures (n = 6) and are representative of two separate experiments. (*) indicates significant differences with respect to the control group fed skim milk ($p \leq 0.05$).

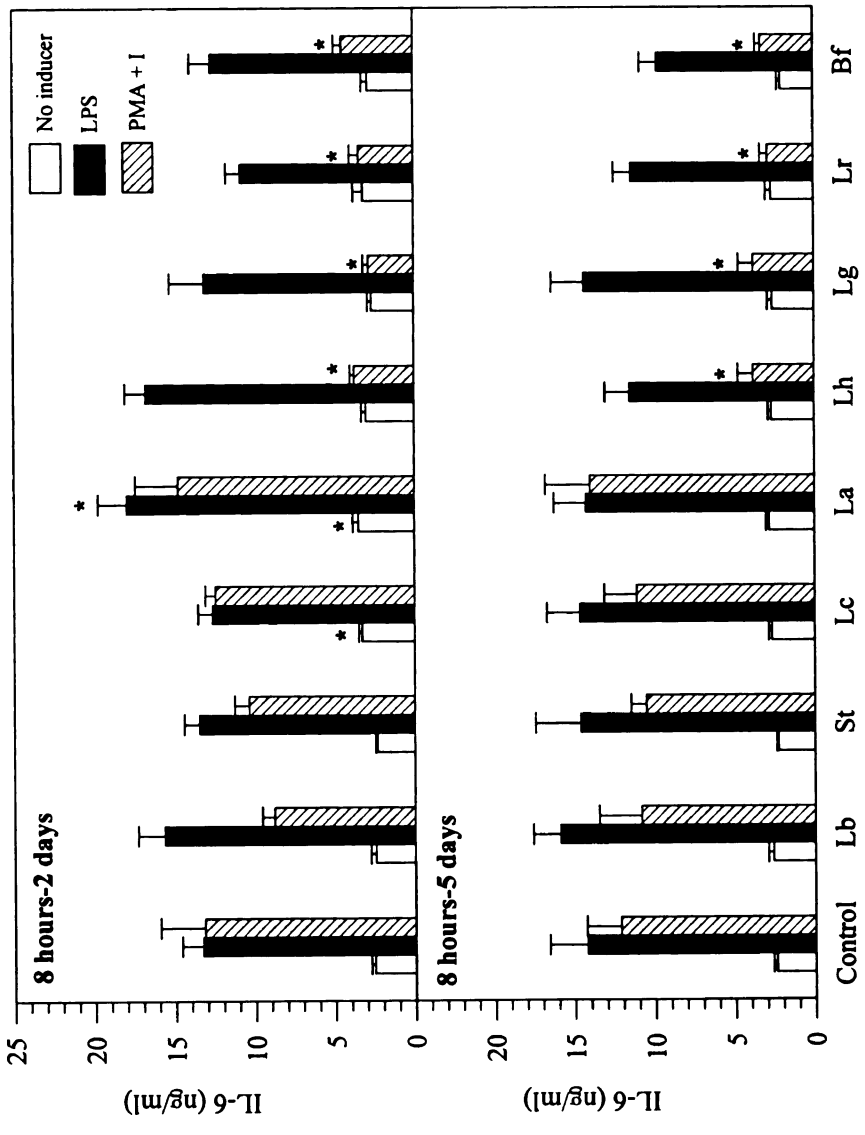
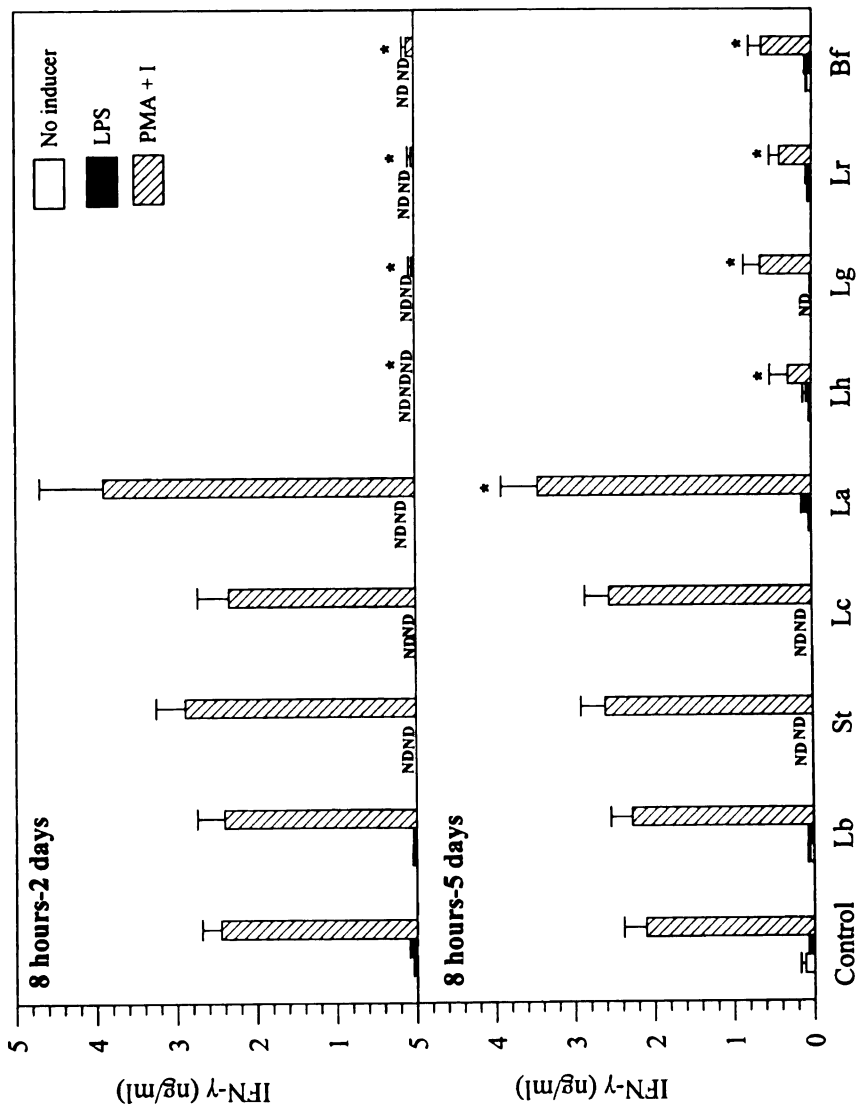

No inducer
LPS
PMA + I
8 hours-2 days
8 hours-5 days
IL-6 (ng/ml)
0
5
10
15
20
25
Control
Lb
St
Lc
La
Lh
Lg
Lr
Bf
*

Figure 2.5. IFN-γ levels in peritoneal cell (1.5 x 10^5 cells/ml) cultures from mice fed a single dose of lactic acid bacteria. Peritoneal cells were obtained **8 h** after administration of lactic acid bacteria as described in methods and cultured in the absence and in the presence of inducing agents (LPS,1 μg/ml; PMA, 10 ng/ml + Ionomycin, 0.5μg/ml). Culture supernatants were collected after 2 and 5 days and assayed for IFN-γ by ELISA. **Lb**= *L. bulgaricus*, **St**= *S. thermophilus*, **Lc**= *L. casei*, **La**= *L. acidophilus*, **Lh**= *L. helveticus*, **Lg**= *L. gasseri*, **Lr**= *L. reuteri*, **Bf**= *Bifidobacterium*. Data are mean ± S.E.M of duplicate cultures (n = 6) and are representative of two separate experiments. (*) indicates significant differences with respect to the control group fed skim milk ($p \leq 0.05$). ND = not detected.


No inducer
LPS
PMA + I
8 hours-2 days
8 hours-5 days
IFN-γ (ng/ml)
IFN-γ (ng/ml)
0
1
2
3
4
5
ND
*
Control
Lb
St
Lc
La
Lh
Lg
Lr
Bf

Peritoneal cells produced more IL-12p40 when stimulated with LPS than when stimulated with PMA + I (Figure 2.6). No consistent effects on IL-12p40 were observed in cultures from treatment mice. IL-12p35 was undetectable in all cases.

NO production was relatively low after incubation of peritoneal cells for 2 d. After 5 days, levels of NO increased for those cells stimulated with PMA + I (Figure 2.7), following a pattern very similar to the one presented for IFN-γ production, with *L. helveticus* Lr-92, *L. gasseri* ADH (NCK 101), *L. reuteri* ATCC 23272, and *Bifidobacterium* Bf-1 inhibiting production of this mediator.

Regarding immunoglobulin production, sera from mice fed lactic acid bacteria revealed no difference in total IgA (Figure 2.8) and IgG (Figure 2.9) levels when compared to control mice fed with NFDM. Similar results were obtained when analyzing for coproantibodies (Figure 2.10). Supernatants from Peyer's patch and spleen cell cultures had undetectable levels of either IgA or IgG.

Figure 2.6. IL-12p40 levels in peritoneal cell (1.5 x 10^5 cells/ml) cultures from mice fed a single dose of lactic acid bacteria. Peritoneal cells were obtained 8 h after administration of lactic acid bacteria as described in methods and cultured in the absence and in the presence of inducing agents (LPS,1 μg/ml; PMA, 10 ng/ml + Ionomycin, 0.5μg/ml). Culture supernatants were collected after 2 and 5 days and assayed for IL-12p40 by ELISA. IL-12p35 was not detected for any of the supernatants tested. **Lb**= *L. bulgaricus*, **St**= *S. thermophilus*, **Lc**= *L. casei*, **La**= *L. acidophilus*, **Lh**= *L. helveticus*, **Lg**= *L. gasseri*, **Lr**= *L. reuteri*, **Bf**= *Bifidobacterium*. Data are mean ± S.E.M of duplicate cultures (n = 6) and are representative of two separateexperiments. (*) indicates significant differences with respect to the control group fed skim milk ($p \leq 0.05$). ND = not detected.

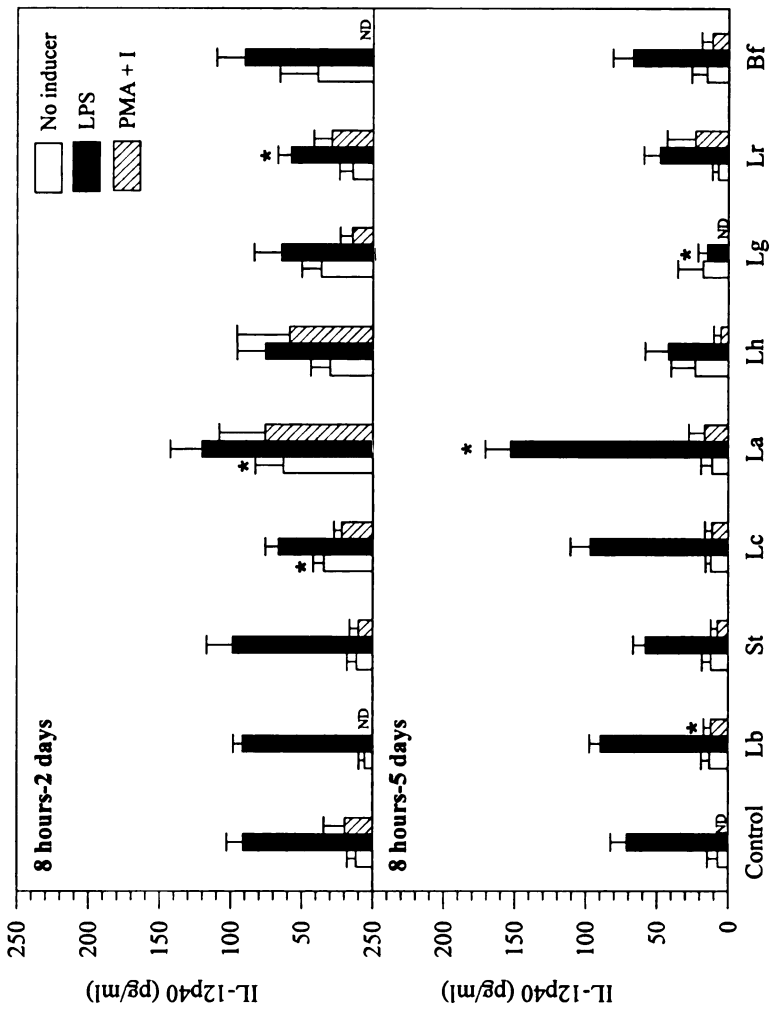

No inducer
LPS
PMA + I
8 hours-2 days
8 hours-5 days
IL-12p40 (pg/ml)
IL-12p40 (pg/ml)
250
200
150
100
50
0
ND
*
Control
Lb
St
Lc
La
Lh
Lg
Lr
Bf

Figure 2.7. Nitric oxide levels in peritoneal cell (1.5 x 10^5 cells/ml) cultures from mice fed a single dose of lactic acid bacteria. Peritoneal cells were obtained 8 h after administration of lactic acid bacteria as described in methods and cultured in the absence and in the presence of inducing agents (LPS,1 μg/ml; PMA, 10 ng/ml + Ionomycin, 0.5μg/ml). Culture supernatants were collected after 2 and 5 days and assayed for nitrate. **Lb**= *L. bulgaricus*, **St**= *S. thermophilus*, **Lc**= *L. casei*, **La**= *L. acidophilus*, **Lh**= *L. helveticus*, **Lg**= *L. gasseri*, **Lr**= *L. reuteri*, **Bf**= *Bifidobacterium*. Data are mean ± S.E.M of duplicate cultures (n = 6) and are representative of two separate experiments. (*) indicates significant differences with respect to the control group fed skim milk ($p \leq 0.05$). ND = not detected.

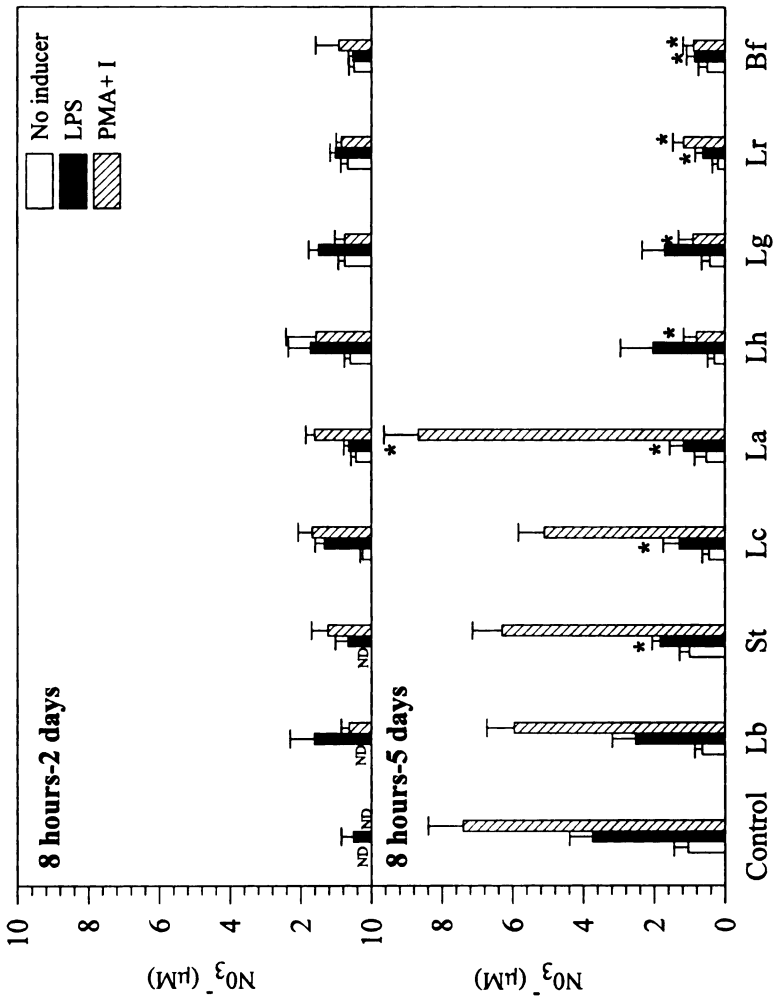

No inducer
LPS
PMA+I
8 hours-2 days
8 hours-5 days
NO_3^- (μM)
ND
*
Control
Lb
St
Lc
La
Lh
Lg
Lr
Bf

Figure 2.8. IgA levels in sera collected from mice after feeding **A.** 1 and 7 doses of *Lactobacillus bulgaricus* 1489 (NCK 231) (1 x 10^9 cells/dose/day) and **B.** 1 dose (1 x 10^9 cells) of several viable lactic acid bacteria (**Lb** = *Lactobacillus bulgaricus* 1489 [NCK 231], **St** = *Streptococcus thermophilus* St-133, **Lc** = *L. casei* ATCC 39539, **La** = *L. acidophilus* La-2, **Lh** = *L. helveticus* Lr-92, **Lg** = *L. gasseri* ADH [NCK 101], **Lr** = *L. reuteri* ATCC 23272, **Bf** = *Bifidobacterium* Bf-1). Data are mean ± S.E.M (n = 6) and are representative of several experiments.

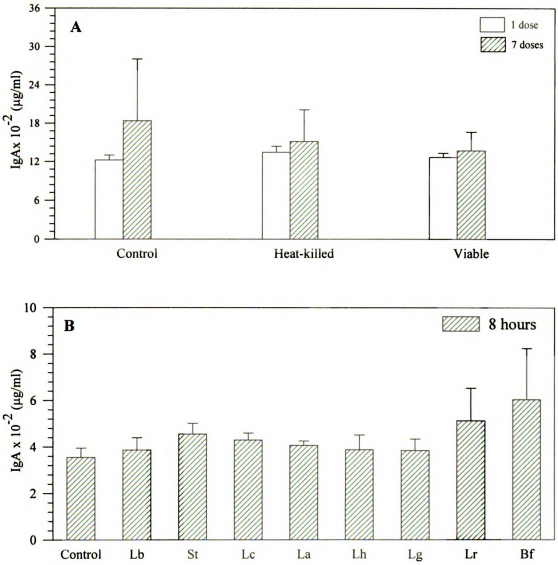
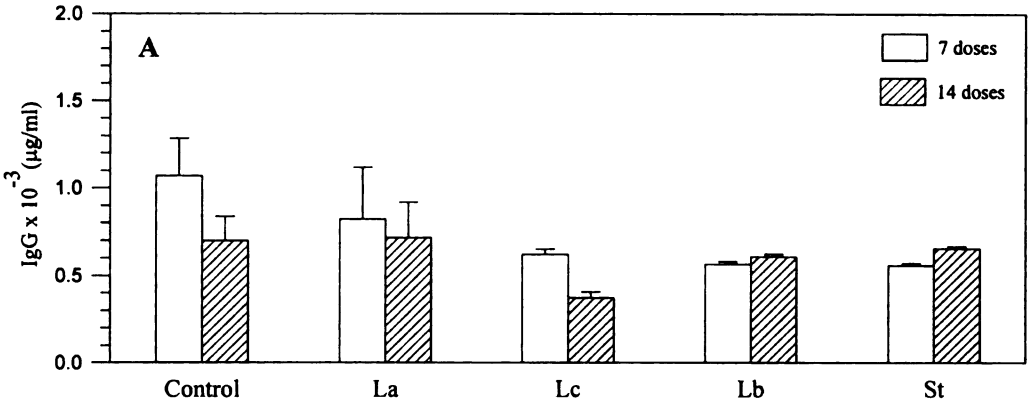

A
1 dose
7 doses
IgAx 10^{-2} (µg/ml)
36
30
24
18
12
6
0
Control
Heat-killed
Viable
B
8 hours
IgA x 10^{-2} (µg/ml)
10
8
6
4
2
0
Control
Lb
St
Lc
La
Lh
Lg
Lr
Bf

Figure 2.9. IgG levels in sera collected from mice after feeding **A.** 7 and 14 doses of **La** = *Lactobacillus acidophilus* La-2, **Lc** = *L. casei* ATCC 39539, **Lb** = *L. bulgaricus* 1489 (NCK 231), and **St** = *Streptococcus thermophilus* St-133 ($1x10^9$ cells/dose/day) and **B.** 1 dose (1×10^9 cells) of several viable lactic acid bacteria (**Lb** = *Lactobacillus bulgaricus* 1489 [NCK231], **St** = *Streptococcus thermophilus* St-133, **Lc** = *L. casei* ATCC 39539, **La** = *L. acidophilus* La-2, **Lh** = *L. helveticus* Lr-92, **Lg** = *L. gasseri* ADH [NCK 101], **Lr** = *L. reuteri* ATCC 23272, **Bf** = *Bifidobacterium* Bf-1). Data are mean ± S.E.M (n = 6) and are representative of several experiments. (*) indicates significant differences with respect to the control group fed skim milk ($p \leq 0.05$).


A
7 doses
14 doses
IgG x 10^-3 (µg/ml)
2.0
1.5
1.0
0.5
0.0
Control
La
Lc
Lb
St

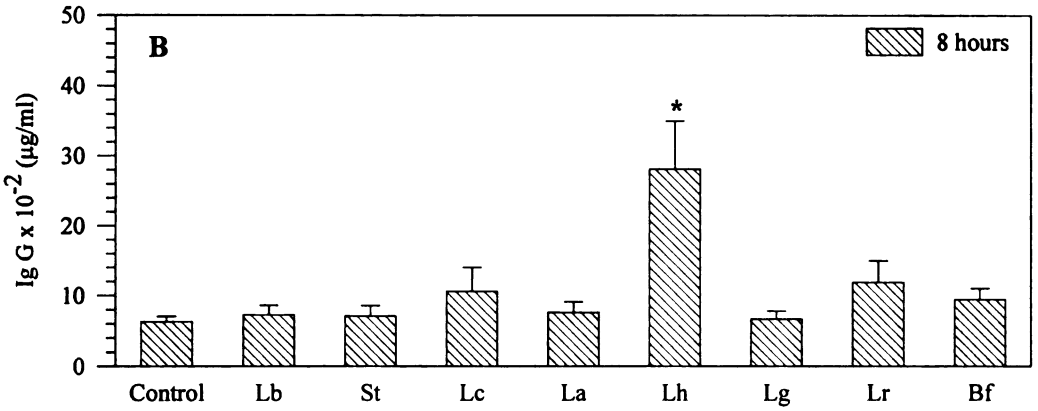
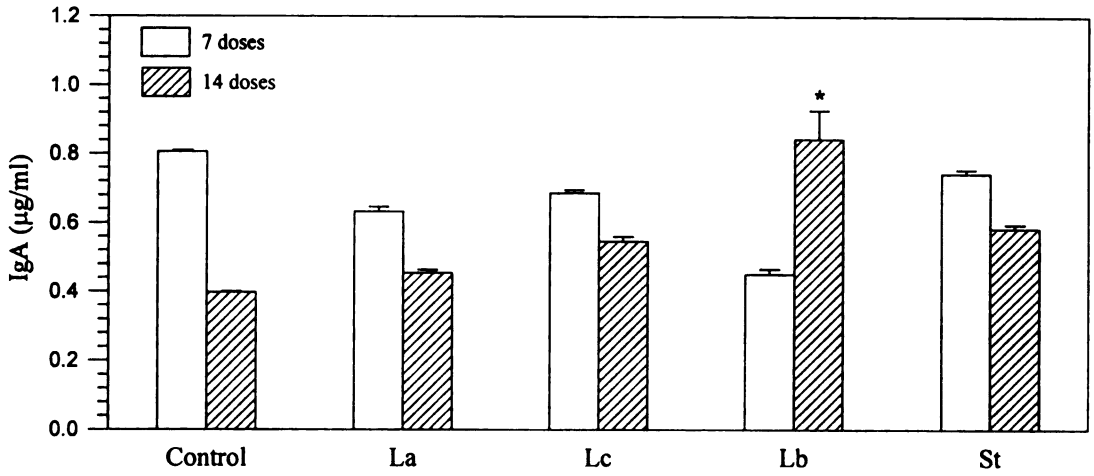

B
8 hours
Ig G x 10^-2 (µg/ml)
50
40
30
20
10
0
*
Control
Lb
St
Lc
La
Lh
Lg
Lr
Bf

Figure 2.10. IgA levels in feces collected from mice after feeding 7 and 14 doses of **La** = *Lactobacillus acidophilus* La-2, **Lc** = *L. casei* ATCC 39539, **Lb** = *L. bulgaricus* 1489 (NCK 231), and **St** = *Streptococcus thermophilus* St-133 (1×10^9 cells/dose/day). Data are mean ± S.E.M (n = 6) and are representative of several experiments. (*) indicates significant differences with respect to the control group fed skim milk ($p \leq 0.05$).


1.2
1.0
0.8
0.6
0.4
0.2
0.0
IgA (μg/ml)
7 doses
14 doses
*
Control
La
Lc
Lb
St

2.5 DISCUSSION

Although several studies have reported the *in vitro* effects of administration of lactic acid bacteria on cytokine production, very little information is available about cytokine production *in vivo*. This study was an attempt to determine potential *in vivo* effects of oral administration of lactic acid bacteria on cytokine gene expression in mucosal and systemic sites. The main findings of study I were that: (1) repeated administration of lactic acid bacteria did not affect the growth rate of mice and, (2) basal mRNA expression of IL-6, IFN-γ and TNF-α was not affected by repeated lactic acid bacteria administration. From study II, it can be concluded that: (1) a single exposure to certain lactic acid bacteria altered subsequent mitogen induced cytokine and nitric oxide production by peritoneal cells, (2) immunoglobulin levels were not affected either at the mucosal or systemic sites.

Freezing between -20^{0}C to -80^{0}C produces variable results depending on bacterial species. The use of NFDM to resuspend bacterial cells has been reported to be successful in long-term preservation for small volumes due to a cryoprotective effect (Silliker et al., 1980). Typically some bacteria can be kept for 6 months to 2 years by this method. Here, I grew cells under optimum conditions and to a sufficient number of cells to provide a good cell suspension. Thus, ordinary freezing proved to be a good means for preserving the viability of my bacterial cultures for studies.

I chose to measure basal cytokine mRNA in spleen, Peyer's patches and mesenteric lymph node because they represent the systemic and mucosal immune compartments. The gut immune response is induce in the Peyer's patches where activation,

switching, proliferation and differentiation of B cells is under the control of T cells, cytokines and other accessory cells (Ogra et al., 1994). As an initial approach I decided to analyze basal cytokine mRNA instead of serum cytokine levels for several reasons: 1) the short half life of some cytokines makes their quantitation a weak indicator of cytokine production *in vivo*; 2) uptake of cytokines by some receptors makes it difficult to accurately measure these proteins. I chose a very sensitive technique, RT-PCR which includes amplification of mRNA present on a tissue sample for a desired cytokine (Svetic et al., 1991).

The *in vivo* results may differ from previous *in vitro* studies because lactic acid bacteria probably do not survive digestion. For those bacteria to target the intestine, they have to tolerate a number of physical and chemical conditions present in the gastrointestinal tract, i.e. gastric acid and small intestinal secretions. Additionally, lactic acid bacteria may need to establish and become active in the gut prior to having immune effects. The influences of various factors of host and of microbial origin, that participate in the formation of an individual gastrointestinal microbial ecosystem, are considerable. This creates great methodological limitations for *in vivo* research (Sanders, 1993). The intestinal microflora, one of the most complex ecological niches, are still not fully understood. Many types of facultative anaerobic bacteria live together, influencing each other. It is very difficult to predict these interactions and obviously there is great difficulty in finding individuals with similar or identical GI ecosystems. Thus, potential beneficial effects of lactic acid bacteria often prove to be statistically insignificant when attempts are made to confirm the conclusions made by different authors.

The result observed in my *ex vivo* study (study II) with peritoneal cells suggests that

macrophages are a key target for lactic acid bacteria immunomodulation activity. Oral lactic acid bacteria administration to mice altered peritoneal cell activity *in vitro*. The effect of various strains relative to their capacity to secrete IL-6, IFN-γ, IL-12 and NO ranged from marginal stimulation or no effect to inhibition. The potential to alter macrophage function is very important for several reasons. Macrophages constitute the second major cell population of the immune system with phagocytosis as their primary function. They originate in bone marrow, and after migration and maturation they settle in tissues as mature macrophages (Janeway and Travers, 1997). They can be activated by a variety of stimuli and their principal function includes phagocytosis of foreign particles, production of cytokines (IL-6, TNF-α, IL-1, IL-12) that recruit other inflammatory cells and act as antigen-presenting cells. Thus, macrophages can participate in humoral immune responses and/or as effector cells of cell-mediated immunity (as antigen presenting cells).

This study examined the effect on peritoneal cells of different mitogenic signals. These include lipopolysaccharide (LPS) from *Salmonella typhimurium*, which is a B-cell and macrophage activator (Beutler and Kruys, 1995), and a combination of phorbol-12-myristate-13-acetate (PMA), which activates protein kinase C (PKC), and ionomycin (I), which increases the concentration of intracellular calcium [Ca^{2+}] (Truneh et al., 1985). The latter two compounds can affect both macrophages and T cells. The peritoneal cavity is frequently used as a concentrated source of mature murine macrophages. Peritoneal lavage yields 2 - 8 x 10^6 cells of which 20 - 40% are macrophages, but there is also contamination by other cell types (Herzenberg et al., 1997). B cells represent 10 - 40 % (Kim et al., 1996), and thus, T cells and other type of cells as natural killer (NK cells) may represent at least

20%. Thus other cells besides macrophages may contribute to the findings observed in peritoneal cell cultures.

It should be noted that vehicle plus dietary factors may influence basal levels in control groups in this study. Casein is a dietary component present in NFDM and in murine diet AIN-93G which could be able to produce a mucosal response in fed-NFDM groups. Peptides derived from casein were shown to contain an immune activating factor (Yamauchi and Suetsuna, 1993). Diets containing between 20-40% of casein increase cellular immunity in spleen cells and macrophages (Ueda et al., 1990).The casein fraction is composed of different proteins (α, β, and κ). Notably, κ casein triggers lymphocyte proliferation (Werfel et al., 1996). Also, β-casein promotes cellular and humoral responses (Cavallo et al., 1996). During enzymatic digestion of human and bovine caseins, peptides are formed and released, which are able to stimulate phagocytic activity of murine and human macrophages and these apparently exert *in vivo* a protective effect against infections (Migliore-Samour et al., 1989).

Other authors have similarly reported that macrophage activation occurs after lactic acid bacteria administration. Saito et al. (1987) found that intramuscular injection to mice with heat-killed *L. casei* LC 99018 increased macrophage function. Perdigon et al. (1986a) observed an enhanced macrophage and lymphocytic activity by administering a mixed culture of *Lactobacillus acidophilus* ATCC 4356 and *L. casei* CRL 431 to mice. This same group later reported a similar conclusion in a feeding study with *L. casei* and *L. acidophilus* to mice infected with a lethal dose of *Salmonella typhimurium* (Perdigon et al., 1990). *L. casei* and *L. bulgaricus* were also studied and found that both given either orally or intraperitoneally were able to activate peritoneal macrophages in mice from day 2 onward

(Perdigon et al., 1986b) and they suggested that these bacteria, when passing through the intestinal tract, may be responsible for an enhanced host immune response. *L. bulgaricus* was generally less effective than *L. acidophilus* increasing the activity of peritoneal macrophages measured by phagocytic assay. Similar results were obtained when using *Streptococcus thermophilus* and *L. acidophilus* (Perdigon et al., 1987) or fermented milks with *L. casei, L. acidophilus* or/and a mixture of both (Perdigon et al., 1988, 1991b, 1992).

Lactic cultures have been shown to stimulate cytokine production in earlier studies. Halpern et al. (1991) reported a rise in serum IFN-γ production in young human adults when they consumed two cups of yogurt daily. Kishi et al. (1996) reported that the oral administration of viable *L. brevis* ssp. *coagulans* produced a significant increase in IFN-α levels in human blood in a dose dependent manner, but heat treated bacteria did not elicit a similar response. Kitazawa et al. (1992, 1994) demonstrated induction of IFNs in murine peritoneal macrophages when stimulated *in vitro* with *L. acidophilus*, and an increase of the expression of mRNA encoding IFN-α in spleen-macrophages and Peyer's patch-adherent cells stimulated with *L. gasseri* DSM 20243. *L. bulgaricus* and *S. thermophilus* added to human peripheral blood lymphocytes have an adjuvant action, potentiating IFN-γ production (De Simone et al., 1986).

In my experiments, cytokine production by lactic acid bacteria (or food component) apparently involves not only macrophages which are capable of producing IL-6, IL-12 and NO, but different types of cells, as indicated by the production of IFN-γ which is not produced by macrophages. Further studies are necessary to elucidate the mechanisms involved in these processes.

IL-6 is a B-cell growth factor that operates in a paracrine manner enhancing immunoglobulin production from previously activated B-cells. IL-6 secretion usually requires a previous stimulation with other cytokines. IFN-γ is known to interfere with the induction of IL-6 mRNA accumulation and protein secretion by macrophages in humans (Cicco et al., 1990).

IFNs are proteins with a variety of physiological effects. They are produced mainly by T lymphocytes, macrophages and natural killer (NK) cells. IFN-α and β are closely related and produced by T cells and macrophages. NK cells are large lymphoid cells with many intracellular granules, which are able to recognize and kill abnormal cells even though they do not bear antigen-specific receptors. IFN-γ is produced exclusively by T lymphocytes and NK cells whereas macrophages do not express the IFN-γ gene (Kirchner, 1986).

IL-12 is a heterodimer and its principal biologic effect is upregulation of the Th1 response via induction of IFN-γ production, T-cell proliferation and NK cell-mediated cytotoxicity (Hendrzak and Brunda, 1995).

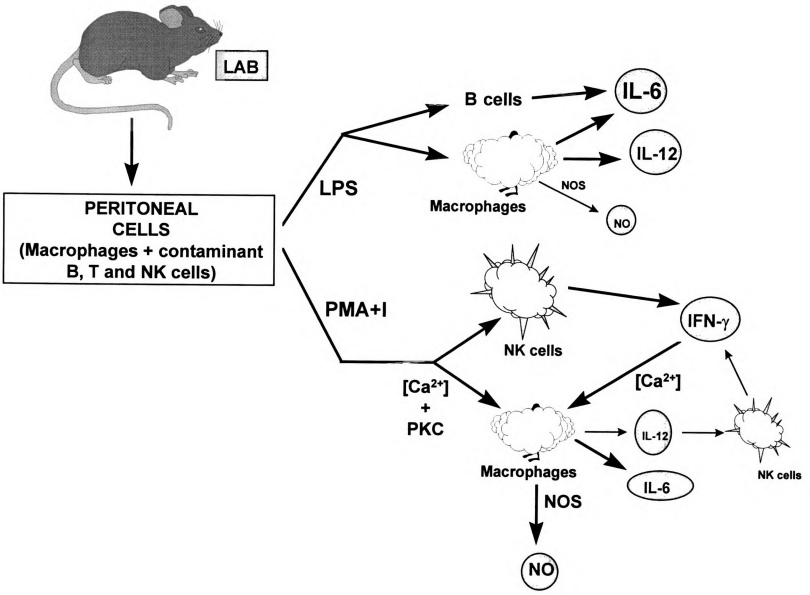
NO is produced by immune cells in response to cytokines such as TNF-α, IFN-γ and IL-2 (Lin et al., 1995) by stimulating the expression of inducible NO synthase and acting in combination with LPS in murine macrophages. IFN-γ activates macrophages and these activated macrophages are able to produce TNF-α, IL-6, IL-12, oxygen radicals and nitric oxide (NO) (Janeway and Travers, 1997). The evidence in the literature suggests that NO participates in inflammatory and autoimmune mediated tissue destruction (Yamashita et al., 1997).

In this study, elevation of IL-6 secretion was observed in LPS-stimulated peritoneal

cell cultures after a single oral exposure to lactic acid bacteria. Since B cells and macrophages are responsive to this mitogen but not T cells, it is likely that they are the cells of choice associated with the lactic acid bacteria immune effect (Figure 2.11). Also, murine peritoneal cells released IL-6 into their culture supernatants after being stimulated with PMA + I combined. The use of PMA + I together stimulates the phosphatidylinositol signal transduction pathway by increasing [Ca^{2+}] and by modulating PKC in murine cells, which is also the functional role of IFN-γ. Since IFN-γ is able to increase [Ca^{2+}] inside murine macrophages stimulating their cytotoxic activity, I believe that stimulation of murine peritoneal macrophages with PMA + I could exert the same effect. Several studies support the fact that the combination of PMA + I is capable of stimulating macrophages by mimicking IFN-γ activity (Klein et al., 1990; Gumina et al., 1991). PMA by itself does not activate macrophages to be cytotoxic what suggests that PKC activity may be required but not sufficient for macrophage activation (Gumina et al., 1991).

The results in study II are not surprising if, as I described above, PMA + I together are capable of activating macrophages. After stimulation with PMA + I, macrophages could be activated to produce IL-6 and IL-12 (Figure 2.11). IL-12 may be able to stimulate IFN-γ production by T cells or NK cells present in the peritoneal cells (Puddu et al., 1997). NO is produced from L-arginine via catalysis by an enzyme that is activated by [Ca^{2+}] (Dietert et al., 1995). Thus PKC and [Ca^{2+}] by PMA + I could increase activity of nitric oxide synthase. In my study, nitrite accumulation was enhanced when IFN-γ was present for more than 48 h following the stimulation with PMA + I. Thus IFN-γ production could be responsible for the induction of NO in murine peritoneal macrophages. In contrast, other authors (Eason and

Figure 2.11. Possible effects of mitogens [LPS from *S. typhimurium* (1 μg/ml), PMA (10 ng/ml) + ionomycin (I, 0.5 μg/ml)] on peritoneal cell cultures extracted from mice fed one dose (1 x 10^9 cells/mouse) of lactic acid bacteria.


LAB
PERITONEAL
CELLS
(Macrophages + contaminant
B, T and NK cells)
LPS
B cells
IL-6
IL-12
Macrophages
NOS
NO
PMA+I
NK cells
IFN-γ
[Ca2+]
+
PKC
[Ca2+]
IL-12
Macrophages
IL-6
NK cells
NOS
NO

Martin, 1995) found this effect when the mitogen used for stimulation was LPS, but not PMA + I alone or in combination, and when using a macrophage cell line and not primary cell culture.

Neither peritoneal cells, spleen nor Peyer's patch lymphocytes responded to LPS or PMA + I by producing TNF-α, typical cytokine produced by activated macrophages. Studies of the kinetic properties on the transcription of mRNA and secretion of IL-6, IL-1 and TNF-α showed that TNF-α mRNA transcription and protein production occur very rapidly (Martin and Dorf, 1990). TNF-α mRNA peaks 1-2 h after the stimulus was produced and the cytokine can be collected in supernatants after 2-4 h culture. IL-6 and IL-1 mRNA peak after 4-8 h and simultaneously but the protein is present at the highest concentration between 8-12 h for IL-6 and 12 h for IL-1. That means that I could have missed TNF-α detection.

The question that remains to be answered is what is the importance of the peritoneum in relation to the immune system. Bellingan et al. (1996) recently showed that macrophages migrate from the peritoneum to the draining lymph nodes. The migration process takes more than 4 h, but once started these macrophages are cleared within 96 h from the peritoneum. Thus, macrophages homing the peritoneal cavity may mediate a strong activation as a consequence of lactic acid bacteria and NFDM administration, perhaps prior to migration within gut associated lymphoid tissues. It is known that in rodents and other animal species some antigen presenting cells (dendritic cells) are able to migrate via afferent lymph into draining lymph nodes or from intestinal spaces, via lymph, to lymph nodes and via blood, to spleen (Herzenberg et al., 1997). Cells from the peritoneum also seem to be able to migrate to Peyer's patches (Heel et al., 1997; Kawabata et al., 1995), which might suggest

a possible role of peritoneal cells activating mucosal immune tissues. Kroese et al. (1989) observed that a high percentage of the IgA-secreting cells located in the lamina propria had their origin in B cells from the peritoneal cavity. There are the so called B-1 cells possessing a CD5 marker, while the conventional cells from Peyer's patches, spleen, and lymph nodes are called B-2 cells. The former have a longer life span and appear to be at a greater activation state, being able to populate the lamina propria inside the gut.

This research presented herein suggests that lactic acid bacteria can alter peritoneal cell function and this may subtly impact immunity. On a speculative level, these effects could be produced by strains possessing specific components in the cell wall or soluble substances which affect immune cells via receptors present mainly in macrophage cell surface, and perhaps in some other cells such as NK cells. Further mechanistic understanding is warranted on how these and other immunologic effects are mediated by lactic acid bacteria.

CHAPTER 3

EFFECTS OF LACTIC ACID BACTERIA AND THEIR EXTRACTS ON CYTOKINE PRODUCTION *IN VITRO*

3.1 ABSTRACT

Cells from a large number of bacterial genera as well as bacterial products have been shown to possess mitogenic and polyclonal activating properties when cultured with cells of the immune system. Notably, gram positive cell walls contain teichoic acid and peptidoglycan which have been shown to induce cytokine production by immune cells and suppress tumor development. In this study, I tested the potentiating effects of representative lactic acid bacteria and their extracts on leukocyte function. Specifically, the effects of *in vitro* exposure to heat-killed cells of *Bifidobacterium, Lactobacillus acidophilus, Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus gasseri, Lactobacillus helveticus*, *Lactobacillus reuteri*, and *Streptococcus thermophilus*, their cell walls, and their cytoplasmic extracts on proliferation, cytokine and intermediate production were examined in the RAW 264.7 macrophage cell line. A similar strategy was applied to spleen and Peyer's patch lymphocytes as well as in peritoneal cells from mice. The results suggest that lactic acid bacteria as well as their cytoplasmic and cell wall fractions are able to stimulate cloned macrophages to produce very significant amounts of TNF-α, IL-6 and nitric oxide. While similar effects were not noted in spleen and Peyer's patch cell cultures from mice, a pronounced enhancement in IL-6 production by peritoneal cells was observed when cultured with those extracts. The results suggested that as a group, the lactic acid bacteria are capable of stimulating macrophages and/or other immune cells to produce cytokines and nitric oxide.

3.2 INTRODUCTION

Cells of a large number of bacterial genera as well as bacterial products have been shown to possess mitogenic and polyclonal activating properties. As mitogens they induce DNA synthesis, blast formation and ultimately division of lymphocytes. One of the most widely studied bacterial products with mitogenic activity is lipopolysaccharide (LPS), which is present in the cell wall of gram negative bacteria. The basic molecule of LPS consists of a lipid component, the lipid A, covalently bound to a heteropolysaccharide of two distinct regions: the core oligosaccharide and the O-specific chain. It can also contain polysaccharide chains of variable lengths. LPS can activate macrophages and induce the release of many mediators and cytokines such as TNF-α, IL-1, IL-6 (Gammage, et al. 1996). Lipopolysaccharide (LPS) has also been shown to exert a mitogenic effect in B cells in mice. Since the structure of LPS is complex and variable even within the same species, the degree of lymphocyte activation can vary. Some bacterial species that represent major human pathogens are gram positive and possess a basic gram positive cell wall structure (streptococci, micrococci, staphylococci, etc.). Gram positive cell walls contain teichoic acid and peptidoglycan. These gram positive components have been shown to induce the TNF-α and IL-6 production of human monocytes (Heumann et al., 1994).

Baricault et al. (1995) reported that the use of fermented milks manufactured with lactic acid bacteria may exert an attenuating effect on cancer cell growth and differentiation. Anti-tumor activity observed with *Lactobacillus bulgaricus* is associated with the bacterial cell wall (Bogdanov et al., 1975), whereas other studies indicate a possible role for the whole

bacteria and/or cell extracts for *Bifidobacterium infantis* (Kohwi et al., 1982). Either viable or intact dead bacteria seem to be able to induce these effects. One explanation may be that tumor suppression observed is mediated through an immune response.

A number of investigations have focused on the capacity of gram positive lactic acid bacteria to alter immune function. Perdigon et al (1986a) observed enhanced macrophage and lymphocyte activity in mice after administering a mixed culture of *Lactobacillus acidophilus* and *L. casei*. This group also reported activation of peritoneal macrophages in mice after oral administration of *L. casei* and *L. bulgaricus* (Perdigon et al., 1986b). Similar result were found for *Streptococcus thermophilus*, *L. acidophilus* orally delivered (Perdigon et al., 1987) and heat-killed *L. casei* administered by injection into mice (Saito et al., 1987). Previous studies conducted in this laboratory found that heat-killed lactic acid bacteria stimulate production of IL-6 and TNF-α in RAW 264.7 macrophages (Marin et al., 1997a, 1997b, 1997c). Although many of the studies regarding lactic acid bacteria are controversial, they seem to intersect in a common result, activation of macrophages after lactic acid bacteria treatment.

In this study, we assessed the potentiating effects of representative lactic acid bacteria and their extracts on leukocyte function. Specifically we evaluated different commercial U.S. dairy strains, either as heat inactivated whole cells or as fractions (cytoplasm and cell wall) for their ability to alter immune functions by measuring effects on secretion of cytokines. Initially I determined the effects of *in vitro* exposure to *Bifidobacterium, Lactobacillus acidophilus, Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus gasseri, Lactobacillus helveticus*, *Lactobacillus reuteri*, and *Streptococcus thermophilus* and their

extracts on proliferation, cytokine and intermediate production by a macrophage cell line. I then examined the same effects on spleen and Peyer's patch lymphocytes as well as by peritoneal cells from mice. The results suggested that lactic acid bacteria as well as their cytoplasmic and cell wall fractions are able to stimulate macrophages to produce large amounts of TNF-α, IL-6 and nitric oxide (NO). Similarly, an enormous increase in production of IL-6 by peritoneal cells is observed. However these effects were not observed in spleen and Peyer's patch cell cultures.

3.3 MATERIAL AND METHODS

3.3.1 Lactic acid bacteria fractionation

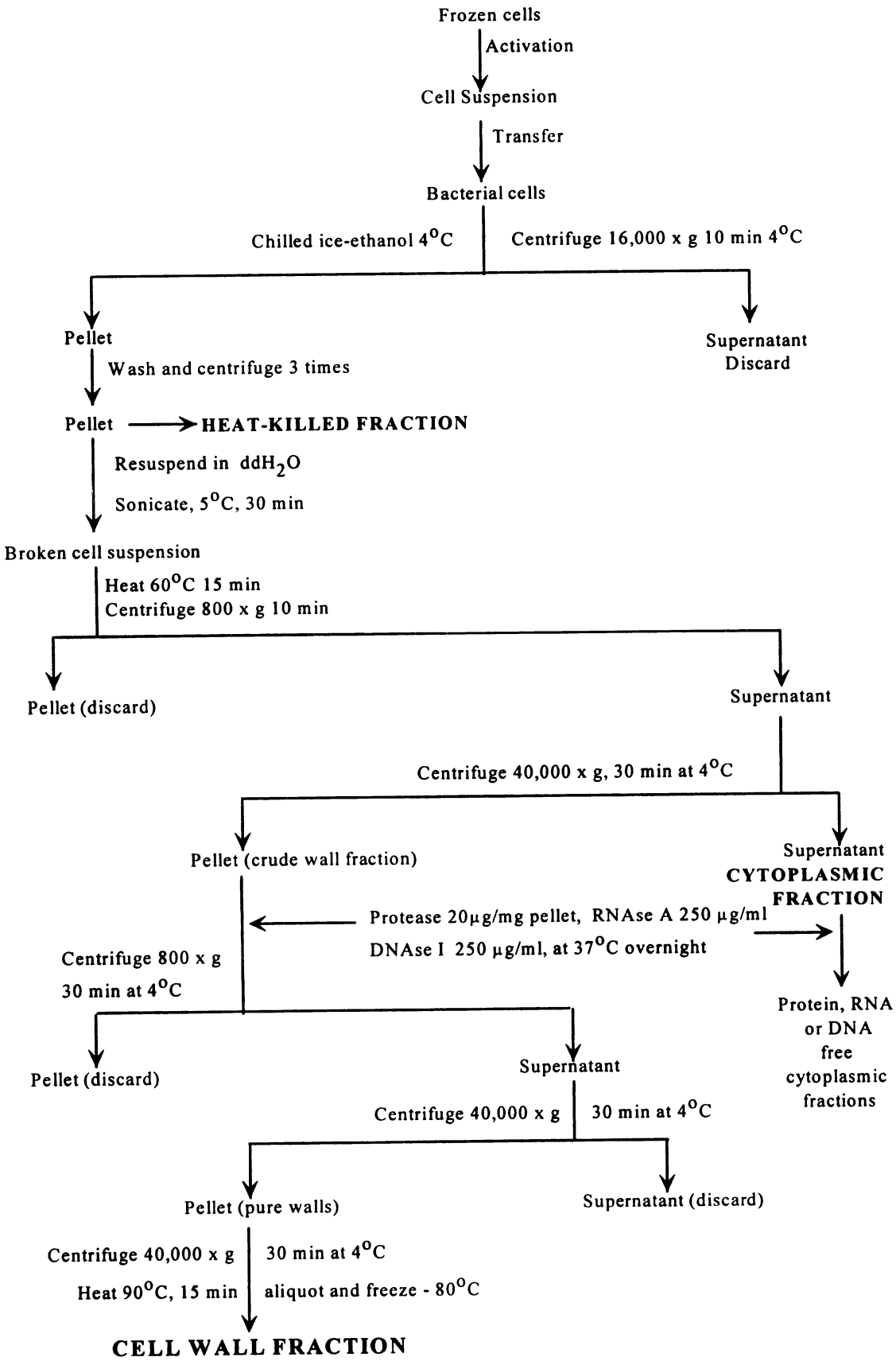
Representative cultures of *Bifidobacterium, L. acidophilus, L. bulgaricus, L. casei, L. gasseri, L. helveticus, L. reuteri, S. thermophilus* were obtained from three different sources: American Type Culture Collection (Rockville, MD), Dr. T.R. Klaenhammer (North Carolina State University) and Sanofi Bio-Industries (Waukesha, WI) (Table 3.1). From our frozen (-80^{0}C) bacterial stocks described in chapter 2, bacteria were thawed with moderate agitation in a 37°C water bath until all the ice melted. Lactobacilli and streptococci were grown in MRS (Difco) broth (De Man et al., 1960) and bifidobacteria in MRS + 5% (w/w) lactose (MRSL) broth at 37°C for 15 h. A small part of each culture (1% v/v) was transferred to fresh broth and then incubated at 37°C until exponential phase was reached. The culture was plated after being serially diluted to assess the number of bacteria per ml. Bacteria were recovered by centrifugation at 1,100 x g for 15 min.

Figure 3.1 shows an schematic representation of the cell fractionation procedure followed. For heat-killed bacterial fractions, bacterial cultures in early stationary phase were quickly chilled to 4^{0}C in an ice-ethanol bath. Bacteria were harvested by centrifugation at 16,000 x g for 10 min at 4^{0}C and then washed with sterile distilled water and centrifuged at 16,000 x g three times. Bacterial yield and number were determined by weight and by plating respectively. After resuspending in Hank's buffer saline solution (HBSS, Sigma Chemical Co., St. Louis, MO) to achieve a concentration of 25 mg/ml, the bacteria were aliquoted in 1 ml vials and heated to a temperature of 100^{0}C for 50 min, then quickly chilled

TABLE 3.1. Cultures used in study

Bacteria	Strain	Source
Bifidobacterium	Bf-1	Sanofi Bio-Industries, Waukesha, WI
Lactobacillus acidophilus	La-2	Sanofi Bio-Industries, Waukesha, WI
Lactobacillus bulgaricus 1489	NCK 23	North Carolina State University, Raleigh, NC
Lactobacillus casei	ATCC 39539	American Type Culture Collection, Rockville, MD
Lactobacillus gasseri ADH	NCK 101	North Carolina State University, Raleigh, NC
Lactobacillus helveticus	Lr-92	Sanofi Bio-Industries, Waukesha, WI
Lactobacillus reuteri	ATCC 23272	American Type Culture Collection, Rockville, MD
Streptococcus thermophilus	St-133	Sanofi Bio-Industries, Waukesha, WI

Figure 3.1. Schematic representation of lactic acid bacteria fractionation.


Frozen cells
Activation
Cell Suspension
Transfer
Bacterial cells
Chilled ice-ethanol 4°C
Centrifuge 16,000 x g 10 min 4°C
Pellet
Supernatant
Discard
Wash and centrifuge 3 times
Pellet
HEAT-KILLED FRACTION
Resuspend in ddH2O
Sonicate, 5°C, 30 min
Broken cell suspension
Heat 60°C 15 min
Centrifuge 800 x g 10 min
Pellet (discard)
Supernatant
Centrifuge 40,000 x g, 30 min at 4°C
Pellet (crude wall fraction)
Supernatant
CYTOPLASMIC FRACTION
Protease 20µg/mg pellet, RNAse A 250 µg/ml
DNAse I 250 µg/ml, at 37°C overnight
Centrifuge 800 x g
30 min at 4°C
Protein, RNA or DNA free cytoplasmic fractions
Pellet (discard)
Supernatant
Centrifuge 40,000 x g
30 min at 4°C
Pellet (pure walls)
Supernatant (discard)
Centrifuge 40,000 x g
30 min at 4°C
Heat 90°C, 15 min
aliquot and freeze - 80°C
CELL WALL FRACTION

and stored at -80^{0}C.

For cell wall and cytoplasmic fraction preparations, bacterial cells were harvested and washed as described above and then subjected to a series of fractionation steps (Work, 1971; Takahashi et al., 1993; Heumann et al., 1994). After resuspending in sterile distilled water, bacteria were disrupted by sonication for 30 min at 5^{0}C in a Branson sonifier W-350 (Branson/Sonic power Co., Danbury, Conn.). The suspension was heated at 60^{0}C for 15 min to inactivate autolytic enzymes. The suspension was centrifuged at 800 x g for 30 min at 5^{0}C and the pellet (unbroken cells) was removed. Cell walls were sedimented from the supernatant by centrifuging at 40,000 x g for 30 min. The supernatant was then aliquoted in 1 ml vials and frozen at - 80^{0}C [cytoplasmic fraction]. The pellet was further treated with protease (20 μg/mg of crude cell wall), ribonuclease and DNAse (250 μg/ml) in a 0.1 M Tris-HCl buffer (pH 7.4) at 37^{0}C overnight to eliminate the contaminating cytoplasmic material. The cell wall fraction was centrifuged at 800 x g for 30 min at 4^{0}C and then at 40,000 x g for 30 min, weighed, heated to 90^{0}C for 15 min, aliquoted at a concentration of 25 mg/ml and then frozen at - 80^{0}C [cell wall fraction]. Aliquots of cytoplasmic fraction or control buffer were treated with protease (20 μg/mg), ribonuclease or DNAse (250 μg/ml) at 37^{0}C overnight to eliminate proteins, RNA or DNA respectively. These were filter sterilized and kept at -80^{0}C until used for assay.

3.3.2 Sugar determination

Sugar content on bacterial fractions was determined using the method described by Dubois et al. (1956). Briefly, a stock solution containing 100 μg glucose/ml was prepared.

From this stock solution, serial dilutions were made and placed in glass tubes ranging from 0 to 90 μg sugar/ml (2 ml of each). Two ml (2 ml) fractions were prepared for heat-killed, cytoplasmic (1/10 dilution) and cell wall fractions (1/100) and placed in glass tubes. Fifty μl of a 80% phenol solution were added to each tube. Concentrated H_2SO_4 (5 ml) was quickly dispensed, mixed and allowed to stand for 10 min. Tubes were shaken and placed in a water bath at 25-30^{0}C for 10-20 min. Absorbance at 490 nm was measured and carbohydrate levels were quantified from a standard curve. The sensitivity of the assay was 10 μg/ml.

3.3.3 Protein determination

Protein determination on bacterial fractions was made in accordance with the method of Bradford (1976) using commercially prepared dye reagent (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin (BSA) standard (Sigma). Briefly, a stock solution containing 1.4 mg BSA/ml was prepared. From this stock solution, serial dilutions were made and placed in glass tubes ranging from 0 to 40 μg protein/ml. Dilutions were prepared for heat-killed (1/100), cytoplasmic (1/500) and cell wall fractions (1/5) and placed in glass tubes and 800 μl of standard or sample were mixed with 0.2 ml of concentrated dye. Tubes were mixed thoroughly. Absorbance at 595 nm was measured and protein concentrations determined from a standard curve. The sensitivity of this assay was 5 μg/ml.

3.3.4 DNA determination

DNA was determined by a modification of the method of Ausubel et al. (1994). Heat-killed bacterial fractions (0.5 ml) were centrifuged at 200 x g for 10 min at 4^{0}C. The

supernatants [vial S] were transferred to a 1.5 ml vial. The pellets [vial B] were resuspended in 0.5 ml of a buffer prepared with 0.01 M Tris-Cl with 1mM EDTA and 0.2% Triton X-100 pH 7.4 (TTE-0.2% Triton). After vortexing vigorously, the lysed cells were centrifuged at 1,100 x g for 10 min at 4^{0}C. These new supernatants [tube T] were transferred to a 1.5 ml vial. TTE-0.2% Triton (0.5 ml) was added to the vial B still containing the pellets. One half (0.5) ml of 25 % (w/v) trichloroacetic acid (TCA) was added to each vial S, T and B and they were vortexed vigorously. Vials were held at 4^{0}C for 18 h to allow the DNA to precipitate.

DNA standards (salmon sperm DNA, 10 mg/ml stock solution), lipopolysaccharide (LPS) from *Salmonella typhimurium* and cell wall fractions (0.5 ml) were centrifuged at 1,100 x g for 10 min at 4^{0}C. The supernatants [tube T] were transferred to a 1.5 ml vial. TTE-0.2% Triton (0.5 ml) was added to the vial B containing the pellets. One half (0.5) ml of 25 % (w/v) trichloroacetic acid (TCA) was added to vials T and B and they were vortexed vigorously. Vials were held at 4^{0}C for 18 h to allow to allow the DNA to precipitate.

Cytoplasmic fractions (0.5 ml) were treated with 0.5 ml of 25% (v/v) TCA, vortexed vigorously, and held at 4^{0}C for 18 h to allow to allow the DNA to precipitate.

After DNA precipitation for all the bacterial fractions and standards, vials were centrifuged at 1,100 x g for 10 min at 4^{0}C and supernatants were aspirated and discarded. Eighty (80) µl of TCA solution (5% v/v) were added to each vial and heated for 15 min at 90^{0}C to hydrolyze DNA. Freshly prepared diphenylamine (DPA; 150 mg DPA powder, 10 ml glacial acetic acid, 50 µl acetaldehyde solution [16 mg/ml]) reagent (160 µl) was added to each vial and also to a blank vial containing 80 µl of TCA 5% (v/v). Vials were vortexed and incubated for 4 h at 37^{0}C for color to develop. One hundred (100) µl of the resulting

colored solution were transferred to a 96-wells plate and absorbance was read at 570 nm using a Vmax kinetic Microplate reader (Molecular Devices' SOFTmax, Molecular Devices Co., Menlo Park, CA). DNA concentrations were determined from a standard curve. Total DNA for each bacterial fraction was calculated by addition of the DNA amounts quantified independently for vials S, T, and B. The sensitivity of the assay was 15 μg/ml.

3.3.5 RAW 264.7 Macrophage culture

A murine macrophage cell line obtained from the American Type Culture Collection (Rockville, MD) was used (RAW 264.7 ATCC TIB 71). RAW cells were maintained in Dubelcco's modified Eagle's medium (DMEM) (Sigma Chemical Co., St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco Laboratories, Chagrin Falls, IL), sodium bicarbonate, non-essential amino acids (1 mM) (Gibco), 1mM sodium pyruvate (Gibco), 10 ml NCTC-135 medium (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). Cell number and viability were assessed by trypan blue (Sigma) dye exclusion using a Neubauer Bright Line counting chamber (American Optical Co., Buffalo, NY). RAW cells were cultured at a final density of 5 x 10^5 cells/ml in 48 well flat bottomed tissue culture plates with and without bacterial fractions. *Salmonella typhimurium* lipopolysaccharide (LPS [Sigma] 1 μg/ml) was used as a positive control for stimulation of RAW cells. Triplicate cultures of this cell line were exposed to 50, 250, 500 μg (wet weight) of heat-killed bacteria, cytoplasm or cell wall fraction/ml and incubated for 48 h at 37^0C in 7% CO_2. RAW cells were also stimulated with 500 μg (wet weight) of the protein, RNA and DNA free cytoplasmic fractions, as well as similarly prepared controls. After suitable time

intervals supernatants were harvested, stored at -80^{0}C for cytokine assay, and cells were used for MTT assay to determine proliferation/differentiation. Results from the protein, RNA and DNA free fractions were compared to the untreated cytoplasmic fractions from each specific microorganism.

Contamination with exogenous endotoxins was ruled out by using a test with polymyxin B. Various concentrations of polymyxin B (Boehringer Mannheim, Germany) (5, 25, 50 μg/ml) were used as the LPS inhibitor to confirm that fractions were not contaminated with lipopolysaccharide. Bacterial fractions were mixed with the various polymyxin B concentrations and after a 30 min incubation at room temperature the mixtures were added to the cultures. After a 48 h incubation, supernatants were harvested and stored at -80^{0}C for cytokine assay, and cells were used for MTT assay to determine proliferation/differentiation.

3.3.6 Leukocyte preparation

Primary cell cultures from B6C3F1 female mice, 8 wk old (Charles River Labs., Raleigh, NC) were used. All animal handling was conducted in strict accordance with regulations established by the National Institutes for Health. Experiments were designed to minimize numbers of animals required to adequately test the proposed hypothesis and approved by Michigan State University Laboratory Animal Research committee. Mice were sacrificed by cervical dislocation under gentle anesthesia. Peritoneal cells were prepared by injecting first 2-3 ml RPMI 1640 medium containing 10% (v/v) heat inactivated fetal bovine serum (FBS) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 5 x 10^{-5}M 2-mercaptoethanol, 25 mM Hepes buffer, 1 mM sodium pyruvate, and 0.1mM nonessential

amino acids (10% FBS-RPMI 1640) into the peritoneal cavity of mice with a 10 ml syringe fitted with a 20 gauge needle. After gentle massage of the abdomen and without withdrawing the needle, the remaining 8-7 ml of medium were injected. Abdomen was massaged again and the liquid was slowly drawn back inside the syringe. After all the fluid was evacuated with the syringe, a small hole was cut in the peritoneum and the remaining fluid was collected with a sterile Pasteur pipette. The lavage fluid was centrifuged at 800 x g for 5-10 min and the supernatant discarded. Ten ml of same medium were added to resuspend the cells. Cells were counted with a Neubauer hemacytometer to determine number of cells per ml and cultured to a final density of 2×10^5 cells/ml.

Peyer's patches were aseptically removed, teased apart, and passed through 85-mesh stainless-steel screen. Cells were suspended in 5 ml 10% FBS-RPMI 1640 medium, washed by centrifugation at 450 x g for 10 min and resuspended in 2 ml of the same medium and counted. Cells were cultured to a final density of 5×10^5 cells/ml.

Spleen was removed aseptically, teased apart with tissue forceps in 10 ml 10% FBS-RPMI 1640, and centrifuged at 450 x g for 10 min. Erythrocytes were lysed for 5 min at room temperature in 5 ml of a buffer containing 9 parts of 0.16 M ammonium chloride plus 1 part 0.17 M TRIS buffer (pH 7.2). Ten ml of fresh 10% FBS-RPMI 1640 were added and cells were centrifuged at 450 x g for 10 min, counted and cultured to a final density of 5×10^5 cells/ml.

Leukocytes were cultured in a 48-well flat bottomed tissue culture plate in triplicate with 500 μg (wet weight) of heat-killed, cytoplasmic and cell wall fraction of *Bifidobacterium*, *L. acidophilus*, *L. bulgaricus*, *L. casei*, *L. gasseri*, *L. helveticus*, *L. reuteri*,

S. thermophilus or *Salmonella typhimurium* LPS 1 μg/ml. Cells from spleen, Peyer's patches or peritoneal cavity were incubated at 37^{0}C in 7% CO_2 and supernatants were harvested at time intervals and stored at -80^{0}C for cytokine assay.

3.3.7 MTT Assay

MTT assay was performed as described by Mosmann (1983). The reagents were prepared fresh weekly and consisted of 5 mg of MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide)(Sigma)/ml in 0.01 M phosphate buffer saline (PBS) at pH 7.4. This solution was sterilized by passage through a 0.2 μm filter and store in the dark at 4^{0}C. MTT reagent (150 μl /well) was added to each well after the supernatant was removed, and plates were incubated for 75 min in a CO_2 incubator at 37^{0}C. Plates were centrifuged at 450 x g for 10 min, MTT reagent was removed from wells using a syringe with a thin needle trying not to disturb the pellet and the crystals formed. Solubilization solution (100% dimethylsulfoxide [DMSO] from Sigma) (450 μl /well) was added to each well and plates were gently agitated to uniformly resuspend the solubilized dye. The solubilized dye (200 μl) was transferred from each well into a 96-well plate and read on a Vmax kinetic microplate reader (Molecular Devices, Menlo Park, CA) at 570 nm. Unstimulated cells values were considered as control cells.

3.3.8 Cytokine quantification

Cytokines were quantitated in supernatants by enzyme-linked immunosorbent assay (ELISA). Immunolon 4 Removawell microtiter strips (Dynatech Laboratories Inc., Chantilly,

VA) were coated overnight at 4^0C with 50 µl/well of a purified rat anti-mouse cytokine capture antibody (PharMingen, San Diego, CA) in 0.1M sodium bicarbonate buffer, pH 8.2 (1 µg/ml [IL-6, TNF-α], 2 µg/ml [IFN-γ] or 0.5 mg/ml [IL-12]). Plates were washed three times with 0.01M phosphate buffered (pH 7.2) saline (PBS) containing 0.2% Tween 20 (PBS-T). Plates were blocked with 300 µl of 3% (w/v) bovine serum albumin (BSA) in PBS (BSA-PBS) at 37^0C for 30 min and washed 3 times with the PBS-T. Standard murine cytokines (PharMingen) or samples were diluted in 10% (v/v) FBS-RPMI 1640, and 50 µl aliquots were added to appropriate wells. Plates were incubated at 37^0C for 60 min, washed 4 times with PBS-T, and 50 µl of biotinylated rat anti-mouse cytokine detection monoclonal antibody (1.5 µg/well; PharMingen) diluted in BSA-PBS were added to each well. Plates were incubated at room temperature for 60 min, washed six times with PBS-T and 1 additional time with distilled water. Fifty µl of streptavidin-horseradish peroxidase conjugate (1.5 µg/well; Sigma Chemical Co., St. Louis, MO) diluted in BSA-PBS were added to each well and plates were incubated at room temperature for 60 min. Plates were then washed 8 times with PBS-T and two more times with distilled water, and 100 µl of substrate [10 mM citric phosphate buffer (pH 5.5), containing 0.4 mM tetramethylbenzidine (TMB; Fluka chemical Corp., Ronkonkoma, NY) and 1.2 mM H_2O_2] were added to each well. The reaction was stopped by adding an equal volume (100 µl) of of 6N H_2SO_4. Absorbance was read at 450 nm on a Vmax kinetic Microplate reader (Molecular Devices Co.) and cytokine concentrations were quantitated by using Vmax Software. The sensitivity of this assay was 0.25 ng/ml for IL-6, 0.4 ng/ml for TNF-α and 1.25 ng/ml for IFN-γ.

3.3.9 Nitric oxide determination

The method of Dietert et al. (1995) was used to detect nitrite production by macrophages. Cells were cultured as described above for 2 and 5 days and nitric oxide (NO) production was assessed by measuring nitrite accumulation, a stable metabolic product of NO, in the culture supernatants. Nitrite concentrations were determined by the Griess reaction. Equal amounts of N-(1-naphthyl) ethylenediamine dihydrochloride (NED; Sigma) [100 mg dissolved in 100 ml of distilled water] and sufanilamide (Sigma) [1 g dissolved in 100 ml of a 5% phosphoric acid solution] solutions were mixed prior to each assay (Griess reagent or chromogenic reagent). Nitrite standards [2 mM stock solution; from 0 to 200 μM] were diluted in the same media in which the cells were suspended. Equal amounts of Griess reagent and $NaNO_2$ standards or samples (100 μl) were placed in a 96 well plate in duplicate and incubated for 5 min at room temperature to allow the chromophore to develop and stabilize. Absorbance was read at 550 nm using the Vmax kinetic Microplate reader (Molecular Devices Co.) and nitrite concentrations were quantitated by using Vmax Software. The sensitivity of the assay was 12 μM.

3.3.10 Statistical methods

One way analysis of variance ANOVA with Dunnett's test for multiple comparison versus a control group was applied to data, using Sigma-Stat Analysis System (Jandel Scientific, San Rafael, CA). A $P \leq 0.05$ was considered statistically significant. Results were expressed as relative change in the cytokine production compared to control by dividing experimental data by control values (fold control).

3.4 RESULTS

3.4.1 Bacteria fractionation process

Lactic acid bacteria yields from MRS broth cultures were between 1 and 1.5 g/100 ml. Wall fractions from bacterial cells were prepared from mechanically broken cells by removal of extraneous material by washing, differential centrifugation and enzymatic digestion. The supernatant collected after centrifugation of the sonicated whole cell fraction represented the cytoplasmic content of the bacteria, and the pellet resulted in the crude cell wall. In our studies the crude cell wall obtained from the whole cell preparation represented 15-30% (wet weight) of our initial bacteria, and this is in agreement with the yield reported in general by the literature (Work 1971). Purified pure cell wall fraction ranged between 36-80 % (wet weight) with respect of the crude cell wall fraction, depending on the bacterial species. By weighing each fraction, total colony forming units for each bacteria strain could be correlated with their wet weight. Those values varied from strain to strain (Table 3.2).

3.4.2 Cell fraction constituents

Heat-killed, cytoplasmic and cell wall fractions were analyzed for protein, total carbohydrate and DNA content (Table 3.3). The highest protein content was found in general in the cytoplasmic fraction. Carbohydrate concentration made up a significant part of the cytoplasmic and cell wall fractions. Heat-killed bacterial fractions possessed also a fairly high carbohydrate content but lower than the corresponding cell wall for that same bacteria. DNA was also detected in both heat-killed bacteria and cytoplasmic fractions.

TABLE 3.2. Bacterial cell equivalents per initial fraction wet weight.

	cells/μg		
	Heat-killed	Cytoplasm	Cell wall
Bifidobacterium Bf-1	2.0×10^4	1.4×10^6	3.6×10^5
L. acidophilus La-2	6.2×10^5	ND	4.0×10^6
L.bulgaricus 1489 NCK 231	1.9×10^5	5.4×10^5	8.6×10^5
L. casei ATCC 39539	2.2×10^5	5.2×10^5	1.4×10^6
L. gasseri NCK 101	2.4×10^5	9.0×10^5	1.1×10^6
L. helveticus Lr-92	1.2×10^4	1.1×10^5	2.0×10^5
L.reuteri ATCC 23272	1.5×10^5	1.0×10^6	1.7×10^6
S. thermophilus St-133	4.4×10^2	2.8×10^2	4.0×10^3

ND = not determined

TABLE 3.3. Chemical composition of bacterial fractions[a].

	Protein μg/mg[b]		
	Whole cells	Cytoplasmic	Cell wall
Bifidobacterium Bf-1	39 ± 9	470 ± 12	169 ± 2
L. acidophilus La 2	12 ± 4	35 ± 0	1 ± 0
L. bulgaricus 1489 NCK 231	25 ± 4	171 ± 12	4 ± 0
L. casei ATCC 39539	37 ± 3	212 ± 4	1 ± 0
L. gasseri NCK 101	14 ± 2	108 ± 25	0 ± 0
L. helveticus Lr 92	15 ± 3	294 ± 10	1 ± 0
L. reuteri ATCC 23272	5 ± 2	200 ± 11	0 ± 0
S. thermophilus St-133	13 ± 3	31 ± 4	0 ± 0

	Carbohydrate μg/mg[b]		
	Whole cells	Cytoplasmic	Cell wall
Bifidobacterium Bf-1	93 ± 5	180 ± 22	246 ± 3
L. acidophilus La 2	28 ± 1	28 ± 1	37 ± 6
L. bulgaricus 1489 NCK 231	40 ± 5	81 ± 10	95 ± 0
L. casei ATCC 39539	32 ± 0	32 ± 0	106 ± 12
L. gasseri NCK 101	46 ± 0	116 ± 0	69 ±1
L. helveticus Lr 92	32 ± 0	128 ± 0	56 ± 3
L. reuteri ATCC 23272	41 ± 2	124 ± 7	39 ± 1
S. thermophilus St-133	28 ± 2	10 ± 1	47 ± 2

	DNA μg/mg[b]		
	Whole cells	Cytoplasmic	Cell wall
Bifidobacterium Bf-1	11 ± 1	0 ± 0	0 ± 0
L. acidophilus La 2	24 ± 0	5 ± 1	1 ± 0
L. bulgaricus 1489 NCK 231	5 ± 0	60 ± 22	4 ± 0
L. casei ATCC 39539	22 ± 1	23 ± 4	7 ± 1
L. gasseri NCK 101	7 ± 1	34 ± 3	1 ± 0
L. helveticus Lr 92	21 ± 1	57 ± 10	1 ± 0
L. reuteri ATCC 23272	5 ± 1	21 ± 7	1 ± 0
S. thermophilus St-133	3 ± 1	5 ± 1	1 ± 0

[a] Values are means ± S.E.M (n = 4).

[b] Relative to wet weight

Recoverable DNA in these two fractions varied with the species, but the cytoplasmic fraction had, in general, a higher concentration of DNA than the whole cells. Surprisingly, cell wall fractions also showed presence of DNA. Since the reagent used for this DNA assay, diphenylamine (DPA), reacts mainly with the sugar portion of the DNA, these values indicating DNA content in cell walls could be reflecting interaction between DPA and any of the sugars present in these wall fractions.

Treatment of whole cell and cytoplasmic fractions with protease was very effective in reducing protein concentrations. Protein concentration (Table 3.4 and 3.5), but not carbohydrate content (Table 3.6 and 3.7), declined drastically after protease treatment as compared to the untreated fractions. This provided whole cell and cytoplasmic fractions that were potentially free only of the protein content that the initial fraction possessed. This type of treatment was repeated using RNAse or DNAse to obtain a derived new fraction free of nucleic acids. The protein content was slightly decreased for RNAse treated fractions, suggesting protein-nucleic acid association. Treatment with RNAse did not affect carbohydrate or DNA content as compared to the untreated fraction (data not shown). Treatment of whole unviable bacterial cells with DNAse removed not only DNA from the original fractions but also some protein. Again, this indicated some DNA association with the membrane or proteins which were dragged out together with DNA after DNAse treatment. Treatment of the cytoplasmic fraction with DNAse did not affect so much protein or carbohydrate content but removed practically all DNA present in the original fractions.

TABLE 3.4. Protein content of the heat-killed fraction (25 mg/ml) of lactic acid bacteria digested or undigested with Protease, RNAse, DNAse.[1]

	Protein concentration (μg/ml)			
	Whole cells	Protease	RNAse	DNAse
Bifidobacterium Bf-1	997 ± 242	150 ± 10	502 ± 140	280 ± 12
Lactobacillus acidophilus La 2	300 ± 106	73 ± 17	280 ± 96	212 ± 88
Lactobacillus bulgaricus 1489 NCK 231	624 ± 111	59± 3	291 ± 14	202 ± 71
Lactobacillus casei ATCC 39539	926 ± 75	0 ± 0	166 ± 30	76 ± 19
Lactobacillus gasseri NCK 101	352 ± 51	42 ± 1	153 ± 31	124 ± 21
Lactobacillus helveticus Lr 92	397 ± 87	11 ± 2	343 ± 32	189 ± 10
Lactobacillus reuteri ATCC 23272	143 ± 58	0 ± 0	191 ± 62	102 ± 25
Streptococcus thermophilus St-133	332 ± 89	20 ± 2	245 ± 10	158 ± 62
Lipopolysaccharide 1 mg/ml (+ control)	5648 ± 515			

[1] Untreated heat-killed fraction was treated with 20 μg protease/mg of bacterial pellet, or 250 μg RNAse/ml or 250 μg DNAse/ml and incubated at 37^{0}C overnight (n = 4).

TABLE 3.5. Protein content of the cytoplasmic fraction (25 mg/ml) of lactic acid bacteria digested or undigested with Protease, RNAse, DNAse.[1]

	Protein concentration (μg/ml)			
	Cytoplasm	Protease	RNAse	DNAse
Bifidobacterium Bf-1	11760 ± 300	0 ± 0	11760 ± 315	12750 ± 375
Lactobacillus acidophilus La 2	885 ± 6	0 ± 0	954 ± 13	1330 ± 192
Lactobacillus bulgaricus 1489 NCK 231	4282 ± 300	1878 ± 62	3504 ± 52	5868 ± 920
Lactobacillus casei ATCC 39539	5312 ± 100	1905 ± 217	6179 ± 24	3013 ± 40
Lactobacillus gasseri NCK 101	2703 ± 628	215 ± 52	3353 ± 592	2295 ± 100
Lactobacillus helveticus Lr 92	7368 ± 264	1020 ± 160	3672 ± 132	3424 ± 128
Lactobacillus reuteri ATCC 23272	5001 ± 297	579 ± 24	3227 ± 174	3486 ± 156
Streptococcus thermophilus St-133	792 ± 109	57 ± 14	937 ± 377	1016 ± 81

[1] Untreated cytoplasmic fraction was treated with 20 μg protease/mg of bacterial pellet, or 250 μg RNAse/ml or 250 μg DNAse/ml and incubated at 37^0C overnight (n = 4).

TABLE 3.6. Carbohydrate content of the heat-killed fraction of lactic acid bacteria digested or undigested with Protease, RNAse, DNAse.[1]

	Carbohydrate concentration (μg/ml)			
	Whole cells	Protease	RNAse	DNAse
Bifidobacterium Bf-1	2326 ± 146	1802 ± 36	1639 ± 48	1776 ± 170
Lactobacillus acidophilus La 2	706 ± 22	732 ± 54	563 ± 55	460 ± 17
Lactobacillus bulgaricus 1489 NCK 231	1011 ± 136	837 ±12	632 ± 54	717 ± 27
Lactobacillus casei ATCC 39539	820 ± 9	478 ± 59	400 ± 12	306 ± 39
Lactobacillus gasseri NCK 101	1162 ± 0	524 ± 36	790 ± 68	741 ± 103
Lactobacillus helveticus Lr 92	805 ± 0	507 ± 12	618 ± 12	677 ± 60
Lactobacillus reuteri ATCC 23272	1034 ± 60	686 ± 48	737 ± 24	907 ± 120
Streptococcus thermophilus St-133	722 ± 63	819 ± 33	782 ± 4	841 ± 101
Lipopolysaccharide 1 μg/ml (+ control)	5457 ± 18			

[1] Untreated heat-killed fraction (25 mg/ml) was treated with 20 μg protease/mg of bacterial pellet, or 250 μg RNAse/ml or 250 μg DNAse/ml and incubated at 37^0C overnight (n = 4).

TABLE 3.7. Carbohydrate content of the cytoplasmic fraction of lactic acid bacteria digested or undigested with Protease, RNAse, DNAse.[1]

	Carbohydrate concentration (μg/ml)			
	Cytoplasm	Protease	RNAse	DNAse
Bifidobacterium Bf-1	4500 ± 555	6300 ± 1290	4110 ± 465	4590 ± 405
Lactobacillus acidophilus La 2	706 ± 22	211 ± 33	165 ± 31	122 ± 8
Lactobacillus bulgaricus 1489 NCK 231	2022 ± 272	1182 ± 18	928 ± 146	1194 ± 196
Lactobacillus casei ATCC 39539	820 ± 9	810 ± 57	718 ± 14	779 ± 11
Lactobacillus gasseri NCK 101	2905 ± 0	1020 ± 55	700 ± 65	698 ± 115
Lactobacillus helveticus Lr 92	3220 ± 0	2116 ± 252	1512 ± 12	1280 ± 52
Lactobacillus reuteri ATCC 23272	3102 ± 180	960 ± 270	732 ± 12	1035 ± 216
Streptococcus thermophilus St-133	243 ± 21	43 ± 13	71 ± 1	67 ± 18

[1] Untreated cytoplasmic fraction (25 mg/ml) was treated with 20 μg protease/mg of bacterial pellet, or 250 μg RNAse/ml or 250 μg DNAse/ml and incubated at 37^0C overnight (n = 4).

3.4.3 Effects of lactic acid bacteria fractions on RAW 264.7 cells.

To assess the effects of lactic acid bacterial fractions and heat-killed cells on macrophage function, RAW 264.7 macrophage cells were stimulated *in vitro* with 50, 250 and 500 μg (wet weight)/ml of each bacterial fraction and with 1 μg/ml LPS, used as a positive control. After culturing these cells for 48 h, cells were used for MTT analysis and supernatants were monitored for cytokines.

Macrophage structure varies with their degree of activation. Figure 3.2 shows the rate of activation reached by stimulation of RAW 264.7 with lactic acid bacteria fractions or LPS. These cells are generally large, irregularly shaped and they often have an eccentrically placed round or kidney-shaped nucleus with one or two prominent nucleoli and finely dispersed nuclear chromatin (Figure 3.2a). Upon activation, the cytoskeleton that surrounds the nucleus extends throughout the cytoplasm to the cell periphery. Actin microfilaments immediately beneath the cell membrane are responsible for the prominent ruffling, locomotion, and pseudopod formation, as well as influencing endocytotic events (Figure 3.2b, 3.2c, 3.2d). The cytoplasm can be seen containing fine and multiple large granules (Figure 3.2c, 3.2d). Vacuoles are frequently seen near the cell periphery, reflecting the active pinocytosis of macrophages.

The proliferation of macrophage cells, measured by MTT assay, was enhanced when RAW cells were cultured with 50 μg and 250 μg of any of the fractions, generally showing a significant increase with respect to the unstimulated cells (control), but this effect decreased when cells were stimulated with 500 μg of either heat-killed cells or cytoplasmic fraction (Table 3.8, 3.9, 3.10). The results suggested that lactic acid bacteria stimulated RAW

Figure 3.2. Effect of incubation with different fractions of *Lactobacillus casei* ATCC 39539 on RAW 264.7 macrophage cells (x 100). A) 24 h incubation without any microbial product (control). B) 24 h incubation with 500 μg/ml cytoplasmic fraction. C) 24 h incubation with 500 μg/ml cell wall fraction. D) 24 h incubation with 1 μg/ml LPS.

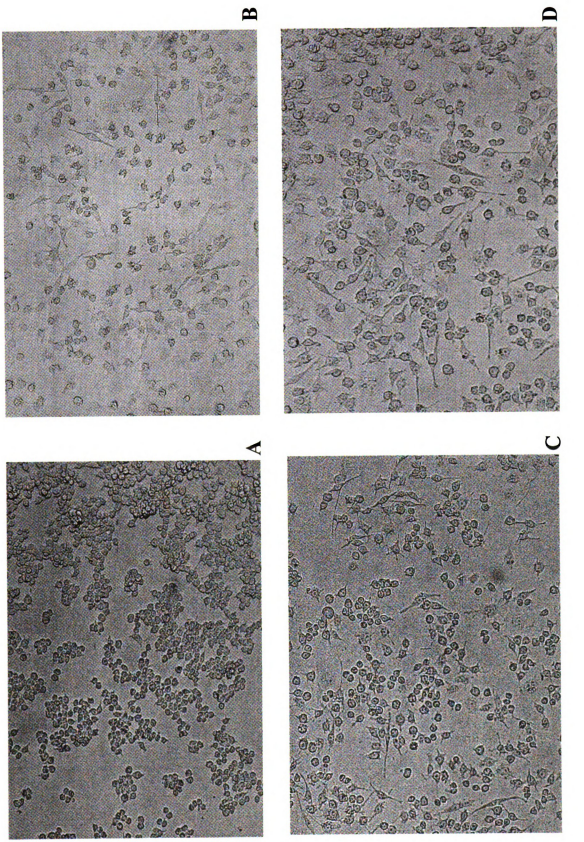

B
D
A
C

TABLE 3.8. Effect of heat-killed lactic acid bacteria on proliferation of RAW 264.7[1] cell line measured by MTT assay.

	fold control [2]		
Bacteria	50 μg	250 μg	500 μg
Bifidobacterium Bf-1	1.41*	1.23*	0.98
Lactobacillus acidophilus La 2	1.35*	1.14*	0.97
Lactobacillus bulgaricus 1489 NCK 231	1.13*	0.88*	0.77*
Lactobacillus casei ATCC 39539	1.26*	1.04	1.03
Lactobacillus gasseri NCK 101	1.25*	1.24*	1.08
Lactobacillus helveticus Lr 92	1.22*	1.00	0.91
Lactobacillus reuteri ATCC 23272	1.35*	0.95	0.58*
Streptococcus thermophilus St-133	1.03	1.08	0.95
Lipopolysaccharide 1 μg (+ control)	0.75*		

[1]RAW 264.7cells (5 x 10^5 cells/ml) were cultured with various concentrations of bacteria for 48 hours.

[2]MTT relative change was calculated by dividing experimental data by control values (n = 4) and are representative of two separate experiments.

*$P < 0.05$

TABLE 3.9. Effect of the cytoplasmic fraction of lactic acid bacteria on proliferation of RAW 264.7[1] cell line measured by MTT assay.

	fold control[2]		
Bacteria	50 μg	250 μg	500 μg
Bifidobacterium Bf-1	1.79*	1.09	0.92*
Lactobacillus acidophilus La 2	1.31*	1.22*	1.05
Lactobacillus bulgaricus 1489 NCK 231	1.29*	1.14*	0.94
Lactobacillus casei ATCC 39539	1.12*	1.09*	0.93*
Lactobacillus gasseri NCK 101	1.05	1.15*	1.17*
Lactobacillus helveticus Lr 92	1.14*	1.05	0.93
Lactobacillus reuteri ATCC 23272	1.36*	1.20*	0.62*
Streptococcus thermophilus St-133	1.38*	1.43*	1.25

[1]RAW 264.7cells (5 x 10^5 cells/ml) were cultured with various concentrations of bacterium fraction for 48 hours.

[2]MTT relative change was calculated by dividing experimental data by control values (n = 4) and are representative.

of two separate experiments.

*$P < 0.05$

TABLE 3.10. Effect of the cell wall fraction of lactic acid bacteria on proliferation of RAW 264.7[1] cell line measured by MTT assay.

	fold control [2]		
Bacteria	50 μg	250 μg	500 μg
Bifidobacterium Bf-1	1.66*	1.68*	1.22*
Lactobacillus acidophilus La 2	1.49*	1.65*	1.32*
Lactobacillus bulgaricus 1489 NCK 231	1.23*	1.15*	1.08*
Lactobacillus casei ATCC 39539	1.47*	1.16*	0.95
Lactobacillus gasseri NCK 101	1.71*	1.40*	1.19
Lactobacillus helveticus Lr 92	1.12*	1.05*	0.91*
Lactobacillus reuteri ATCC 23272	1.49*	1.36*	1.27*
Streptococcus thermophilus St-133	1.28*	1.56*	1.23*

[1]RAW 264.7cells (5 x 10^5 cells/ml) were cultured with various concentrations of bacterium fraction for 48 hours.

[2]MTT relative change was calculated by dividing experimental data by control values (n = 4) and are representative.

of two separate experiments.

*$P < 0.05$

264.7 cells exhibited enhanced proliferation that seemed to be concentration-dependent particularly for stimulation with the cell wall fraction of these bacteria.

Exposure of RAW 264.7 cells to lactic acid bacteria fractions also increased TNF-α and IL-6 levels in a concentration-dependent manner. For heat-killed cells, production of TNF-α was significantly higher than in control cells with all bacteria demonstrating a great activity. In general TNF-α levels were higher than the levels produced by stimulation with a positive control, (LPS, 1μg/ml) (Table 3.11). Similar trends were found when analyzing for IL-6, except that the levels here were lower as compared to LPS (Table 3.12). For heat-killed bacteria fractions, pre-treatments with protease, DNAse or RNAse did not considerably affect the production of cytokines by RAW 264.7 cells (data not shown). Since non-viable bacteria were still intact, protease or nucleases may have had difficulties diffusing inside the cell.

Cytoplasmic fractions from lactobacilli and streptococci induced marked TNF-α production (Table 3.13), with significant increases being observed for all concentrations. *L. helveticus*, *L. reuteri* and *Bifidobacterium* showed an opposite effect, the latter producing considerable levels of TNF-α at 50 μg and 250 μg but decreasing dramatically at 500 μg, and lactobacilli showing a lower level at the highest concentration. Most of the cytoplasmic fractions induced lower levels of this cytokine than LPS. In general, similar results were found for IL-6 (Table 3.14).

For cell wall fractions, TNF-α levels significantly increased with concentration for all species. In general, these increases were lower than the ones found for heat-killed bacteria (Table 3.15). Stimulation with cell wall seemed to be more effective than with cytoplasmic

TABLE 3.11. Effect of heat-killed lactic acid bacteria on TNF-α production by RAW 264.7[1] cells.

	TNF-α fold control[2]		
Bacteria	50 μg	250 μg	500 μg
Bifidobacterium Bf-1	199.0	336*	395*
Lactobacillus acidophilus La 2	142.0	739*	816*
Lactobacillus bulgaricus 1489 NCK 231	388*	456*	648*
Lactobacillus casei ATCC 39539	369*	669*	738*
Lactobacillus gasseri NCK 101	274*	329*	235*
Lactobacillus helveticus Lr 92	549*	756*	1010*
Lactobacillus reuteri ATCC 23272	125*	241*	199*
Streptococcus thermophilus St-133	51*	166*	340*
Lipopolysaccharide 1 μg (+ control)	221*		

[1]RAW 264.7cells (5 x 10^5 cells/ml) were cultured with various concentrations of bacteria for 48 hours

[2]TNF-α relative change was calculated by dividing experimental data by control values (n = 4) and are. representative of two separate experiments.

*$P < 0.05$

TABLE 3.12. Effect of heat-killed lactic acid bacteria on IL-6 production by RAW 264.7[1] cells.

	IL-6 fold control[2]		
Bacteria	50 μg	250 μg	500 μg
Bifidobacterium Bf-1	41*	173*	226*
Lactobacillus acidophilus La 2	2.0	9*	61*
Lactobacillus bulgaricus 1489 NCK 231	37*	58*	83*
Lactobacillus casei ATCC 39539	3.0	27*	53*
Lactobacillus gasseri NCK 101	56*	107*	112*
Lactobacillus helveticus Lr 92	27*	58*	85*
Lactobacillus reuteri ATCC 23272	61*	112*	195*
Streptococcus thermophilus St-133	7*	36*	52*
Lipopolysaccharide 1 μg (+ control)	204*		

[1]RAW 264.7cells (5 x 10^5 cells/ml) were cultured with various concentrations of bacteria for 48 hours

[2]IL-6 relative change was calculated by dividing experimental data by control values (n = 4) and are. representative of two separate experiments.

*$P < 0.05$

TABLE 3.13. Effect of the cytoplasmic fraction of lactic acid bacteria on TNF-α production by RAW 264.7[1] cells.

	TNF-α fold control[2]		
Bacteria	50 μg	250 μg	500 μg
Bifidobacterium Bf-1	100*	89*	3
Lactobacillus acidophilus La 2	100*	125*	169*
Lactobacillus bulgaricus 1489 NCK 231	134*	257*	295*
Lactobacillus casei ATCC 39539	125*	237*	296*
Lactobacillus gasseri NCK 101	42*	124*	170
Lactobacillus helveticus Lr 92	307*	266*	180*
Lactobacillus reuteri ATCC 23272	64*	160*	103*
Streptococcus thermophilus St-133	5	19*	28*
Lipopolysaccharide 1 μg (+ control)	221*		

[1]RAW 264.7cells (5 x 10^5 cells/ml) were cultured with various concentrations of bacterium fraction for 48 hours

[2]TNF-α relative change was calculated by dividing experimental data by control values (n = 4) and are representative of two separate experiments.

*$P < 0.05$

TABLE 3.14. Effect of the cytoplasmic fraction of lactic acid bacteria on IL-6 production by RAW 264.7[1] cells.

	IL-6 fold control[2]		
Bacteria	50 μg	250 μg	500 μg
Bifidobacterium Bf-1	0	65*	49*
Lactobacillus acidophilus La 2	63*	195*	215*
Lactobacillus bulgaricus 1489 NCK 231	22	134*	160*
Lactobacillus casei ATCC 39539	11	79*	211*
Lactobacillus gasseri NCK 101	7	38	110*
Lactobacillus helveticus Lr 92	26*	68*	85*
Lactobacillus reuteri ATCC 23272	31	151*	154*
Streptococcus thermophilus St-133	0	11	6
Lipopolysaccharide 1 μg (+ control)	204*		

[1]RAW 264.7cells (5 x 10^5 cells/ml) were cultured with various concentrations of bacterium fraction for 48 hours

[2]IL-6 relative change was calculated by dividing experimental data by control values (n = 4) and are representative of two separate experiments.

*$P < 0.05$

TABLE 3.15. Effect of the cell wall fraction of lactic acid bacteria on TNF-α production by RAW 264.7[1] cells.

	TNF-α fold control[2]		
Bacteria	50 μg	250 μg	500 μg
Bifidobacterium Bf-1	17*	59*	99*
Lactobacillus acidophilus La 2	41	49*	148*
Lactobacillus bulgaricus 1489 NCK 231	308*	500*	679*
Lactobacillus casei ATCC 39539	28	239*	418*
Lactobacillus gasseri NCK 101	34*	77*	110*
Lactobacillus helveticus Lr 92	303*	369*	405*
Lactobacillus reuteri ATCC 23272	44	149*	228*
Streptococcus thermophilus St-133	33*	45*	142*
Lipopolysaccharide 1 μg (+ control)	221*		

[1]RAW 264.7cells (5 x 10^5 cells/ml) were cultured with various concentrations of bacterium fraction for 48 hours

[2]TNF-α relative change was calculated by dividing experimental data by control values (n = 4) and are representative of two separate experiments.

*$P < 0.05$

material and/or LPS relative to TNF-α production, especially for *L. bulgaricus*, *L. casei*, *L. helveticus*. Production of IL-6 in this case was also significantly increased for all bacteria species, with *L. bulgaricus* and *L. reuteri* showing the greatest effect (Table 3.16). In general, values were slightly lower than the ones found for cytoplasmic and heat-killed fractions.

The above described immunopotentiating activity in this fractions could be due to nucleic acid. Table 3.17 represents a typical result for cytoplasmic fractions free of protein showing in general the highest activity when compared to those free of RNA or DNA for IL-6 production. Cytokine production by cells stimulated with cytoplasmic fraction was in general not significantly decreased by the protease treatment respect to the untreated fraction.

Nitrite levels were used to measure production of nitric oxide (NO), another important macrophage mediator. Table 3.18 and 3.20 summarize the effects of stimulation with heat-killed and cell wall fractions respectively on NO production by a macrophage cell line. In general, NO production increased in a concentration-dependent manner reaching values similar to the ones exhibited by LPS stimulation. *L. reuteri* and *S. thermophilus* exhibited an opposite effect, where levels of nitric oxide decreased with the concentration of heat-killed fraction used to stimulate cells. Stimulation of cells with the cytoplasmic fraction of lactic acid bacteria disclosed an inverse effect than the observed for heat-killed bacteria and cell wall (Table 3.19). NO levels decreased in a concentration-dependent manner for all bacteria except for *S. thermophilus*.

To rule out the possibility that TNF-α, IL-6 and NO production was induced by LPS contaminants within the bacteria extracts, RAW 264.7 cells were cultured with up to 50 μg/ml polymyxin B and with the bacterial fractions or LPS. Polymyxin B is an antibiotic

TABLE 3.16. Effect of the cell wall fraction of lactic acid bacteria on IL-6 production by RAW 264.7[1] cells.

	IL-6 fold control[2]		
Bacteria	50 μg	250 μg	500 μg
Bifidobacterium Bf-1	0	3	4
Lactobacillus acidophilus La 2	1	2*	5*
Lactobacillus bulgaricus 1489 NCK 231	66*	121*	175*
Lactobacillus casei ATCC 39539	1	9*	29*
Lactobacillus gasseri NCK 101	0	20*	51*
Lactobacillus helveticus Lr 92	7	35*	72*
Lactobacillus reuteri ATCC 23272	0	38	116*
Streptococcus thermophilus St-133	0	0	3*
Lipopolysaccharide 1 μg (+ control)	204*		

[1]RAW 264.7cells (5 x 10^5 cells/ml) were cultured with various concentrations of bacterium fraction for 48 hours

[2]IL-6 relative change was calculated by dividing experimental data by control values (n = 4) and are representative of two separate experiments.

*$P < 0.05$

TABLE 3.17. Effect of the cytoplasmic fraction of lactic acid bacteria after treatment with protease, RNAse or DNAse on IL-6 production by RAW 264.71 cells.

	IL-6 relative change (% untreated fraction)[2]		
	Protease	RNAse	DNAse
Bifidobacterium Bf-1	200	10	10
Lactobacillus acidophilus La 2	110	0	0
Lactobacillus bulgaricus 1489 NCK 231	150	170	190
Lactobacillus casei ATCC 39539	190	80	150
Lactobacillus gasseri NCK 101	130	50	110
Lactobacillus helveticus Lr 92	120	0	0
Lactobacillus reuteri ATCC 23272	210	140	140
Streptococcus thermophilus St-133	460	250	190

[1]RAW 264.7cells (5 x 10^5 cells/ml) were cultured with various concentrations of bacterium fraction for 48 hours

[2]IL-6 relative change was calculated by dividing experimental data by control values (untreated cytoplasmic fraction) x 100 (n = 4). Note: Values above 100 reflect an increased activity with respect to the untreated fraction while values close to 0 reflect no stimulation capability. Results are representative of two separate experiments.

TABLE 3.18. Effect of heat-killed lactic acid bacteria on NO_3^- production by RAW 264.7[1] cells.

	NO_3^- fold control[2]		
Bacteria	50 μg	250 μg	500 μg
Bifidobacterium Bf-1	11*	12*	15*
Lactobacillus acidophilus La 2	7*	18*	22*
Lactobacillus bulgaricus 1489 NCK 231	10*	9*	17*
Lactobacillus casei ATCC 39539	13*	19*	18*
Lactobacillus gasseri NCK 101	20*	14*	15*
Lactobacillus helveticus Lr 92	14*	15*	17*
Lactobacillus reuteri ATCC 23272	16*	10*	7*
Streptococcus thermophilus St-133	1*	4*	6*
Lipopolysaccharide 1 μg (+ control)	15*		

[1]RAW 264.7cells (5 x 10^5 cells/ml) were cultured with various concentrations of bacteria for 48 hours

[2]NO_3^- relative change was calculated by dividing experimental data by control values (n = 4) and are representative of two separate experiments.

*$P < 0.05$

TABLE 3.19. Effect of the cytoplasmic fraction of lactic acid bacteria on NO_3^- production by RAW 264.7[1] cells.

	NO_3^- fold control[2]		
Bacteria	50 μg	250 μg	500 μg
Bifidobacterium Bf-1	5*	6*	2
Lactobacillus acidophilus La 2	17*	12*	7*
Lactobacillus bulgaricus 1489 NCK 231	40*	15*	18*
Lactobacillus casei ATCC 39539	16*	15*	14*
Lactobacillus gasseri NCK 101	16*	15*	16*
Lactobacillus helveticus Lr 92	16*	14*	14*
Lactobacillus reuteri ATCC 23272	16*	5	0
Streptococcus thermophilus St-133	1	5*	8*
Lipopolysaccharide 1 μg (+ control)	15*		

[1]RAW 264.7cells (5 x 10^5 cells/ml) were cultured with various concentrations of bacterium fraction for 48 hours

[2]NO_3^- relative change was calculated by dividing experimental data by control values (n = 4) and are representative of two separate experiments.

*$P < 0.05$

TABLE 3.20. Effect of the cell wall fraction of lactic acid bacteria on NO_3^- production by RAW 264.7[1] cells.

	NO_3^- fold control[2]		
Bacteria	50 μg	250 μg	500 μg
Bifidobacterium Bf-1	0	5*	11*
Lactobacillus acidophilus La 2	0	10*	16*
Lactobacillus bulgaricus 1489 NCK 231	20*	19*	19*
Lactobacillus casei ATCC 39539	4*	16*	18*
Lactobacillus gasseri NCK 101	5*	16*	17*
Lactobacillus helveticus Lr 92	18*	19*	17*
Lactobacillus reuteri ATCC 23272	10*	18*	17*
Streptococcus thermophilus St-133	0	5*	13*
Lipopolysaccharide 1 μg (+ control)	15*		

[1]RAW 264.7cells (5 x 10^5 cells/ml) were cultured with various concentrations of bacterium fraction for 48 hours

[2]NO_3^- relative change was calculated by dividing experimental data by control values (n = 4) and are representative of two separate experiments.

*$P < 0.05$

capable to induce outer membrane permeability in gram negative bacteria (Nikaido and Vaara, 1985). The basic molecule of LPS consists of a lipid component (lipid A) covalently bound to a heteropolysacharide (Stewart-Tull et al., 1985). Lipid A is responsible for the mitogenic activity attributed to LPS. Polymyxin B is able to inactivate lipid A by attaching to it affecting overall LPS activity. The results indicated a constant stimulation effect of bacterial extracts independently of the polymyxin B concentration (Figure 3.3). On the contrary, LPS activity declined when concentration of polymyxin B was increased, affecting proliferation of cells and decreasing production of TNF-α.

The effects of lactic acid bacterial stimulation were determined in cultures prepared from RAW 264.7 macrophage cells at different times of exposure (Figure 3.4). Cells were incubated with *L. casei* ATCC 39539 fractions for 1, 6, 12, 24, and 48 h. The capacity for elevated cytokine production was observed in RAW 264.7 cells as early as 1 h for TNF-α and 6 h for IL-6. Levels of these two cytokines were constantly increasing for all treatments regardless the fraction used to stimulate the cells. Detectable levels of NO appeared after incubating for more than 24 h and these levels could be related with the considerable boost experienced by TNF-α after incubation for 12-24 h.

3.4.4 Effects of lactic acid bacteria fractions on peritoneal cells.

Peritoneal, spleen, and Peyer's patch cells were incubated with 500 μg of heat-killed, cytoplasmic or cell wall fractions from lactic acid bacteria as well as with 1 μg LPS for a period of 48 h and analyzed as described before. Enhanced proliferation of peritoneal cells was more evident than activation based on microscopy (Figure 3.5, 3.6). Spleen and Peyer's

Figure 3.3. Effect of polymyxin B on *Lactobacillus casei* ATCC 39539 fractions and LPS stimulation properties. A) MTT assay B)TNF-α production.

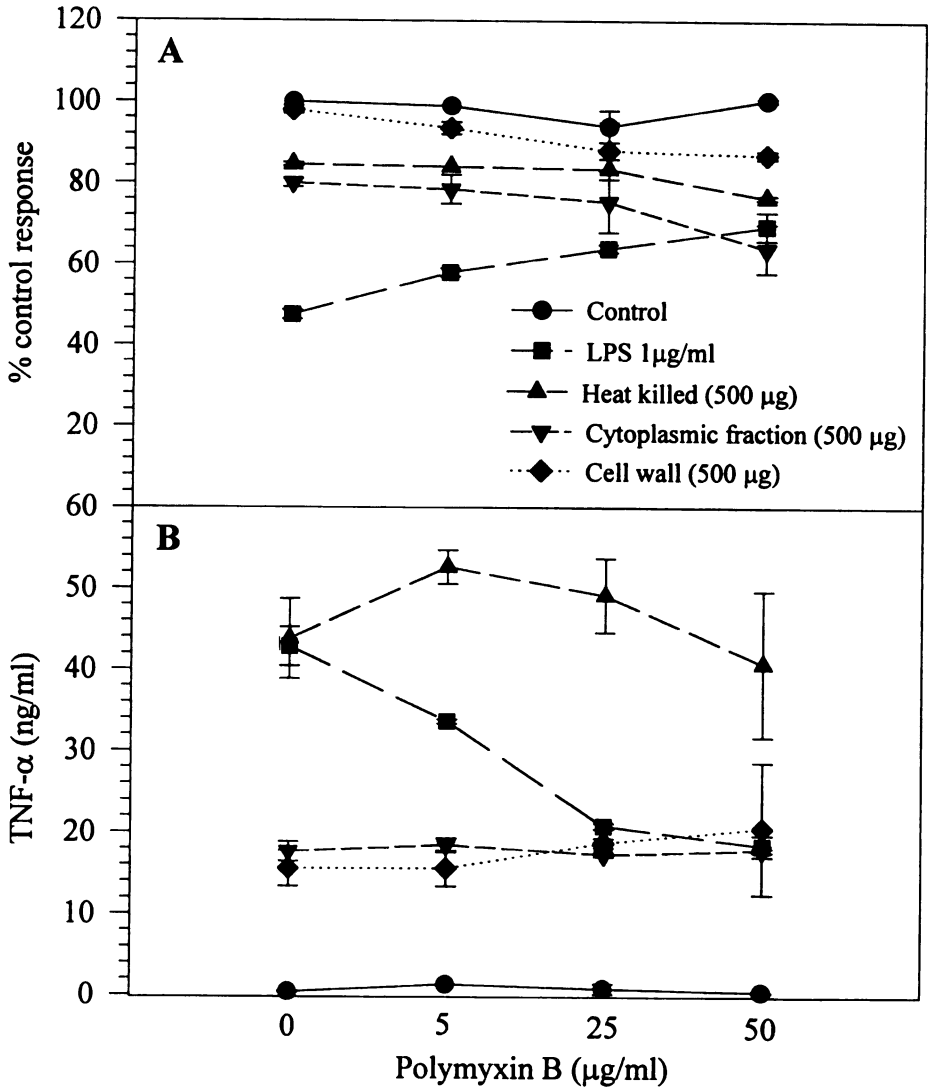

A
B
% control response
TNF-α (ng/ml)
Polymyxin B (μg/ml)
Control
LPS 1μg/ml
Heat killed (500 μg)
Cytoplasmic fraction (500 μg)
Cell wall (500 μg)
120
100
80
60
40
20
60
50
40
30
20
10
0
0
5
25
50

Figure 3.4. Kinetics of IL-6, TNF-α and NO after stimulation of RAW 264.7 macrophage cells with *Lactobacillus casei* ATCC 39539 fractions for 1, 6, 12, 24, and 48 h. ND=non detected.

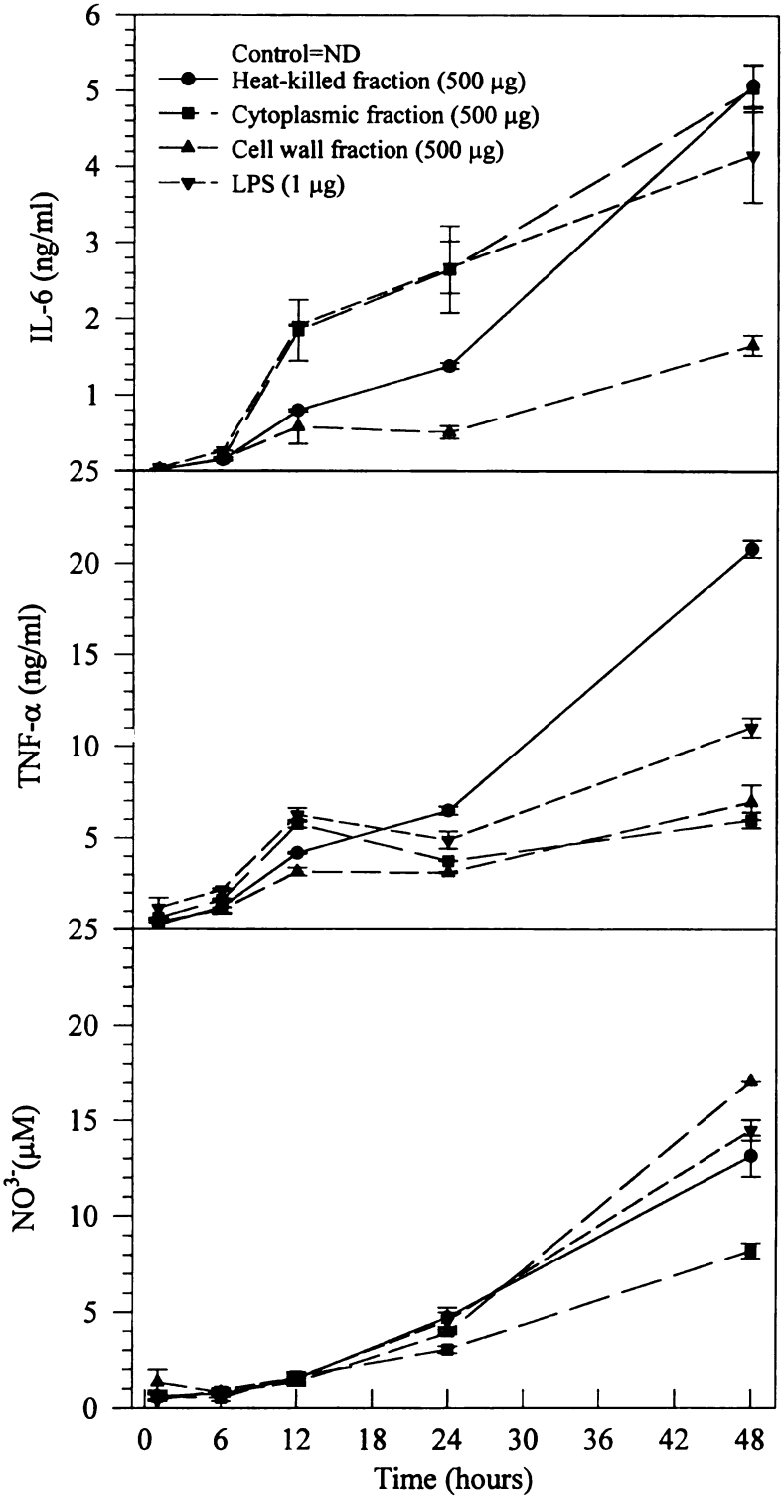

Control=ND
Heat-killed fraction (500 μg)
Cytoplasmic fraction (500 μg)
Cell wall fraction (500 μg)
LPS (1 μg)
IL-6 (ng/ml)
TNF-α (ng/ml)
NO3- (μM)
Time (hours)
0
6
12
18
24
30
36
42
48

Figure 3.5. Effect of incubation of peritoneal macrophages with different fractions of *Lactobacillus casei* ATCC 39539 (x 100). A) 24 h incubation without any microbial product (control). B) 24 h incubation with 500 μg/ml cytoplasmic fraction. C) 24 h incubation with 500 μg/ml cell wall fraction. D) 24 h incubation with 1 μg/ml LPS.

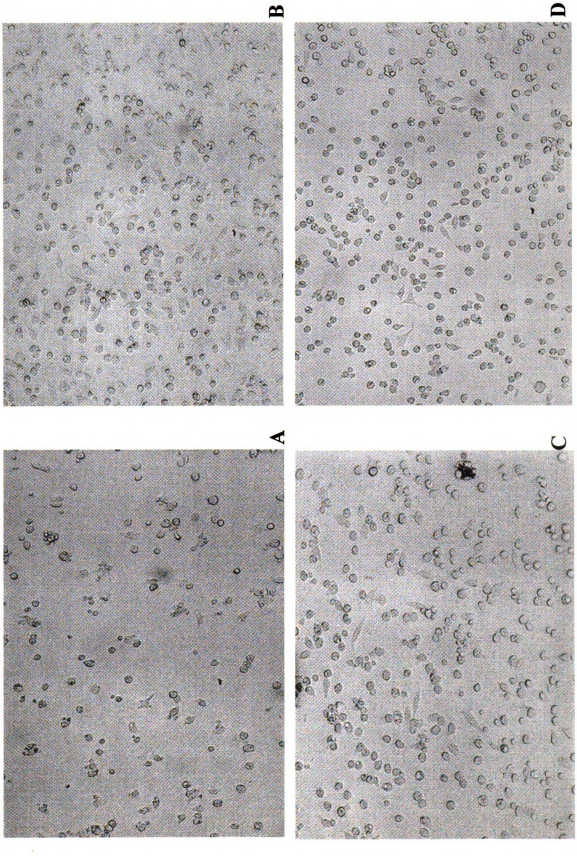
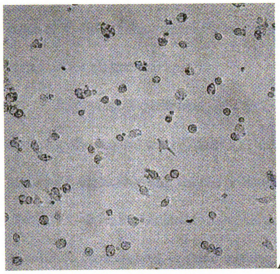
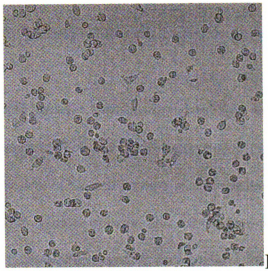
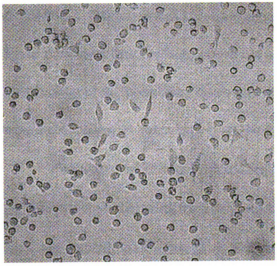
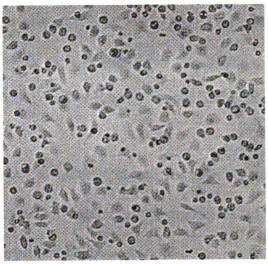

B
D
A
C

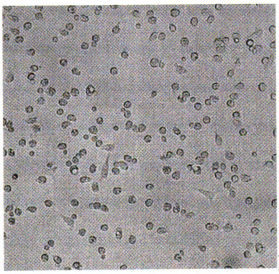
Figure 3.6. Effect of incubation of peritoneal macrophages with different fractions of lactic acid bacteria (x 100). A) 24 h incubation without any microbial product (control). B) 24 h incubation with 500 μg/ml *Lactobacillus acidophilus* cytoplasmic fraction. C) 24 h incubation with 500 μg/ml *Lactobacillus acidophilus* cell wall fraction. D) 24 h incubation with 500 μg/ml *Lactobacillus bulgaricus* cytoplasmic fraction. E) 24 h incubation with 500 μg/ml *Lactobacillus reuteri* cell wall fraction. F) 24 h incubation with 500 μg/ml *Streptococcus thermophilus* cytoplasmic fraction.

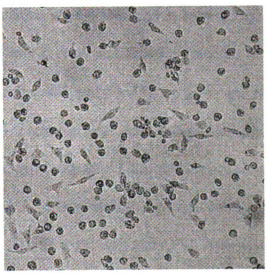

A


B


C


D


E


F

patch cells did not show any difference respect to the unstimulated cells. Similar results were obtained for all bacteria species.

Exposure of peritoneal cells to lactic acid bacterial fractions increased IL-6 levels respect to the control cells (Table 3.21). Production of IL-6 was significantly higher for all bacterial species, showing very large levels regardless of the cell fraction used. Stimulation with cell wall and cytoplasmic material seemed to be more effective than stimulation with heat-killed bacteria and, in some instances, LPS. *Bifidobacterium*, *L. bulgaricus*, *L. casei*, *L. gasseri*, *L. helveticus* and *L. reuteri* appeared to have a high stimulation rate on these cells. Since peritoneal macrophages were not specifically isolated from other type of cells, we cannot discard the possibility that some other cells (i.e. T or B cells) besides macrophages were contributing to these high IL-6 levels.

Production of TNF-α by peritoneal cells was not detected in any case. Similar results were found when analyzing for IFN-γ and NO. Spleen and Peyer's patch lymphocytes did not show any indication of either IL-6, TNF-α, IFN-γ or NO production.

The effects of lactic acid bacteria stimulation were determined in spleen, Peyer's patch and peritoneal cell by incubating cultures with *L. casei* ATCC 39539 fractions for 1, 6, 12, 24, and 48 h. Supernatants were collected and analyzed for TNF-α, IFN-γ and NO, showing no production of any of these cytokines or intermediates. Interestingly and only in peritoneal cells, IL-6 was very rapidly produced, appearing after 1 h in considerable levels and escalating until incubation was maintained for 12 h. In general IL-6 peaked either at 12 h (for cytoplasmic and heat-killed fractions) or 24 h (for cell wall and LPS). Afterwards, levels of IL-6 started to decline slowly (Figure 3.7). Spleen and Peyer's patch cell cultures

did not produce detectable IL-6.

TABLE 3.21. Effect of heat-killed, cytoplasmic and cell wall fraction (500 μg) of lactic acid bacteria on IL-6 production by peritoneal macrophages.

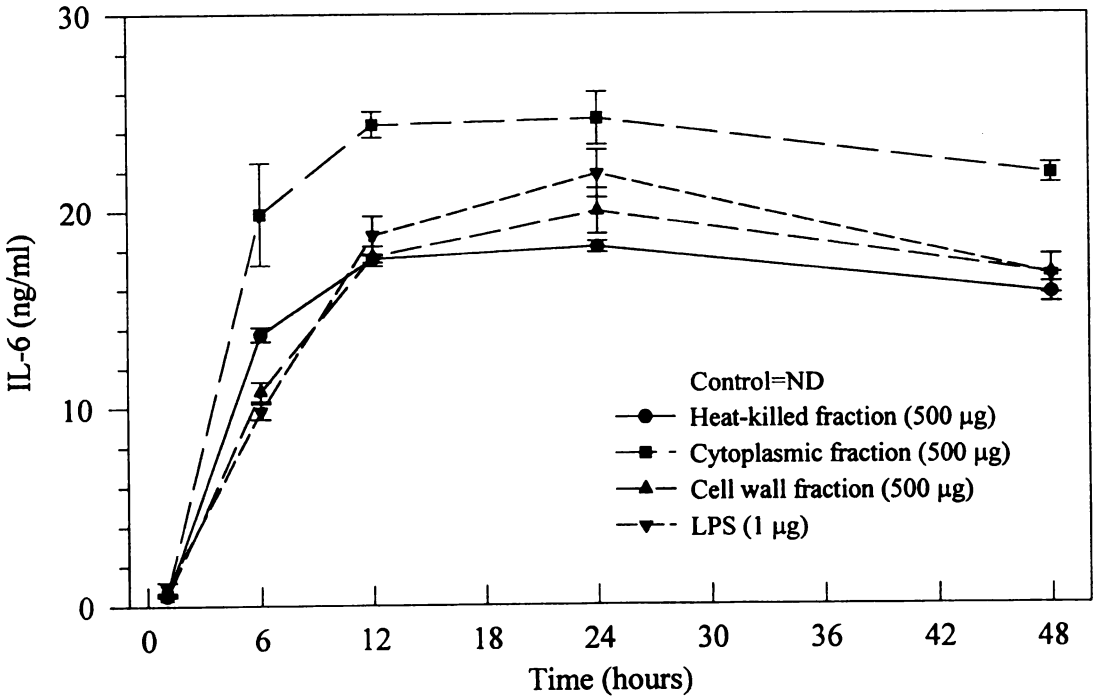
	IL-6 fold control[2]		
	Whole cells	Cytoplasm	Cell wall
Bifidobacterium Bf-1	490*	575*	900*
Lactobacillus acidophilus La 2	588*	650*	430*
Lactobacillus bulgaricus 1489 NCK 231	400*	700*	400*
Lactobacillus casei ATCC 39539	780*	1100*	840*
Lactobacillus gasseri NCK 101	490*	1110*	750*
Lactobacillus helveticus Lr 92	865*	1265*	910*
Lactobacillus reuteri ATCC 23272	635*	1385*	1000*
Streptococcus thermophilus St-133	795*	635*	685*
Lipopolysacharide (1 μg/ml) (+ control)	875*		

[1]RAW 264.7cells (5 x 10^5 cells/ml) were cultured with 500 μg of bacterial fraction for 48 hours.

[2]IL-6 relative change was calculated by dividing experimental data by control values (untreated cells) (n = 4)and are representative of two separate experiments.

*Statistically significant respect to the control (no stimulation) $P<0.05$

Figure 3.7. Kinetics of IL-6 after stimulation of peritoneal macrophages with *Lactobacillus casei* ATCC 39539 fractions for 1, 6, 12, 24, and 48 h.
ND=non detected.


30
20
10
0
IL-6 (ng/ml)
0
6
12
18
24
30
36
42
48
Time (hours)
Control=ND
Heat-killed fraction (500 µg)
Cytoplasmic fraction (500 µg)
Cell wall fraction (500 µg)
LPS (1 µg)

3.5 DISCUSSION

This research showed that in cloned macrophages lactic acid bacteria and their fractions altered cells morphology as evidenced by pseudopod formation and increased number or density of fine granules near the cell periphery and inside their cytoplasm. Macrophages belong to the myeloid lineage and they play a key role in inflammation and in host defense (Abbas et al., 1994). Functions including phagocytosis, antigen presentation, mediator production, anti-microbial and tumoricidal activity contribute to its role in host defense. They are controlled by numerous dynamic stimuli from the tissue microenvironment. The effects of these stimuli on the macrophages are usually associated with specific alterations in gene expression after a series of intracellular signals initiated by ligand-receptor interaction (Abbas et al., 1994). The cellular spreading observed in RAW 264.7 cells could be an early indication of an increased cellular activity. This possibility is supported by the observation that lactic acid bacteria and their cytoplasmic and cell wall fractions were capable of stimulating RAW 264.7 macrophages to produce very significant amounts of TNF-α, IL-6 and NO. In general, bacterial fractions stimulated macrophages and increased their proliferation.

The multifunctional cytokines IL-6 and TNF-α are major effector molecules in bacterially mediated local tissue destruction and necrosis. TNF-α plays a role in such augmented toxicity inducing gene expression of several cytokines. TNF-α has been reported to be induced in mouse peritoneal cells 3 h after the intraperitoneal injection of *Bifidobacterium* cell wall preparation (Sekine et al., 1995). TNF-α responses involve

increased rates of transcription of particular target genes, often through activation of NF-κB or AP-1 transcription factors. At low concentrations, TNF-α acts as a paracrine and autocrine regulator making cell surface more adhesive. Also, TNF-α stimulates other cell types to produce cytokines such as IL-1, IL-6 and TNF-α itself (Janeway et al. 1997). At greater quantities TNF-α enters the blood stream as an endocrine hormone and act as an endogenous pyrogen and it is able to induce the expression of nitric oxide synthase (NOS), which results in conversion of arginine to citrulline and NO. Interleukin 6 (IL-6) is synthesized by macrophages and other cells in response to TNF-α, among others. IL-6 serves as a growth factor for activated B cells during B cell differentiation (Abbas et al., 1994). It can act as an autocrine factor and also as a cofactor with other cytokines. Previous research conducted in this laboratory using heat-killed lactic acid bacteria to stimulate this same cell line, produced IL-6 and TNF-α (Marin et al., 1997a, 1997b, 1997c). Our findings are similar to Miettinen at al. (1996) who reported an induction of the proinflammatory cytokines TNF-α and IL-6 by viable lactic acid bacteria and fixed in 2.5% glutaraldehyde (non viable) lactic acid bacteria when used to stimulate human peripheral blood mononuclear leukocytes. In other studies where RAW 264.7 macrophages were cultured with microbial spores, induction of TNF-α, IL-6 and NO secretion was observed (Hirvonen et al. 1997). Purified cell walls from *Streptococcus mitis* were also seen to induce TNF-α *in vitro* in whole blood of LPS sensitive and LPS resistant mice (Le Roy et al., 1996).

Several investigations have examined the effects of lactic acid bacterial fractions (especially *Bifidobacterium* strains), on immunopotentiating activity. For example, using sonicated cells of a strain of *Bifidobacterium adolescentis*, Lee et al., (1993) showed that

those cells stimulate lymphocytes from Peyer's patch and lymph nodes. Another species of *Bifidobacterium*, *B. breve*, accelerate proliferation of Peyer's patch cells, particularly B cells. The proliferation of B cells is enhanced when the supernatant of plastic adherent cells cultured with *B. breve* (either whole cell or a cell wall preparation) is added. This indicates that *B. breve* activates plastic-adherent cells and that these cells secrete a soluble factor that enhanced proliferation of B cells (Yasui et al., 1991). Takahashi et al. (1993) investigated the interaction of cell fractions of lactic acid bacteria with the immune system, using *Bifidobacterium longum* and *Lactobacillus acidophilus*. In mice fed *B. longum* for more than 8 weeks, a strong antibody response was detected to the cytoplasmic fraction, but not the cell wall fraction. In mice fed *L. acidophilus* for more than 6 weeks an antibody response was detected against the cytoplasmic and cell wall fractions. Sekine et al. (1994) demonstrated that cell wall preparations from *Bifidobacterium infantis* induced polymorphonuclear cells and macrophages when injected in peritoneal cavity. These cells inhibited the growth of tumor cells *in vitro*.

During fractionation procedures of bacterial cells that contain large amounts of lipids, disintegration is impaired by clumping, and they are best subjected to preliminary solvent extraction at room temperature (Work, 1971). However, these types of fractionation procedures have the disadvantage that there is considerable destruction of sugars and presence of organic solvent residues, which is not desirable due to the possibility of stimulatory effects or cell damage. Less drastic methods of wall isolation from cells were used in this research, such that polymers (peptidoglycan), mucopeptides, teichoic acids, polysaccharides and possibly some other components were present together and possibly

interacting with each other.

Heat-killed, cytoplasmic and cell wall concentrations needed in this study to elicit maximal cytokine production were substantially higher than the concentrations of LPS (1 μg/ml) that induce comparable levels of TNF-α and IL-6. Some components of gram positive bacteria (streptococci, micrococci, staphylococci, etc.) produce significant amounts of TNF-α and IL-6 at concentrations above 100 ng to 1 μg of cell walls per ml with a maximal production requiring 10 to 100 μg of cell wall material per ml (Heumann et al., 1994). In those cases the wall of those pathogens may contribute to the septic shock induced by gram positive bacteria. Whole cells were effective as mitogens and activators, but their use may make interpretation of results more difficult than purified products because the presence of diverse components in intact cell, which may act via different mechanisms (Ringden et al. 1977).

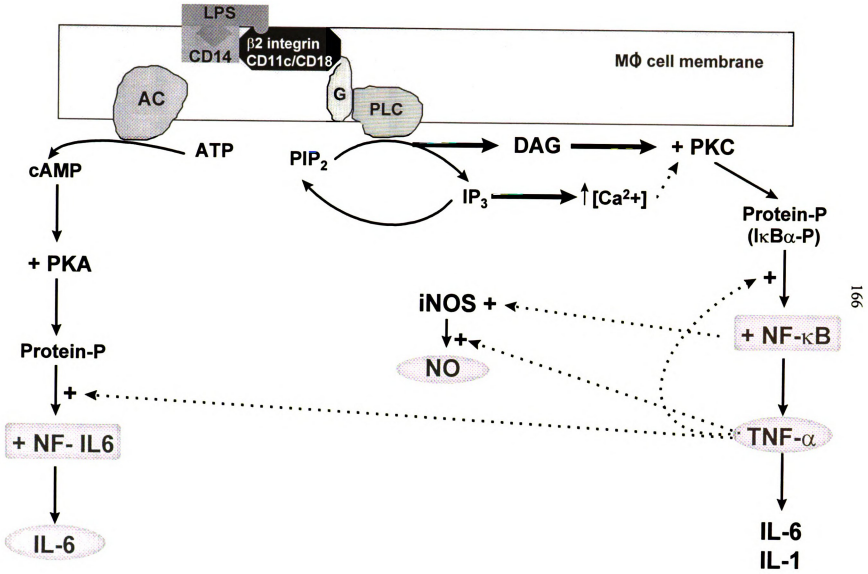
When I explored the kinetics of TNF-α, IL-6 and NO production in macrophages, I saw that lactic acid bacteria induced RAW 264.7 cells to produce significant amounts of TNF-α and IL-6 after just 1 h or 6 h of incubation, respectively, and those levels were further increased, while significant increases in NO occurred only after a substantial amount of TNF-α and/or IL-6 was secreted. These results follow a similar pattern to the effect of cell stimulation with LPS, which can serve as model for cell activation by microbial products.

LPS has numerous important cellular effects. When macrophages are exposed to LPS in small concentrations they secrete not only TNF-α but also IL-6, IL-1, IL-8 and IL-12. These cytokines activate other immune effector cells (Janeway et al., 1997). The LPS-induced signal transduction pathway has not been completely defined. However, one of the

signaling pathways initiated by LPS in macrophages involves the breakdown of polyphosphoinositides and the subsequent generation of intracellular calcium. LPS is recognized by high-affinity receptors (Gammage et al., 1996) in soluble and cell membrane form (CD14 and the β2-integrin CD11c/CD18). The highest affinity receptor, CD14, is located in macrophages and it is a cell surface protein. LPS binds to macrophage CD14 and this complex stimulates the induction of cytokine gene transcription and eventually TNF-α release (Figure 3.8). Mouse TNF promoter/enhancer region contains four functional NF-κB sites (Beutler et al., 1995). In spite of a completely different chemical structure, Gram positive bacterial extracts and LPS can induce similar biological effects and this might activate the same mediator system. Relatedly, peptidoglycan present in gram positive bacteria has been reported to induce inflammation (Stewart-Tull et al., 1985). Peptidoglycan also stimulates macrophage activity inducing the release of numerous macrophage products including endogenous cytokines, intermediates and prostaglandins (Abbas et al. 1994). Their induced secretion might well have important implications with respect to the pathogenesis of the inflammatory process that occurs after exposure to peptidoglycan (Dziarski, 1982). Like LPS, peptidoglycan is a T cell independent B cell mitogen and polyclonal activator.

The cell walls of Gram positive bacteria (not lactic acid bacteria) at a dose of 10 μg (1×10^6 cells) produced, *in vitro*, maximum activation of the peritoneal macrophages of guinea-pigs due to the presence of both peptidoglycan and peptidoglycolipid (Takada et el., 1979). It has been also reported that induction of cytokines is produced after stimulation with cell wall, acting on B cells through T cell mediation. Isolated peptidoglycan-polysaccharide polymer fractions from cell walls have been shown to produce also these type of effects

Figure 3.8. LPS signaling pathways in macrophages


LPS
CD14
β2 integrin
CD11c/CD18
MΦ cell membrane
AC
G
PLC
ATP
cAMP
PIP2
DAG
+ PKC
IP3
↑[Ca2+]
Protein-P
(IκBα-P)
+ PKA
iNOS +
+
+ NF-κB
Protein-P
NO
+
+ NF- IL6
TNF-α
IL-6
IL-6
IL-1

(Novakovic et al., 1994). In staphylococcal species, the peptidoglycan seems to be a B cell mitogen and polyclonal activator which possesses equally high activity in both mice and humans and is equally effective in the induction of DNA synthesis and secretion of polyclonal antibodies (Dziarski et al., 1979). Recently, De Ambrosini et al. (1996) found that specific strains of *L. casei*, *L. acidophilus* and *Lactococcus lactis* contained glycerol teichoic acids and A4α type murein on their peptidoglycan. When they tried to induced phagocytosis in peritoneal macrophages, only the *L. casei* peptidoglycan showed some increased phagocytic activity. This biological activity associated with lactic acid bacteria may not always be due to the peptidoglycan structure.

Most of the studies of the immunoactive components of bacteria have been limited mainly to the cell wall components, such as peptidoglycan, polysaccharides or soluble factors produced by bacteria (Hosono et al. 1997). Experimental evidence suggests that β-glucans enhance the host immune system by activating macrophages, NK cells and killer T cells (Ohno et al. 1996), their action being somehow related to their molecular weight, degree of branching and conformation. Incubation of peritoneal macrophages with β-glucans in presence of TNF-α or IL-6 augmented NO synthesis. It has been demonstrated that some type of glycolipids from gram positive bacteria activated human monocyte IL-6 expression by an identical pathway to LPS involving NF-κB and NF-IL-6 (Zhang et al., 1994). Those glycolipids were unable to stimulate murine resident peritoneal macrophages to release TNF-α. Proteins (Gomez-Flores et al., 1997) and teichoic acids stimulate macrophage cells to release cytokines and NO. Loss of an acyl group or any other modification could mean the abolition in some cases of the cytokine stimulating activity (Henderson et al., 1996).

Besides protein and carbohydrates present in the cell wall, cytoplasmic or heat-killed bacteria fractions, DNA might also have immunomodulatory effects. This possibility is supported by our findings that fractions treated with RNAse or DNAse exhibited less activity. There is an increasing evidence that mammalian immune system can distinguish between bacterial and vertebrate DNA, with bacterial DNA activating immune cells. Bacterial DNA has been reported to induce murine macrophage and B cell proliferation as well as immunoglobulin production after intraperitoneal injection. This effect has not been observed with mammalian DNA (Krieg et al., 1995). The difference between these sources of DNA is the presence of CpG nucleotides in bacterial DNA at a very high frequency. In addition to this CpG suppression, the cytosine residue in CpG dinucleotides is highly methylated in vertebrates, but not in bacteria. CpG motifs appeared to stimulate NK cells and protect B cells against anti-IgM-induce growth inhibition. CpG motifs were nonstimulatory if they were immobilized, so that indicates that cell uptake is required for activation.

Stacey et al. (1996) observed that DNA was taken up by RAW 264.7 macrophage cells. Also, characteristic bacterial DNA sequences as unmethylated CpG motifs activated a signaling cascade ending in the activation of NF-κB and inflammatory gene induction. TNF-α is strongly induced after 1 h treatment with DNA (20 μg/ml). Treatment of cells with DNA also increases NF-κB activation. This transcription factor, when activated in macrophages is involved in the induction of TNF-α transcription. IL-6 gene was reported also as being induced in response to bacterial DNA (Yi et al., 1996; Pisetsky et al., 1993), as well as IL-12 and IFN-γ (Klinman et al., 1996). Foreign and synthetic RNAs (poli I:poli C) stimulate immune responses using possibly similar mechanisms to DNA (Pisetsky 1996).

Nitric oxide production by macrophages was measured based in a metabolic pathway in the cell that converts L-arginine to reactive nitrogen intermediates, via an oxidative enzymatic pathway involving a nitric oxide synthase (NOS) (Dietert et al. 1995). Nitric oxide produced *in vivo* reacts with molecular oxygen to generate other reactive oxides of nitrogen, such as dinitrogentrioxide (N_2O_3) and dinitrogentetroxide (N_2O_4). These can react with water to form nitrite, which is further oxidized to nitrate *in vivo*. It is known that NO is an important mediator in the nonspecific host-defense against microbes and tumors. NO is one of the cytotoxic agents by which activated macrophages can kill bacteria, tumor cells and a variety of other pathogens, but also normal tissue cells during auto-immune reactions (Laskin et al., 1994). NO is a free radical messenger that serves also as a neurotransmitter and vasodilator. Chemically, NO is a small lipophilic molecule, so few microbes can block its entry. While large amounts of NO may inhibit production of cytokines, smaller amounts may induce it. An excessive production of NO plays a role in promoting the classical signs of inflammation as well as tissue injury (Laskin et al., 1994). Recently there is a great deal of interest focused on the possible role of NO as an immune defense molecule. Its role in host defense still needs to be defined.

There are constitutive and inducible forms of NO (MacMicking et al., 1997). There are three genes encoding NOS. Primary macrophages appear to express only NOS2 and lymphocytes only rarely express any NOS. NOS2 is independent of elevated intracellular Ca_2^+ and it is often called iNOS (inducible) (MacMicking et al., 1997). The pathway where iNOS is expressed is usually associated with inflammation and infection. iNOS in mouse macrophage can produce NO for as long as 5 days when care is taken to replenish both the

inductive stimuli and the L-arginine substrate. iNOS can bind to calmodulin (CaM) so tightly that CaM copurifies as a subunit in the absence of added Ca_2^+. In the induction of iNOS gene, two regulatory regions placed in the promoter site of this gene are known to be involved. One of them contains an element for transcriptional induction of iNOS which involves the NF-κB site. NF-κB site is the enhancer region acting as a functional regulatory element for the maximal inducibility of iNOS promoter in LPS alone or IFN-γ plus LPS treated RAW 264.7 macrophages. The other region mediates the synergistic induction of iNOS transcription by IFN-γ for which requires IRF-1 binding to interferon regulatory factor element (IRF-E) (Kim et al., 1997). It was suggested also the presence of an enhancer region containing another LPS responsive element with similarities in sequence to the NF-κB region.

Numerous cytokines and microbial products, sometimes acting synergistically stimulate iNOS expression. In mouse peritoneal macrophages, IFN-γ seems to be responsible for this stimulation (Kim et al., 1997). In RAW 264.7, LPS also stimulates iNOS expression through induction of NF-κB. Ingestion of microorganisms generally elicit TNF-α production and this cytokine exerts an autocrine stimulus increasing the antimicrobial action of the macrophage, in particular by inducing the production of NO. Some other studies found that rodent phagocytes exposed to inflammatory mediators or pathogens produce large amounts of NO (Chesrown et al., 1994), but not human phagocytes. Eigler et al. (1995) showed that LPS-stimulated murine RAW 264.7 cells produce both NO and TNF-α and that NOS inhibition increased TNF-α production suggesting that endogenous NO could suppress TNF-α production.

Taken together, these data strongly suggest that lactic acid bacteria can stimulate macrophages to produce cytokines. Elevated cytokine production, especially that of IL-6 and TNF-α, was well consistent with later increased NO synthesis. Further purification of these lactic acid bacteria fractions used in our experiments may result in the isolation and characterization of stimulatory compounds. It was reported that bacterial cell wall components can persist in tissues for long periods of time (Dziarski, 1982). If activation by these bacterial fractions play any role *in vivo*, one key factor that could influence this effect might be the requirement for prolonged exposure of leukocytes to these stimulants. Thus, cell wall may be the best choice to provide a continuous stimulus required for activation. Under normal conditions, these cell wall components are immediately in contact with cells from the intestine or the peritoneum. These cells are activated either directly by bacterial products or by cytokines secreted by peritoneal macrophages or possibly other immune cells. These release inflammatory mediators, such as TNF-α, IL-6, which can act on the neighboring cells and quickly amplify the cellular response. Following diffusion in the tissue, these cytokines may activate local macrophages, fibroblasts and endothelial cells which also produce mediators and thus contribute to the classical inflammatory reaction.

3.6 FUTURE STUDIES

We have found, using peritoneal cells as well as a macrophage cell line, that *in vitro* exposure to lactic acid bacteria stimulates the production of IL-6, TNF-α and NO. The mechanisms involved in the immunostimulative effect of lactic acid bacteria fractions on macrophage cytokine production have to be determined yet but they could be similar to the mechanisms followed by LPS. Measurement of intracellular concentration of NF-κB in macrophages after lactic acid bacteria exposure and comparison with LPS-mediated signal transduction in macrophages could give us an approximation of how similar these mechanisms of activation are and the possible differences between stimulation with gram positive and gram negative bacterial components. Bacterial extracts need further purification and chemical identification to determine exactly which component exerts the best immunostimulatory effect on immune cells. Finally, to provide additional support for the involvement of nitric oxide in the conditioned immunomodulation, we could assess the effect of addition of a competitive inhibitor of oxidative L-arginine metabolism to cultures of cells from animals exposed to lactic acid bacteria.

After purification and characterization of bacterial extracts, several studies could be conducted to assess the effects of these purified fractions on *in vivo* and *ex vivo* functions.

CHAPTER 4

ENHANCED MUCOSAL AND SYSTEMIC IgA RESPONSES TO CHOLERA TOXIN IN MICE FED YOGURT CONTAINING *LACTOBACILLUS ACIDOPHILUS* AND *BIFIDOBACTERIUM* SP.

4.1 ABSTRACT

Lactic acid bacteria have been reported to have benefits for the prevention and treatment of some forms of diarrhea and related conditions. I hypothesized that a possible mechanism for these effects is direct stimulation of the gastrointestinal immune response. To test this hypothesis, I determined whether administration of yogurt enhances mucosal and systemic immunity in mice leading to increased levels of antibodies against an orally presented immunogen, cholera toxin. Yogurts were manufactured with starter cultures containing different species/strains of lactic acid bacteria. Mice were fed these yogurts for three weeks during which they were also orally immunized twice with 10 μg cholera toxin. Blood was collected at day 0 and 21 and fecal pellets were collected weekly. Upon sacrifice, spleen and Peyer's patches were aseptically removed, and the lymphocytes from these organs were isolated and cultured. An enzyme-linked immunosorbent assay designed for cholera toxin was used for titration and quantification of coproantibodies, serum antibodies and cell supernatant antibodies against cholera toxin of the IgA and IgG isotype. Mice immunized orally with cholera toxin responded by producing specific intestinal and serum IgA-anti cholera toxin. Antibody responses of the IgA isotype were significantly increased in mice fed yogurts made with starters containing *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (yogurt bacteria) supplemented with *L. acidophilus* and *Bifidobacterium* spp. (*B. bifidum, B. infantis*). Yogurt manufactured with starters containing only yogurt bacteria produced decreased IgA-anti cholera toxin when compared to the control group fed non-fat dry milk. Although a strong response was also observed for IgG-anti cholera toxin in serum,

the response did not differ among groups. Thus, administration of yogurt supplemented with *L. acidophilus* and *Bifidobacterium* spp. enhanced mucosal and systemic IgA responses to the immunogen cholera toxin.

4.2 INTRODUCTION

Lactic acid bacteria have been documented to have benefits in the prevention and treatment of some forms of diarrhea and related conditions as well as in enhancing immune function (Sanders, 1993). Preservation of the integrity of the normal intestinal flora, colonization resistance, adherence, production of antibacterial substances appear to be important for these effects. Although the precise mechanisms of action are still unclear, yogurt consumption has increased significantly in recent years, presumably because of these perceived health benefits. Conventional yogurt is a fermented milk produced by classical addition of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* to milk (Trapp et al., 1993). Based on increasing evidence that other lactic acid bacteria such as *L. acidophilus* and the bifidobacteria have therapeutic properties, these species have also been added to conventional yogurt, or alternatively used as main starter culture for production of new types of yogurt or fermented milks (Mitsuoka, 1990; Robinson, 1991). These latter bacteria are advantageous because while conventional yogurt bacteria have a very poor intrinsic resistance to acid and bile (Marteau and Rambaud, 1993), *L. acidophilus* and *Bifidobacterium* can tolerate a pH 3 and 2-8% of bile acid concentrations (Gilliland, 1989). Both bacteria can be added in an optimal ratio before inoculation that result in 8-9 x 10^8 acidophilus cells/ml and 5-8 x 10^8 bifidobacteria cell/ml (Alm et al., 1993).

There is evidence that ingestion of lactic acid bacteria exert an immunomodulatory effect in the gastrointestinal system of either humans or animals (Solis-Pereyra and Lemonnier, 1993; Perdigon et al., 1986a, 1986b, 1987). The gastrointestinal system

possesses specialized elements that react upon exposure to antigens coming from diet resulting in immune reactions (Pestka, 1993). These elements constitute the mucosal immune system and essentially they are lymphoid tissue containing a full array of immune cells necessary for induction of an immune response, that is, B, T, macrophage and accessory cells. The tissues that represent the gut mucosal immunity are Peyer's patches, mesenteric lymph nodes, lamina propria and intraepithelial lymphocytes (Janeway and Travers, 1994). The systemic immune compartment is formed by all tissues involved in defense of the internal environment from invading microorganisms, and consists of spleen, thymus, bone marrow and lymph nodes throughout the body. The mucosal and systemic immune compartments, can overlap in some of their specific activities. This connection is mediated via circulation of blood and lymph, where B and T cells can migrate from one compartment to the other. The mechanisms by which lactic acid bacteria might activate the mucosal and systemic compartments are still unknown.

One potent antigen that stimulates both the mucosal and systemic compartments is cholera toxin (CT) (Ogra et al., 1994). CT induces an intestinal secretory IgA response to itself and to additional co-administered antigens (Epple at al., 1997; Robbins et al., 1989). A strong local IgA response has been reported in the small intestine as demonstrated by the detection of antibodies against CT in intestinal secretions (Snider, 1995). A strong serum IgG antibody response is also induced by CT immunization, and the cells secreting this isotype appeared to be located in Peyer's patches, spleen and lamina propria. CT is known to act as an intestinal adjuvant for many common proteins, viruses or bacterial polysaccharides, creating a memory response with strong IgA antibody response to antigens (Ogra et al.,

1994), and maybe signaling by secretion of certain cytokines.

In this study, the effect of yogurt ingestion on gastrointestinal immune system of the mouse was assessed using CT immunization as a model. Feeding individuals with different types of yogurt made with *L. bulgaricus*, *S. thermophilus* with or without *L. acidophilus* and *Bifidobacterium* spp. and using CT through oral immunizations as a protein antigen, I was able to demonstrate the adjuvant activity of yogurt containing *L. acidophilus* and *Bifidobacterium* spp. The results indicated that ingestion of yogurts supplemented with *L. acidophilus* and *Bifidobacterium* spp. enhanced the mucosal and systemic IgA response to CT.

4.3 MATERIAL AND METHODS

4.3.1 Yogurt preparation

Four commercial yogurt starter cultures were obtained by Sanofi Bio-Industries (Waukesha, WI), Chr. Hansen's Laboratories Inc. (Milwaukee, WI) and Rhone Poulenc Inc. (Madison, WI). Table 4.1 summarizes the type of culture present in each starter and their source. The four yogurts were made using pasteurized 12 % (w/v) non-fat dry milk (NFDM) (Michigan Milk Producers Association, Ovid, MI) according to the instruction of the culture suppliers. NFDM was inoculated with the starter and mixed. The inoculated mixtures were aliquoted into 50 ml sterile conical polyethylene centrifuge tubes and incubated for 6-8 h at 37^0C developing a typical yogurt consistency. Yogurts were rapidly cooled and stored at 4^0C and ingested within 21 d. Numbers of total aerobes, streptococci and bifidobacteria were determined throughout storage.

4.3.2 Enumeration of yogurt bacteria

Total aerobic cells, streptococci and bifidobacteria counts were performed at 0, 1, 2, 3 and 4 wks of storage to determine the viability of the cultures. De Mann-Rogosa-Sharpe medium for lactobacilli (MRS) (DeMan et al., 1960) + 5 % (w/v) lactose (MRSL) agar plates, modified ST agar (Lee's agar) (Lee et al., 1974) and neomycin sulfate, paromomycin sulfate, nalidixic acid, lithium chloride (NPNL) agar (Teraguchi et al., 1978; Rasic and Sad, 1987) were used for total aerobic, *Streptococcus* and *Bifidobacterium* counts, respectively. After the appropriate dilutions were made, samples were plated using the media mentioned

TABLE 4.1. Composition of lactic acid bacteria in commercial yogurt starter cultures.

Yogurt culture	Lactic acid bacteria	Supplier
Ultra-Gro Direct yogurt culture	*S. salivarius* ssp. *thermophilus* *L delbrueckii* ssp. *bulgaricus*	Sanofi Bio-Industries Waukesha, WI
Sbifidus Direct yogurt culture	*S. salivarius* ssp. *thermophilus* *L delbrueckii* ssp. *bulgaricus* *L. acidophilus* *Bifidobacterium* spp.	Sanofi Bio-Industries Waukesha, WI
PY-3 Redi-Set yogurt	*S. salivarius* ssp. *thermophilus* *L delbrueckii* ssp. *bulgaricus* *L. acidophilus* *B. bifidum*	Chr. Hansen Inc. Milwaukee, WI
DPL yogurt Quick Start ABY-2C	*S. thermophilus* *L bulgaricus* *L. acidophilus* *B. infantis*	Rhone-Poulenc Madison, WI

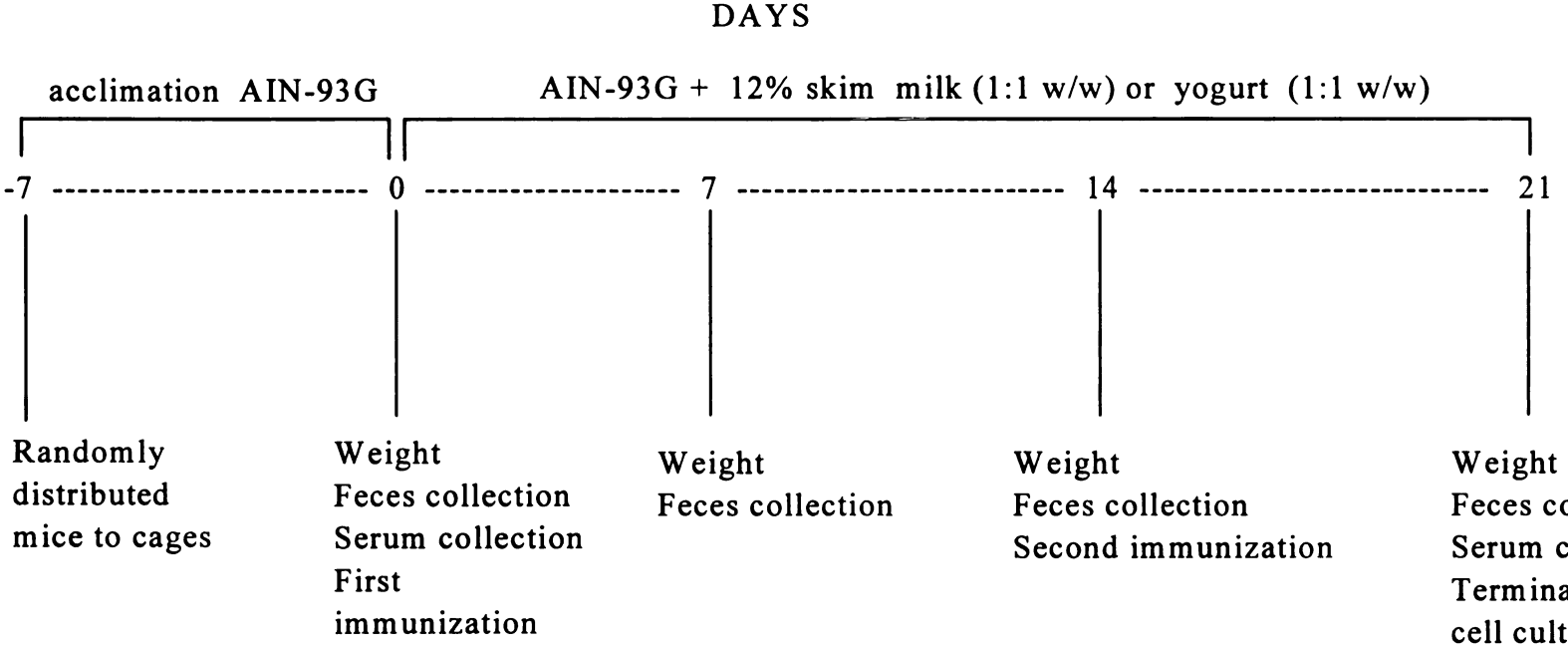
above and incubated for 48 h at 37^{0}C, aerobically for total aerobic and *Streptococcus*, and anaerobically using anaerobe jars and an anaerobic Gas Pak® (Becton Dickinson Co., Cockeysville, MD) system for *Bifidobacterium*. The colonies were counted using a Quebec colony counter (Fisher Scientific, Pittsburgh, PA.).

4.3.3 Animal model and diet

Female B6C3F1 mice (C57BL/6 female x C3H/HeN male), 8 week old, were obtained from Charles River Labs. (Raleigh, NC). Ten mice per experimental group were employed. All animal handling was conducted in strict accordance with regulations established by the National Institutes for Health. Experiments were designed to minimize numbers of animals required to adequately test the proposed hypothesis and approved by Michigan State University Laboratory Animal Research committee. Mice were housed (5 per cage) in a windowless room at 25-27^{0}C with a 12:12 hr light/dark cycle and a negative-pressure ventilated area, in protected-environment cages (Nalgene, Rochester, NY) that include a transparent polycarbonate body with filter cover and stainless-steel wire lid. Distilled water was provided *ad libidum* and changed every 3 days.

Mice were acclimatized to housing and fed nutritionally complete modified semipurified diet as described by the American Institute of Nutrition (AIN-93G) (Reeves et al., 1993) for at least one wk prior to starting experiments. The study lasted 21 d. Yogurt or NFDM control were mixed (1:1) with AIN-93G (ICN Nutritional Biochemical, Cleveland, OH). Yogurt and control diets were prepared fresh and provided daily during the experiment in clean powder feeders with stainless steel grids and lids to reduce spilling. Figure 4.1

Figure 4.1. Experimental design for assessing effects of yogurt ingestion on immunoglobulin response to cholera toxin.


DAYS
acclimation AIN-93G
AIN-93G + 12% skim milk (1:1 w/w) or yogurt (1:1 w/w)
-7
0
7
14
21
Randomly distributed mice to cages
Weight
Feces collection
Serum collection
First immunization
Weight
Feces collection
Weight
Feces collection
Second immunization
Weight
Feces collection
Serum collection
Termination and cell culture

summarizes feeding and treatment schedules during the experimental period. Weight changes were recorded and fecal samples were collected weekly. Fecal pellets were placed in sterile plastic containers, immediately processed and stored at - 80^{0}C until analyzed.

4.3.4 CT immunization

Mice were deprived of food for 2 h before oral immunization. Just prior to immunization, they were gavaged with 0.5 ml of a solution consisting of 8 parts Hanks' balanced salt solution (Sigma Chemical Co., St. Louis, MO) and 2 parts 7.5 % sodium bicarbonate to neutralize stomach acidity. Thirty minutes later, 10 μg of CT (Sigma Chemical Co., St. Louis, Mo) was administered in 0.25 ml of filter-sterilized phosphate-buffered saline (PBS). Groups of mice were immunized on days 0 and 14. Five experimental groups were used in the study (control, Ultra-Gro Direct, Sbifidus Direct, PY-3 Redi-Set, and DPL Yogurt Quick Start ABY-2C). Food and water were restored immediately after immunization.

4.3.5 Fecal pellet and serum preparation

Fecal samples were prepared as described previously (de Vos and Dick, 1991). Briefly, feces were picked, aseptically weighed and placed into centrifuge tubes. Ten ml PBS per gram of feces (v/w) were added, and the mixture was incubated for 15 min at room temperature. Samples were mixed by vortexing until suspended, left to settle for 15 min, mixed again, and then centrifuged at 22,000 x g for 10 min. The supernatant was removed and stored at - 80^{0}C for immunoglobulin measurement.

Blood was obtained from anesthetized mice from the retroorbital plexus. Serum was obtained after overnight incubation at 4^0C and centrifugation at 1,000 x g for 15 min. Serum samples were aliquoted, stored at - 80^0C prior to monitoring for IgA and IgG anti-cholera toxin antibody responses.

4.3.6 Lymphocyte culture

Mice were sacrificed one week after the second and last immunization with cholera toxin by cervical dislocation under gentle anesthesia. Spleen and Peyer's patch cultures were chosen to represent systemic and mucosal immune responses, respectively.

Peyer's patches were removed aseptically, placed in a small Petri dish containing 5 ml RPMI 1460 medium (Sigma chemical Co., St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco Laboratories, Chagrin Falls, IL), 2-mercaptoethanol (50 μM), non-essential amino acids (1 mM) (Gibco BRL, Life Technologies, Grand Island, NY), sodium pyruvate (100 mM) (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma), and teased using two sterile cover slides. The cell suspension was passed through a 70 μm nylon membrane that was fixed on top of a 15 ml sterile centrifuge tube. Five ml of fresh RPMI 1640 were added to wash the plate and membrane. Cells were centrifuged at 450 x g for 8-10 min, resuspended in 2 ml of fresh medium and counted using 0.4 % trypan blue stain (Sigma) and a hemacytometer. Cell suspensions were kept on ice at all times.

Spleens were removed aseptically, placed in a Petri dish containing 10 ml RPMI 1460 medium as above, teased thoroughly and cell suspensions passed through a sterile 85 mesh screen to remove tissue debris. Lymphocytes were then placed into a 50 ml sterile centrifuge

tube, left to settle for 5 min on ice and then transferred to another sterile tube. The suspension was centrifuged at 450 x g for 8 - 10 min and supernatant was discarded. Erythrocytes were lysed for 3 min at room temperature in 5 ml of a buffer containing 9 parts of 0.16 M ammonium chloride plus 1 part 0.17 M TRIS buffer (pH 7.2). Fresh RPMI 1640 (10 ml) was added, cells were mixed, centrifuged at 450 x g for 10 min, resuspended in 20 ml of fresh medium and counted. Cell were kept on ice at all times.

Peyer's patch and spleen cells (1 x 10^5/ml) were cultured in supplemented RPMI 1640 medium in flat-bottomed 48 well (1 ml) tissue culture plates (Fisher Scientific Co., Corning, NY) at 37^0C in a 7% CO_2 humidified incubator. Duplicate cultures were stimulated with or without 20 μg/ml lipopolysaccharide (LPS) from *Salmonella typhimurium* (Sigma Chemical Co., St. Louis, MO). Supernatant was collected at 7 days and stored at - 80^0C until analysis.

4.3.7 Enzyme-linked immunosorbent assay (ELISA)

Antibody titers in serum, fecal extracts and cell culture supernatants were determined by an ELISA as described by Jackson et al. (Jackson et al., 1993) with some modifications. Immunolon IV Removawell microtiter strip wells (Dynatech Laboratories Inc., Chantilly, VA) were coated with 100 μl of CT (5 μg/ml) in 0.1 M carbonate buffer pH 9.6. Plates were incubated overnight at 4^0C in a humid atmosphere and washed three times with phosphate-buffered saline (PBS) plus Tween 80 (PBS-T). Plates were blocked with 200 μl of 1% bovine serum albumin (BSA) in PBS for an hour at 37^0C. The plates were washed again three times with PBS-T. Fifty microliter aliquots of serial dilutions of serum (1:50 to 1:6400), fecal

extracts (1:2 to 1:320) or supernatants in 1% BSA-PBS were added in duplicate, and the plates were incubated for 75 minutes at 37^{0}C. Preimmune serum and fecal extracts at similar dilutions were used on the same plates as controls. Plates were washed three times with PBS-T. The secondary antibody consisted of 50 μl/well of horseradish peroxidase conjugated to goat IgG fraction to anti-mouse IgA-α chain (26 μg/ml) or IgG-γ chain (40 μg/ml) (Cappel, ICN Pharmaceuticals, Inc., Aurora, OH) in 1% BSA-PBS. Plates were then incubated at 37^{0}C for 75 minutes and then washed six times with PBS-T. Bound peroxidase was determined by adding 100 μl of 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) substrate [0.4mM ABTS,50 mM citrate buffer (pH 4.0), and 1.2 mM hydrogen peroxide] as described previously by Pestka et al. (1980). Absorbance was measured at 405 nm on a Vmax kinetic microplate reader (Molecular Devices, Menlo Park, CA). End-point titers were expressed as the last dilution which gave an OD at 405 nm of ≥ 0.2 OD units above the OD of preimmune serum and fecal extract.

CT-specific IgA and IgG were quantitated gravimetrically in serum, fecal extracts and cell culture supernatants as described by Pestka et al. (1990). Concurrently with the ELISA described above, 50 μl of serially diluted standard mouse reference serum were added to anti-Ig wells that flanked CT wells for use in isotype reference curves. Fifty μl of serum or fecal sample (1:50 and 1:3 dilution respectively) were added to the wells coated with CT. Plates were then incubated and washed as described for titration above. Immunoglobulins bound to CT were assigned concentrations (ng/ml) based on comparison of absorbance to values obtained in isotype reference curves that were run in parallel.

4.3.8 Statistical Analysis

Data were analyzed by Student's *t* test using SigmaStat Statistical Analysis System (Jandel Scientific, San Rafael, CA). A *p* value of less than 0.05 was considered statistically significant.

4.4 RESULTS

4.4.1 Viability of the refrigerated yogurt during storage

When viability of the yogurt was assessed over a four week period (Figure 4.2), total aerobic and *Streptococcus* counts ranged from 1 x 10^9 to 5 x 10^8 CFU/g of yogurt. Numbers of viable bifidobacteria declined at a more rapid rate. For DPL yogurt Quick start ABY-2C and PY-3 Redi-Set yogurt, *Bifidobacterium* counts declined from 1.2 x 10^8 to 2.3 x 10^6 CFU/g and from 6.9 x 10^7 to 3.7 x 10^6 CFU/g, respectively, over a 3 week period. For Sbifidus Direct yogurt, counts declined from 2.5 x 10^8 to 1.1 x 10^7 CFU/g. Nevertheless, viability of lactic acid bacteria and bifidobacteria in yogurt remained above 1 x 10^6 CFU/g, which is within normal values reported in commercial milk or yogurt (Shin, 1997).

4.4.2 Body weight and feed intake

All animals remained healthy during the feeding trial. Diarrhea was not observed in any treatment or control group. No differences in initial mean body weight among the groups (20.5 ± 0.4 g) were detected. After 3 weeks, body weights of the treatment groups were comparable to those of control group (Figure 4.3). No statistically significant differences in body weights were observed among the treatment groups and/or control group at any time during the study.

4.4.3 Specific Immunoglobulin production

Intestinal and serum anti-CT responses were measured in mice immunized orally with

Figure 4.2. Bacterial counts. Total aerobic, Streptococci, and Bifidobacteria counts in yogurts manufactured for feeding trial. UG= Ultra-Gro Direct yogurt culture, SBIF= Sbifidus Direct yogurt culture, PY3= PY-3 Redi-Set yogurt, ABY= DPL yogurt Quick Start ABY-2C.

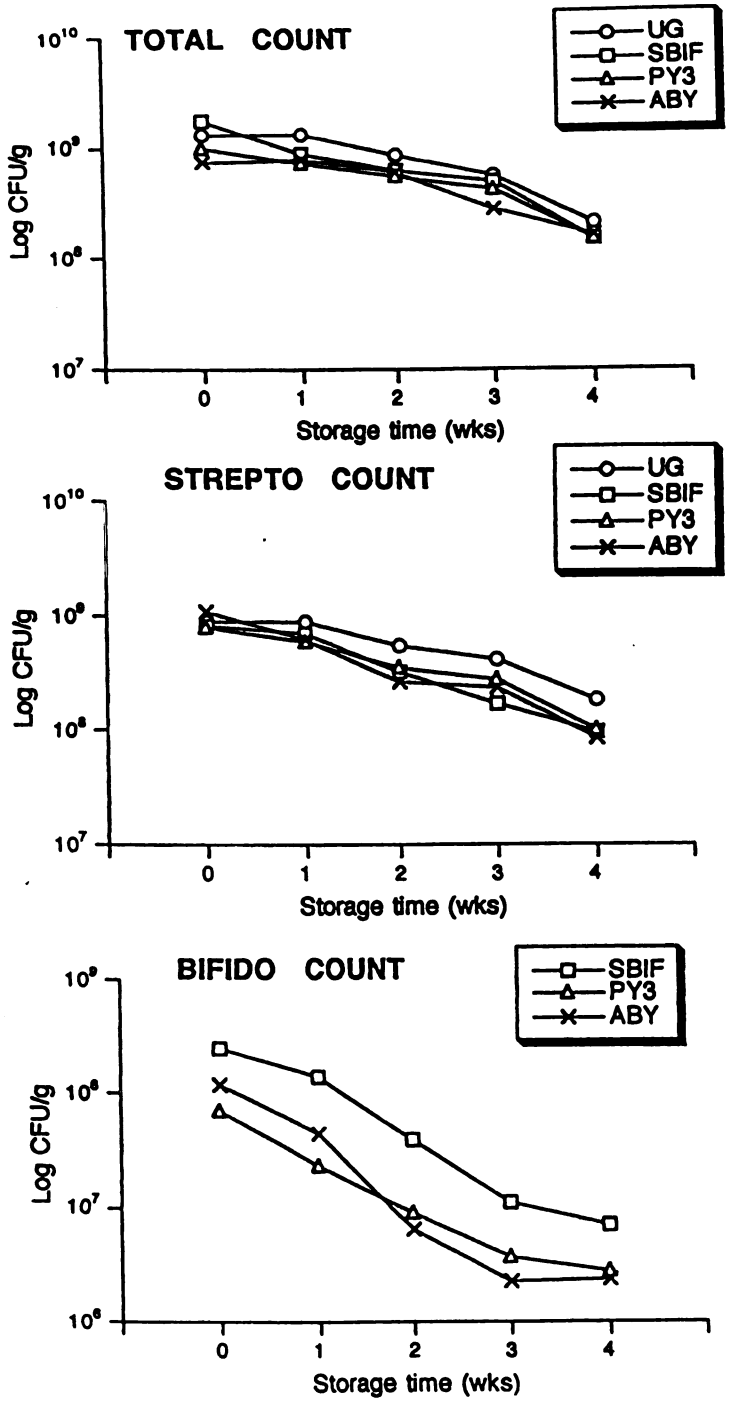
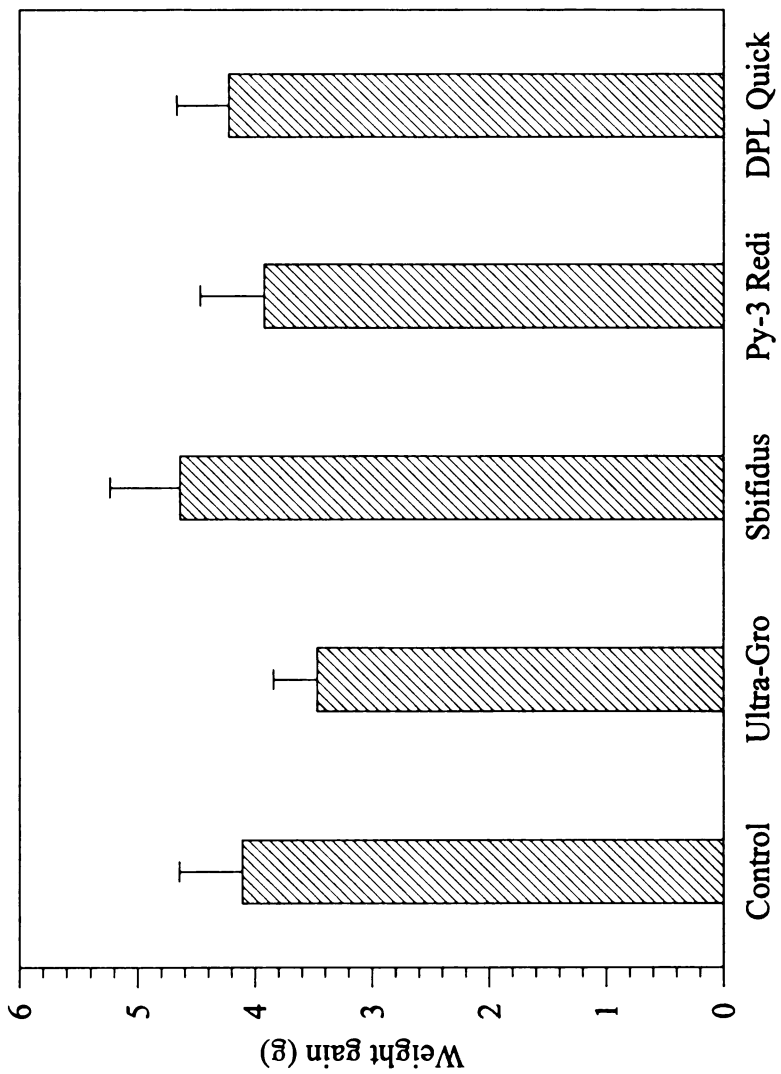

TOTAL COUNT
UG
SBIF
PY3
ABY
Log CFU/g
Storage time (wks)
STREPTO COUNT
UG
SBIF
PY3
ABY
Log CFU/g
Storage time (wks)
BIFIDO COUNT
SBIF
PY3
ABY
Log CFU/g
Storage time (wks)

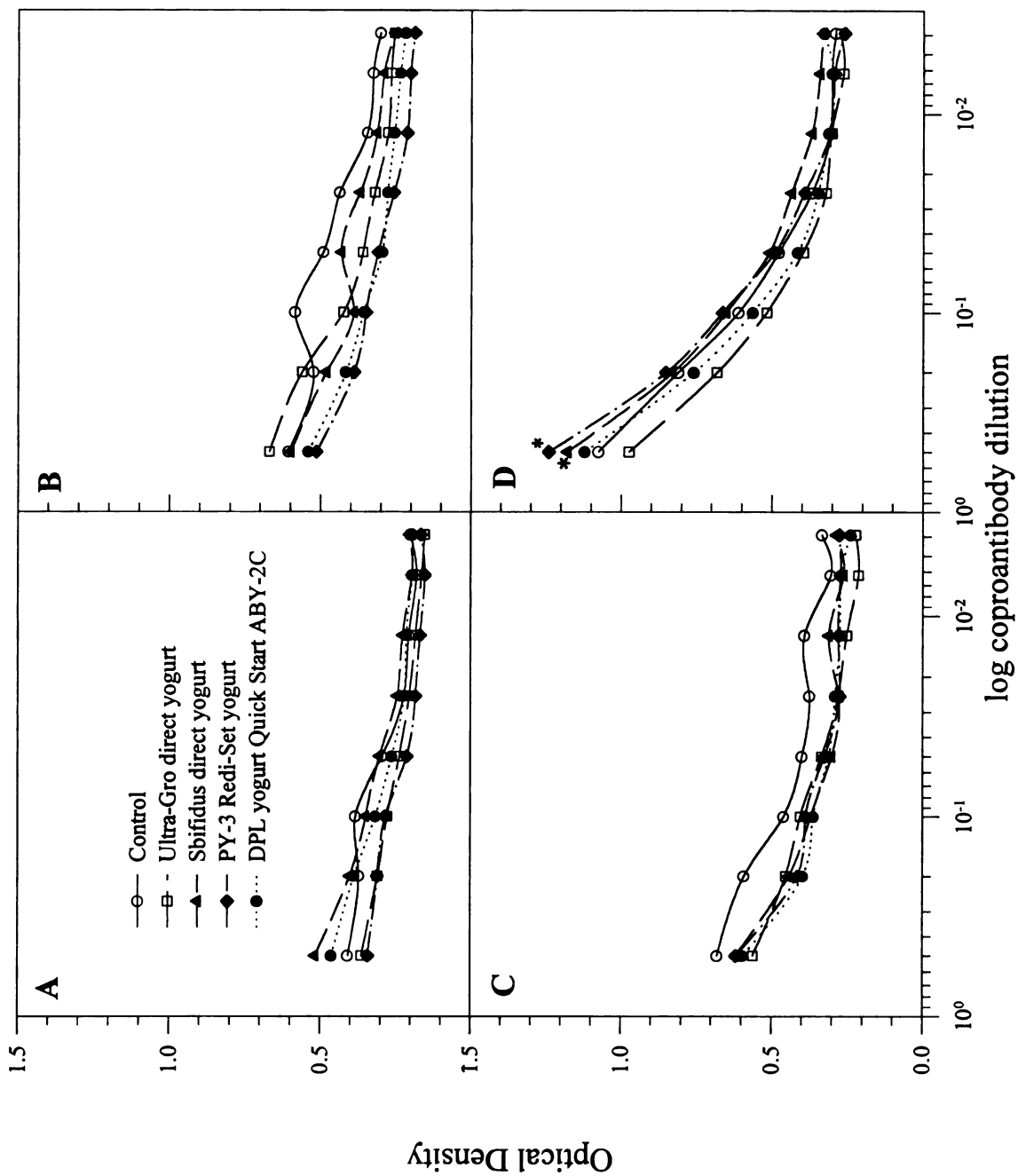
Figure 4.3. Weight gain for mice fed skim milk or yogurt made with lactic acid bacteria and bifidobacteria for 3 wk (n=10). Mice were weighed at the beginning of the feeding trial and change in weight was monitored weekly until the end of the experiment. Data represent the mean ± S.E.M.


Weight gain (g)
0
1
2
3
4
5
6
Control
Ultra-Gro
Sbifidus
Py-3 Redi
DPL Quick

10 μg CT twice (day 0 and 14) and simultaneously fed with the experimental yogurts. To measure intestinal response, fecal pellets were collected from the various experimental groups and extracts were tested by ELISA for specific IgA anti-CT (Figure 4.4). Endpoint titers for CT from all groups of mice before immunization and yogurt feeding showed very low antibody levels against CT (Figure 4.4A). Anti-CT IgA levels slightly increased one week after control or treatment mice received the first CT dose, but there were no significant differences among the groups (Figure 4.4B). Levels appeared to decrease during the second week for the four treatment groups while the control group presented a slightly higher level (Figure 4.4C). One week after a second dose of CT was given, endpoint anti-CT IgA titers differed markedly among all groups (Figure 4.4D). Based on optical density measurements, mice fed yogurt made with Sbifidus Direct yogurt culture and PY-3 Redi-Set yogurt culture (containing conventional yogurt bacteria supplemented with *L. acidophilus* and *Bifidobacterium* spp.) exhibited significantly higher titers than the control group ($p \leq 0.05$), whereas the group fed yogurt made with Ultra-Gro Direct yogurt culture (i.e conventional yogurt bacteria) exhibited a decreased specific IgA levels respect to the control diet. Gravimetric estimates of CT-specific IgA calculated were significantly higher in groups fed Sbifidus Direct yogurt culture, PY-3 Redi-Set yogurt culture, and DPL yogurt Quick Start ABY-2C when compared with the control (Table 4.2). Interestingly, group Ultra-Gro Direct yogurt culture exhibited significantly lower specific IgA than the corresponding control group and also with respect to the rest of yogurt treatment groups.

Mice immunized orally with CT also responded with specific serum antibody responses of IgA and IgG isotype. As with coproantibodies, levels of anti-CT IgA in serum

Figure 4.4. Specific IgA-anti-cholera toxin in fecal samples (coproantibodies). Fresh fecal pellets were collected from each mouse in each group (n=10, 5 groups) at the times indicated: A= day one of feeding, B= 1 wk after first immunization with cholera toxin and feeding yogurt, C= 2 wks after first immunization with cholera toxin and feeding yogurt, D= 1 wk after second immunization with cholera toxin and 3 wks feeding yogurt. Extracts were analyzed by ELISA for specific IgA-anti cholera toxin. Data represent the mean ± S.E.M. * Significantly different from control group ($p \leq 0.05$).


A
B
C
D
Control
Ultra-Gro direct yogurt
Sbifidus direct yogurt
PY-3 Redi-Set yogurt
DPL yogurt Quick Start ABY-2C
Optical Density
log coproantibody dilution
1.5
1.0
0.5
0.0
10^{0}
10^{-1}
10^{-2}

were significantly higher in yogurt treatment groups than in control group except again for group Ultra-Gro Direct yogurt culture which lacked *L. acidophilus* and *Bifidobacterium* spp. (Figure 4.5). Gravimetric estimates of these specific antibodies were higher in serum than in fecal samples as expected (Table 4.2). Mice also elicited a strong response for specific anti-CT IgG (Figure 4.6) but titers were lower for IgG than for IgA and there were no significant differences between group of mice fed yogurt and control diet except for Ultra-Gro Direct yogurt culture (i.e., conventional yogurt bacteria) which showed a significantly lower specific IgG levels than control group and other yogurt treatments (Table 4.2). Together these results showed that this protocol of oral immunization was effective in eliciting CT-antibody responses and suggested that yogurt made with *L. acidophilus* and *Bifidobacterium* spp. enhanced mucosal and systemic IgA responses.

4.4.4 Specific immunoglobulin production in cell cultures

The effects of yogurt feeding on immunoglobulin production were determined in cultures prepared from Peyer's patches and spleen which are representative mucosal and systemic lymphoid tissues, respectively. Peyer's patch and spleen cells (1×10^5/ml) were stimulated with or without 20 μg/ml lipopolysaccharide (LPS) from *Salmonella typhimurium* and supernatants collected and analyzed after 7 days for specific IgA and IgG. Specific IgA levels were similar in Peyer's patch cell cultures for all treatments with respect to the control. A trend towards increased IgA anti-CT levels was observed in spleen cell cultures stimulated with LPS from groups treated with yogurt as compared to control group but these effects were not significant (data not shown). Similar results were obtained when analyzing for CT-

specific IgG.

Figure 4.5. Specific IgA-anti cholera toxin in serum samples. Samples were analyzed by ELISA for specific IgA-anti-cholera toxin. Data represent the mean ± S.E.M. Preimmune serum O.D = 0.12. * Significantly different from control group ($p \leq 0.05$).

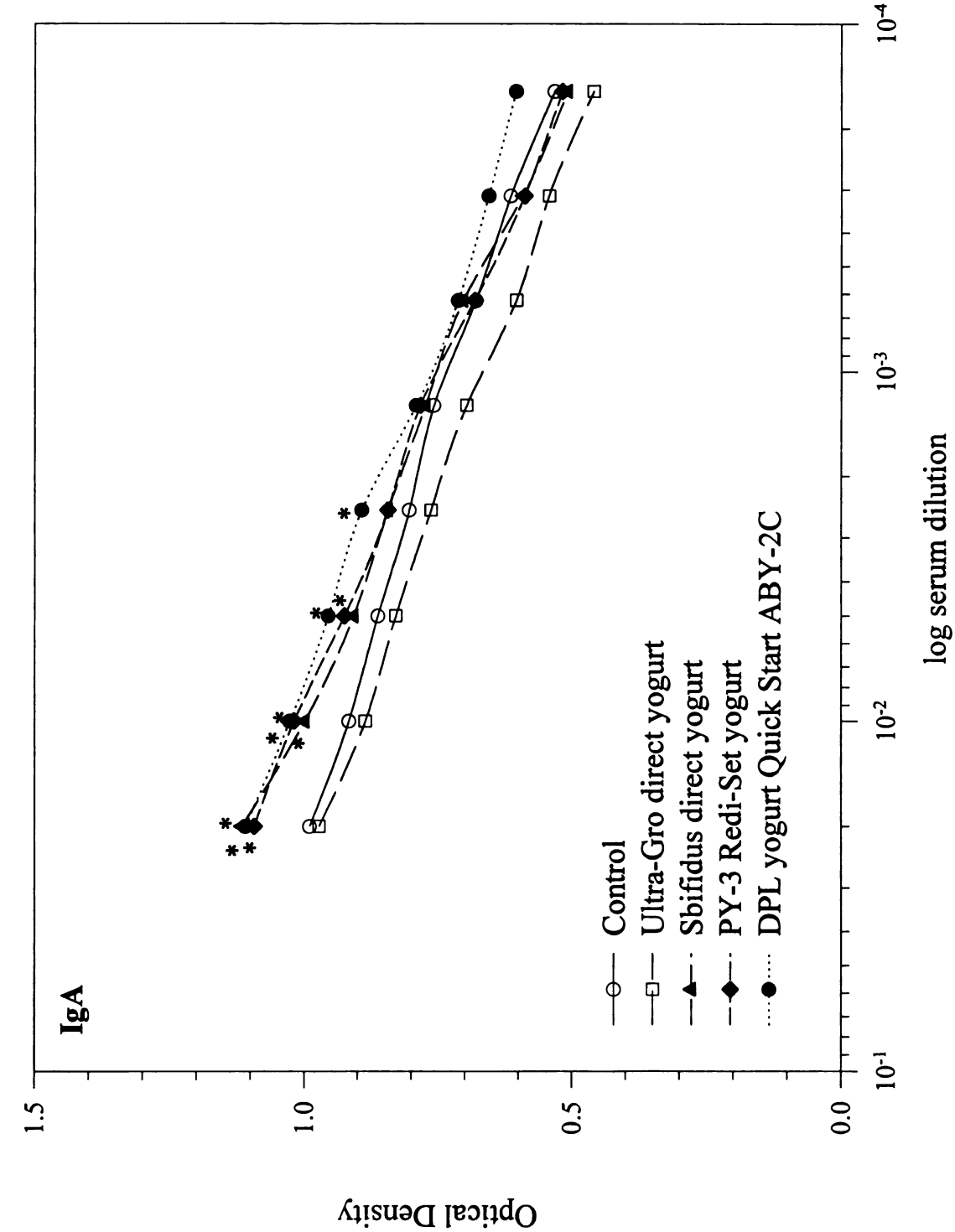

IgA
Control
Ultra-Gro direct yogurt
Sbifidus direct yogurt
PY-3 Redi-Set yogurt
DPL yogurt Quick Start ABY-2C
Optical Density
log serum dilution
0.0
0.5
1.0
1.5
10^{-1}
10^{-2}
10^{-3}
10^{-4}

Figure 4.6. Specific IgG-anti cholera toxin in serum samples. Samples were analyzed by ELISA for specific IgG-anti-cholera toxin. Data represent the mean ± S.E.M. Preimmune serum O.D = 0.10. * Significantly different from control group ($p \leq 0.05$).

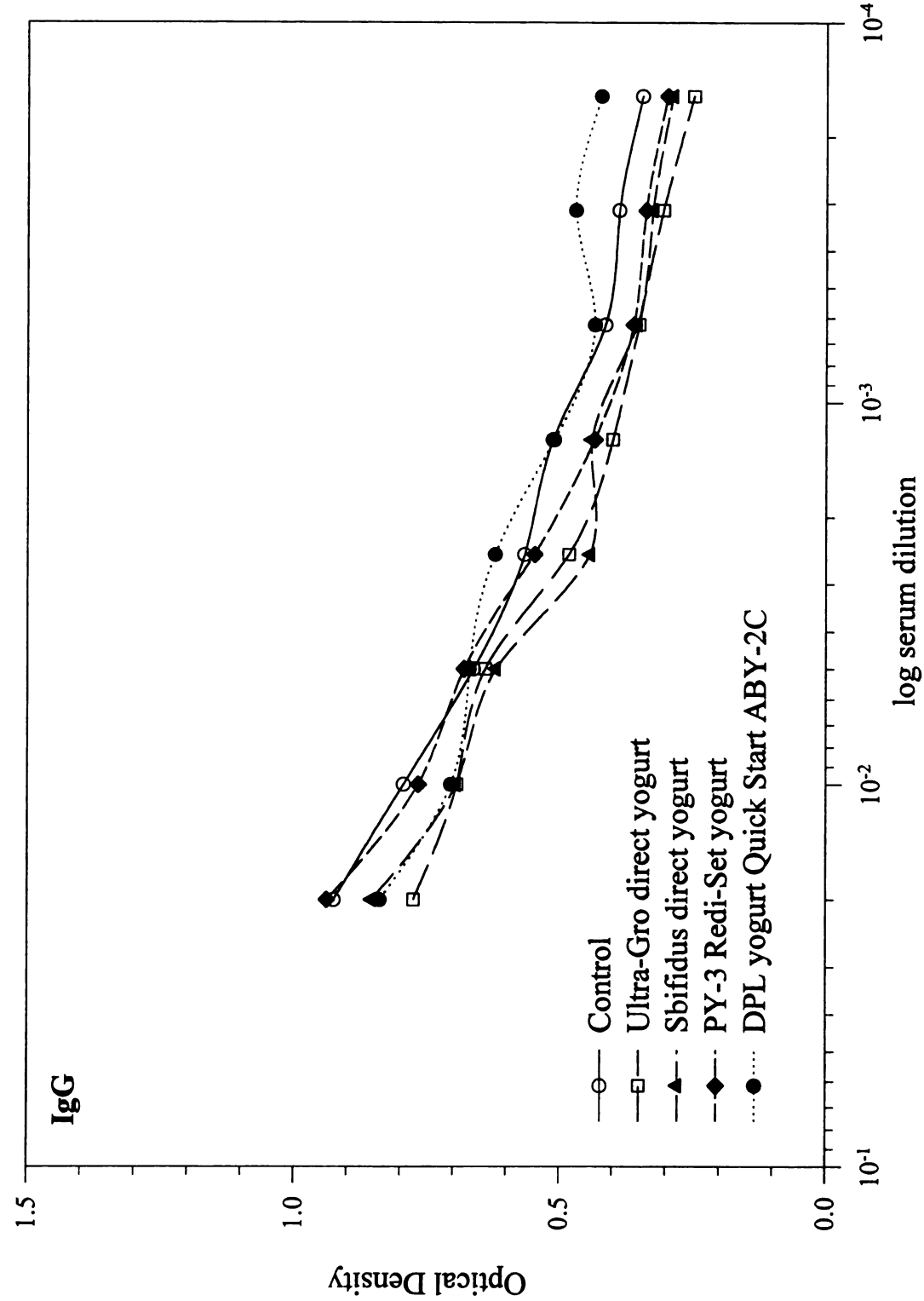

IgG
Optical Density
1.5
1.0
0.5
0.0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
log serum dilution
Control
Ultra-Gro direct yogurt
Sbifidus direct yogurt
PY-3 Redi-Set yogurt
DPL yogurt Quick Start ABY-2C

TABLE 4.2. Gravimetric estimates of cholera toxin specific immunoglobulins after three weeks of oral yogurt administration[a]

	IgA anti-cholera toxin		IgG anti-cholera toxin
Yogurt treatment	Feces[b] (μg/g)	Serum[b] (μg/ml)	Serum[b] (μg/ml)
Control	2.4 ± 0.1	28.8 ± 0.2	1.1 ± 0.1
UltraGro	1.6 ± 0.0*[c]	25.1 ± 1.0	0.4 ± 0.0*[c]
Sbifidus	4.5 ±0.5*	53.3 ± 6.1*	0.8 ± 0.2
PY-3 Redi	7.0 ± 0.7*	71.8 ± 19.5*	1.2 ± 0.2
ABY-2C	3.1 ± 0.8*	48.0 ± 2.4*	0.8 ± 0.3

[a] Groups of 10 mice were orally immunized with 10 μg of cholera toxin on days 1 and 14 of yogurt feeding. Samples of serum and feces were assayed on day 21.

[b] Dilution plated for antigen-specific immunoglobulin quantitation by comparison to a standard mouse reference serum curve for serum 1:50, for fecal samples 1:3.

[c] Statistically significant respect to other yogurt treatments ($p < 0.05$)

* $p < 0.05$, statistically significant respect to control treatment (12% skim milk + AIN 93G [1:1])

4.5 DISCUSSION

This study demonstrated that administration of yogurt differentially affected mucosal and systemic IgA responses to CT *in vivo*. Specifically, yogurt starter cultures containing *L. acidophilus* and any type of *Bifidobacterium* had an effect on mice that was reflected by specific IgA anti-CT production as compared to conventional yogurt starter cultures containing only *L. bulgaricus* and *S. thermophilus*. Thus, the bacteria present in the starter culture seemed to be critically important for immunomodulation. This suggests that several factors specifically related to a bacteria species might play a role in the extent to which yogurt alters immune function.

To exert a maximal influence on gastrointestinal function and to be able to act as probiotics, lactic acid bacteria may need to be present in high number in fermented milks, and survive the digestive process (Klaenhammer, 1982). In this experiment, the viability of bacteria in all yogurts remained high and within the normal values for commercial fermented milks (Shin, 1997). This is important because the ability of *L. bulgaricus* and *S. thermophilus* to persist in the gut after administration in mice is extremely doubtful and because there is a general consensus that continuous ingestion is needed to maintain colonization in animal models (Fuller, 1991). The capacity to tolerate low pH and high bile concentrations is advantageous for the survival of *L. acidophilus* and *Bifidobacterium bifidum* inside the gut of animals (Alm, 1991). Since a mouse model was used, it is possible that starter cultures containing *L. acidophilus* and *Bifidobacterium* spp. were able to persist in the mouse gut while the traditional yogurt bacteria, *L. bulgaricus* and *S. thermophilus*, were washed out

faster.

B6C3F1 mice were chosen for this study because of increased hardiness and longevity characteristics of heterosis and because the wider genetic diversity is more characteristic of human populations (Festing, 1979). This hardiness is especially critical for conducting feeding studies and the use of genetically identical mice minimizes variability encountered in immunological experiments. No diarrhea, food refusal or discomfort were observed at any time during the feeding trial. We have previously shown that the average feed intake for yogurt produced with these same starters ranged from 5.4 to 5.8 g/mouse/day (Ha, unpublished data).

NFDM or yogurt were provided with a semipurified powder diet (AIN-93G) at a 1:1 ratio and the gain in body weight was not significantly different between control and treatment groups. Puri et al. (1996) have similarly found no difference in the growth rate of mice fed classical yogurt (*L. bulgaricus* and *S. thermophilus*) or milk for 4 weeks. This yogurt diet was also prepared by mixing with powdered pellets at a 1:1 ratio. In contrast with these latter findings, better growth has been observed in rats fed freeze-dried yogurt (Hitchins et al, 1983) and classical yogurt (McDonough et al., 1985) when compared to milk fed rats. This discrepancy among studies might be explained because a different animal model was employed and because my study used yogurt mixed with a powdered food while some others (Hitchins et al., 1983; McDonough et al., 1985) fed only unsupplemented yogurt or milk.

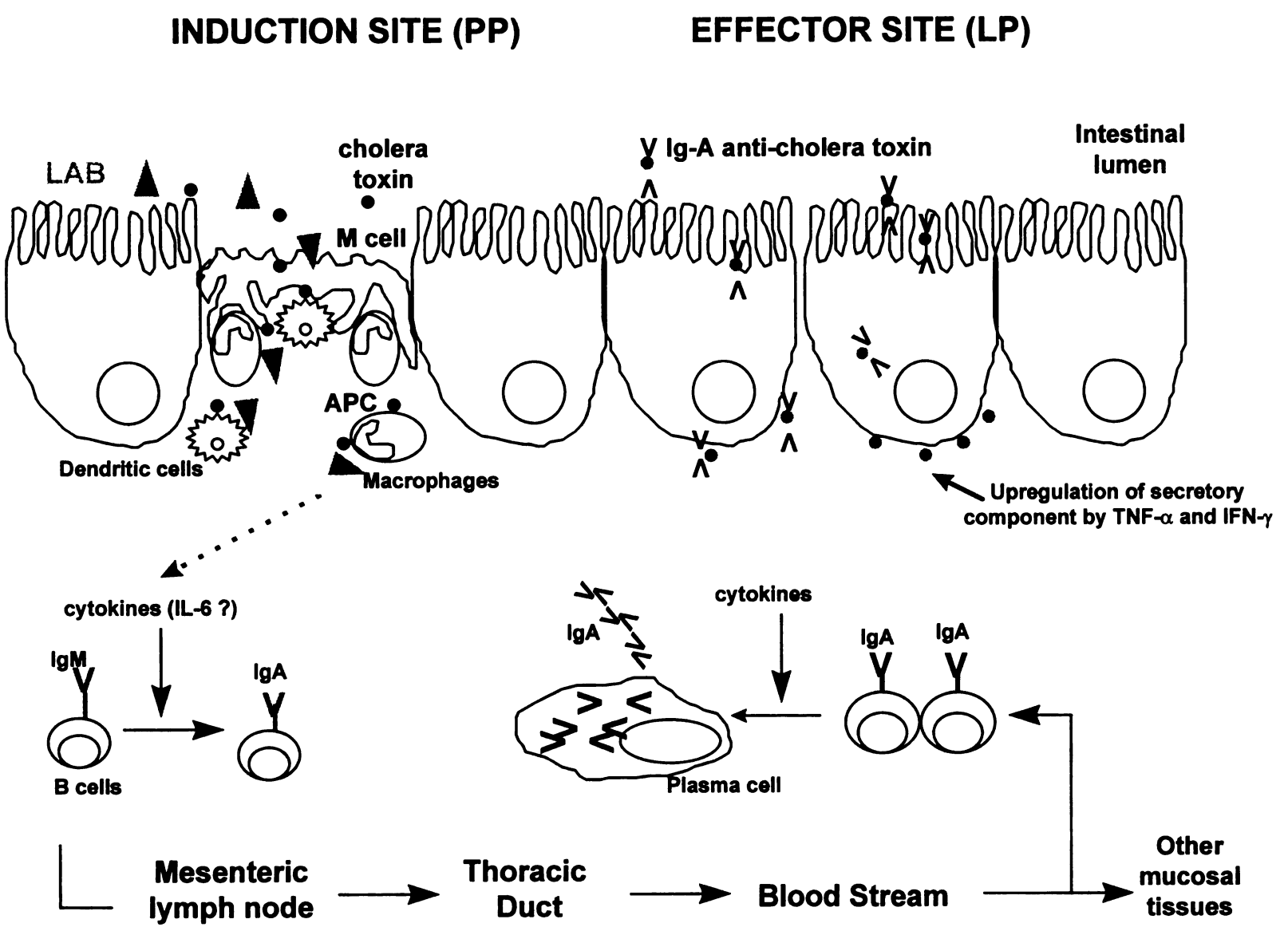
The observation that fecal and serum IgA anti-CT levels were higher in mice fed yogurts with bifidobacteria and *L. acidophilus* when compared to the control-fed group is

likely to be related to enhanced stimulation of the gut mucosal immune system. In support of this contention, feeding mice with *L. casei* increases IgA to enteropathogens (Perdigon et al., 1990) and induces a protective effect against *E. coli*, *L. monocytogenes* (Nader de Macias et al., 1993; Nomoto et al., 1985) and *Mycobacterium bovis* (Saito, 1988). Other studies have reported that feeding mice fermented milks containing *L. casei* (Paubert-Braquet et al., 1995), *L. acidophilus* and/or yogurt cultures (*L. bulgaricus* and *S. thermophilus*) (Perdigon et al., 1991b) exerts a protective effect against intestinal pathogens and increases production of immunoglobulins and activated lymphoid follicles (Bourlioux, 1986). Also, serum IgA levels after challenging with *Salmonella* were significantly higher in mice fed classical yogurt when compared to milk-fed controls (Puri et al., 1996). In a related study, mice fed yogurt fermented by *L. bulgaricus* and *S. thermophilus*, the identical heated product and a *L. casei* fermented milk and vaccinated with partially purified CT (three times intraperitoneally [40-100-200 μg/mouse] and once orally [100 μg/mouse]) at weekly intervals (Portier et al., 1993). When sera were analyzed by vibriocidal test for specific antibodies against two highly correlated serotypes (Ogawa strain and Inaba strain) of *V. cholerae*, significant differences to Ogawa but not to Inaba serotype were found. Thus, in some cases it seems that classic yogurt has adjuvant properties and the capacity to stimulate the systemic immune system. This contrasts with the findings in this study where yogurt made with *L. bulgaricus* and *S. thermophilus* had no effect for IgA anti-CT at the systemic level. This contradiction could be attributed to the different extract of toxin used, the different dose or immunization protocol followed and/or the different assay used to measured specific antibodies. It has been reported that mice orally immunized three times at weekly

intervals with 10 μg CT produced maximum IgA anti-CT responses in fecal samples and IgG anti-CT in serum on day 21 (Xu-Amano et al., 1993). Knowing of the capacity of CT to act as a potent mucosal adjuvant for other antigens or vaccines I reduced the immunization schedule and administered just two doses. This was because the goal was not to achieve a peak antibody response to CT alone but to determine whether yogurt treatment facilitates in any way the induction of antibody responses to the co-administered antigen given orally.

The Peyer's patches are the central focus for the induction of T and B-cell responses following an oral immunization (Pestka 1993). These organs lie below a specialized layer of epithelial cells called M cells (Figure 4.7). Once the antigen has traversed the M cells, antigen presenting cells (APC) within the Peyer's patch can take up the antigen and present it to nearby T cells. There are two different clones of T cells: T-helper cells can be classified as Th1 (producing IL-2, IL-3 and IFN-γ) and Th2 (producing IL-6, IL-4, IL5) (Pascual et al., 1996). Some of these cytokines are able to activate B cells and mediate proliferation, switching and differentiation of these cells to become committed to secrete IgA (Shanahan, 1994). B cells within the follicles expressing IgM or IgD on their surface upon stimulation can proliferate and differentiate into lymphoblasts expressing IgA on their surface. B cell lymphocytes pass into the efferent lymphatics to the mesenteric lymph nodes and from there, they can enter the systemic circulation via thoracic duct and enter distant effector sites as lamina propria of the intestine. At these effector sites B cells proliferate and mature into IgA plasma cells in response to certain signaling by cytokines produced by T cells and macrophages. Plasma cells produce polymeric IgA that is then secreted across the epithelial cell into the lumen (Pestka, 1993). IgA secreted by B cells in the lamina propria can be

Figure 4.7. Gastrointestinal immune response following oral immunization


INDUCTION SITE (PP)
EFFECTOR SITE (LP)
LAB
cholera toxin
M cell
Ig-A anti-cholera toxin
Intestinal lumen
APC
Dendritic cells
Macrophages
Upregulation of secretory component by TNF-α and IFN-γ
cytokines (IL-6 ?)
IgM
IgA
B cells
cytokines
IgA
IgA
IgA
Plasma cell
Mesenteric lymph node
Thoracic Duct
Blood Stream
Other mucosal tissues

released to the intestine following later the bowel content or goes to the general circulation raising the serum IgA levels.

CT is composed of subunit A (posttranslationally cleavaged into toxigenic A1 and A2 peptides) and subunit B. CTB is a homopentamer that serves as a carrier for the CTA, by binding to monosialoganglioside GM1 present in the intestinal cells. A conformational change in this GM1 allows A1 peptide to penetrate the cell. The mechanism is unknown but it could involve some type of endocytosis (Ogra et al., 1994) or direct translocation of A1 component through the lipid bilayer (Fishman, 1990). Internalized CT drives B cells towards IgA-committed precursors. CT can change the isotype pattern from IgM to IgG and IgA in Peyer's patch B cells primed for an unrelated hapten after antigen-dependent clonal expansion *in vitro* (Elson and Dertzbaugh, 1994). CT might affect Peyer's patch B cells either directly by contact or indirectly by activation of macrophages and T cells, which might produce cytokines that induce differentiation of B cells and immunoglobulin switching. Stimulated B cells in Peyer's patches migrate to lamina propria to populate IgA in that organ, or travel through blood to spleen where they become IgA or G secreting plasma cells.

The mechanisms by which *Bifidobacterium* and *L. acidophilus* yogurt stimulate the gut immune system are unclear. One possible mechanism by which certain yogurts might be able to alter the immune response on GALT could involve the generation of a local signal at the intestinal mucosal surface either by the bacteria strain present in yogurt, bacterial moieties with immunomodulatory activity, substances produced during fermentation, or translocation of these substances or microorganisms through the mucosal barrier to another host compartment.

Some bacterial components with immunomodulatory activities seem to be lipoteichoic acids, endotoxic lipopolysaccharide and peptidoglycans which are species and strain specific. Related to these bacterial fractions, other studies conducted by this laboratory showed that whole non-viable lactic acid bacteria cells (Marin et al., 1997a, 1997b, 1997c) and their cell wall and cytoplasmic fractions stimulated macrophages *in vitro* to release TNF-α, IL-6 and nitric oxide (see chapters 2 and 3). It has been suggested that *L. acidophilus* or some of its microbial components are able to cross the epithelial membrane of human intestinal cells in culture, but the mechanism is still unknown (Coconnier et al., 1997).

Gangliosides are glycosphingolipids which contain sialic acid and neuraminic acid (important component of bacterial cell walls) (Pelczar et al., 1993). They are found in apical membranes of all intestinal epithelial cells. Most pathogens, including *Helicobacter pylori*, are able to attach themselves to epithelial cell surfaces through specific structures namely GM3 gangliosides, and damage the mucus coat by protease activity (Slomiany and Slomiany, 1992). *L. casei* was also found to bind in the intestinal tract to some specific glycosphingolipids possessing short sugar chains and galactosyl moiety (Yamamoto et al., 1996). Yohe and Ryan (1986) observed that both ganglioside composition and sialic acid composition of macrophages are profoundly altered with presence of gram negative endotoxin. Present also in milk and other dairy products, they seem able to inhibit enterotoxin activity from *Vibrio cholerae* and *E. coli*, *in vitro* and *in vivo* in human studies (Lægreid et al., 1986). Conventional yogurt might present a higher amount of these gangliosides which would exert an inhibitory effect on the ability of CT to be internalized.

Regarding the possible internalization of microorganisms or fermentation products

through the mucosal barrier to another host compartment, it is known that M cells present in the intestine are able to translocate bacteria, immunogens from the intestinal lumen to underlying lymphoid tissue (Pestka, 1993). Some studies showed that the proportion of M cells and lymphocytes was significantly higher in mice fed with live *L. bulgaricus* and *S. thermophilus* as compared to controls (Muscettola et al., 1994). *L. acidophilus* and *Bifidobacterium* spp. might either affect the proportion and permeability of M cells which translocate CT from intestinal lumen to internal gut lymphoid tissues more efficiently than some other species of lactic acid bacteria, or penetrate the epithelial barrier of the intestines themselves providing an antigenic stimulus to the local immune mechanisms of the gut, activating immune cells to function as APC.

The potential signal produced by any of the above events might initiate a cascade involving (1) activation of gut-immune cells (macrophages, T and B cells), (2) cytokine production and (3) eventually translation of this effect into proliferation of B cells and their differentiation to plasma cells committed to secrete IgA against CT, as it was observed.

Macrophage activation is one of the general mechanisms of adjuvant action as well as generation of inflamation, stimulation of B cell isotype, switching, proliferation, differentiation and stimulation of increased cytokine production. Macrophages are normally in a resting state and a variety of stimuli can activate them, increasing their phagocytic activity, secreting cytokines (IL-6, IL-1, TNF-α), increasing microbicidal activity, secretion of inflammatory mediators and ability to activate T cells. In addition, activated macrophages also function as APC.

Lactic acid bacteria may activate macrophages directly. Administration of fermented

milks with lactic acid bacteria (*L. bulgaricus* and *S. thermophilus*, *L. casei*, *L. acidophilus*, *Bifidobacterium* spp.) apparently enhances immune response in animal studies by activating macrophages and lymphocytes (Perdigon et al., 1988). Notably, it has been observed that classical yogurt induced less phagocytic activity in macrophages than *L. acidophilus* and *Bifidobacterium* fermented milks (Moineau et al., 1989; Moineau and Goulet, 1991). Oral administration of *L. acidophilus* strain La 1 and *Bifidobacterium bifidum* strain Bb 12 at doses of $7x10^{10}$ CFU/day (Schiffrin et al., 1997) to humans increases phagocytic activity in blood. Activated macrophages play an important role in the resistance of the host to infections and tumors (Perdigon et al., 1986a, 1986b, 1987). If a mixture of *L. casei* and *L. acidophilus* is administered orally an increased lymphocytic activity and *in vitro* peritoneal macrophage phagocytic activity is found (Perdigon et al., 1986a). Macrophages and lymphocytes are also activated with administration of *L. acidophilus* and/or *S. thermophilus* (Perdigon et al., 1987). When administered to animals in a yogurt form, these bacteria seem to increase the number of spleen germinal centers, T and B lymphocytes and decrease preexisting enterobacterial infections (De Simone et al., 1987a, 1988a, 1988b, 1992). In humans fed yogurt, research showed an increase of B and NK-cells in lymph nodes and IFN-γ production (De Simone et al., 1986, 1988b, 1988c, 1989, 1993).

Administration of lactic acid bacteria or their fermented milks have been reported to have an effect on production of cytokines. The administration of 1×10^8 CFU/day for 15 days of *L. bulgaricus* and *S. thermophilus* used in the manufacture of fermented milk products, either in mice (Solis-Pereyra et al., 1991) or humans (Solis-Pereyra and Lemonnier, 1993), resulted in increased levels of IL-1β, TNF-α, IFN-γ. This effect was even higher

when using *L. casei*, *L. acidophilus* and *Bifidobacterium* spp. fermented milks (Solis-Pereyra and Lemonnier, 1993). Recently, in this laboratory the effects of dietary yogurt manufactured with different commercial starters were studied regarding the cytokine gene expression in mice. Interestingly, the yogurts suppressed some cytokine mRNA either at mucosal or systemic level (Ha, unpublished data) but bacterial strain specificity was not detected.

Given the reported therapeutic action of *L. acidophilus* (Perdigon et al., 1990, 1991b; Nader de Macias et al., 1993; Nomoto et al., 1985; Saito, 1988; Paubert-Braquet et al., 1995; Bourlioux, 1986) and *Bifidobacterium* spp. (Mitsuoka, 1990, Robinson, 1991; Moineau et al., 1989; Moineau and Goulet, 1991), a question that remains to be answered is: "why not manufacture a yogurt or fermented milk using only *L. acidophilus* and *Bifidobacterium* spp. as starter cultures?". First of all, for a commercially fermented milk to be called yogurt, it has to contain active *L. bulgaricus* and *S. thermophilus* (Trapp et al., 1993). Second, by excluding the conventional yogurt bacteria from the starter culture, we could be also eliminating some interactions or formation of by-products which could have an influence in the outcome of the reported effects. Finally, fermented milks have been produced with *L. acidophilus* attempting to produce a yogurt-like product. However, these microorganisms do not produce acetaldehyde which gives the characteristic flavor of regular yogurt (Sellars, 1991). As a result, fermented milk with only *L. acidophilus* is tart and plain. Furthermore, fermented milk produced only with *Bifidobacterium* also presents texture and flavor problems. Thus, the food industry has started adding bifidobacteria and *L. acidophilus* cultures to milk and its by-products (yogurt in particular) (Rasic and Kurmann, 1983; Wood and Holzapfel, 1995) and the normal practice is to include *L. acidophilus* and

Bifidobacterium in conventional yogurt starter cultures. The most common species used are *B. bifidum*, *B. longum* and *B. breve* often combined with cultures of *L. acidophilus* and *S. thermophilus* to facilitate acidification. Introducing lactobacilli and bifidobacteria into the food chain to exert the specific therapeutic properties experimentally reported may be difficult. To achieve high microorganism counts, the ingested portion should be 500 ml of commercial yogurt or fermented milk per day (Alm et al., 1993), and this fermented milk must be taken regularly for an unlimited time period as the administrated lactobacilli are washed out after approximately 14 days from the intestinal tract if not continually administered.

In summary, a murine model has been established in which the adjuvant activity of yogurt containing *L. acidophilus* and *Bifidobacterium* spp. was demonstrated by generating a strong gut mucosal IgA anti-CT response. Yogurt manufactured with starters containing only yogurt bacteria *L. bulgaricus* and *S. thermophilus* produced decreased IgA-anti CT when compared either to the NFDM fed control group or to other groups fed different types of yogurt made with *L. bulgaricus* and *S. thermophilus* supplemented with *L. acidophilus* and *Bifidobacterium* spp.

4.6 FUTURE WORK

Since the effect if *L. acidophilus* and *Bifidobacterium* spp. has been reported to be strain dependent and host specific, development of adequate screening tests are needed to select strains of these microorganisms for use as dietary adjuncts in dairy products or other foods, which produce the desired effects at a maximum level. The mechanistic bases for the findings in this study require further research. The change in permeability of intestinal cells, increased immunogen translocation or uptake by APC and involvement of other immune cells and cytokines would be interesting to decipher, to give a complete picture of the mechanism of action. The fact that a potential increase in secretion of CT-specific IgA and IgG was observed on Peyer's patch and spleen cell cultures coming from mice treated with yogurts containing *L. acidophilus* and *Bifidobacterium* spp., but without statistically significant differences, might probably be attributed to limitations on the culture system. In this study, the absence of a direct CT challenge to the cell culture resulted in a lack of proliferation and/or differentiation to plasma cells capable of immunoglobulin secretion. These cells probably would need to be co-cultured with the same antigen (CT), and if immunoglobulin secretion is detected, that could be indicating a local memory B cell reaction. Other immunogens should be also tested.

CHAPTER 5

SUMMARY

In this study, I evaluated the potential immunomodulatory activity of lactobacilli, streptococci, bifidobacteria and their extracts in the gastrointestinal system and in the immune cells by using the mouse model. *In vivo* feeding studies as well as *ex vivo* and *in vitro* studies were conducted in an attempt to show such an activity and identify the cellular components that possess the immunostimulatory properties.

This investigation suggests that lactic acid bacteria may alter peritoneal cell function *in vivo*, and this may subtly impact immunity. I demonstrated that lactic acid bacteria stimulate mainly macrophages to produce cytokines (IL-6 and TNF-α) and NO, as well as some other immune cells (such as NK cells) to produce IFN-γ. The results pointed to a possible relation between peritoneum and gastrointestinal system, suggesting a role of peritoneal cells in the activation of mucosal immune tissues by migration of cells via lymph and/or blood to Peyer's patches or spleen. Further mechanistic understanding is needed on how these and other immunologic effects are mediated by lactic acid bacteria.

Activation of macrophages to produce very significant amounts of these cytokines and NO occurred under stimulation of either whole cell, cytoplasmic or cell wall fractions, showing a similar pattern to stimulation produced with endotoxin, LPS. Further purification of lactic acid bacteria fractions is required in an attempt to isolate and characterize the stimulatory components. The mechanisms involved in the immunomodulatory effect might be close to the mechanisms followed by LPS. Measurement of intracellular concentration of calcium and/or NF-κB in macrophages after lactic acid bacteria exposure and comparison with LPS-mediated signal transduction could provide an approximation of how closely related these mechanisms are, and clarify the possible differences between stimulation with

Gram positive and Gram negative bacterial components.

Finally, I was able to establish a murine model in which the adjuvant activity of a fermented milk containing specific lactic acid bacteria was demonstrated by generating a strong gut mucosal IgA response against an antigen (CT) used through oral immunizations, what suggests that species/strains of lactic acid bacteria are critically important for immunomodulation. Since the effect of *L. acidophilus* and *Bifidobacterium* spp. may be strain dependent and host specific, development of adequate screening tests are needed to select strains for use as dietary adjuncts in dairy products or other foods. The involvement of other immune cells and cytokines would be interesting to decipher after challenging with this same immunogen and also with some others.

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