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PROBIOTIC *BIFIDOBACTERIUM LACTIS* HN019 ENHANCES THE RESISTANCE AND IMMUNITY AGAINST ENTERIC PATHOGENS

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ABSTRACT

Probiotics are defined as a group of live microorganisms, including some microbial stimulants that exert health promoting effects, such as the maintenance of a normal intestinal microbiotia, increased nutritional value of foods, anticarcinogenic activity, reduction of serum cholesterol levels, alleviation of lactose intolerance and stimulation of the immune system. Some of strains of lactic acid bacteria (LAB) are representative probiotics. The objective of this study was to examine the immunomodulatory and antiinfection properties of a new identified LAB strain- *Bifidobacterium lactis* using two animal models.

Two experiments were conducted and reported in this thesis. In the first experiment, a piglet weaning diarrhoea model was used to test the efficacy of *Bifidobacterium lactis* HN019 protecting against diarrhoea associated with Rotavirus and *E. coli*. 17 three-week-old piglets were allocated into two groups balanced for liveweight and litter of origin. The first group (n=8) was orally administered *B. lactis* HN019 (10^9 cfu/piglet/day) through the experiment; the second group (n=9) was not given *B. lactis* HN019 (control). After one week, the animals were penned individually and weaned onto a weaner diet. Blood samples were taken to measure the antibody responses, cell proliferation, and phagocytic activity of leukocytes (monocytes and neutrocytes). Also the effect of *B. lactis* HN019 on weaning diarrhoea was assessed by monitoring the severity of diarrhoea, feed intake and liveweight gain of the piglets on the weaner diet. Compared to the controls, piglets receiving *B. lactis* HN019 had lower severity of weaning diarrhoea, higher survival rate and feed conversion efficiency (or liveweight gain). The protection was associated with lower levels of faecal rotavirus and *E. coli* shedding, higher phagocytic activities and cell proliferative response to mitogens, and higher specific antibody titers. These results suggest that dietary *B. lactis* can reduce the severity of weaning diarrhoea associated with rotavirus and *E. coli*, and the probiotic is associated with enhanced immune responsiveness.

In the second experiment, the protective effects of *Bifidobacterium lactis* HN019 against *E. coli* O157:H7 and associated immunological parameters were investigated using murine models. After one week acclimatisation on a skim milk powder (SMP)-based diet, eighty-six

BALB/c and C57 mice were selected and randomised to two treatment groups. One group was fed on the SMP-based diet until the end of the experiment, while the other group was fed the SMP-based diet supplemented with *B. lactis* HN019 (3 x 10^8 cfu/g). After one week on these diets, mice were intragastrically inoculated with 0.1 ml *E. coli* O157:H7 suspension (10^9 cfu/ml). Protection against *E. coli* O157:H7 infection was assessed by monitoring the morbidity, feed intake, bacterial translocation to visceral tissues (spleen and liver) and immune responsiveness. Phagocytic activities of blood and peritoneal cells, and antibody titres against *E. coli* O157:H7 in intestinal content were also measured. The results showed that *B. lactis* HN019-fed mice conferred a significant degree of protection against *E. coli* O157:H7 challenge in comparison to the control mice that did not receive *B. lactis* HN019. Protection included lower morbidity and higher post-challenge feed intake, reduced pathogen translocation to blood, spleen and liver, as well as significantly higher phagocytic activities of blood and peritoneal cells and anti-*E. coli* IgA level in gut content. These results suggest that *B. lactis* HN019 can enhance the host resistance to *E. coli* O157:H7 and that the protection is associated with enhanced immune functions.

In summary, potential immune enhancing effects of *B. lactis* HN019 were investigated in one pig trial and one mice trial. The results showed that supplement of *B. lactis* HN019 relieved diarrhoea associated with rotavirus and *E. coli* infection in piglets and enhance the host resistance to *E. coli* O157:H7 challenge in mice. Immunological measurements indicated *B. lactis* HN019 fed groups had significant higher phagocytosis and anti-*E. coli* IgA levels. And the pathogen shedding was also reduced in *B. lactis* HN019 fed groups. As concluded, *B. lactis* HN019 can provide a protective role against special enteric pathogen infection by its immunomodulatory effects.

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ABBREVIATIONS

B. lactis HN019	*Bifidobacterium lactis* HN019 (DR10^{TM})
BHI	brain heart infusion broth
ConA	concanavalin A
DTH	delayed-type hypersensitivity
EaggEC	enteroaggregative *E. coli.*
EHEC	enterohaemorrhagic *E coli*
ELISPOT	enzyme-linked immunospot assay.
EPEC	enteropathogenic *E coli.*
ETEC	enterotoxigenic *E.coli*
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
FMLP	N-formyl-methionyl-lircyl-phenylalamine).
GALT	gut associated lymphoid tissue
GIT	gastrointestinal tract
GRAS	generally recognized as safe
HLA	human leukocyte antigens
ICAM-1	intercellular adhesion molecule-1
IFNs	interferons
IFU	infectious forming units
IgA	immunoglobulin A
IL	interleukin
LAB	lactic acid bacteria
LGG	*L. casei subsp. casei* strain GG
LPS	lipopolysaccharide
M cells	membranous cells
M-CSF	monocyte colony-stimulating factor
NK cells	natural killer cells
NZDRI	New Zealand Dairy Research Institute
PBL	peripheral blood lymphocytes
PBMCs	human peripheral blood mononuclear cells

PBS	phosphate buffered saline
PFC	plaque-forming cells
PHA.	phytohaemoagglutinin
PMNs	polymorphonuclear cells
PP	Peyer's patches
RA	rheumatoid arthritis
SCFA	short chain fatty acids
SI	stimulation index
SIgA	secretory IgA
SLE	systemic lupus erythematosus
SMP	skim milk powder
SRBC	sheep red blood cells
TNF	tumour necrosis factor
TNF-α	tumour necrosis factor-α
WHO	World Health Organization

CHAPTER 1 General Introduction

Recently, study relating to the health benefits and therapeutic effects of some harmless live microorganisms has become one of the hot topics in nutritional science. The term "probiotic " is used to describe those live microorganisms, and also some microbial stimulants that exert health promoting effects, such as the maintenance of a normal intestinal microbiotia, increased nutritional value of foods, anticarcinogenic activity, reduction of serum cholesterol levels, alleviation of lactose intolerance and stimulation of the immune system (Naidu *et al.*, 1999; Holzapfel *et al.*, 1998; Fuller, 1995).

Lactic acid bacteria (LAB) include many representative microorganisms with probiotic effects (Alander *et al.*, 1999; Fuller, 1995). They have been used in fermented food products to benefit health for several centuries (Fuller, 1995). Studies on LAB have developed rapidly in recent years. There are at least three reasons that may help to explain this increase. Firstly, the gastrointestinal tract is not only an organ of the body but also a complex ecosystem in which the intestinal microflora play an important role in digestion, absorption and intestinal defence against infection. The microorganisms in the colon, such as LAB strains for example, can ferment foods that are not digested in the small intestine, and provide important nutrients and energy resources such as short chain fatty acid, as well as exert immunomodulatory effects (Halzapfel *et al.*, 1998). Therefore, supplementation of foods with LAB can improve the intestinal microbiological environment by influencing the composition of intestinal microflora (Fuller, 1995). Secondly, LAB have also been successfully used as biotherapeutic agents to treat infectious and noninfectious diarrhoea (Halzapfel *et al.*, 1998; Elmer *et al.,* 1996). Today biotherapy, using "good bacteria" to resist infection induced by harmful bacteria, is being re-emphasised in clinical management as a means to counteract increasing development of antibiotic resistance (Bengmark, 1998; Elmer *et al.,* 1996; Saavedra, 1994). In addition to the risk of leading to resistance, excessive use of antibiotics can cause the destruction of the intestinal microbial balance, and possibly even serious organ damage (Bengmark, 1998). Thirdly, health care costs are on the rise. Nutraceuticals and functional foods provide a low cost approach for maintaining health (Gibson, 1998).

Bifidobacteria is a group of the most important LAB that constitute 90-95% of the normal microflora in a human infant, and play a key role in protecting against enteric infection (Park *et al.*, 1999; Fuller and Gibson, 1997). Supplementation with bifidobacteria in animals and humans were demonstrated to have a protective role against viral and bacterial infections (Macfarlane *et al.,* 1999; Naidu *et al*, 1999; Gill *et al*, 1998; Isolauri *et al.,* 1998; Tejada-Simon *et al.*, 1998). The mechanisms of this protective role are related to competition with other flora for nutrients, acidification of intestinal contents, production of inhibitory or antimicrobial substances against pathogens, effective colonisation in gastrointestinal mucosa that limit pathogenic bacteria adhesion and growth, and immunomodulatory effects (Naidu *et al.*, 1999; Alander *et al.*, 1999; Bengmark, 1998; Gill, 1998; Saavedra, 1995).

Bifidobacterium lactis HN019 (*B. lactis* HN019) (DR10^{TM}) is a newly identified LAB strain. Studies in murine models and human clinical trials have shown that *B. lactis* HN019 is safe and has immune-enhancing effects in animals (Gill *et al.*, 2000; Shu *et al.*, 1999a & b and 2000; Zhou *et al.,* 2000a & b; Prasad *et al.*, 1998) and humans (Chiang, *et al.* 2000). A study by Shu *et al.* (2000) further indicated that *B. lactis* HN019 reduced the risk of *Salmonella typhimurium* infection in murine models, and also enhanced natural and acquired immunity.

The aim of this project is to further investigate and confirm the protective effects of *B. lactis* HN019 against enteric pathogens, and its immune enhancing properties on host using a pig model and a mouse model. It was hypothesized that *B. lactis* HN019 could enhance host immunity and resistance to rotavirus and *E. coli* infections, and that the protective effects of *B. lactis* HN019 against the enteric infections were associated with the enhanced immunity.

The main measurements carried out in two animal models included body weight, feeding intake, diarrhoea score, and immune responses-phagocytosis, lymphocyte proliferative responses to T-cell mitogens (Con-A), and specific antibody responses.

The literature review deals with background information about studies on probiotics in which immunomodulatory effects of probiotics were elucidated in detail.

Chapter 3 and Chapter 4 report the two independent animal trials that were undertaken to test the hypotheses. In the first animal trial, a pig model was used to investigate the protective efficiency of *B. lactis* HN019 against weaning diarrhoea associated with rotavirus and pathogenic *E.coli* infection. In the second animal trial, a mouse model was designed to study the protective role of *B. lactis* HN019 against *E.coli* O157 challenge. The experimental design and methods for each animal trial are described in each chapter.

Concluding remarks in Chapter 5 sum up the results of the two animal trials and discuss these results in relation to the general hypotheses that formed the basis for this work.

CHAPTER 2 Literature Review

The aim of this chapter is to review background knowledge about probiotics, their major health benefits and the immunomodulatory mechanisms that contribute to their effectiveness.

2.1. The microbial ecosystem of the human gut

Recent scientific developments have opened new frontiers and enabled a better understanding of the gastrointestinal tract (GIT) as a complex and delicately balanced ecosystem (Umesaki *et al*, 1997; Wang, *et al*., 1997; Bry *et al*., 1996; Shanahan, 1994). This complex ecosystem in the human consists of more than 400 bacterial species including both facultatively aerobic and anaerobic microorganisms (Moore and Holdeman, 1974). Bacterial numbers and composition vary considerably along the human gastrointestinal tract. The total bacterial count in gastric contents is usually below 10^3/g due to the acid lumen pH. In the small intestine, bacterial numbers range from approximately 10^4/ml contents to about 10^6-10^7/ml at the ileocaecal region (Salminen *et al.,* 1998). The main factors limiting growth in the small bowel are the rapid transit of contents and secretion of bile and pancreatic juice (Gibson and Macfarlane, 1995).

The human large intestine is an intensively populated microbial ecosystem in which typical bacterial numbers are about 10^{11}-10^{12}/g (Fuller, 1995). Table 2.1 shows bacteria commonly isolated from the human colon. The majority of these bacteria are strict anaerobes. Quantitatively, the most important genera of intestinal bacteria in animals and man are the *Bacteroides* and bifidobacteria, which can account for 30% and 25% of the total anaerobic counts respectively (Salminen *et al.,* 1998; Cummings and Macfarlane, 1991).

Most human large intestinal microorganisms have a strictly anaerobic metabolism by which these microorganisms can ferment carbohydrate and protein, and metabolise a wide range of endogenous and exogenous molecules, including bile acids, fats and drugs (Edwards, 1994). The main substrates for the bacteria of the endogenous microflora are dietary fibre (non-starch polysaccharides), unabsorbed sugars and oligosaccharides and

resistant starch that have escaped digestion in the small intestine (Englyst, *et al*, 1992). In addition, proteins and amino acids can be effective as growth substrates for colonic bacteria. These include elastin, collagen and albumin, as well as bacterial protein released following cell lysis.

Table-2.1. Bacteria, their substrates and products in the human large intestine (adapted from Macfarlane *et al*., 1995)

Bacteria	Description*	Concentration (log_{10}/g dry weight faeces)	Substrate	Fermentation Products*
Bacteroides	G– rods	11.3	Saccharolyitc	A,P,S
Eubacteria	G+ rods	10.7	Saccharolytic, some amino acid fermenting species	A,B,L
Bifidobacteria	G+ rods	10.2	Saccharolyitc	A,L,f,e
Clostridia	G+ rods	9.8	some amino acid fermenting species	A,P,B,L,e
Lactobacilli	G+ rods	9.6	Saccharolyitc	L
Ruminococci	G+ cocci	10.2	Saccharolyitc	A
Peptostreptococci	G+ cocci	10.1	As for *Clostridia*	A,L
Peptococci	G+ cocci	10.0	Amino acid fermenter	A,B,L
Methanobrevibacter	G+ cocci Bacilli	8.8	Chemolithotrophic	CH_4
Desulfovibrios	G– rods	8.4	Various	A
Propionibacteria	G+ rods	9.4	Saccharolyitc Lactate fermenting	A,P
Actinomyces	G+ rods	9.2	Saccharolyitc	A.L.S
Streptococci	G+ rods	8.9	Carbohydrate and amino acid fermenting	L,A
Fusobacteria	G– rods	8.4	Amino acid fermentation, carbohydrate also assimilated	B,A,L
Escherichia	G– rods	8.6	As for S*treptococci*	Mixed acids

* G+, Gram-positive; G-, Gram-negative; A, acetate; P, propionate; B, butyrate; L. lactate; S, succinate; f, formate; e, ethanol.

According to host welfare, intestinal bacteria may be divided into harmful and beneficial species shown in figure 2.1 (Fuller and Gibson, 1997; Gibson and Roberfroid, 1995).

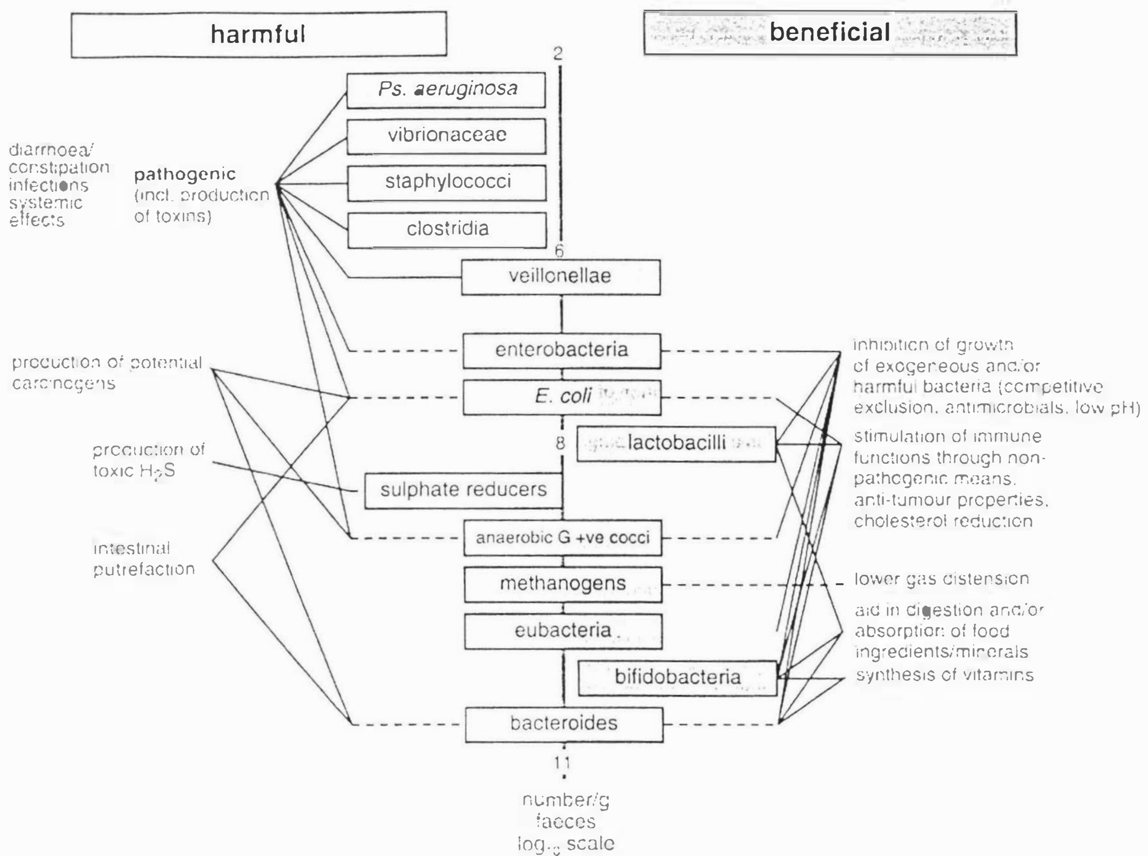

Figure 2.1. Beneficial, putatively harmful and overtly pathogenic attributes of bacteria growing in the human large intestine. SCFA, short chain fatty acids. (adapted from Gibson and Roberfroid, 1995).

Harmful bacteria produce pathogenic effects including diarrhoea, infections, liver damage and carcinogenesis. These invasive microorganisms can colonize the gut and reside for a short period after entering the gastrointestinal tract and manifest a wide variety of virulence factors that enable them to overcome host defences (Gibson and Roberfroid, 1995). Invasive microorganisms such as Enteroinvasive *E. coli, Salmonillae, Shigellae, Yersiniae, Campylobacters, Vibrios* and *Aeromonads,* can multiply within enterocytes or colonocytes and ultimately cause cell death. Toxigenic microorganisms such as enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) strains of *E.coli* are also cytotoxic. Enterotoxins secreted by *Shigellas* and enterotoxigenic *E.coli* (ETEC) can affect intestinal salt and water balance. Some other bacteria that cause acute

inflammation in the intestine are adhesive microorganisms such as ETEC and enteroaggregative *E. coli.* (EaggEC) that bind tightly to the gut mucosa (Gibson and Roberfroid, 1995).

Beneficial bacteria, as main microbial flora in the gastrointestinal tract, exert health promoting effects such as the inhibition of growth of harmful bacteria, stimulation of immune functions, lowering of gas distention problems, improved digestion/absorption of essential nutrients and systemic effects on blood lipids. In the past two decades, these beneficial bacteria such as lactic acid bacteria have been widely studied. As a result, the probiotic concept was further formed and developed (Fuller, 1995).

2.2. Probiotics

2.2.1. The definition of probiotics

Lactic acid bacteria (LAB) have been used in various fermented foods for thousands of years. They are recognised to have both preservative effects and health benefits for human consumption (Fuller, 1995). Early in 1907, Metchnikoff proposed there were health benefits related to the regular consumption of fermented milks containing"*Bulgarian bacillus*", an organism closely related to *Lactobacillus delbreuckii ssp. bulgaricus* (Naidu *et al.,* 1999). In the past century, various different microorganisms have been tested for their ability to prevent and cure diseases in both humans and animals. Microorganisms have also been added to domestic animal feed to enhance growth (Goldin, 1998). Based on these new applications, the word "probiotic" was first used by Lilley and Stillwell in 1965, to describe substances secreted by one microorganism to stimulate the growth of another (Lilley and Stillwell, 1965). In 1989, Fuller defined a probiotic as " a live microbial feed supplement, which beneficially affects the host animal by improving its microbial balance" (Fuller, 1989). Recently probiotics have been suggested to include not only live microorganisms such as bacteria, yeasts, fungi, viruses and bacteriophages, but also microbial stimulants that affect the endogenous microflora of the recipient. Probiotic effects on the host's immune system have also been widely confirmed. These include the modulation of cell mediated

immunity, activation of the reticulo-endothelial system, augmentation of cytokine pathways, and stimulation of the pro-inflamatory pathways, such as regulation of interleukins and tumor necrosis factors (Gill, 1998; Fuller, 1995).

Thus, probiotics are identified as a group of live microorganisms, including some microbial stimulants that exert health promoting effects, such as the maintenance of a normal intestinal microbiotia, increased nutritional value of foods, anticarcinogenic activity, reduction of serum cholesterol levels, alleviation of lactose intolerance and stimulation of the immune system (Naidu *et al*., 1999; Fuller and Gibson, 1997)

2.2.2. Types of LAB as probiotics

Probiotics are consumed through many types of products available from commercial industries. These products contain one or more of the following probiotic strains: *L. delbreucki ssp. bulgaricus, L. acidophilus, L. casei. L. fermentum, L. plantarum, L brevis, L lactis, L. reuteri, Lactococcus lactis, Leuconostoc mesenteroides, Streptococcus thermophilus, and Bifidobacteium spp.* Table 2.2 shows the types of probiotics most commonly used in health industries.

2.2.3. The benefits of LAB to the host

A number of studies have shown that the probiotic activities of LAB play a beneficial role in the ecosystem of the human gastrointestinal tract, and has led to the development of a variety of foods and feeds containing LAB cells in man and animals (Naidu *et al*., 1999; Macfarlane *et al*, 1999; Sanders, 1998; Bengmark, 1998; Goldin, 1998; Salminen, *et al*., 1998; Holzapfel *et al*, 1998). The probiotic activities of LAB include nutritional, physiological, antimicrobial, anti-tumor and immunomodulatory effects (Naidu *et al*., 1999).

2.2.3.1. Improved host digestive functions

The metabolic products of LAB range from simple short chain fatty acids (SCFA) to essential organic acids such as folic and orotic acid. They create an acidic milieu in the gut by dropping the pH, and meanwhile modulate a series of physiological events

Table-2.2. Types of probioitcs, desirable bacteria in probiotics and antimicrobial metabolites produced by these bacteria. (adapted from Ray,1996)

Probiotics	**Bacteria**	**Metabolites**
(predominant)	(Most commonly used)	(In the products)
A. Fermented dairy products	*L. bulgaricus*	Lactate, acetate, diacetyl,
(Yoghurt, buttermilk,	*Str. thermophilus*	HCO3, H2O2,
bacteriocins,		
acidophilus milk, etc.)	*L. lactis reuterine*	
	Leu. mesenteroides	
	L. acidophilus	
	L. casei	
	Bifidobacterium spp.	
	L. reuteri	
B. Supplemented foods	*L. acidophilus*	can supply
(pasteurized milk, drinks)	*Bifidobacterium spp.*	β-galactosidase.
	L. reuteri	the indigenous types
	L. bulgaricus	establish and produce
	Str. thermophilus	metabolites.
C. Pharmaceuticals	*L. acidophilus*	can supply
(tablets, capsules,	*L. bulgaricus*	β-galactosidase.
granules)	*Bifidobacterium spp.*	the indigenous types
		establish and produce
		metabolites.
D. Health food products	*L. acidophilus*	None, except in liquid
		(liquid, capsules,
		powders)
	Bifidobacterium spp.	fermented products.
	(many other lactobacilli,	can supply
	currently not recognised	β-galactosidase.
	as species, are also used)	

to benefit the host (Holozapfel *et al.*, 1998). In the colon, for example, the lowering of gut pH as result of LAB metabolic activities, could stimulate intestinal mobility and may relieve constipation (Kasper, 1998; Bennet and Eley, 1976).

Various enzymes released into the intestinal lumen by LAB exert potential synergistic effects on digestion and hence alleviate the symptoms of intestinal malabsorption. Lactose intolerance, for instance, is present in approximately 70% of the population in the world (Goldin, 1998; Gilliland and Kim, 1984). Because of deficiency of lactase, subjects may experience bloating, cramps, and diarrhoea after ingesting milk, due to the unhydrolyzed and poorly absorbed lactose. A certain strain of LAB has been suggested to alleviate these symptoms. Kilara and Shahani (1975) reported that yoghurt containing *L. bulgaricus* and *S. thermophilus* had a beneficial effect for lactose intolerant individuals because of the endogenous lactase. These findings were further supported by the research of Gilliand and Kim (1984), Kolars *et al.* (1984) and Marteau *et al.* (1990) and were reviewed extensively by Sanders (1994) and Naidu *et al.* (1999).

2.2.3.2. The Supply of nutrients for the host

Numerous studies have indicated that the fermentation of food with LAB cultures increases the quantity, availability, and digestibility of nutrients. Yoghurt is a good source of protein, riboflavin, folic acid, and calcium (Gorbach, 1990). Fermentation has been reported to increase the folic acid content in a variety of products, including yoghurt, bifidus milk, and Kefir (Alm, 1982; Deeth and Tomine, 1981; Shahani and Chandan, 1979). There have also been studies showing an increase in niacin and riboflavin in the fermentation of products such as yoghurt, B_{12} in cottage cheese, and B_6 pantothenic in Cheddar cheese (Alm, 1982; Deeth and Tomine, 1981). The increase of calcium absorption has also been demonstrated by some studies (Smith *et al.*, 1985; Cochet *et al.,* 1983). In addition, short chain fatty acids (SCFAs) produced by LAB are important energetic resources in the colon. Principal SCFAs such as lactic acid, propionic acid and butyric acid are readily absorbed and metabolised by the colonic epithelium (butyrate), liver (propionate), and muscle (acetate)(Cummings and Branch, 1986), which

contribute 40%-50% of the available energy of the carbohydrate to the host (Naidu *et al*, 1999; Roediger, 1982).

2.2.3.3. The reduction of cholesterol levels

Gilliland *et al*. (1985) reported that the consumption of *L. acidophilus* RP32 significantly inhibited increases of serum cholesterol level in pig fed on a high cholesterol diet. De Rodas *et al*. (1996) also demonstrated that both *L. acidophilus* and calcium could cause the reduction of serum cholesterol in pigs fed on a high cholesterol diet. In addition, Fukushima and Nakano (1996) reported that a probiotic mixture of *L. acidophilus* and *S. fecalis* decreased the synthesis of cholesterol in the liver, and increased the loss of cholesterol components-steroids from the intestine in rats. A number of human studies have also demonstrated the potential lipid-lowering effect of various yoghurts (Richelsen *et al*., 1996; Agerbaek *et al*., 1995; Mann and Spoerry, 1974).

The hypocholesterolemic effect is probably exerted by two ways. 1) LAB inhibit 3-hydroxy-3-methylglutaryl CoA reductase, which is a rate limiting enzyme in the body's endogenous cholesterol biosynthesis; and 2) LAB promote the excretion of dietary cholesterol in feces as a result of coprecipitation in the intestine and absorption by the organisms (Naidu *et al*, 1999).

2.2.3.4. Antimicrobial effects

Lactic acid is the major end product of carbohydrate metabolism. It is generated from pyruvate by lactic acid dehydrogenase. The accumulation of lactic acid and the concomitant reduction in pH of the milieu, results in a broad spectrum inhibitory activity against gram positive and gram negative bacteria. The factors determining the inhibitory activity of lactic acid and acetic acid in the milieu include the acidic pH, dissociation constant (pK value) and molar concentration (Holzapfel *et al*., 1998; Ingram *et al*., 1956).

In the presence of oxygen, LAB can produce hydrogen peroxide (H_2O_2) through electron transport via flavin enzymes (Park *et al*, 1999; Hillier, *et al*., 1992). Then superoxide anions of H_2O_2 form destructive hydroxy radicals. This process may induce peroxidation

of membrane lipids (Morris, 1979), increased membrane permeability (Kong and Davison, 1980), and the destruction of nucleic acids and cell proteins (Dahl *et al.*, 1989). H_2O_2 increased by certain LAB also enhances the antimicrobial system in phagocytes and tissue fluids as the system is composed of peroxidase, H_2O_2, and a halide (Naidu *et al*, 1999; Balasubramanya *et al.*, 1995).

LAB also produce a wide range of similar antagonistic factors that include metabolic products, antibiotic-like substances, and bactericidal proteins, that are collectively termed bacteriocins (Naidu *et al.,* 1999; Sanders, 1993; Havenaar *et al.*, 1992). And these antimicrobial agents are species specific and exert their lethal activity through absorption to specific receptors located on the external surface of sensitive bacteria. This is then followed by metabolic, biological and morphological changes, which result in the killing of such bacteria (Ganzle *et al.* 1999; Fuller, 1995). For instance, the pediocin-like bacteriocin produced by *Pediococcus acidilactici* has strong anti-listeria effects (Naidu *et al.,* 1999). The bacteriocin reuterin produced by *L..reuteri* also can inhibit Gram-negative (*Salmonella and Shigella*) and Gram-positive (*Clostridia* and *Listeria)* bacteria (Naidu *et al.,* 1999; Fuller and Gibson, 1997).

A number of LAB can produce CO_2 from malate and citrate (London, 1990; Fleming, *et al.*, 1986) by metabolizing arginine via the arginine deaminase pathway (Poolman, 1993). The decarboxylation of amino acids (histidine, tyrosine) can also result in CO_2 formation. The CO_2 also contributes to the antimicrobial activity by creating an anaerobic environment. Moreover, the CO_2 can decrease extra and intracellular pH and destroy cell membranes of bacteria. Therefore, the CO_2 creates a potent inhibitory system against a wide variety of microorganisms in gastrointestinal tract (Eklund, 1984; Clark and Takacs, 1980).

Diacetyl (2,3-butanedione) is an end product of pyruvate metabolism (Condon, 1987). It is formed by citrate-fermenting LAB (Hugenholtz, 1993). Diacetyl elicits a potent antimicrobial activity against various food-borne pathogens and spoilage microorganisms

(Jay, 1982). Furthermore, diacetyl is more effective against gram negative bacteria, yeasts and moulds than against gram positive organisms (Jay, 1996).

2.2.3.5. Microbial interference

Colonisation resistance is defined as the mechanism whereby the intestinal microbiota are protected against incursion by new and occasionally pathogenic microorganisms (Holzapfel *et al.*, 1998; Gorbach *et al.*, 1988). Hawthorn and Reid (1990) reported that precoating of polymers with certain LAB strains reduced the binding of uropathogenic coagulase negative *Staphycocci* and *E.coli* to the biomaterial surface. Chan *et al.* (1984) reported complete or partial inhibition of adherence of several gram-negative uropathogens, by preincubation of human uroepithelial cells *in vitro* with several LAB species of the normal flora. Because the colonisation is an essential stage of pathogenic microorganisms infection in gatrointestinal tract, colonisation resistance from a certain LAB is of significance in the prevention of diarrhoea.

The ability of oral *Lactobacilli* to congregate with *Streptococci* and *Actinomycetes* was reported by Wilcox *et al.* (1993). Live and heat killed *L. acidophilus* strain LB, which strongly adheres to Caco-2 cells, were reported to inhibit both cell association and invasion of Caco-2 cells by *Salmonella typhimurium* and enteropathogenic *E. coli* (Chauviere, *et al.* 1992). Kabir *et al.* (1997) demonstrated the inhibitory effects of *Lactobacilli* on *H. pylori* attachment to murine and human gastric epithelial cells. Those reports revealed an additoinal protective mechanism of LAB against intestinal infection.

By competitive growth, LAB in the gut can also produce inhibitory effects on some microorganisms such as non-indigenous organisms. Nutrient depletion and a decrease in the redox potential produced by LAB could lead to competitive antagonism (Kmet *et al*, 1995). To extend the shelf life, *L. lactis ssp. lactis var. diacetylactis* is often used to dress cottage cheese (Fredrickson, 1977). Haines and Harmon (1973) reported an inhibitory effect on other microorganisms from LAB by competitively intake of the essential vitamins- niacin and biotin. Several investigations have demonstrated that various species of LAB exert antagonistic action against intestinal and food borne pathogens (Gibson *et al.*, 1997). LAB are capable of preventing the adherence, establishment, replication,

and/or pathogenic action of specific enteropathogens (Saavedra, 1995). Much evidence thus supports the expectation that probiotic bacteria can be effective weapons for preventing and treating many microbial infections (Brashears *et al*., 1998; Fujiwara *et al*., 1997; Duffy, *et al*., 1994; Bernet *et al*., 1994 and 1993; Boudraa, *et al*., 1990; Chan *et al*., 1984; Iandolo *et al*, 1965).

With emergence of antibiotic resistance, microbial interference treatment by probiotics is being reconsidered as an effective way to prevent diseases and reduce antibiotic use. The World Health Organization (WHO, 1994) recently reported that antibiotic resistance is at present a major public health problem in both developed and developing countries throughout the world. Therefore, WHO (1994) recommends global programs to reduce the use of antibiotics in animals, plants and fishes for promoting livestock growth. In human medicine, WHO (1994) also recommends increased efforts to prevent disease through increasing immunization coverage with existing vaccines, and through the development of newer, more effective and safer vaccines. In addition, several older forms of therapy, including bacteria interference, serum therapy and the use of macrophages to kill organisms, may be worth reconsidering (WHO, 1994).

2.2.3.6. Antitumour effects

Nitrosamines are a type of mutagens. LAB, by absorbing nitrites, can reduce the formation of nitrosamines from nitrites (Hosoda, *et al.,* 1992; Fernandes *et al.* 1987). *In vitro* studies revealed that LAB also have the ability to absorb cooked food mutagens (Lidbeck *et al*., 1992). Colon cancer patients given *L. acidophilus*-fermented milk showed a significant increase in the number of intestinal *Lactobacilli* and significant decline in the levels of both soluble fecal bile acids and fecal bacterial enzymes that are two risk factors for colon cancer (Hosoda, *et al.,* 1996; Hosono, *et al.,* 1986; Goldin *et al.,* 1980).

The incidence of colon cancer is also associated with effects of fecal procarcinogenic enzymes produced by the intestinal bacteria. These procarcinogenic enzymes such as β-glucuronidase, azoreductase, nitroreductase, 7-α-dehydroxylase, and 7-α-dehydrogenase

could convert precarcinogenic compounds into carcinogens. Several researchers have investigated the ability of LAB to decrease the activity of these enzymes (Sanders, 1994; Fernandes and Shahani, 1987). Goldin *et al.* (1980) found that supplementing adults with viable *Lactobacillus* cultures significantly reduced β-glucuronidase activity. Ling *et al.* (1994) demonstrated that glucuronidase activities were lower following the administration of a *Lactobacillus*-fermented whey drink, compared with the results from a placebo drink.

The antitumor and tumour inhibiting effects of dietary LAB have also been demonstrated to relate to diminished neoplastic enzyme activity or immunostimulation of the host (Naidu, *et al.*, 1999). For example, a peptidoglycan isolated from *B. infantis,* produced 70% tumour regression in a mouse study (Sekine *et al.*, 1985). Sekine *et al.*, (1995,1994a) further demonstrated that the cell wall fractions of peritoneal exudative cells of mice are the biological response modifiers that activate neutrophils and macrophages in tumour-bearing animals. Therefore, specific LAB may play a role as immune modulators in the intestines of humans and animals, and in the maintenance of health (Sekine *et al.*, 1995).

Comparatively few studies have examined the relationship between immuno-stimulation induced by orally fed lactic cultures and resistance to tumours. Feeding yoghurt for 7 and 10 days inhibited the development of intestinal carcinoma in mice (Perdigon *et al.*, 1995). The anti-tumour effects were associated with the stimulation of immune responses, as indicated by a significant increase in the IgA producing cells and T lymphocytes in the large intestine. Non-specific host immuno-activation has also been suggested to mediate anti-tumor effects against Sarcoma 180, L1210 Leukaemia and MCA K-1 in mice given *L. casei* (Kato *et al.*, 1981). Fernandes and Shahani (1987) reported a marked reduction in the anti-tumour effects of *L. acidophilus* in mice treated with cortisone (Immunosuppressed). In humans, administration of *L. casei* strain Shirota to patients with Dukes A colorectal cancer increased the percentage of T helper cells and NK cells and decreased the proportion of suppressor cells (Sawamura *et al.*, 1994). Kato *et al.*, (1981) suggested that the anti-tumour effects of LAB were mediated through activated

macrophages. The authors found that administration of carrageenan, an anti macrophage agent, was able to reduce the anti tumour effects of *L. casei* against Sarcoma-180 in mice. In a word, probiotic use is showing much promise of success in the cancer therapy (Holzapfel *et al.*, 1998; Salminen *et al.*, 1996; 1998).

2.2.3.7. Immunomodulatory effects

A number of studies showed that LAB may affect both mucosal and systemic immune responses (Naidu *et al.*, 1999; Gill, 1998; Fuller and Gibson, 1997; Gerritse *et al.* 1990). And the immunomodulatory mechanisms of LAB are wide and complicated, including modulation of various cytokines, modulation of several phagocytic cells, and modulation of mucosal and systemic antibody levels. Famularo *et al.,* (1997) summed up the effects of probiotics on systemic and mucosal immunity (Table 2.3).

Table 2.3. Effects of probiotics on systemic and mucosal immunity
(adapted from Famularo, *et al.,* 1997)

- Modulation of IL-1, IL-6, TNF-alpha, IL-2, IFNs (gamma, alpha, beta), M-CSF production
- Modulation of antigen- and mitogen-driven mononuclear cell proliferation
- Modulation of monocyte-macrophage phagocytic and killing activities
- Modulation of autoimmunity
- Modulation of immunity to *Salmonella typhimurium*
- Modulation of immunity to *Cryptosporidium parvum*

IL, interleukin; TNF, tumour necrosis factor; IFNs, interferons; M-CSF, monocyte colony-stimulating factor.

The main health benefits in the immune enhancing ability of LAB include protection against enteric infections, use as an oral adjuvant, immunopotentiation in malnutrition, and the prevention of chemically induced tumors. Table 2.4 and Table 2.5 show some important relative studies in animals and human. This part will be further discussed in detail in the review.

Table 2.4. Examples of animal studies related to the effects of LAB ingestion on humoral and cell mediated immune responses (adapted partially from Gill, 1998).

Treatment with LAB culture	Effects	Reference
Normal and immunosuppressed mice given *L. casei, L. acidophilus, L. delbrueckii subsp. bulgaricus* or *S. salivarius subsp. thermophilus* and infected with *Candida albicans* .	↑ PFC and DTH responses and protection in immunosuppressed mice given *L. casei* and *L. bulgaricus. L. acidophilus* less effective and *S. thermophilus* ineffective.	De Petrino *et al*., (1995)
Well-nourished & malnourished mice fed with 1.2×10^9 cfu/d *L. casei*, then challenged with *S. typhimurium*.	Malnourished: ↑ IgA-producing cells. Slight ↑ in circulating leukocytes. No protection against *S. typhimurium*. Well nourished: ↑ in phagocytic activity of macrophages.	Perdigon *et al*., (1995)
Mice fed conventional diet plus yoghurt supplements (2×10^8 cfu/ml).	↑ IgA-secreting B cells in the small and large intestines. Dose-dependent.	Perdigon *et al*., (1994)
Mice fed with *L. casei*, then injected with fibrosarcoma cells; or mice inoculated with *tumour* cells, then given *L. casei*.	↑ phagocytosis. ↑ inhibition of *tumour* growth, both preventively and therapeutically.	Perdigon *et al*., (1993)
Mice fed with *L. casei* fermented milk, then vaccinated against *Vibrio cholerae*.	↑ specific antibody responses in the serum.	Portier *et al*., (1993)
Mice fed with a lyzozyme lysate from *L. bulgaricus* for 10d. At d 6, injected with *Klebsiella pneumoniae* or *Listeria monocytogenes*	↑ spreading ability of macrophages. ↑ phagocytosis of *E. coli*. ↑ IL-1 production. ↓ mortality after challenge with *Klebsiella pneumoniae* and *Listeria monocytogenes*.	Popova *et al*., (1993)
Mice fed with *B. longum* or *L. acidophilus* (about 8×10^{10} nonviable cells/d) for 12 weeks.	*B. longum*: cytoplasm only induced proliferative response in mucosa and serum, but not spleen. *L. acidophilus*: both cell wall and cytoplasm induced proliferative response.	Takahasi *et al*., (1993)
Mice fed milk fermented with *L. casei* and *L. acidophilus* and challenged with *Shigella sonnei*	↑ Serum anti-*Sh. Sonnei* antibody concentration in serum and intestinal fluid	Nader de Marcia, *et al*., (1992)
Mice fed *B. breve* and immunized with cholera toxin	↑ IgA production, ↑ Anti-cholera IgA antibody levels.	Yasui & Ohwaki (1991)
Mice fed yoghurt and *Kefir* and inoculated with tumour cells	↑ In plaque-forming cells (PFC) in the spleen ↓ Delayed-type hypersensitivity (DTH)	Furukawa *et al*., (1991)
Mice fed milk fermented with *L. casei* and/or *L. acidophilus* and challenged with *S. typhimurium*	↑ Survival ↑ Anti-*S.typhimurium* antibody response in serum and intestinal fluid.	Perdigon *et al*., (1990)
Mice fed fermented milk containing *B. breve*	↑ Anti-*B. breve* antibody levels	Yasui *et al*., (1989)
Mice fed milk fermented with *L. casei* and /or *L. acidophilus* and inoculated intraperitoneally with sheep red blood cells (SRBC)	↑ IgM plaque-forming cells against SRBC ↑ Anti-SRBC circulating antibodies	Perdigon *et al*., (1988)
Mice fed powdered milk, yoghurt containing live cultures or heat-treated yoghurt	↑ Lymphocyte proliferation to PHA. ConA and PWM in mice fed live yoghurt ↑% of Thy 1.2 cells in spleen	Vesely *et al*., (1985)

Table 2.5. Examples of human studies related to the effect of LAB ingestion on humoral and cell mediated immune responses (adapted partially from Gill, 1998).

Treatment with LAB culture	Effects	Reference
54 infants Ingested of freeze-dried *L. casei* for 5 d. On d 1: administration of D x RRV live oral rotavirus vaccine.	↑ rotavirus-specific IgM-secreting cells ↑ rotavirus IgA seroconversion	Isolauri *et al.*, (1995)
Children with acute rotavirus gastroenteritis given *L. casei subsp. casei* strain GG (LGG), *L. casei subsp. rhamnosus* or *S. thermophilus* and *L. delbreuckii subsp. bulgaricus*.	↓ Duration of diarrhoea ↑ Rotavirus-specific IgA secreting cells ↑ Serum IgA antibody to rotavirus cells convalescent stage in LGG treated children	Majamaa, *et al.*, (1995)
Patients with Dukes A colorectal cancer given *L. casei shirota*	↑ % Helper T cells ↓ % Suppressor T cells	Sawamura *et al.*, (1994)
30 healthy adults Ingested for 3 weeks of milk fermented with *L. acidophilus*, *Bifidobacteria*, and other LAB. *S. typhimurium* oral vaccine administered at day 0, 2, 4.	↑ specific serum IgA antibody ↑ total serum IgA values.	Link-Amster *et al.*, (1994)
Ex vivo incubation of human blood mononuclear cells with several LAB	↑ production of IL-1, α, β TNF, γ-IFN	Solis *et al.*, (1993)
Healthy adults Ingested of lyophilized dietary *lactobacilli* for 28 d.	↑ IFNg, B lymphocytes, and natural killer cells.	De Simone *et al.*, (1993)
25 healthy adults Ingested lyophilized *B. bifidum* and *L. acidophilus* for 28 d.	↑ colonic inflammatory infiltration. ↑ B cell frequency in the peripheral blood. ↑ TNF-alpha	De Simone *et al.*, (1992)
Children with gastroenteritis ingested milk fermented with *L. casei* for 5 days.	↑ IgA, IgG, IgM with return to low levels 3 weeks later	Kaila *et al.*, (1992)
48 adults with resected bladder after superficial bladder cancer ingested viable *L. casei* for around 1 year.	↑ 50 % recurrence-free interval (350 days vs 195 days) ↑ production of TNF.	Aso *et al.*, (1992)
Healthy elderly subjects given LAB for 28 days.	↑ Frequency of B cells in the peripheral blood. ↑ Colonic inflammatory infiltration	De Simone *et al.*, (1991)

2.2.4. Safety of probiotics

LAB have a long history of consumption in the general population. Most probiotics have been designated as "generally recognised as safe" (GRAS) (Goldin, 1998). Salminen and Danahue (1996) reviewed the safety data and found that no evidence suggested that probiotics were involved in human infections. A number of animal experiments and large scale of human studies have supported this conclusion (Naidu *et al*., 1999; Salminen and Danahue, 1996). For instance, a total of 5192 blood cultures isolated from patients with bacteriaemia, were studied in southern Finland (Goldin, 1998). The results showed only twelve isolates contained lactobacilli. These lactobacilli did not correspond to *Lactobacilli GG* or any other *Lactobacilli* used in dairy products or pharmaceutical preparations (Salminen and Danahue, 1996). Nevertheless, there were occasional reports showing that in severely immunocompromised individuals, bacteriaemias and endocarditis were associated with *Lactobacillus* (Salminen and Danahue, 1996).

Therefore, the International Union of Microbiological Societies (IUMS) subcommittee on *Bifidobacterium, Lactobacilli,* and related organisms concluded that the suggested risk posed by LAB and related organisms is not substantiated by data on ingestion, or of a clinical nature. However, as new probiotic preparations such as bifidobacteria, enterococci, propionibacteria, and even saccharomyces strains are being used, it is important to verify their safety (Naidu *et al.,* 1999). Table 2.6 shows a scheme for probiotic safety assessment proposed by Salminen and Danahue in 1996.

With more and more evidence showing that probiotics are safe and beneficial to health, they are being used in clinical management. For example, probiotics can down regulate hypersensitivity reactions and promote endogenous barrier mechanisms. The use of formula supplemented with probiotic bacteria supplemented formula can further reduce antigenicity of substitute formulae, and consequently have an important role in the development of specific therapy for patients who have food allergies (Salminen *et al.,* 1996). Management of infantile diarrhoea requires infant formulae with efficient lactic acid bacteria. Other alternatives are to supply oral rehydration solutions with similar bacteria (Isolauri *et al.,* 1995). In clinical nutrition, elemental diets are being used for the

treatment of intestinal inflammation, radiation enteritis and inflammatory bowel disease (Salminen *et al,* 1996). Lactic acid bacteria treatment is being taken by more and more people.

Table-2.6. Safety Assessment Scheme (adapted from Salminen and Danahue, 1996)

Type of property studied:	Safety factors to be assessed:
Intrinsic properties of LAB	adhesion factors, antibiotic resistance, existence of plasmid transfer potential, harmful enzyme profile.
Metabolic products	Concentrations, safety and other effects
Toxicity	Acute and subacute effects of ingestion of large amounts of tested bacteria.
Mucosal effects	Adhesion, invasion potential, intestinal mucus degradation, infectivity in immunocompromised animals (e.g. following lethal irradiation)
Dose response effects	Dose response studies by oral adminstration in volunteers
Clinical assessment	Potential for side effects, careful evaluation in healthy volunteers and disease specific studies
Epidemiological studies	Surveillance of large population following introduction of new strains and products.

2.2.5. Bifidobacteria as probiotics

Bifidobacterium spp. are non-pathogenic, gram-positive and anaerobic bacteria, which inhabit the intestinal tract of humans and animals. The main species of the genus *Bifidobacterium* characteristic of the infant intestinal flora are *Bifidobacterium bifidum, B. infantis, B. breve, and B. longum* (Mitsuoka, 1992). Bifidobacteria are a group of important LAB (Fuller, 1995). In this project, a newly identified bifidobacteria strain-*B. lactis* HN019 was used to investigate the immune-enhancing ability of a probiotic.

Bifidobacteria, in human infants, are the predominant component of the intestinal flora. In adults, they are decreased to about 25% of total microflora in the gastrointestinal tract

(Fuller, 1995). Furthermore, there are obvious differences in biological properties between various strains (Ray, 1996; Macfarlane *et al.,* 1995). Therefore, the supplementation with selected bifidobacteria with probiotics is of significance. Characterisation of probiotic bifidobacteria is their ability to survive, transit and colonise the digestive tract. It has been demonstrated that a single strain of bifidobacteria can efficiently survive and colonise the intestinal tract, especially the large intestine (Naidu *et al.*, 1999; Fuller and Gibson, 1997; Marteau *et al.*, 1997).

As lactic acid producing bacteria (LAB), *Bifidobacterium spp.* have been widely used in fermented commercial products in dairy industry. The main probiotic effects on the host include maintenance of intestinal microflora balance, improvement of lactose intolerance, reduction of serum cholesterol concentrations, synthesis of vitamins, anti-carcinogenic activity, and stimulation of the immune system (Gopal *et al.*, 1996; Sanders, 1993; Kurmann and Raric, 1991; Yamazaki *et al.*, 1991; Homma, 1988).

Bifidobacteria apparently enhance several immune functions, including macrophage and lymphocyte activation (Sekine *et al.*, 1994 b & c; Hatcher and Lambreche, 1993), antibody production (Link-Amster *et al.*, 1994; Lee *et al.*, 1993; Yasui *et al.*, 1992; Yasui and Ohwaki, 1991), and the proliferative responses in the spleen and Peyer's patches (Hosono *et al.*, 1997; Lee *et al.*, 1993; Takahashi *et al.*, 1993; Yasui and Ohwaki, 1991). Bifidobacteria ingestion has been proposed to enhance resistance to infection by pathogenic organisms (Yasui *et al.*, 1995; Duffy *et al.*, 1994; Sasaki *et al.*, 1994) and potentially prevent cancer (Sekine *et al.*, 1995, 1994a). Cell components of *Bifidobacterium,* which function as immunomodifiers of the host, reportedly include peptidoglycan, intracellular and extracellular polysaccharide products, cell free extracts, and cell wall preparations (Hosono *et al.*, 1997; Sekine *et al.*, 1995, 1994a; Hatcher and Lambreche, 1993; Gomez *et al.*, 1988; Namioka, 1985). However, at the present time there is not yet a clear understanding of the molecular and cellular basis for immuno-stimulation by bifidobacteria.

2.3. Host immune system and immune enhancing properties of probiotics

The effect of LAB on the immune system is the focus of much ongoing research (Salminen *et al* 1998; Perdigon *et al*., 1995). LAB and fermented milks have been found to modulate certain parameters of both the non-specific and specific immune responses. This section will mainly deal with the immune system, potential probiotic effects on the immune system, and possible mechanisms involved.

2.3.1. The host immune system

The immune system is the main host defence system that contains wide and complicated mechanisms. The host immune system is usually classified into two basic aspects: specific and non-specific immunity according to antigen recognition (Fuller, 1995; Perdigon and Alvarez, 1992). In addition, the host immune system can also be differentiated as systemic and mucosal immunity to better understand local immunity such as the gastrointestinal immunity related to probiotic efficacy (Fuller, 1995; Perdigon and Alvarez, 1992).

2.3.1.1. Non-specific immune system

Non-specific immune responses are the first line of host defence (Roitt, 1994). These responses are induced by various stimuli such as pathogens. The major cellular effectors of non-specific immunity include mononuclear phagocytes (monocytes and macrophages), polymorphonuclear leukocytes (mainly neutrophils), and natural killer cells (NK cells) (Roitt, 1994). The important functions of mononuclear phagocytes and neutrophils are phagocytosis and killing of microbial pathogens. The process of phagocytosis initiates a series of intracellular events that produce a variety of microbicidal products, e.g. reactive oxygen and nitrogen species, enzymes, TNF and IL-1. These products then can inhibit and damage and remove invasive microorganisms. The above process is called the inflammatory response (Roitt, 1994). It is characterised by extensive neutrophils infiltration, phagocytosis and release inflammatory mediators such as cytokines. The released substances then attract macrophages that further inactivate antigens by phagocytosis. Once the antigens are degraded, some peptides of these

antigens can be expressed on the macrophage membrane. Then the macrophage presents the antigen to lymphocytes, which indicates the start of the specific immune responses (Gill, 1998; Perdigon *et al.*, 1995).

2.3.1.2. Specific immune system

Specific immune responses consist of two broad categories: humoral immunity and cellular immunity (Holzapfel, 1998; Perdigon *et al.*, 1995). Humoral immunity is represented by the production of antigen-specific antibodies. These antibodies can bind specifically to antigenic epitopes on the surface of pathogenic organisms, which recognise and neutralise particular pathogen. antigens. In addition, the combination of antigen and antibody also activates a series of complement proteins that are able to kill pathogens (Roitt, 1994).

Specific classes of antibodies have specific functions (Roitt, 1994). IgA antibodies predominate at mucosal surfaces and prevent adherence of pathogens to the gut mucosa. IgG and IgM are involved in systemic neutralization of bacterial toxins, and promote phagocytosis by monocyte macrophages. Antibodies are effective at neutralising or eliminating extracellular pathogens and antigens (Fuller, 1995).

Cellular immunity is mediated by T lymphocytes (Roitt, 1997). When exposed to antigens or pathogens, T lymphocytes of pre-determined clones proliferate and produce cytokines. A number of T lymphocytes directly kill pathogens and damage tumour cells by phagocytosis and cytotoxic effects. Moreover, through cytokines that are produced, T cells also influence the activities of other immune cells, e.g. augmenting the ability of macrophages to kill intracellular pathogens and tumour cells. In addition, T cell subsets act as helper cells ($CD4^+$) to enhance antibody production, mediate delayed type hypersensitivity (DTH) or execute cytotoxic effects ($CD8^+$) against virus infected cells and cancer cells; cell mediated immunity is more effective against intracellular pathogens and tumour cells than humoral immunity (Gill, 1998; Perdigon *et al.*, 1995).

2.3.1.3. Mucosal immunity

Non-specific mucosal immunity generally includes two aspects (Wells *et al.*, 1996; McGhee, *et al.,* 1992). The first aspect is the barrier effect of the mucous membrane located on the surface of the gastrointestinal tract, respiratory tract, and urogenital tract. The mucous membrane is a single layer of cells consisting of several cell types such as goblet cells, ciliated cells, and membranous cells (M cells). Goblet cells secrete mucus to form a protective layer against the invasion of bacteria. M cells are overlaid on lymphoid aggregates. They take up various antigens such as viruses, bacteria and protozoa, and soluble proteins in the human gut, in an indiscriminate way and release them in an ungraded form into the subepithelial area for antigen presentation. Ciliated cells mainly take over absorption and excretion of nutrients. The second aspect is an inhibitory effect from lysozyme, lactoferrin, and lactoperoxidase that are contained in mucus (Figure 2.2) (Wells *et al.*, 1996; Fuller, 1995).

Specific mucosal immunity is executed by the gut associated lymphoid tissue (GALT). GALT are specialised lymphoid aggregates of the gastrointestinal tract, including the tonsils and adenoids, peyer's patcher (PP), appendix and solitary lymphoid nodules. GALT are regarded as the major inductive sites for gastrointestinal mucosal immune responses, and are responsible for the synthesis of secretory IgA (SIgA) (McGhee *et al.*, 1989). Figure 2.2 describes the process of specific immune responses which occur in the Peyer's patches (PP) of the gastrointestinal tract and the migratory pathways of mucosal lymphocytes (Wells *et al.*, 1996). SIgA plays a major role in antigen exclusion. It has a protective role against viral infections (Abraham and Ogra, 1994). Moreover, in the presence of lysozyme and complement, secretory IgA may also lyse and opsonize bacteria, and interfere with the adherence of bacterial antigens to mucosal surfaces, thus, limiting bacterial colonisation and enhancing the elimination of bacteria (Abraham and Ogra, 1994).

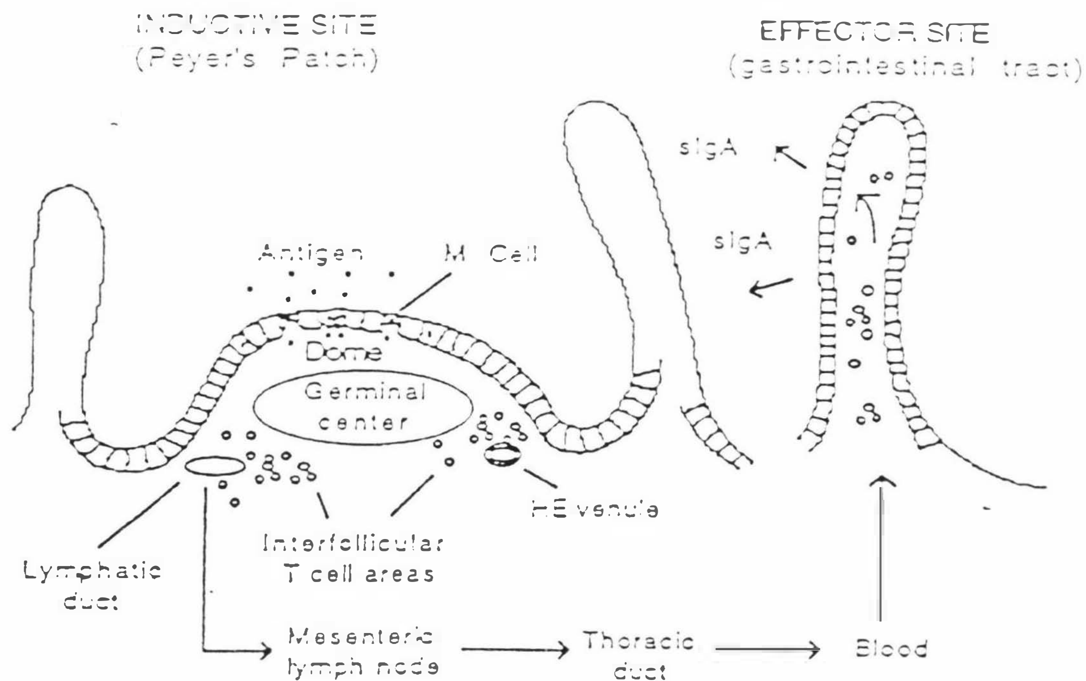

Figure 2.2 Schematic representation of the uptake of antigens by M cells in the Peyer's patches (PP) of the gastrointestinal tract and the migratory pathways of mucosal lymphocytes. Stimulated lymphocytes from the PP migrate into the submucosal lymphatics and then via the thoracic duct into the bloodstream. The cells then localise in the mucosal effector sites where polymeric IgA produced by plasma cells is transported through the epithelium via its interaction with a receptor (the secretory component) on the basal membrane of epithelial cells. The influx of lymphocytes to the PP occurs across post capillary high endothelial venules which have receptors recognised by lymphocyte adhesion molecules. The dome region and germinal center or the PP lymphoid follicles are indicated (adapted from Well *et al.*, 1996)

2.3.1.4. Systemic immunity

The non-specific systemic immune response factors in the host include transferrin, phagocytes, complement and mannose binding protein (Roitt, 1997). Transferrin can competitively bind iron, so as to limit iron supply for bacteria. Phagocytes (particularly polymorphonuclear leucocytes and macrophages) engulf and destroy bacteria. Phagocytes also release IL-6, a cytokine that triggers the production of mannose-binding protein and hence activates the complement cascade (Roitt, 1997). The complement

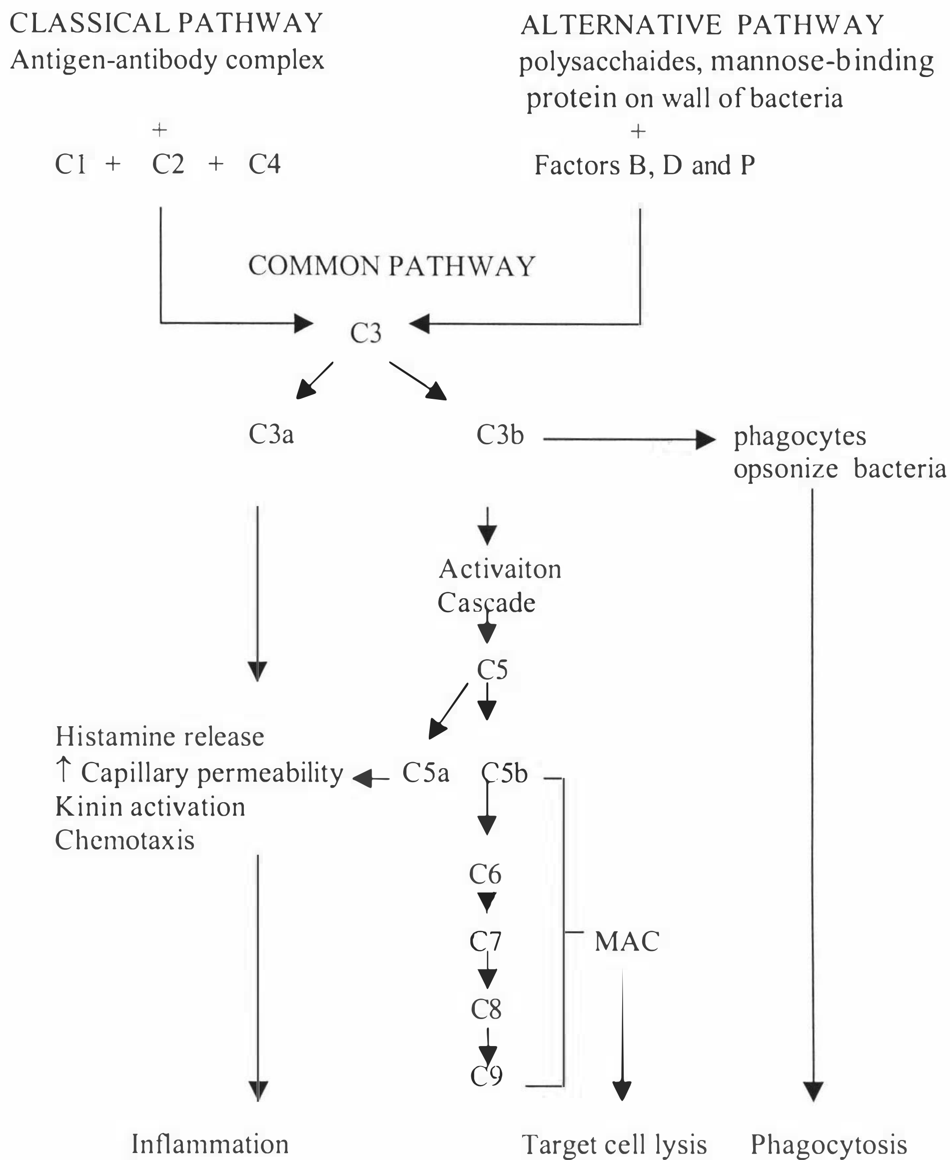

Figure 2.3 Activation and actions of complement and phagocytosis (Hubbard, J and Mechan, D., 1997)

Classical pathway (specific immunity): complement is activated by the interaction between an antigen antibody complex and complement proteins C1, C2 and C4 (complement fixation). **Alternative pathway** (non-specific immunity): complement is activated by the interaction between plasma proteins (factors B, D and P) with polysaccharides on the cell wall of some bacteria and fungi. **Common pathway**: C3 is cleaved into C3a and C3b. C3bbinds to the target cell membrane and activates the remaining complement proteins as well as enhancing phagocytosis (opsonization). C5-C9 form the membrane attack complex (MAC) causing lysis of the target cell. C3a and other complement proteins enhance histamine release, increase capilary permeability, activate kinins and promote chemotaxis

cascade is a set of proteins that are normally inactive but are activated by a set of proteolytic cleavage reactions (Roitt, 1997). Activation is triggered by bacterial surface molecules, mannose-binding protein or binding of the same activated complement components (figure 2.3). These activated components attract phagocytes to the site of invasion (C5a), enhance phagocyte killing activities (C3a), opsonize bacteria so that phagocytes can engulf them (C3b), and form a complex (C5b-C9; MAC) that kills Gram negative bacteria. The combination of complement activation and phagocyte action is called the inflammatory response (Roitt, 1997). Phagocytes kill bacteria by engulfing them in a phagosome, which then fuses with lysosomes. Phagolysosome fusion results in the release of hydrolytic enzymes (lysozyme, proteases) and defensins, and production of reactive forms of oxygen and nitrogen. Release of lysosomal enzymes and toxic products into the surrounding environment can occur and cause damage to cells (Roitt, 1997). In some diseases, damage caused by phagocytes rather than direct action of toxic bacterial products, is responsible for the symptoms of the disease (Fuller, 1995).

A specific 'systemic immune response' starts from antigen presentation (Roitt, 1997). When antigens are presented to lymphocytes, these lymphocytes produce a special memory in response to the antigens (Roitt, 1997). Once exposed to the antigens again, the lymphocytes can recognise the antigens and produce rapid proliferation responses (Roitt, 1997). The processed antigens are expressed on the surface of antigen presenting cells like macrophages through two kinds of human leukocyte antigens (HLA), i.e. HLA class I and HLA class II (Roitt, 1997; Perdigon *et al.*, 1995). HLA class I antigens are expressed in all cells of the body. HLA class II antigens are expressed in the immune cells and some non-immune cells such as epithelial cells. HLA class I molecules generally present cytoplasmic antigens (endogenous antigens) that are primarily synthesised by infected cells (Roitt, 1997). HLA class II molecules primarily present peptides derived from exogenous antigens such as soluble protein antigen derived from microorganisms (Roitt, 1997). In the specific immune response, if the antigens are associated with HLA class I molecules, the immune responses will be operated by cytotoxic T cells (cell mediated immunity) that have the antigen marker $CD8^+$. In

contrast, if the antigens are associated with HLA class II, the immune response will be mediated by T helper cells which have the antigen marker $CD4^+$ (Gill, 1998; Perdigon *et al*., 1995). T helper cells stimulate B lymphocytes to produce specific antibodies (humoral immunity) (Perdigon *et al*., 1995).

Antibodies are proteins that bind specifically to foreign antigens (Roitt, 1997). IgG and IgM are the antibodies responsible for the protection of skin and tissue against bacterial infections, whereas SIgA is responsible for protection of mucosal surfaces (Mestecky and Russell 1998, Roitt, 1997; Perdigon *et al*., 1995; McGhee, *et al*., 1989). IgM and IgG but not Ig A activate complement. A major role of IgG is the opsonization of bacteria. Another important role of all three types of antibodies is the neutralisation of bacterial toxins (Mestecky and Russell, 1998; McGhee *et al*., 1989).

2.3.2. The mechanisms of probiotics in stimulating immune systems

2.3.2.1. The modulation of gut associated lymphoid tissue

Physiological indigenous microflora have a key role in influencing the appropriate development of the mucosal immune system (De Simone *et al*. 1993). This effect of microflora may be mediated by the adhesion of bacteria to the gut surface epithelium, which results in the active stimulation of GALT (Fuller, 1997). LAB have been shown to modulate several functions of GALT.

Peyer's patches cell (PP cell) are a group of organized lymphoid follicle aggregates along the small intestine and an important part of mucosal immune system in gastrointestinal tract. For instance, M-cells in Peyer's patches as specialized transport cells can phagocytose both soluble antigens and microorganisms (Salminen *et al*., 1998). Also PP cells are more accessible to microorganisms than other epithelial surfaces of the gut (Salminen *et al*., 1998). De Simone *et al*. (1993) demonstrated that PP cells suspension cultures from LAB fed BALB/c mice had a strong increase in blastogenic proliferative responses to mitogens such as phytohaemoagglutinin (PHA) and lipopolysaccharide (LPS) when compared with controls). There is also evidence that supplementation with LAB may abolish the suppression of lymphocyte responsiveness to mitogens seen in

tumour bearing mice. Furthermore, the increased cell proliferation in response to LPS, which is mainly a mitogen for B lymphocytes, was correlated with an expansion of the B-lymphocyte pool in PP (De Simone *et al.*, 1993). This data has been further supported by a recent report. The report demonstrated that the content of immunoglobulin synthesizing cells in the jeujunal lamina propria of germ free mice was significantly increased following oral and intraperitoneal administration of killed *L.acidophilus* strains (Smeyanov *et al.*, 1992).

Similar results have been obtained in the mitogen driven splenocyte proliferation. Mice fed living LAB had a strong increase of splenocyte proliferation to lectins (De Simone *et al.*, 1993). These effects were correlated with an expansion of the T-lymphocyte pool in the spleen (De Simone *et al.*, 1993).

LAB in the gastrointestinal tract may act as adjuvants to the humoral immune response. Volunteers who consumed a fermented milk containing *L.acidophilus* and bifidobacteria over a period of three weeks during which an attenuated *S. typhimurium* was administrated to mimic an enteropathogenic infection had a significant increase in the titre of specific serum IgA in comparison to the control group (Link-Amster *et al.*, 1994). An increase in total serum IgA was also observed. Remarkably, these changes correlated with an increase in *L. acidophilus* and *Bifidobacterial* counts in samples of faecal flora (Link-Amster *et al.*, 1994). Although the mechanisms accounting for these effects of LAB on the humoral immune response remain to be fully established, it has been suggested that the increased production of IFN-gamma and the enhancement of macrophage functions following the administration of LAB probably play a critical role (Fuller, 1995).

2.3.2.2. The modulation of phagocyte functions

Phagocytic cells play a central role in protection against microbial infections (Gill, 1998; Roitt, 1997; Fuller, 1995). In addition, macrophages are involved in antigen presentation, tissue repair and also play an important role in the regulation of other immune responses (Roitt, 1997). Many experimental studies have shown that certain strains of LAB can:

- Enhance phagocytic activity of peritoneal and pulmonary macrophages (Paubert-Braquet *et al*, 1995; De Petrino *et al.*, 1995; Perdigon *et al.*, 1988)
- Increase secretion of lysosomal enzymes (Paubert-Braquet *et al.*, 1995; Perdigon *et al.*, 1988), reactive oxygen and nitrogen (Gill, 1998; Balasubramanya *et al.*, 1995), and monokines by phagocytic cells (Gill, 1998).
- Promote *in vivo* clearance of colloidal carbon, an indicator of phagocyte function of the reticuloendothelial system (Perdigon and Alvarez, 1992; Perdigon *et al.*, 1988).

Ingestion of fermented milk containing *Lactobacillus acidophilus* strain *Lal* or *Bifidobacterium bifidum* strain Bb12 for 3 weeks increased phagocytic activity of peripheral blood leukocytes in human subjects (Schiffrin *et al.*, 1997). An *in vitro* study found that macrophage and granulocyte phagocytosis of *E. coli.* were enhanced in healthy volunteers given a fermented milk product supplemented with LAB (Schiffrin *et al.*, 1995). This enhancement was coincidental with faecal colonization by LAB and persisted for 6 weeks after ingestion of the fermented products ceased (Schiffrin *et al.*, 1995). In another study, Mikes *et al*, (1995) noted a significant increase in the ability of neutrophils from human subjects given *Enterococcus faecium* for 6 weeks to produce oxygen radicals following incubation with zymosan. The increase of oxidation in neutrophils accelerated the destruction of phagocytized antigens (Mikes *et al*, 1995).

The mechanisms involved in LAB improvement of phagocytic activities need to be clarified. Some studies (Fuller, 1995) suggested that LAB might mediate these effects through modulation of cell surface molecules that are involved in bacterial uptake by leukocytes. A serum factor, such as an opsonin, may also play a role in mediating this biological effect (Keller *et al.*, 1994).

Several factors influencing the effects of LAB on phagocyte cell functions are suggested. Firstly, The ability to enhance phagocyte cell function is species and strain dependent (Hatcher and Lambreche, 1993). For example, Mice receiving *Str. thermophilus* also had a lower ability to clear colloidal carbon than mice receiving *L. acidophilus*, *L. casei* and *Lactobacillus delbruekii subspp. bulgaricus* (Perdigon and Alvarez, 1992; Perdigon *et al.*,

1986). And macrophages from mice fed fermented milk containing *Lactobacillus casei,* 0.were more effective at enhancing the secretion of lysosomal enzymes than cells from mice fed fermented milk containing *Lactobacillus acidophilus* and *Streptococcus thermophilus* (Perdigon and Alvarez, 1992). Secondly, Structural differences in the cell wall composition of different LAB strains are possibly responsible for differences in their efficacy although the differences of cell wall composition between different strains are to be clarified. Schiffrin *et al* (1997) demonstrated that oral administration of LAB alters the function of blood phagocytes and found the effect may be mediated by the modulation of cell surface molecules involved in bacterial uptake by leukocytes. Furthermore, strains that are able to survive in the gastrointestinal tract, adhere to the gut mucosa and persist above a critical level, are more efficient at stimulating phagocytic cells (Schiffrin *et al*., 1997; Perdigon and Alvarez, 1992). Thirdly, the consumption of fermented milk containing LAB is more efficient at enhancing phagocyte cell function than unfermented milk. The studies showed that phagocytic activity, secretion of lysosomal enzymes, and carbon clearance rates were all greater in mice fed fermented milk containing LAB than in mice fed unfermented milk containing the same LAB (Perdigon *et al*., 1988; 1986). The mechanism is still not clear. It is possibly associated with the presence of immunoactive peptides generated during fermentation from milk proteins (Fiat *et al*., 1993).

2.3.2.3. The modulation of NK cell activity by LAB

NK cells are the principal effectors of anti-tumour immunity and also participate in the destruction of virus infected cells (Fuller, 1995). Comparatively few studies have examined the effect of LAB consumption on NK cell activity. De Simone *et al*. (1989) reported a progressive increase in the percentage of NK cells in the peripheral blood of human volunteers following regular consumption of yoghurt for 28 days. Oral administration of milk based diets containing some LAB strains were suggested to enhance NK cell activity of mouse spleen cells against tumour cells (Fuller, 1995). Augmentation of NK cell activity of peripheral blood lymphocytes (PBL), following pre incubation of PBL stimulated with ConA in the presence of yoghurt, has also been

observed (De Simone *et al.*, 1986). The potentiating effect was attributed to the interferon-γ (IFN-γ) present in the culture supernatants (De Simone *et al.*, 1986).

2.3.2.4. The modulation of cytokine production

The ability of LAB to modulate cytokine production *in vivo* and *in vitro* has been widely reported (Tables 2 and 3). De Simone *et al.* (1989) found higher serum IFN-γ levels in human subjects fed lyophilised lactobacilli and plain yoghurt, compared with those of the control group. Augmented secretion of IFN-γ *in vivo* in healthy subjects given yoghurt was indicated by increased levels of 2-5A synthetase activity in circulating mononuclear cells (Aattouri and Leminnier,1997). Similar effects of yoghurt consumption on IFN secretion in human subjects have been reported (Gill, 1998; Solis Pereyra and Lemonnier 1993; Halpern *et al.* 1991).

LAB consumption also influences IFN-α production in humans. Kishi *et al.*, (1996) reported an increase in virus-induced production of IFN-α in healthy human subjects fed *L.breves subspp. coagulans (Labre)* for different lengths of time. The response was dose dependent, with a higher intake of LAB resulting in a more rapid increase in IFN-α production. Consumption of heat-treated *Labre* also enhanced IFN secretion, but the increase was not statistically significant. Furthermore, ingestion of *Labre* was more efficient at enhancing IFN secretion at the start of the study. Miettinen *et al.*, (1996) noted that LAB strains differed in their ability to stimulate cytokine production. It has also been observed that live bacteria are more potent inducers of cytokine release than fixed or dead bacteria (De Simone *et al.*, 1986). Whether this suggests a role for metabolites produced by live cells, or whether it was due to changes in cell wall composition of LAB induced by heat or fixation treatment, is not clear.

The mechanisms by which LAB induce cytokine secretion are not yet clear. Two possible mechanisms have been suggested (Fuller, 1995). Firstly, cytokine secretion may be triggered following antigen (derived from LAB) presentation by antigen presenting cells to T cells (Fuller, 1995). Secondly, it may result from a direct interaction between LAB or their cell wall components, and the immunocompetent cells (Fuller, 1995). The

presence of specific receptors for peptidoglycan, a cell wall component of LAB, on lymphocytes and macrophages has been found by Dziarski (1991). The ability of peptidoglycan to induce secretion of IL-1, IL-6 and TNF-α by monocytes, and IFN-γ by lymphocytes, also has been reported by Bhakdi *et al.* (1991), Tufano *et al.*, (1991), and Heumann *et al.* (1994). The ability of lipoteichoic acid from *E. faecalis* to induce TNF-α secretion *in vitro* has also been demonstrated recently (Keller *et al.*, 1994).

2.3.2.5. The modulation of autoimmunity by LAB

The composition of endogenous intestinal microflora has an important role in the expression of systemic autoimmunity in both human and animal models (Fuller, 1995). Bacterial products may cause polyclonal B-cell activation, but may even induce the production of anti-ds-DNA antibodies, on the basis of cross reactivity between bacterial and human DNA (Fuller, 1995). The possibility has been suggested that antibacterial antibodies from systemic lupus erythematosus (SLE) patients are sequested in immune complexes (Fuller, 1995). This might indicate a possible role for antibacterial antibodies in exacerbation of SLE (Apperloo-Renkema *et al.*, 1995). Colonization resistance is regarded as the defense capacity of the endogenous microflora against colonization of the gut by foreign bacteria. The observation has shown colonization resistance was reduced in SLE patients. It indicated that, in SLE patients, more and different bacteria are translocated across the gut wall, resulting in a higher chance of subsequent DNA cross-reaction antibacterial antibody production (Apperloo-Renkema *et al.*, 1995).

In addition to the SLE model, other evidence suggests that intraluminal microflora are a pivotal factor, even in the inflammatory process of Crohn's disease (Dumonceau *et al.*, 1994). Diversion of the faecal stream after ileal resection can prevent recurrence and lowering luminal bacterial concentrations improves active disease (Wellmann *et al.* 1986). The deleterious action of bacteria could be due to a few of their products exhibiting potent proinflammatory activity, such as LPS (lipopolysaccharide) and FMLP (N-formyl-methionyl-lircyl-phenylalamine). Bacterial overgrowth in the small intestine has a role in the pathogenesis of rheumatoid arthritis (RA) (Struthers, 1986; Thompson and Elson, 1993; Kanerud *et al.*, 1994). Although further studies are needed, the

confirmation of the above hypothesis would support the use of oral bacterotherapy with LAB in the treatment of active RA (Fuller, 1995).

2.3.3. Correlation between the antiinfective effects of probiotics and stimulated immune responses

Protective effects of LAB against enteric infections have been suggested (Aso *et al.*, 1995; Saavedra, 1995; Hentages, 1992; Kanbe, 1992; Adachi, 1992). However, the relative importance of each immune mechanism in host protection is not clear. Numerous studies have shown that consumption of some strains of LAB results in the stimulation of the immune system, and that this may be responsible for enhanced resistance to disease. Nader de Macias *et al.*, (1992) reported that enhanced resistance to *Shigella* infection in mice fed fermented milk was associated with high levels of anti-*Shigella* antibodies in sera and intestinal secretions. Perdigon *et al.* (1990) also noted an association between augmentation of host immune responses and resistance to challenge infection with *Salmonella* in mice given lactic cultures. Similar observations were made by Paubert-Braquet *et al.*, (1995) regarding the effect of specific lactic cultures against *Salmonella typhimurium*. Consumption of LAB has also been found to reverse corticoid induced immuno-suppression in mice. Oral administration of *L. casei* and *L. bulgaricus* enhanced protection against *Candida albicans* infection in corticoid-immuno-suppressed animals (De Petrino *et al.*, 1995).

A relationship between LAB induced immuno-stimulation and enhanced resistance to disease has also been demonstrated in humans. Kaila *et al.*, (1992) reported that *Lactobacillus GG* promotes recovery from rotavirus diarrhoea in children with acute rotavirus gastroenteritis, through augmentation of host immune responses. Children receiving *Lactobacillus GG* had reduced duration of diarrhoea, and a higher anti-rotavirus IgA response. A similar association between the protective effects of *Lactobacillus* GG and enhancement of specific immune responses was reported by Majamaa *et al.*, (1995). These studies demonstrate a correlation between enhanced specific and non-specific immune responses and resistance to diseases.

2.3.4. Animal models for probiotic immune response studies

The principal methodology used to study *in vivo* immune responses influenced by probiotics involves the use of germ-free animals (Salminen *et al*, 1998). Bacteria isolated from the digestive flora, or used as probiotics, can be analysed by inoculating the gut of germ free animals. These germ free animals are also called gnotobiotic animals (Salminen *et al*, 1998). Recently, germ-free mice associated with human flora have been developed to study functional properties of probiotics and prebiotics *in vivo* (Salminen *et al*, 1998). The advantages of gnotobiotic animal studies are to determine which kind of immune response such as a non-specific and/or specific immune response, or an inductive or suppressive immune response is exerted when a given bacteria is established in the gut, (Salminen *et al*, 1998). The disadvantages of the gnotobiotic model are the expensive animal facilities and animal species used. Moreover, it is not certain if a positive result observed in gnotobiotic conditions can be repeated effectively in conventional conditions (Salminen *et al*, 1998). Thus, conventional animal models are still commonly used to observe therapeutic effects of antiinfective agents and confirm host immune responses (Perdigon *et al.,* 1995). Based on current knowledge, both humoral and cellular immune responses can be assessed at the intestinal level in conventional animal models (Benlounes, *et al.,* 1996; Sutas, *et al.,* 1996a and b). These include the induction of protective IgA antibody responses at the mucosal level (immune exclusion), and the suppression of humoral and cellular immune responses to chronically administered antigens at the systemic level (immune regulation) (Benlounes, *et al.,* 1996; Sutas, *et al.,* 1996 a & b; Weiner *et al.,* 1994). Main methodologies relating to immune responses in animal models have been summarised in Table 2.7.

2.4. Factors that influence the efficacy of LAB

2.4.1. Oligosaccharides

It has been suggested that certain indigestible oligosaccharides on oral ingestion, could lead to changes in the colonic bacterial ecosystem (Gibson *et al*., 1995). In healthy

Table 2.7. Selected methods to study intestinal immune function (adapted from Salminen *et al*., 1998)

Immune response	*Methodology*	*Advantages*	*Disadvantages*
Proliferative assays	*In vitro*:measurement of the proliferation of systemic or intestinal lymphoid cells after or stimulation with mitogens or cell components.	Easy for systemic assays: from blood samples (H).	Not easy for intestinal assay in human subjects: need correlation with *in vivo* assays to give a biological significance.
Cytokine production	*In vitro*: after proliferative assays.	Development of new methodologies (H) Biomarkers (H).	Need correlation with in vivo assays to give a biological significance.
Phagocytic activity	*In vitro*: peritoneal cells, circulating cells.	Easy for systemic assays: from blood samples (H).	Correlation with specific immune response poorly understood technical difficulties for intestinal phagocytes.
Modulation of molecular expression in intestinal cell lines	*In vitro*: HT-29, CaCo-2 cell line cultures. FACS analysis, histochemical methods.	Cell lines originated from human intestine Specialised line cells.	Adenocarcinoma lines absence of correlation with intestinal cellular environment.
IgA antibody responses	Measured by ELISA or ELISPOT at several levels:	Biomarkers.	
	1) In serum: soluble IgA antibodies.	Easy in human subjects (H).	Does not reflect the intestinal response
	2) in blood: circulating IgA-producing cells.	Reflects intestinal response (H).	Does not reflect transepithelial transport into gut lumen.
	3) In faeces.	Easy, allows kinetic studies (H).	Individual and daily variations, proteolytic activity, reflects only colonic response.
	4) In saliva.	Easy (H).	Individual and daily variations.
	5) Whole gut lavage fluid..	Invasive but avoids intestinal biopsy (H).	Needs hospitalisation
Oral tolerance to dietary antigens	*Ex vivo*: proliferative assays of lymphoid blood cells with specific antigen.	Direct measure of the unresponsiveness state (H).	
	Inflammatory cytokine production by lymphoid blood cells.	Biomarkers: TNF-α (H).	Cause or consequence of oral tolerance breakdown?
	In vivo: intestinal permeability.	Increase in food hypersensitivities (H).	Not specific to immunological changes.
	In vitro: Using chamber.	Direct correlation with antigen.	Invasive and difficult technology.

[H], applicable to human studies; IgA, immunoglobulin A; TNF-α, tumour necrosis factor-α; FACS, fluorescence-activated cell sorting; ELISPOT, enzyme-linked immunospot assay.

humans, these oligosaccharides are virtually not digested by the intestinal enzymes and reach the colon. In the colon, they are fermented by a limited number of colonic bacteria such as bifidobacteria. Meanwhile they also stimulate bacterial growth (Bouhnik *et al*., 1997; Gisbon *et al*., 1995). In addition, there is striking evidence to indicate that human milk oligosaccharides are also potent inhibitors of bacterial adhesion to epithelial surfaces at the initial stage of the infective processes (Ashoor and Monte, 1983).

2.4.2. Whey

Petschow and Talbott (1991 a & b) examined the growth-promoting activity of human milk, cow's milk, and their whey and casein fractions, for five strains of *Bifidobacterium* species isolated from faeces of human infants. The results showed that whey promoted the growth of *B. bifidum sero-var pennsylvaicus* and *B. bifidum.* Whey dominant formulas promoted better growth of *B.bifidum serovar pennsylvanicus*, *B. bifidum*, and *B. infantis* than casein dominant formulas. The data also identified a direct relationship between the quantity of whey specific factors, and the ability to promote growth of clinically relevant strains of *Bifidobacterium* species by human milk, cow's milk, and cow based infant formula (Petschow and Talbott 1991 a & b).

2.4.3. Glycosylated components

Romond *et al*.,(1990) detected growth factors for *B. bifidum* in the faeces of axenic mice. Most of these factors were found in the nondialyzable fraction obtained after aqueous extracton and dialysis. Electrophoresis and gel filtration revealed that many glycosylated components had bifidogenic activities. Intestinal colonization of mice by *B. bifidum* involved the utilization and eventually the disappearance of these intestinal bifidogenic factors-glycosylated components (Romond *et al*.,1990).

2.4.4. Additional factors

Poch and Bezkorovainy (1988) found that, except *B. adolescentis* and *B. longum,* all other *bifidobacteria* required growth promoters such as inorganic salts, vitamins, nitrogen, and carbon. Several other intestinal bacteria such as bacteroides, enterobacter,

and enterococcus may also release a growth stimulator for bifidobacteria and play an important role in the regulation of a bifidobacterial population in colonic microflora. For instance, a bifidobacterial growth stimulator was identified in the cell free filtrate of *Propionibacterium freudenreichii* 7025 culture, and in the methanol extract fraction of the cells (Kaneko *et al*., 1994).

The diversity of LAB results in a large variation between various LAB strains in the ability of LAB to impact on the immune system (De Petrino *et al*., 1995; Paubert-Braquet *et al*., 1995; Perdigon and Alvarez, 1992). Live cultures are more efficient at enhancing certain aspects of immune function than killed cultures (Portier *et al*., 1993; De Simone *et al*., 1986; Versely *et al*., 1985). Moreover, lactic cultures in fermented products induce a superior response compared to cultures in unfermented products (Perdigon *et al*., 1986). Host age, physiological status and dietary intake also influence the efficacy of LAB. They need to be further studied (Gill, 1998).

In addition, a variety of transition metals and divalent cations were shown to be growth inhibitors of *B. bifidum var. pennsyvanicus* as they interfered with bacterial calcium and iron metabolism by antagonism (Naidu, *et al*., 1999; Topouzian, *et al*., 1984). It has been demonstrated that some inhibition could be reversed fully or partially by 0.5mM to 1.0mM ferric iron in the presence of Zn^{2+}, Cu^{2+}, Au^{3+}, Pt^{4+}, La^{3+}, Cr^{3+}, Mn^{2+}, Ni^{2+}, and Cd^{2+} (Topouzian, *et al*., 1984).

2.5. Summary

In summary, probiotics are a group of live microorganisms including some microbial stimulants that exert health promoting effects, such as the maintenance of a normal intestinal microbiotia, increased nutritional value of foods, anticarcinogenic activity, reduction of serum cholesterol levels, alleviation of lactose intolerance and stimulation of the immune system. Lactic acid bacteria (LAB) are safe and widely used probiotic strains, which may prevent adherence, establishment, and replication of enteric mucosal pathogens. LAB also can release various enzymes into the intestinal lumen and assist intestinal digestion and absorption. Certain strains of LAB may elicit anti-tumour effects.

These effects are associated with inhibition of mutagenic activity, decrease of the generation of carcinogens, mutagens and tumour promoting agents, and suppression of tumours. One of the important mechanisms underlying these functions may be LAB's immunomodulatory effects including increase of phagocytosis, augmentation of cytokine pathways and enhancement of humoral and cell mediated immunity. The following chapters will report the results of studies on the anti-infection and immune enhancement properties of a probiotic strain *B. lactis* HN019 in piglet and murine models.

CHAPTER 3 Effects of supplementation with *Bifidobacterium lactis* HN019 on weaning diarrhoea associated with rotavirus and *Escherichia coli* infection in a piglet model

3.1. Introduction

In the literature review, lactic acid bacteria (LAB) were suggested to have many health benefits. In particular, immunomodulatory effects of LAB were described in detail. The hypothesis that a single LAB strain could protect a host against infectious diarrhoea had been well supported by a number of studies. To further test the hypothesis, a new probiotic LAB strain (*Bifidobacterium lactis* HN019) was used in a pig model to investigate and confirm its protective effects of LAB against enteric infections. The results of this study are reported and discussed in this chapter.

As reviewed in Chapter 2, lactic acid bacteria (LAB), such as lactobacilli and bifidobacteria, are ubiquitous Gram-positive commensals of the normal human intestinal tract. LAB are also the predominant microbes used in the manufacture of fermented dairy products, and following oral delivery, some well-defined LAB strains have been reported to beneficially enhance host immunity and increase resistance to enteric pathogens (Gill 1998; Majamaa *et al*., 1995; Saavedra *et al.,* 1994; Kaila *et al*., 1992; Perdigon *et al*., 1990). In particular, feeding of defined probiotic (gut-colonising) LAB strains has been reported to reduce the duration, severity and/or incidence of infant diarrhoea in some cases (Majamaa *et al*., 1995; Saavedra *et al.,* 1994; Kaila *et al*., 1992). It is possible, therefore, that well-defined strains of probiotic LAB may offer promise as safe and effective means of combating infant diarrhoea, via dietary supplementation and subsequent modification of the intestinal microflora.

Several mechanisms have been proposed to explain enhanced resistance to gastrointestinal tract (GI) pathogens conferred by probiotic LAB, including inter-microbial competition with pathogens for intestinal attachment sites, the production of

substances that are directly microbicidal for pathogens, and enhancement of protective immune responses of the hosts (Shu *et al*., 2000; Gill, 1998; Kaila *et al*., 1992; Sato *et al*., 1988). While no theory is mutually exclusive, well-defined LAB strains such as *Lactobacillus rhamnosus* GG have been shown to enhance immune responses pertinent to the control of microbial infection (Majamaa *et al*., 1995; Kaila *et al*., 1992), and may therefore be reliant wholly or partially on this mechanism for conferring enhanced protection against diarrhoea-causing pathogens. Recent research in our laboratory has identified a new probiotic LAB strain, *Bifidobacterium lactis* HN019, that has immune-enhancing properties in mice and human (Gill *et al*., 2000; Shu *et al*., 2000; Arunachalam *et al*., 2000; Chiang *et al*., 2000). Further studies in murine models have also indicated that *B. lactis* HN019 can be utilised as an effective biological agent to reduce the severity of *Salmonella typhimurium* infection (Shu *et al*., 2000).

The objective of the present study was to investigate the potentially protective effects of *B. lactis* HN019 in an animal model of infant diarrhoea, by studying naturally-acquired rotavirus and *E. coli* infection in a weaning piglet model. It is well known that infectious diarrhoea is a major paediatric health problem worldwide. The predominant pathogens implicated in infectious diarrhoeal disease in neonates are rotavirus and *Escherichia coli* (Nabuurs 1998; Yasui *et al*., 1995; Levine, 1987). The annual death rate of infants associated with rotavirus in developing countries alone has been estimated as more than 1 million (Yasui *et al*., 1995). In industrialised countries, rotavirus and *E. coli* can cause significant morbidity associated with diarrhoea, and are frequently encountered as clinical infections in neonatal nurseries and paediatric hospitals (Fujiwara *et al*., 1997; Kaplan *et al.,* 1982; Von Bonsdorff *et al*., 1978). Effective and safe means of combating diarrhoea-causing microbial pathogens are therefore much sought-after (Brunser *et al*., 1989).

Weaning piglets have been suggested to be an ideal model for human gastrointestinal tract infection as: 1) the young of humans and pigs are of similar bodyweight (5~7 kg) at the comparable developmental stage of peak lactation in the dam; 2) naturally-acquired piglet weaning diarrhoea is associated with both rotavirus and *E. coli* infection (Benfield

et al., 1988; Leece *et al.,* 1983); 3) the piglet has been suggested as an appropriate monogastric animal model for studying nutrition, gastroenteritis, immunology, and other aspects of human infants (Darragh *et al.*, 1995; Rowan *et al.*, 1994; Miller *et al.*, 1987; Tumbleson 1986). In comparative studies of the digestive process in milk-fed piglets and human infants, it has been concluded that the digestive tracts of piglets and human infants are very similar in respect to anatomy, histology, and aspects of digestive physiology (Moughan *et al.,* 1992; Pond and Houpt, 1978).

In the present study, three-week-old piglets (bodyweight within 5~7 kg) were used. The efficacy of feeding with the probiotic strain *B. lactis* HN019 for reducing the severity of naturally-acquired weaning diarrhoea was assessed by monitoring the daily fecal consistency (diarrhoea score), feed conversation efficiency, and the quantities of rotavirus and *E. coli* fecal shedding during weaning. Blood cell phagocytic activities and lymphocyte proliferative responses, as well as fecal antibody titers, were also measured as indices of the animals' concurrent immune status.

3.2. Materials and Methods

3.2.1. Animals and feeding

Seventeen three-week-old male suckling piglets (bodyweight within 5~7 kg, fecal rotavirus test positive), were randomly selected from a production farm (Waitakera farm Ltd., NZ), and allocated into 2 groups balanced for live weight and litter of origin (on day –5). The first group (n=8) was administered *B. lactis* HN019 (10^9 cfu/piglet) daily via oral gavage throughout the experiment; the second group (n=9) was not given a probiotic. All piglets stayed with their mothers until they were weaned (day 0). On day 0, they were re-located to Massey University Pig Research Unit (Palmerston North, New Zealand) and housed individually in standard pens at a controlled temperature (29 ± 1°C) with free access to water at all times. Piglets were fed twice daily on a weaner diet ('Commercial Weaner', Unifeeds Ltd., New Zealand; 11% metabolic body weight) throughout the rest of the experiment. The animal use in the experiment had been approved by Massey University Animal ethics Committee (AEC98/179).

3.2.2. Bifidobacterium lactis HN019

Freeze-dried *Bifidobacterium lactis* HN019 was obtained from the starter unit of the New Zealand Dairy Research Institute (NZDRI, Palmerston North, New Zealand). *B. lactis* HN019 powder was reconstituted in skim milk prior to feeding at a concentration of 10^8 cfu/ml. The viability of *B. lactis* HN019 powder was checked on MRSC (MRS broth + cysteine-HCL) agar by incubating at 30° C for 72 hours in an anaerobic jar before and after reconstitution. Skim milk without *B. lactis* HN019 was used as a control. Each dose volume was 10 ml/piglet daily.

3.2.3. Experimental procedures

Health appearance and diarrhoea score were closely monitored during the period of the experiment (from day –5 to 9). Severity of diarrhoea was scored according to the following criteria: 1, hard and formed pellets; 2, non-formed pellets; 3, soft faeces; 4, very soft containing small amount of water-like faeces; 5, semisolid containing more than half water-like faeces; 6, watery-like faeces; a score of 5 and 6 was considered as severe diarrhoea. Feed intake of each animal was measured daily by weighing the feed refusal, and the animals were weighed on days -5, 1 and 8 using an electrical scale (Mettler Toledo, Switzerland). Feed conversion efficiency was calculated using the following formula:

$$\text{Feed conversion efficiency} = \frac{\text{Daily live weight gain (kg)}}{\text{Daily feed intake (kg)}}$$

Blood samples were collected prior to (day –5) and after weaning (day 9) for measuring changes in phagocytic cell activity and lymphocyte proliferative responses to a T-cell mitogen. Blood was sampled from anaesthetised piglets via cardiac puncture using 0.9 x 25mm needles and vacutainers (Becton Dickinson, USA). Fresh fecal samples were collected on days -5, 2 and 9 for measuring shedding of rotavirus and *E. coli*, and specific antibody responses to rotavirus and *E. coli* in the gastrointestinal tract of piglets. The fecal samples were weighed and diluted 1:10 in PBS (pH7.4). After homogenisation, aliquots of the samples were centrifuged for 10 minutes (at low speed [200 g] for enumerating rotavirus and *E. coli;* at high speed [10,000 g] for measuring antibody

titres), and the supernatants were collected and stored at –70°C until use. Aliquots of the fecal supernatants were thawed and serially diluted for further analysis. A second aliquot of the fresh faeces from each piglet was weighed and dried at 105°C in an oven for measuring fecal dry matter content.

3.2.4. Detection of rotavirus

Rotavirus particles in the fecal supernatants were determined using a commercial rotavirus detection kit (IDEIA^{TM} Rotavirus-DAKO Diagnostics Ltd., UK) and the ELISA protocol described in the manufacturer's instructions. The principle of the IDEIA^{TM} Rotavirus test was to utilize a polyclonal antibody in a solid-phase sandwich enzyme immunoassay to detect group specific antigen present in Group A rotaviruses (the instruction for IDEIA^{TM} Rotavirus kit, DAKO Diagnostics Ltd., UK). In the experiment, serial dilutions of a standard rotavirus suspension were also included with each plate as a calibrated reference. Assay results were expressed as viral infection forming units (IFU) per gram of fecal dry matter.

3.2.5. Enumeration of E. coli

E. coli-selective agar (Fort Richard Laboratories, New Zealand) was used to detect *E. coli*. The agar was a chromogenic media in which *E. coli* colonies showed purple colour due to the action of β-glucuronidase produced by most *E. coli* (Bell, C. and Kyriakides, A.,1998). In the experiment, serial dilutions of test samples were plated on the *E. coli*-selective agar in triplicate and incubated at 37°C for 48 hours. *E. coli* colonies were identified in accordance with the manufacturer's instructions. The *E. coli* numbers were expressed as cfu/gram fecal dry matter.

3.2.6 Blood phagocytosis

One hundred microlitres of EDTA-treated whole blood from each piglet was added to a 2 ml glass tube containing 10 μL heat-killed fluorescein isothiocyanate (FITC)-labeled *E. coli*. The tubes were incubated for 30 minutes at 37°C in a 5% CO_2 incubator. Phagocytosis was stopped by the addition of 100 μl of 8% formaldehyde, and erythrocytes were lysed by the addition of 1ml ice cold water. After centrifugation, the

supernatant was discarded and the pellet resuspended in 0.5mL PBS. Fifty microlitres of trypan blue was added (to quench fluorescence of non-internalized bacteria) and phagocytosis was assessed using a FACSCalibur flow cytometer (Becton Dickinson, USA.) (Gill *et al*., 2000). The flow cytometer was to measure and record fluorescent values produced by phagocytes containing heat-killed fluorescein isothiocyanate (FITC)-labeled *E. coli.* after phagocytosis. Data were expressed as the percentage of cells showing phagocytic activity in the cell preparation.

3.2.7. Blood cell proliferation assay

In vitro proliferation responses of blood cells to mitogens were determined using a commercial cell proliferation kit (Boehringer Mannheim, Mannheim, Germany) as previously described by Gill *et al.* (2000). Briefly, fifty microlitres of heparinized whole blood (1:4 diluted in complete RPMI-1640 medium) was added in triplicate to wells of ninety-six-well flat-bottomed tissue culture plates (Nunc, GIBCO BRL, Life Technologies Ltd., NZ) and cultured in the presence or absence of a T-cell mitogen (concanavalin A, Con A; 2.5 μg/ml; Sigma, USA). Control wells received fifty microlitres of complete RPMI-1640 medium. These plates were cultured for 72 hours at 37°C in a humidified CO_2-air (5:95, v/v) atmosphere. Cellular proliferation was determined via incorporation of ^{3}H-thymidine over the final 18h of culture. The radiometric values of each well were read using a Betaplate counter. A stimulation index (SI) was used to measure the degree of cell proliferation (SI in this experientment was expressed using mean value of cells with mitogen- mean values of cells without mitogen).

3.2.8. Measurement of antibody by ELISA

Antibody responses were determined using an ELISA protocol based on the method described by Shu, *et al* (2000). Briefly, ninety-six well ELISA plates (Nunc, USA) were coated overnight with either inactived rotavirus (5 x 10^3 IFU/ml, from IDEIA™ rotavirus detection kit) or formalin-killed *E. coli* K88 (10^8 cells/ml) in carbonate-coating buffer (pH 9.6). High speed-centrifuged faecal supernatant samples were serially diluted in 1,

1/4, 1/16 and 1/64 and added to wells (100 μl/well) in duplicate as the source of primary antibody. Mouse anti-porcine IgG, IgA or IgM serum (1:500; Serotec, UK) and alkaline phosphatase-conjugated sheep anti-mouse immunoglobulin (Dako, USA) were employed as secondary and tertiary antibodies; binding was visualized using an alkaline phosphatase substrate (Bio-Rad Laboratories, CA, USA). The absorbance was read using a CERES 900 Bio-Tec microtiter plate reader (CERES 900,Bio-Tec Instrument Inc., USA), at 450 nm by which OD values for the samples were established in the range of 0.1-2.5; and titer end-point was calculated as the highest titration OD value > the mean plus 2 standard deviations of the mean control OD values (ODs obtained using PBS in place of a piglet fecal supernatant sample).

3.2.9. Statistical analyses

Significant differences between the experimental and control groups were determined using ANOVA [SAS (r) Proprietary Software Release 6.12, SAS Institute Inc., Cary, NC, USA.] except that significance in diarrhoea score and the incidence of severe diarrhoea were tested by non-parametric (Wilcoxon) and binomial proportions (Fisher's exact) tests, respectively. For statistical analysis, data for fecal rotavirus and *E. coli* as well as the cell proliferation index were transformed by $\log_{10}$ and the antibody data were transformed by $\log_{10}(1+x)$. The following descriptors are used to indicate statistical significance as follows: ns, $P > 0.05$; *, $0.05 \geq P > 0.01$; **, $P \leq 0.01$.

3.3. Results

3.3.1. Severity of diarrhoea, feed conversion efficiency and survival rate

The piglets in both control and *B. lactis* treatment groups experienced naturally-acquired diarrhoea after relocating from the farm to the pig research unit, corresponding to the shift onto a weaner diet. However, a significantly higher ($P<0.01$) mean diarrhoea score was observed in the control group on days 1 and 2 in comparison to the *B. lactis* group (Figure 3.1A). There was no statistically significant difference in diarrhoea score between the two groups on the subsequent days ($P>0.05$).

Three (on day 1) and five (on day 2) piglets in the control group exhibited severe diarrhoea (score 5 or 6), while no animals in the *B. lactis* group had severe diarrhoea on days 1 and 2. The incidence of severe diarrhoea (percentage accumulative number of animals with diarrhoea scored 5 or 6) in the test and control groups is summarized in figure 3.1B. Throughout the experiment, 87.5% piglets in the control group showed severe diarrhoea, compared to 62.5% in the *B. lactis* group (diarrhoea scores of 5 or 6). The *B. lactis* HN019-fed piglets had a significantly lower incidence of severe diarrhoea on days 1, 2, and 3 than the controls (P<0.01).

One piglet from the control group died (before death, the piglet showed life-threatening clinical signs including severe diarrhoea, dehydration, lying down and lethargic, non-reactive to stimulus, and was treated with antibiotics for humane reasons). All the animals in the *B. lactis* group recovered well after weaning.

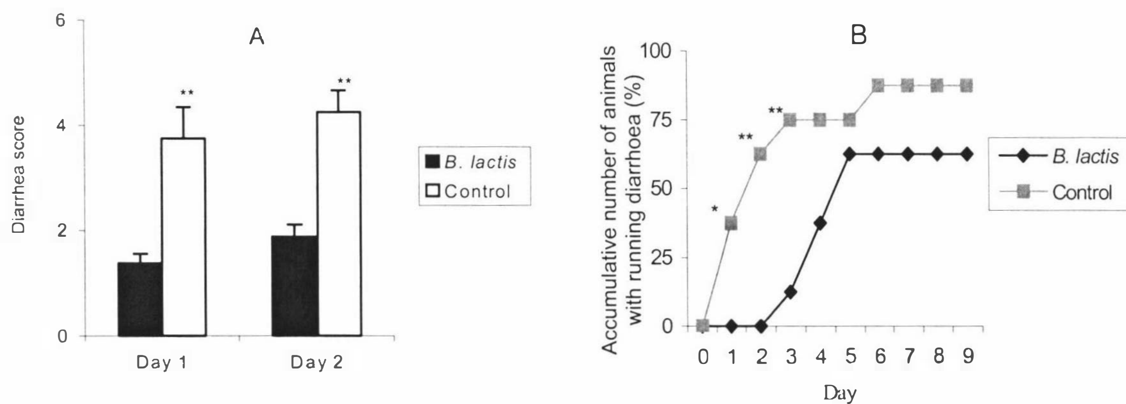

Figure 3.1 Mean diarrhoea score (A) and accumulative number of animals with severe diarrhoea (B) during the period of weaning. Vertical error bars represent standard errors of the means. *B. lactis* group had significantly less severity of diarrhoea than the controls on days 1 and 2 (**P<0.01). *B. lactis* group also had significantly fewer accumulative number of animals with severe diarrhoea than the control group on days 1, 2, and 3 (*P<0.05).

The animals fed *B. lactis* HN019 had significantly higher (P<0.05) post-weaning feed conversion efficiency than the non-*B. lactis* controls (Figure 3.2).

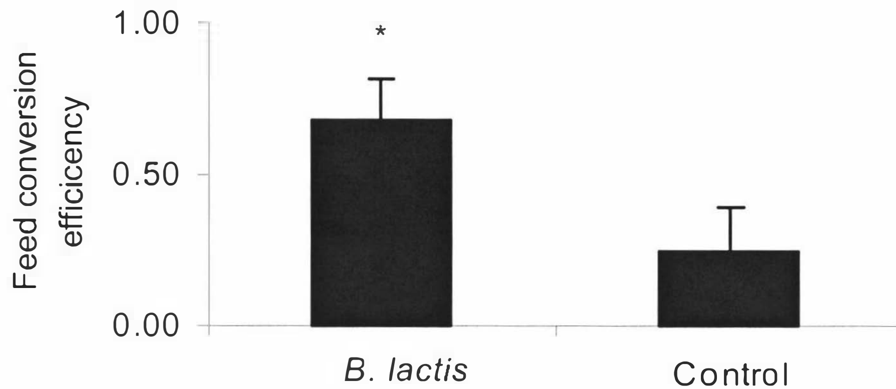

Figure 3.2 Mean feed conversion efficiency of the *B. lactis*-fed and control piglets on the weaner diet during days 1~8. Vertical error bars represent standard errors of the means. *B. lactis* group maintained significantly higher feed conversion efficiency than the controls.
*P<0.05

3.3.2. Fecal rotavirus and E. coli

Feces from *B. lactis* treatment and control piglets were positive for rotavirus and *E. coli* before weaning, but there was no difference (P>0.05) in fecal counts of either pathogen on day –5 between groups. After weaning, significantly higher concentrations of rotavirus and *E. coli* were recorded in the control group on both sampling days (days 2 and 9) in comparison to the *B. lactis* group (Figure 3.3).

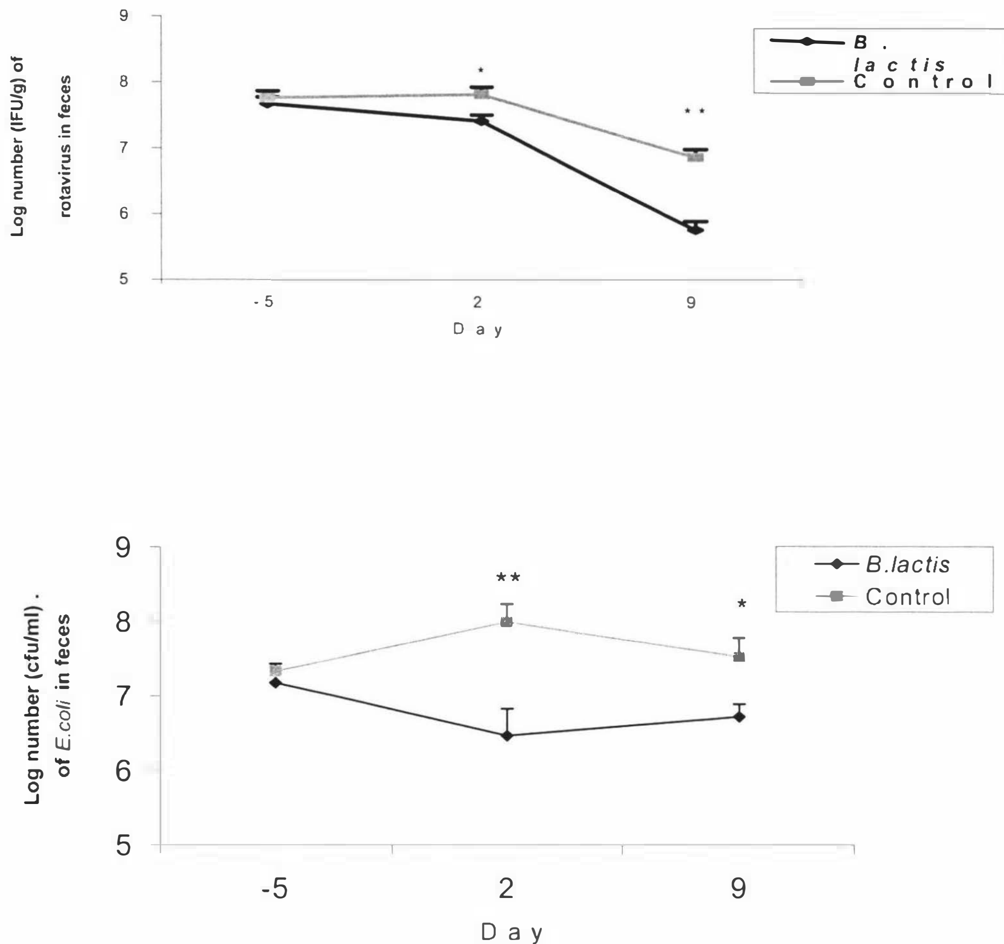

Figure 3.3 Changes in rotavirus and *E. coli* numbers in feces of piglets throughout the experiment. Weaning commenced on day 0. Vertical error bars represent standard errors of the mean fecal pathogen counts. *B. lactis* group had significantly lower counts of fecal rotavirus and *E. coli* than the controls on days 2 and 9. The rotavirus shedding in both groups significantly decreased from day 2 to 9 (**P<0.01). The *E. coli* shedding in the control group significantly increased from day –5 to 2 (*P<0.05).

3.3.3. Cellular immune responses

Throughout the experiment the mean phagocytic activity (i.e. percentage of phagocytically-active cells) among peripheral blood leukocytes in *B. lactis*-fed piglets increased 4.74%, while that in the control group decreased 10.8% (Table 3.1). The

change of phagocytic activity (from day –5 to 8) between the two groups was significantly different (P<0.05). Prior to weaning (on day –5), there was no difference (P>0.05) in blood lymphocyte proliferative response to Con A of *B. lactis*-fed piglets, compared with control piglets. After weaning, significantly higher blood lymphocyte proliferative responses to Con A were recorded in the *B. lactis* group (on day 8) in comparison to the control group (Table 3.1).

Table 3.1 Blood phagocytic activity and lymphocyte proliferative response to T-cell mitogen (Con A) throughout the experiment

	B. lactis HN019	Control	Significance
Change of % cells with phagocytic activity †	+4.74 (SE =4.69)	-10.8 (SE =4.84)	*
Cell proliferative response to mitogen ††	4.93 (SE =0.15)	3.98 (SE =0.43)	*

† Due to large intra-group variations, data were standardized as percentage change in response between day –5 (pre-weaning) and day 8 (post-weaning); i.e. % cells with phagocytic activity on day 8 minus % cells with phagocytic activity on day –5. SE=standard error

†† Cell proliferation index on post-weaning day 8 (log_{10} transformed). There was no significant difference in cell proliferation index on pre-weaning day –5 (P>0.05). SE=standard error

* P<0.05

3.3.4. Antibody levels in feces

Following probiotic feeding (on days 2 and 9), piglets fed *B. lactis* HN019 had significantly higher mean titer end-points of specific antibodies (IgA, IgM, IgG) for rotavirus and *E. coli* in fecal supernatants compared with piglets in the control group (figure 3.4).

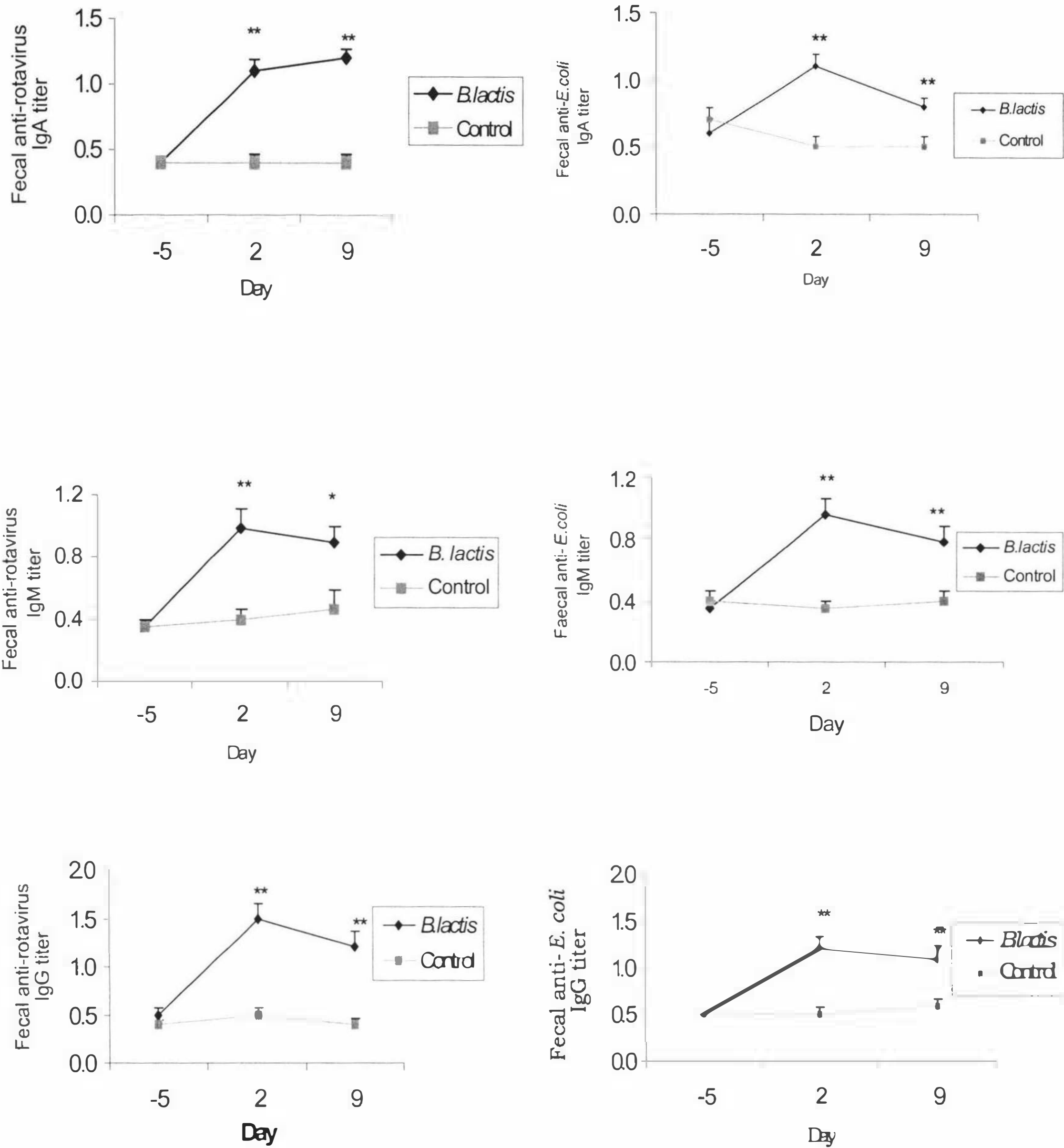

Figure 3.4 Specific antibody titers in feces. Data are mean (± SE) anti-*E. coli* and anti-rotavirus antibody titer end-points (inverse titer end-point, $\log_{10}(1+x)$-transformed) in fecal samples among control and *B. lactis*-fed piglets. The *B. lactis* group had significantly higher fecal anti-*E. coli* and anti-rotavirus IgG, IgA and IgM titers than the controls. All the antibody titers in the *B. lactis* group increased significantly from day-5 to 2 (P<0.01).
*P<0.05; **P<0.01

3.4. Discussion

The results of the present study have shown that oral feeding of the probiotic LAB strain *B. lactis* HN019 is able to reduce the severity of weaning diarrhoea in piglets. *B. lactis* HN019-fed animals also maintained a higher feed conversion efficiency, and displayed a reduction in fecal shedding of rotavirus and *E. coli* compared to the control group. All the animals fed *B. lactis* HN019 recovered well after weaning but one piglet from the control group died. These results, together, suggest that oral feeding of *B. lactis* HN019 enhances resistance to weaning diarrhoea. Enhanced resistance was accompanied with higher cellular immunological activity in the peripheral blood, and higher fecal antibody titers against rotavirus and *E. coli*. These results are consistent with our previous observations that dietary supplementation of mice with *B. lactis* HN019 can enhance immunity and concomitantly increase host resistance to enteric pathogen challenge (Shu *et al.,* 2000).

Rotavirus and pathogenic *E. coli* are recognized as major causes of gastrointestinal infection and diarrhoea disease in neonatal animals and human infants worldwide (Naidu *et al*., 1999; Paul, 1994; Esters *et al*., 1983; Synder and Merson 1982; Black *et al*., 1981; Anderson *et al*., 1980). In this study, we have utilized the weaning piglet as a model to study the protective efficacy of a probiotic against infant diarrhoea. The sequence of naturally-acquired weaning infection in the piglet model has been proposed as follows (Leece *et al*., 1983): (1) the piglets are weaned in a commercial piggery environment that is usually contaminated by a number of enteric pathogens such as rotavirus and pathogenic *E. coli*; (2) the gut becomes more susceptible to pathogen infection at weaning because it is no longer supplied with protective antibody from sow's milk; (3) rotavirus infects and destroys absorptive epithelium, thereby producing a malabsorption of nutrients; (4) the malabsorption further creates an enteric environment that favors the colonization of the intestines by enteropathogenic *E. coli,* and further exacerbates infection of enterocytes by rotavirus; (5) the more burdensome the dietary regimen, the more efficient reinfection by rotavirus and the more persistent diarrhoea and shedding of rotavirus. Accordingly, an important mechanism for controlling weaning diarrhoea could be to enhance the local antibody levels against rotavirus and *E. coli*. Several studies have been conducted using piglet models to investigate immunity and host resistance to enteric

pathogens (Lanza *et al*., 1995; Apgar, *et al*., 1993; Bernard *et al*., 1990; Moxley *et al*., 1989; Shirai *et al*., 1988; Crouch, 1985; Askaa *et al*., 1983). It has also been demonstrated using a murine model that a LAB strain-*B. bifidum* (ATCC 15696, obtained from the American Type Culture Collection) conferred protection against diarrhoea, which was related to the reduction of rotavirus and enhancement of host immune responses (Duffy *et al*., 1994; Duffy *et al*., 1993). In human study, Saavedra *et al*.(1994) also demonstrated the effectiveness of *B. bifidum* in prevention of infant diarrhoea and shedding of rotavirus; Correlation between protection against rotavirus diarrhoea and enhanced immune responses has been demonstrated in other studies as well (Offit, 1996; Ward, 1996). In general, immune-enhancing LAB stimulate immune responses that are pertinent to the control of rotavirus and *E. coli* at the mucosal surfaces such as pathogen-specific antibody production (Yasui *et al*., 1995; Perdigon *et al*., 1991; Perdigon *et al*., 1990; Yamazaki *et al*., 1985), and at remote sites from the site of delivery, e.g. phagocytic and lymphoproliferative activity of blood leukocytes (Moineau, *et al*., 1989; Perdigon *et al*., 1986). The results of the present study are consistent with the previous observation that some probiotic strains of LAB may reduce weaning diarrhoea and the shedding of rotavirus, and can concomitantly enhance mucosal specific antibody levels (acquired immunity) and/or blood cell phagocytic activities (innate immunity) and lymphoproliferative responses. Results from the present study have thus confirmed that *B. lactis* HN019 stimulated both innate (natural) and acquired immune responses against rotavirus and *E. coli*.

The mucosal immune response is critical to protect the host against enteric pathogens, and the antibody elicited must be of a particular immunoglobulin isotype in order to contribute maximally to host defense against the pathogen (Kerlin and Watson, 1988; Watson, 1987). Normally, secretory IgA is the dominant isotype of antibody present in monogastric mammalian intestinal secretions. In the present study, we detected significantly higher titers of specific IgA, as well as IgG and IgM, in the fecal samples of animals fed with *B. lactis* HN019 compared to the controls. These results suggested that *B. lactis* HN019 may have a pluripotent stimulatory effect on the gut immune system.

It is recognized that immunological control of microbial pathogens is dependent on the function of both innate and specific immune responses. Therefore, the enhanced natural and acquired immune responses in the piglets after feeding *B. lactis* HN019 might play an important role in reducing the shedding of rotavirus and *E. coli*. Logically, the enhanced immunity may contribute to a reduction in the severity of weaning diarrhoea via reduction of the enteric rotavirus and *E. coli* burdens. In this study, the reduction of enteric rotavirus and *E. coli* was indicated by a significant decrease in fecal shedding of rotavirus and *E. coli* in the *B. lactis* HN019 treatment group in comparison with the control group. However, although *B. lactis* HN019 conferred a significant degree of protection against weaning diarrhoea during the early post-weaning period, complete protection was not observed in this study.

In summary, the results of the current study indicate that *B. lactis* HN019 conferred a significant degree of protection against weaning diarrhoea associated with rotavirus and *E. coli* in a piglet model of neonatal gastrointestinal infection. This, together with the results obtained in our previous studies in mice and humans (Gill *et al*., 2000; Shu *et al*., 2000; Arunachalam *et al*., 2000; Chiang *et al*., 2000), suggests that *B. lactis* HN019 has health-promoting effects to combat intestinal pathogens and may be efficient at reducing diarrhoea disease in human infants, especially at times of abrupt changes in dietary habits, such as weaning.

CHAPTER 4 Effect of *Bifidobacterium lactis* HN019 supplementation on *Escherichia coli* O157:H7 infection in mice

In chapter 3, *Bifidobacterium lactis* HN019 (*B.lactis* HN019) was found to enhance the resistance of piglets to naturally acquired *E.coli* infection in weaning diarrhoea model. This chapter further express the efficacy of *B.lactis* HN019 on protecting against *Escherichia coli* O157:H7 challenge in a mouse model.

4.1. Introduction

Escherichia coli O157:H7 is recognised as an important food-borne pathogen inducing hemorrhagic colitis, hemolytic uremic syndrome, and/or diarrhoea in humans and animals (Dundas *et al.*, 1999; Shere *et al.*, 1998; Haruo *et al.*, 1996; Su and Brandt, 1995; Martin *et al*, 1990; Ostroff *et al.*, 1990). Many cases of infections and outbreaks caused by *E. coli* O157:H7 have been reported world-wide. For example, a massive outbreak involving more than 9,500 cases and nine deaths occurred in Japan in 1996 (O'Loughin, 1997). Thus, measures to limit the occurrence or impact of *E. coli* O157:H7 are of great significance to public health.

A recent outbreak of *E. coli* O157:H7 in the United States was associated with consumption of unpasteurized apple juice (Venkitanarayanan *et al.*, 1999). A wide variety of foods including meat, milk, fruit juices, and vegetables have been implicated as potential vehicles of *E. coli* O157:H7 (Venkitanarayanan *et al.*, 1999). Compared with other food-borne pathogens, *E. coli* O157:H7 is suggested to have a higher resistance to acid and heat environment (Ellajosyula *et al.*, 1998; Deng *et al.*, 1999), and is thus a serious public health risk from environmental and food sources that are reliant on existing hygiene practices.

Current therapeutic measures to control *E. coli* O157:H7 infection rely on the use of anti-microbial agents. Due to complications associated with antibiotics, such as the

emergence of drug-resistant strains and the potential for chronic toxicity, the widespread use of antibiotics is not acceptable as a preventive or therapeutic control measure (Dundas ***et al.***, 1999). There is therefore a desire to develop alternative, non-pharmaceutical strategies for controlling *E. coli* O157:H7 infection. One possibility is the use of live, oral biomodulatory agents, such as probiotic lactic acid bacteria (LAB). LAB (comprising *Lactobacilli* and *Bifidobacterium*) are commensal Gram-positive constituents of the normal human intestinal microflora, and also represent the predominant microbes in fermented foods. Previously, it has been demonstrated that certain LAB strains are capable of enhancing host immunity and conferring increased protection against enteric pathogens in both animals and human following oral delivery (Holzapfel *et al.*, 1998; Mestecky and Russell, 1998; Yasui *et al.,* 1995; Isolauri *et al.,* 1994; Perdigon *et al.,* 1990 & 1991). Therefore, certain LAB strains may be effective dietary additives for human to combat *E. coli* O157:H7 infection.

Recent research in our laboratory has shown that dietary supplementation with the probiotic *Bifidobacterium lactis* HN019 can enhance immune function in mice and humans (Arunachalam *et al.,* 2000; Gill *et al.*, 2000). In a recent study, *B. lactis* HN019 was also shown to enhance protection in mice against an oral challenge infection of *Salmonella typhimurium*, and the degree of protection was shown to correlate closely with immune enhancement conferred by the probiotic (Shu *et al.*, 2000). The major aim of the present study was to further investigate the protective effects of supplementation with *B. lactis* HN019 against *E. coli* O157:H7 infection in mice. Protection against oral challenge was assessed by monitoring morbidity and feed intake, as well as measuring pathogen translocation to visceral tissues (spleen and liver) and specific and non-specific immune responses of the hosts; Specific and non-specific immune responses were assessed by measuring cellular and humoral parameters relevant to the immunological control of *E. coli*.

4.2. Materials and Methods

4.2.1. Microorganisms

Bifidobacterium lactis strain HN019 (*B. lactis* HN019), originally isolated from yoghurt and maintained as lyophilised seed stock at the New Zealand Dairy Research Institute (NZDRI), Palmerston North, New Zealand, was supplied as frozen dry culture (stored at –20 °C). For feeding, the probiotic diet was subsequently prepared by mixing the dry culture to the desired concentration (3×10^8 cfu/g) in a skim milk powder (SMP)-based diet. The concentration of *B. lactis* HN019 was confirmed on MRSC (MRS broth + cysteine-HCL) agar by incubating at 30° C for 72 hours in an anaerobic jar. According to the designed formula, the *B. lactis* HN019 supplemented SMP-based diet was prepared everyday to maintain the viability of *B. lactis* HN019. And the desired concentration and viability of *B. lactis* HN019 in the probiotic diet were also rechecked and confirmed on MRSC agar after feeding.

E. coli O157:H7 strain 2988 (ATCC35150, obtained from NZ-CDC) was grown overnight in a brain heart infusion broth (BHI, Difco) at 37° C, washed in sterile phosphate buffered saline (PBS) and resuspended to the desired concentration (10^9 cfu/ml).

4.2.2. Experimental procedures and measurements

4.2.2.1. Animals

Six to eight week-old BALB/c and C57BL/6 male mice were housed in pairs at a controlled temperature (22 ± 2°C) with a 12 hour light/dark cycle. Animals were fed *ad libitum* with standard mouse diet along with free access to water at all times. For an acclimatisation period of 7 days, prior to commencement of feeding experiments, the mice were fed *ad libitum* on a SMP-based diet, which was replenished daily. Mice were randomly allocated into two groups (*B. lactis* group and control group). Control animals (20 BALB/c mice, and 20 C57 mice) continued to be fed the SMP-based diet as before, while animals in the *B. lactis* group (24 BALB/c mice, and 22 C57 mice) were fed the SMP-based diet containing *B. lactis* (3×10^8 cfu/g). After seven days on the test diets, all

animals were challenged orally with *E. coli* O157:H7 suspension (0.1 ml/mouse, administered via a pipette). Mice in *B. lactis* group then were maintained on *B. lactis* HN019-supplemented SMP-based diet until the end of experiment following the challenge. And mice in control group were still fed the SMP-based diet as before. Throughout the experiment, feed intake was assessed daily by measuring the remaining (unconsumed) food. The animal use in the experiment was approved by Massey University Animal ethics Committee (AEC98/129).

The health appearances (ostensibly normal/abnormal) of all mice were carefully monitored twice daily over the period of experimentation. The criteria used for normal and abnormal appearance were: 1) Normal appearance: mouse bright-eyed and alert, has a smooth coat with a sheen, responds to stimulus, shows interest in its environment without diarrhoea; 2) Abnormal appearance: fur ruffled, a loss of sheen to the coat; less alert or active, and less interested in environment outside of cage, signs of hyperventilating when handled; hunched over and lethargic; non-reactive to stimulus; agitated or displaying diarrhoea. Morbidity was calculated based on the number of animals with abnormal appearance to the total number of animals in each group. It was pre-determined that, after challenge with *E. coli* O157:H7, animals would be withdrawn from the trial and euthanased by isofluorane overdose for reasons of welfare when the following appearance was observed: mouse non-reactive to stimulus, fur has a "bottle brush" appearance, mouse hunched over preferring to sleep than react to environment.

At the end of the trial (one week post-challenge), twenty mice which were ostensibly normal (ten from each group; five BALB/c and five C57 mice) were randomly selected for measuring *E. coli* O157 translocation to blood, spleen and liver and to investigate specific and non-specific immune responses. Mice were euthanased by isofluorane overdose. Approximately 1 ml of blood was withdrawn via cardiac puncture, and used for assessing bacterial translocation and leukocyte phagocytic activity. Peritoneal cells were collected by lavage from each mouse for measuring their phagocytic activity. The spleen and liver were removed from each mouse aseptically to assess bacterial translocation. The small intestine was recovered, and the contents flushed with 1 ml

PBS; particulate material was removed by centrifugation and the remaining supernatant fluid was used to measure mucosal antibody response to *E. coli* (Ig A and IgG).

*4.2.2.2. Culture of E. coli O157:*H7 *in blood, spleen and liver*

Immediately after sampling, the liver and spleen of each mouse were homogenised individually in 0.1% peptone water. The tissue homogenates were serially diluted in peptone water, and then plated in triplicate on *E. coli* O157 selective agar (CHROM agar O157, Fort Richard Laboratories Ltd, New Zealand). After incubation at 37°C for 48 hours, the colonies on the agar were enumerated. Blood samples were cultured in BHI (Brain Heart Infusion) broth overnight and then plated on the *E. coli* O157 selective agar.

4.2.2.3. Phagocytosis assays using whole blood or peritoneal cells

Phagocytosis was assessed via flow cytometric analysis of the uptake of fluoresceinated formalin-killed *E. coli*, as described by Gill *et al.* (2000). Results were expressed as the phagocytic capacity, i.e. the proportion (percentage) of phagocytically-active cells in each sample.

4.2.2.4. ELISA measurement of antibody

The assays for determining antibody titers are based on those described by Shu *et al.* (1999 and 2000). Antigen-binding onto ninety-six well ELISA plates utilised overnight incubation of whole formalin-killed *E. coli* O157:H7 (5×10^7 cells/ml) in 100 μl carbonate coating buffer (pH 9.6). Anti-*E. coli* antibody responses were assessed in serially diluted samples of intestinal fluid (1, 1/2, 1/4, 1/8 and 1/16) in triplicate wells; antibody binding was visualised using alkaline phosphatase conjugated sheep anti-mouse immunoglobulin (IgA or IgG) antibody (Serotec, UK) and an alkaline phosphatase substrate (Bio-Rad Laboratories, CA, USA). Results were read at 405 nm on an ELISA reader CERES 900, Bio-Tec Instrument Inc., USA), by which OD value was optimised in the range of 0.1-2.0, and titer end-point was calculated as the highest titration OD > the mean plus 2 standard deviations of control intestinal fluid (derived from mice that had not been challenged with *E. coli*).

4.2.3. Statistical analyses

Differences in mean feed intake, phagocytic activity, and antibody titers between *B. lactis* HN019 treatment and control groups were statistically analyzed using student's t tests to compare two sample means in a population (Riffenburgh, 1999). Antibody titer end-points were transformed by log 10.

4.3. Results

4.3.1. Morbidity and feed intake

Both BALB/c and C57 mice fed a diet containing *B. lactis* HN019 showed lower cumulative morbidity rates following infection with *E. coli* O157:H7 in comparison to control group mice (Figure 4.1). The mean feed intake (post-challenge) of animals fed *B. lactis* HN019 was also higher (P<0.01) than the controls (Figure 4.2).

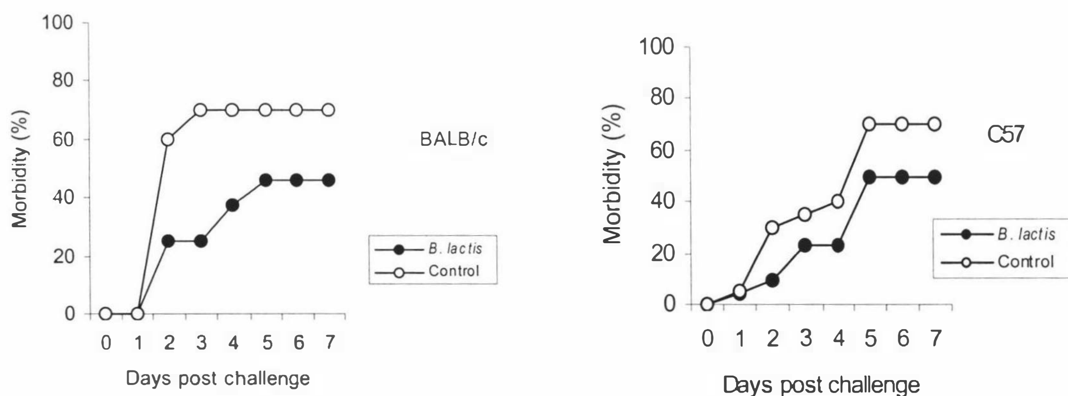

Figure 4.1 Cumulative morbidity in *E. coli* O157 H:7-challenged BALB/c and C57BL/6 mice

Data are expressed as the cumulative percentage of animals with abnormal appearance. Abnormal appearance was expressed as: fur ruffled, a loss of sheen to the coat; less alert or active, and less interested in the external environment, signs of hyperventilating when handled; hunched over and lethargic; non-reactive to stimulus; agitated or showing signs of diarrhoea.

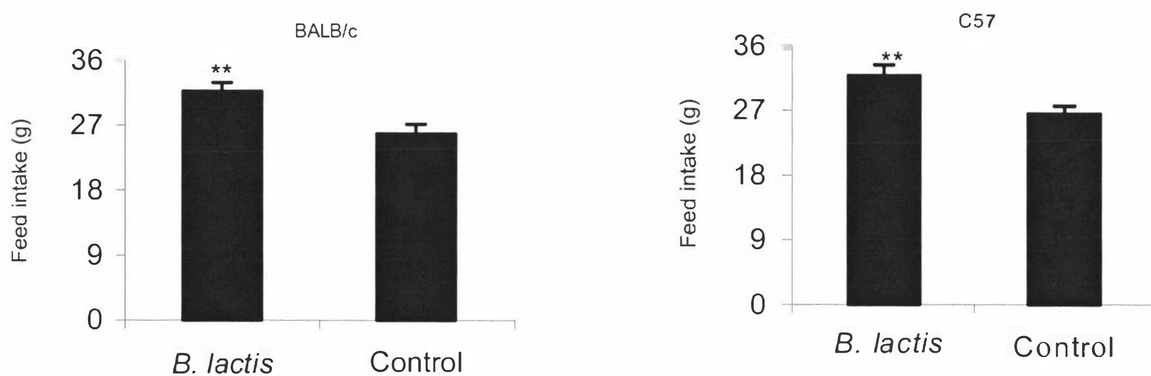

Figure 4.2 Feed intake of BALB/c and C57BL/6 mice over a period of one week after challenged with *E. coli* O157:H7
Data are mean ±SE. ** P<0.01.

4.3.2. Effect of *B. lactis* HN019 on bacterial translocation

All the blood samples collected from the animals in the *B. lactis* HN019-fed group proved negative for *E. coli* O157. However, a blood sample from one of the C57 control mice was *E. coli* O157 positive. *E. coli* O157 was also detected from one spleen and two liver samples from the *B. lactis* HN019 treatment group, and from three spleens and 5 livers collected from the control group (Table 4.1). Mean log bacterial burdens per infected mouse were 4.6 and 6.1 in control mice, compared to 2.1 and 4.1 in the *B. lactis* HN019-fed mice (values for BALB/c and C57 mice, respectively).

Table 4.1 Translocation of *E. coli* O157:H7 to blood, spleen and liver in *B. lactis*-fed and control mice. Numbers of *E. coli* O157 were expressed as $\log_{10}$ value of colony forming unit (cfu).

Group	Mice	Number of animals with *E. coli* O157-positive culture			Total number of mice with *E. coli* O157-positive culture
		Blood	Spleen	Liver	
B. lactis	BALB/c (n=5)	0	0	1 (2.1)*	1 (20%)**
	C57 (n=5)	0	1 (1.5)	1 (2.6)	1 (20%)
Control	BALB/c (n=5)	0	1 (3.3)	2 (1.8~4.1)	2 (40%)
	C57 (n=5)	1	2 (2.1~3.8)	3 (1.9~6.0)	3 (60%)

* Bacterial count was expressed as $\log_{10}$ cfu/organ and shown in a bracket.

** The rate of positive culture mice in each group was shown in a bracket.

4.3.3 Effect of *B. lactis* HN019 treatment on phagocytic capacity and IgA antibody titers

Mice in the *B. lactis* treatment groups had significantly greater phagocytic capacity (i.e. percentage of phagocytically-active cells) in both the blood and peritoneal cell preparations in comparison to non-*B. lactis* controls ($P<0.01$) (Figures 4.3 and 4.4). Mice fed *B. lactis* HN019 had significantly higher mean anti-*E. coli* IgA titer end-points in comparison to the control mice ($P<0.05$) (Table 4.2). The difference in IgG titers between the *B. lactis* and control groups was not statistically significant ($P>0.05$)

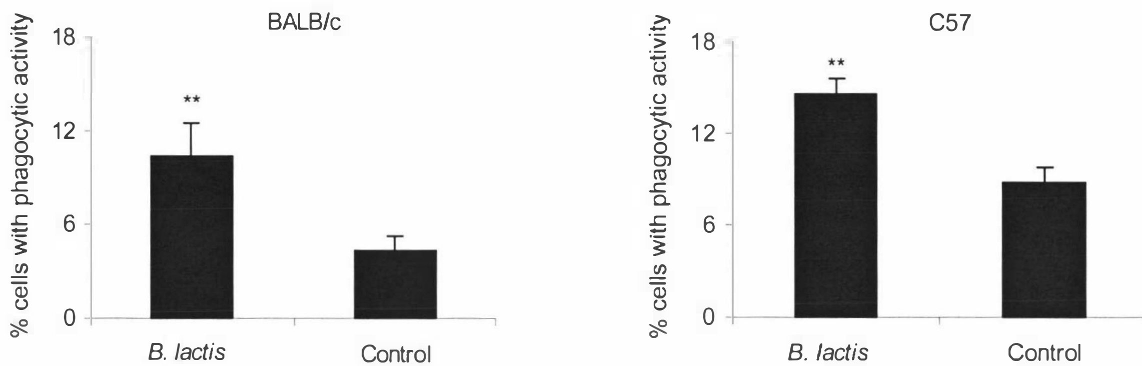

Figure 4.3 Phagocytic activities of blood leukocytes in BALB/c and C57BL/6 mice
Data are mean (± SE) percentages of cells showing phagocytic activity, for 5 *B. lactis*-fed and 5 control mice. *B. lactis*-fed mice vs controls: **P < 0.01.

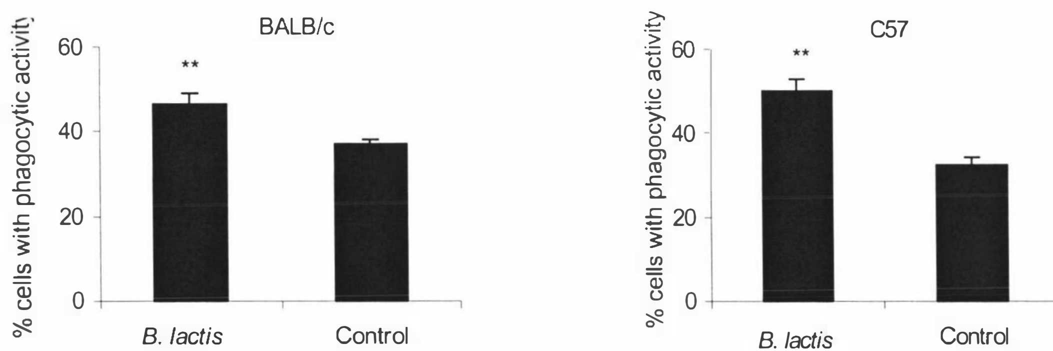

Figure 4.4 Phagocytic activities of peritoneal macrophages in BALB/c and C57BL/6 mice
Data are mean (± SE) percentages of cells showing phagocytic activity, for 5 *B. lactis*-fed and 5 control mice. *B. lactis*-fed mice vs controls: **P < 0.01.

Table 4.2 Anti-*E. coli* mucosal antibody responses of mice in *B. lactis*-fed and control groups.

	B. lactis	Control	Significance
Ig A			
BALB/c	1.20 (0.19)	0.54 (0.15)	$P < 0.05$
C57	0.84 (0.24)	0.24 (0.11)	$P < 0.05$
IgG			
BALB/c	0.72 (0.31)	0.24 (0.18)	not-significant
C57	0.84 (0.29)	0.48 (0.23)	not-significant

Data are mean (SE) anti-*E. coli* antibody titer end-points (inverse titer end-points, $\log_{10}$-transformed), of 5 *B. lactis*-fed and 5 control mice. *B. lactis*-fed mice vs controls:

4.4. Discussion

The results of this study demonstrate that dietary supplementation with *B. lactis* HN019 can reduce the severity of *E. coli* O157:H7 infection in mice. After oral challenge with the pathogen, *B. lactis* HN019-fed BALB/c and C57 mice exhibited lower cumulative morbidity, and maintained significantly higher feed intake, than their respective control groups. These effects were accompanied by higher phagocytic capacities of blood leukocytes and peritoneal macrophages, and superior intestinal IgA antibody responses against *E. coli* in the *B. lactis* HN019 treatment group, compared with the control groups. This is consistent with our previous observation that *B. lactis* HN019 can enhance murine protection against oral challenge with an enteric pathogen (*Salmonella typhimurium*), and that this effect is associated with enhanced specific and non-specific immune responses (Shu *et al.*, 2000). This study also demonstrates that reduction in the severity of infection, and enhancement of potentially protective immune responses, can be identified in BALB/c and C57 mice that represent broadly different patterns of immune protection against intracellular pathogens.

Although there is little information on the protective effects of LAB against *E. coli* O157:H7 infection *in vivo*, several studies have examined the protective effects *in vitro*. Brashears *et al.* (1998) studied the antagonistic action of *Lactobacillus lactis* toward *E. coli* O157:H7 on refrigerated raw chicken meat, and found that samples inoculated with *Lactobacillus lactis* exhibited a significant decline in *E. coli* O157:H7 counts after three days of storage. The decline in *E. coli* O157:H7 counts was associated with hydrogen peroxide produced by *L. lactis*. Massa *et al.* (1997) also demonstrated inhibition of the growth of *E. coli* O157:H7 by yoghurt containing *Bifidobacterium bifidum*. Our results further provide *in vivo* evidence that some LAB strain can reduce disease severity due to *E. coli* O157:H7 infection. The mechanisms by which *B. lactis* HN019 conferred this effect against *E. coli* O157:H7 may include 1) inter-microbial competition with pathogens for intestinal attachment sites, 2) the production of substances that are directly microbicidal for pathogens, and/or 3) enhancement of host immunity. The present study was not designed to explore the first two aspects but measured cellular and humoral responses that are pertinent to the immunological control of *E. coli* O157:H7 infection.

The ability to translocate, survive and proliferate in extra-intestinal tissues is a critical determinant of the potential for intestinal pathogenic organisms to cause infection (Ford *et al.*, 1996; Berg, 1983; Steffen and Berg, 1983). Previous studies in experimental animals have indicated that some LAB strains can reduce the translocation of enteric pathogens to extra-intestinal tissues, which is an important step in prevention against progressive infection (Shu *et al.*, 2000; Isolauri *et al.*, 1998; Perdigon *et al.*, 1990). It has also been reported that translocation of pathogenic bacteria to the spleen reaches a peak level in 7 days after infection in mice, and declines thereafter (Perdigon *et al.*, 1990). For this reason, in this study bacterial translocation was studied in mice at one week after challenge with *E. coli* O157:H7. The *B. lactis* HN019-fed mice showed a trend towards a lower incidence of translocation and lower mean bacterial burdens in *E. coli* O157:H7-positive animals. The subjective index of lower cumulative morbidity and higher post-challenge feed intake, suggested an increased degree of protection in these mice.

In addition to *E. coli* O157:H7, protective effects of orally-delivered LAB against other enteropathogenic *E. coli* strains have been demonstrated in several studies (Macfarlane, 1999; Naidu *et al*, 1999; Isolauri, 1998; Tejada-Simon, 1998; Perdigon *et al*., 1991). It has been suggested that the protective effect could be associated with the enhancement of various immune defense mechanisms pertinent to host protection (Naidu *et al*., 1999; Fuller, 1997; Perdigon *et al*. 1991). Cross *et al*. (1995) indicated that complement-mediated bacteriolysis, macrophage- and neutrophil- mediated opsono-phagocytic killing, and cytokine-mediated responses, are important host defense mechanisms against enteropathogenic *E. coli* infection (Cross *et al*, 1995; Karpman *et al*., 1997). Most bacteremic *E. coli* are susceptible to opsono-phagocytic killing (Cross *et al*., 1984). Selected bifidobacteria and lactobacilli have been demonstrated in general studies to be able to stimulate phagocytic activities of peripheral blood leucocytes and macrophages and to increase production of various cytokines (Naidu *et al*., 1999; Marin *et al*., 1998; Fuller, 1997; Solis-Pereyra *et al*., 1997; Sasaki *et al*, 1996; Perdigon *et al*., 1988). In the pig trial described in Chapter 3, *B. lactis* HN019 also significantly increased phagocytic activities. Combined with earlier studies in this laboratory, *B. lactis* HN019 was demonstrated here to significantly enhance the phagocytic capacity of both blood leukocytes and peritoneal macrophage preparations (Gill *et al*., 2000; Arunachalam *et al*., 2000; Shu *et al*., 2000), and this may have contributed to the increased protection observed in these mice.

Another important aspect of the immune response against enteric pathogens is the specific IgA response in the gut (Fukushima, 1999; Mestecky and Russell, 1998). Significant increases in intestinal IgA antibody responses against *E. coli* as a result of *B. lactis* HN019 feeding were also observed in the present studies. This is an important result, since IgA is the predominant mucosal antibody and plays an important role in protection against intestinal pathogens. Our previous studies (Shu *et al*., 2000) had demonstrated a significant correlation between the degree of immune enhancement and level of protection against *Salmonella typhimurium* in *B. lactis* HN019-fed mice. Furthermore, Perdigon *et al*. (1991) have previously demonstrated enhanced gut mucosal IgA responses among mice that had been fed *Lactobacillus casei* and challenged with the

enterotoxigenic *E. coli* strain O111 K58. In the present study, we consider it likely that enhanced immune responses, conferred by feeding *B. lactis* HN019, at least contributed to a greater degree of protection against *E. coli* O157.

The enhanced immunity and reduced disease severity conferred by *B. lactis* HN019 in this study, together with evidence from previous studies of immunity-enhancing and protective effects (Gill *et al*., 2000; Shu *et al*., 2000), suggest that dietary supplementation with *B. lactis* HN019 may represent an effective biotherapeutic/prophylactic means of countering gastrointestinal infection in humans (Elmer *et al*., 1996). This is consistent with the previous reports that probiotic supplementation of the diet is a potentially valuable means of combating diarrhoea infections (Majamaa *et al*., 1995; Saavedra *et al*., 1994; Kaila *et al*., 1992), and that immunoregulatory LAB have the potential to be incorporated into foodstuffs (e.g. yogurt) and used as a non-pharmaceutical means of boosting immunity and enhancing protection (Salminen *et al*., 1998; Hull *et al*., 1992). *B. lactis* HN019 has been shown to enhance immunity in humans when used as a dietary supplement (Arunachalam *et al*., 2000; Chiang *et al*., 2000), and thus it remains to be determined whether this effect corresponds to increased protection against important gastrointestinal pathogens in humans as well.

It is worth noting that the maintenance of *B. lactis* HN019 viability is an important limit factor when used as a supplement for foods (Naidu *et al*., 1999). As mentioned in chapter 2, *B. lactis* HN019 is an anaerobic bacteria. The viability of *B. lactis* HN019, to some degree, relies on whether it was kept in an anaerobic environment when prepared and fed to host. In this study, the reconstitution diet were prepared everyday and kept in the sealed containers before fed to mice. And the desired concentration and viability of *B. lactis* HN019 in the diet were rechecked before feeding and confirmed after feeding.

In summary, the protective effect of the probiotic *Bifidobacterium lactis* HN019 against *Escherichia coli* O157:H7 was investigated in murine challenge infection models. BALB/c or C57BL/6 mice were fed milk-based diets supplemented with *B. lactis* HN019 (3×10^8 cfu/g) for 7 days prior to and following oral challenge with *E. coli* O157:H7.

Behavioral parameters (morbidity, feed intake) were measured for 7 days following challenge; immunological responses (phagocytosis, antibody) and pathogen translocation were measured in a sub-sample of ostensibly healthy animals one week post-challenge. Results showed that *B. lactis* HN019-fed mice maintained significantly higher post-challenge feed intake and exhibited a lower cumulative morbidity rate, compared to control mice which did not receive the probiotic. Significantly higher proportions of phagocytically active cells in the blood and peritoneum, and higher intestinal tract IgA anti-*E. coli* antibody responses, were recorded among *B. lactis* HN019-fed mice compared to controls. Among *B. lactis* HN019-fed mice, pathogen translocation was identified in 1/5 BALB/c and 2/5 C57 mice; the comparative figures in control mice were 2/5 and 3/5, respectively, and the mean bacterial burdens in these mice were over 100-fold higher than in *B. lactis* HN019-fed mice. These results demonstrate that *B. lactis* HN019 can reduce the severity of infection due to the enterohemolytic pathogen *E. coli* O157:H7, and suggest that this reduction may be associated with enhanced immune function conferred by the probiotic.

CHAPTER 5 Concluding remarks

In this thesis, the anti-infection properties of *B. lactis* HN019 were tested in two animal models: 1) naturally-acquired weaning diarrhoea associated with rotavirus and *E. coli* in piglets and 2) artificial challenge infection with *E. coli* O157: H7 in mice. The results showed that *B. lactis* HN019 reduced the severity of the enteric infections in both animal models, and enhance both non-specific and specific immune responses. This is consistent with our previous observation that *B. lactis* HN019 provides protection against *Salmonella typhimurium* in mice, which is positively correlated with enhanced immune responses (Shu, *et al.*, 2000). These results provided further evidence to support the hypothesis that *B. lactis* HN019 may reduce enteric pathogen infection and that the protection may be associated with enhanced non-specific and specific immune responses.

Although significant protection was achieved in both trials, complete protection against either naturally acquired weaning diarrhoea or challenge infection was not attained. For example, the results in the pig trial showed that the animals in the *B. lactis* group still experienced weaning diarrhoea, in which *B. lactis* HN019 supplement delayed the occurrence of weaning diarrhoea and reduced the severity of diarrhoea. This may be due to the extreme experimental conditions such as relocation of animals from the farm to the animal house, individual housing, and weaning onto a solid diet. Weaning diarrhoea probably resulted from not only infectious agents, but also other factors such as stress, solid food intake and premature digestive functions, that may be, to a lesser degree, influenced by the probiotic LAB strain in young piglets (Naidu *et al.* 1999). Another reason may be that the dose of *B. lactis* HN019 (10^9 cfu/piglet/day used in this trial) was not high enough. It would be useful, in future studies, to examine the efficacy of a higher dose of *B. lactis* HN019 (such as 10^{10} cfu/piglet/day) against weaning diarrhoea.

The piglet study showed that supplementation with *B. lactis* HN019 significantly enhanced the feed conversion efficiency (liveweight gain per Kg feed consumption). This is consistent with other observations that probiotics are beneficial in promoting growth in young animals and humans (Holzapfel *et al.*, 1998). Young rats fed a liquid diet

consisting either of milk, yogurt or milk fermented with *S. thermophilus,* showed a significantly higher weight gain when fed fermented products (Wong *et al.,* 1983; Hargrove and Alford, 1980). In four weeks, the animals fed milk gained 116g in contrast to 131.3g when fed yogurt and 136.3g when maintained on a *S. thermophilus* fermented milk diet. A similar study has been conducted in humans (Robinson and Thompson, 1952). Bottle fed infants during the first month of life gained on average 21.9 ozs when fed a standard formula, and 26.5 ozs when *L. acidophilus* was added to the formula. The increase in liveweight gain may largely result from the reduced severity of infection (Goldin, 1998). It is logical that severe infection may induce a poor appetite and a low digestive and absorptive function, as result of low gastric acid, intestinal epithelial damage and diarrhoea, which may prevent the growth of animals. Due to health and environmental concerns, the widescale use of feed-additive antibiotics in animal production industries may soon be banned. There is therefore an urgent need to develop alternative strategies for disease control, in order to maintain high levels of health and performance in farmed animals. The improvement of feed conversion efficiency suggests that *B. lactis* HN019 has promise for use in the animal industry, as an alternative to antibiotics, to maintain wellbeing and high performance.

Although used in human for generations, only recently have probiotics been widely studied in clinical studies (Naidu *et al*., 1999). After a number of studies have shown their prophylactic and therapeutic effectiveness on the severity and the duration of intestinal infections in infant and adult population, probiotics are being used in clinical management of diarrhoea and further expanded into anti-tumor treatment (Naidu *et al*., 1999; Elmer *et al*., 1996).

Recent global marketing trends of probiotics are based on the expectations of a prophylactic effect and in many cases as an alternative to conventional pharmaceutical preparations. Many studies focus on the selection of target-specific probiotics for prophylactic and therapeutic health benefits (Naidu *et al*., 1999; Macfarlane, 1999; Sanders, 1998; Bengmark, 1998; Goldin, 1998; Salminen, *et al*., 1998; Holzapfel *et al*., 1998; Perdigon, *et al*., 1995). These results, together with our previous studies (Gill *et*

al., 2000; Shu *et al*., 1999a and 2000; Zhou *et al.,* 2000a & b; Prasad *et al*., 1998), suggest that *B. lactis* HN019 could be used as a useful probiotic strain for functional foods and feeds.

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