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Safety Studies on Probiotic Strains *Lactobacillus rhamnosus* HN001, *Lactobacillus acidophilus* HN017, and *Bifidobacterium lactis* HN019

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To my wife Lily, sons Punan and Daniel

Abstract

Lactic acid bacteria (LAB) have been consumed in foods by human beings for several centuries without any obvious adverse effects. But the safety of consumption of these organisms, especially novel strains, which are added to foods as probiotics, has been questioned recently due to occasionally reported infections implicated with some particular LAB strains. Evaluation of the safety or potential toxicity of probiotic candidate strains, especially novel strains for which no prior safety data exist, is highly recommended. The LAB strains *Lactobacillus rhamnosus* HN001 (DR20TM), *Lb. acidophilus* HN017 and *Bifidobacterium lactis* HN019 (DR10TM) are three newly identified probiotic organisms with immune-enhancing properties. Their safety/potential toxicity was investigated in this study through a series of both *in vitro* and *in vivo* experiments.

The mucus layer coating the surface of the gastrointestinal tract plays an important role in the gut mucosal defence system. Platelet activation and /or aggregation is a critical factor in the pathogenesis of infective endocarditis (IE). In the first part of this study, the potential pathogenicity of LAB strains was examined by *in vitro* mucin degradation (HN001, HN017, and HN019) and platelet aggregation (HN001 and HN019) assays. Following incubation with hog gastric mucin (HGM) in a minimal medium, the mucin degradation activity of test strains was determined via changes in the carbohydrate and protein concentration of the culture media and molecular weight changes of mucin glycoproteins (SDS-polyacrylamide gel electrophoresis, SDS-PAGE). The mucinolytic activity of test strains was also measured in an agarose petri dish assay. The results from these experiments suggested that HN001, HN017 and HN019 had no ability to degrade HGM *in vitro*. Flow cytometry analysis using platelet specific monoclonal antibodies demonstrated an inability of the test strains HN001 and HN019 to induce or enhance human platelet aggregation. These experiments indicated that the test strains are unlikely to degrade the mucin layer of the gastrointestinal mucosal surface or participate in the pathogenesis of endocarditis.

Resistance of LAB strains to commonly used antibiotics has caused safety concerns regarding the genetic stability of these resistance properties. The antibiotic

susceptibility and plasmid profiles of test organisms were investigated in another series of experiments. The susceptibility of the test strains to 18 antibiotics in common clinical use was examined by disk diffusion method. No extraordinary antimicrobial resistance was detected among the test strains (HN001, HN017, HN019, and HN067), and there were several antibiotics that efficiently suppressed the growth of test bacterial cells. A plasmid screening experiment demonstrated that all LAB strains examined were plasmid-free, this was verified by Southern blotting and DNA hybridisation techniques. These results indicate that the probiotic organisms tested here do not express or carry plasmid-associated antibiotic resistance, so their antibiotic resistance attributes are unlikely to disseminate to other clinically significant strains.

To investigate the oral toxicity of test strains (HN001, HN017, and HN019), conventional BALB/c mice were inoculated with a high dose (10^{11} cfu/mouse/day) of the test probiotic LAB strains for 8 consecutive days. The feed and water intake, body weight gain, and general health status, of the mice were monitored. The potential translocation of inoculated LAB strains and gut mucosal histological changes following feeding were also investigated. Random amplified polymorphic DNA (RAPD) fingerprinting techniques were used for bacterial identification. Results showed that the test LAB strains had no adverse effects on the parameters observed; no viable bacteria were recovered from blood or tissue samples (mesenteric lymph nodes, liver, and spleen). These results suggest that the test strains had no acute toxicity and had no potential to result in infection in normal mice at the high dose applied in this study.

To observe the consequences of longer-term consumption of test LAB strains, groups of BALB/c mice were orally administered with test LAB strains (HN001, HN017 and HN019) at doses of 5×10^7 , 10^9 or 5×10^{10} cfu/mouse/day for 4 weeks. In addition to the indicators observed in the acute toxicity study, the animals' haematological parameters; total and differential leucocyte counts; and blood biochemistry (plasma total protein, albumin, cholesterol, and glucose) were also investigated. Similar results to those of the acute toxicity study were obtained, i.e. 4 weeks consumption of HN001, HN017, and HN019 had no significant effects on the animals' general health status, haematology, blood biochemistry, or gut mucosal histological parameters. No dose-related effects were detected for any of the observed indicators. Translocation of test

LAB strains was not observed. These results suggest that longer-term consumption of test strains is unlikely to cause any obvious health problems in host animals.

In the final stage of this study, the potentially detrimental effects of HN001 and HN019 on hosts with sub-optimal immune functions were tested. To characterise the potential infectivity of test strains in immune deficient hosts, a group of adult male BALB/c mice pre-treated with dexamethasone (200µg/mouse/48 hrs) were fed with freshly cultured living HN001 or HN019 at doses of $1.5 \sim 2.5 \times 10^7$ cfu/mouse/day for 7 days; similar safety indicators to those outlined above were monitored. Results showed that no significant changes were noted in any of the safety parameters measured. No translocation of dietary LAB or systemic infection was detected. These findings suggest that HN001 and HN019 are well tolerated in immunocompromised mice without any significant safety concerns.

To investigate the effects of consumption of test LAB strains in hosts with a pre-existing immunological dysfunction, a group of female CBA/CaH mice (6 to 8 weeks) with experimentally induced autoimmune thyroiditis (EAT) were fed with freshly prepared probiotic preparations (HN001 4.2×10^8 cfu/mouse/day; HN019 2.16×10^8 cfu/mouse/day) for 5 to 8 weeks. Probiotic feeding was commenced one week prior to the immunization with auto antigens (MTg, mouse thyroglobulin). Antibody titres and spleen cell proliferative responses to the autoimmune inducing antigens (MTg) were determined via *in vitro* immunoassays. Lymphocyte (or mononuclear leucocyte) infiltration into thyroid tissue was also examined. Results showed that HN001 or HN019 feeding did not exacerbate spleen cell proliferative responses to MTg or lymphocyte infiltrations in thyroid tissues. These results indicate that feeding of HN001 or HN019 had no adverse effect on the induction or progress of autoimmune responses in CBA/CaH mice.

Overall, the combined results from these studies suggest that the probiotic LAB strains HN001, HN017, and HN019 are non-pathogenic for experimental animals and are likely to be safe for human consumption.

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List of abbreviations

AAD	antibiotic associated diarrhoea
ACF	aberrant crypt foci
ACNFP	Government's Advisory Committee on Novel Foods and Processes
ADI	acceptable daily intake
ADP	adenosine 5'-diphosphate
AOM	azoxymethane
AKP	alkaline phosphatase
ATCC	American Type Culture Collection
AXN	alloxan
BALT	bronchus - associated lymphoid tissues
BBN	N-butyl-N- (4-hydroxybutyl) nitrosamine
BHI	brain and heart infusion
BSA	bovine serum albumin
BT	bacterial translocation
CFA	complete Freund's adjuvant
CFU	colony forming unit
CHO	carbohydrate
CI	cellular immunity
CT	computed tomography
CV	coefficient of variation
DEX	dexamethasone
DMH	1, 2-dimethylhydrazine
DTH	delayed type hypersensitivity
EAT	experimental autoimmune thyroiditis
Eh	redox potential
EH	epithelial cell height
EPN	epinephrine
FC	flow cytometry
FCS	foetal calf serum

FI	feed intake
FITC	fluorescein-isothiocyanate
FPLC	fast protein liquid chromatography
GALT	gut-associated lymphoid tissues
GCC	glucocorticosteroids
GCS	glucocorticoides
GF	germ free
GHS	general health score
GI	gastric intestine
GIT	gastrointestinal tract
GLM	general linear models (SAS programme)
GRAS	generally recognised as safe
H & E	haematoxylin and eosin
HB	haemoglobin
HGM	hog gastric mucin
HI	humoral immunity
HIG	human intestinal glycoprotein
HIV	human immunodeficiency virus
HN001	<i>Lactobacillus rhamnosus</i> HN001 (DR20 TM)
HN017	<i>Lactobacillus acidophilus</i> HN017
HN019	<i>Bifidobacterium lactis</i> HN019 (DR10 TM)
HN033/Lb. GG	<i>Lactobacillus rhamnosus</i> GG (Lb. GG)
HN067	<i>Lactobacillus rhamnosus</i> HN067
HRP	horseradish peroxidase
HT	haematocrit
IBS	irritable bowel syndrome
IE	infective endocarditis
IFA	incomplete Freund's adjuvant
IFN	interferon
JCA	juvenile chronic arthritis
KD	kilo Daltons
LAB	lactic acid bacteria
LD ₅₀	50% of lethal dose
LDL	low density lipoprotein

LM	lactose maldigestion
LPS	lipopolysaccharide
LWG	live weight gain
MAC	macrophages
MAF	macrophage activating factor
MALT	mucosal associated lymphoid tissues
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
2-ME	2-mercaptoethanol
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIC	minimal inhibition concentration
MLN	mesenteric lymph node
MLS	macrolide-linocosamide-streptomgramin
MoAbs	monoclonal antibodies
MQ-H ₂ O	Milli-Q plus system purified water
MRS	Mann-Rogosa-Sharpe
MRS-C	MRS supplemented with cysteine-HCl
MT	mucosal thickness
MTg	mouse thyroglobulin
NCCLS	National Committee for Clinical Laboratory Standards
NCTC	National Collection of Type Cultures (UK)
NK	natural killer
NMS	normal mouse serum
NOD	non-obese diabetes
ODC	ornithine decarboxylase
OECD	Organisation for Economic Co-operation and Development
PAS	periodic acid-Schiff
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PBS-T	PBS supplemented with tween-20
PCR	polymerase chain reaction
PCV	packed cell volume

PE	phycoerythrin
PEC	peritoneal exudate cells
PGE	prostaglandin E
PI	pathological index
PLC	platelet count
PRO	protein
PRP	platelet rich plasma
PTg	porcine thyroglobulin
RAPD	random amplified polymorphic DNA finger-printing
RBC	red blood cells
RT	room temperature
SALT	skin - associated lymphoid tissues
S.C.	subcutaneous
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGR	specific growth rate
SHIME	simulator of the human intestinal microbial ecosystem
SIID	sucrose-isomaltase deficiency
SLE	systemic lupus erythematosis
SMP	skim milk powder
SPF	specific pathogen free
SRBC	sheep red blood cells
SWI	spleen weight index
TD	traveller's diarrhoea
TNF	tumour necrosis factor
TPO	thyroperoxidase
VH	villus height
VLDL	very low density lipoprotein
WBC	white blood cells
WI	water intake

Related publications and conference presentations

- Acute oral toxicity and bacterial translocation studies on potentially probiotic strains of lactic acid bacteria, *Food and Chemical Toxicology* 2000, 38, 153-161.
- Potential probiotic lactic acid bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) do not degrade gastric mucin *in vitro*, *International Journal of Food Microbiology* 2001, 63 (1-2), 81-89.
- Safety assessment of potential probiotic lactic acid bacterial strains *Lactobacillus rhamnosus* HN001, *Lb. acidophilus* HN017, and *Bifidobacterium lactis* HN019 in BALB/c mice, *International Journal of Food Microbiology* 2000, 56, 87-96.
- Probiotic lactic acid bacteria (*Lactobacillus acidophilus* HN017, *Lb. rhamnosus* HN001 and *Bifidobacterium lactis* HN019) have no adverse effects on the health of mice, *International Dairy Journal* 2000, 9, 831-836 (co-author).
- *Lactobacillus rhamnosus* HN001, *Bifidobacterium lactis* HN019, and *Lactobacillus acidophilus* HN017 do not degrade gastric mucin *in vitro*. In: “Dairy Foods in Health” (Abstract Book), International Dairy Federation Nutrition Week, Wellington, New Zealand, March 1998, pp 24.
- Toxicity and dose-response studies of potential probiotic bacterial strains in mice (oral presentation), Probiotics—The Good Millennium BUGs Symposium. Sydney, Australia, August 12 –13, 1999 (Awarded “Best Student Presentation Award”).
- Antibiotic susceptibility profiles and plasmid mapping of potential probiotic bacterial strains, IXth International Congress of Bacteriology & Applied Microbiology, Sydney, Australia, August 16 – 20, 1999, ppBPO3.06.

- *In vivo* evidence: DR10 and DR20 are safe probiotic strains, IXth International Congress of Bacteriology & Applied Microbiology, Sydney, Australia, August 16 – 20, 1999, pp BPO3.07.
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- *Bifidobacterium lactis* HN019 enhances antibody responses against *Escherichia coli* K-88 in piglets, Australasian Society for Immunology Annual Meeting, University of Otago, New Zealand, December 5-8, 1999, pp10.2.
- Bacterial translocation in immune-compromised mice following oral feeding of probiotic lactic acid bacteria, Microbiology Beyond 2000—The New Zealand

Microbiological Society Inc, University of Otago, New Zealand, November 23-26, 1999, pp p39.

- Investigation of the Potential Association of Probiotic LAB Strains *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* HN019 with Platelet Aggregation, International Congress of Probiotics Medicine, Los Angeles, California, July 6 to 8, 2001.
- Probiotic lactic acid bacteria have no oral toxicity in autoimmune mice, 9th International Congress of Toxicology, Brisbane, 8-12 July 2001.

1 General introduction

Lactic acid bacteria (LAB) have been consumed by humans via fermented foods for thousands of years for their unique metabolic characteristics (which give the fermented foods longer shelf life, better palatability, and higher digestibility)(Caplice & Fitzgerald, 1999). Since 1910 when Metchnikoff first hypothesised the association of the longevity of Bulgarians with their consumption of large quantities of soured milks containing lactic acid cultures, this concept has been developed further over the intervening decades and has led to a burgeoning activity in the elucidation of the role of LAB and cultured milk products in the alleviation of human and animal gastrointestinal tract (GIT) disorders (Fuller, 1992; Huis in't Veld, 1998; Sanders, 2000).

It is becoming increasingly accepted that the colonic microflora may play an important role in the maintenance of the host's health (Gibson & Roberfroid, 1995; Mitsuoka, 1996; Holzapfel *et al.*, 1998). The healthy human GIT, particularly the large intestine, contains a very large and diverse population of microorganisms that can be categorised into potentially pathogenic or health-promoting groups (Gibson & Roberfroid, 1995; Gibson *et al.*, 1997). The bacteria from the former group may have adverse effect(s) on the health of their host; whereas the organisms from the 'health-promoting' group, such as lactobacilli and bifidobacteria, may confer some general health-promoting benefits (Hamann *et al.*, 1998; Blum *et al.*, 1999; Hanson *et al.*, 1999). In healthy subjects, these two groups of microorganisms are well balanced and beneficial bacteria dominate (Mitsuoka, 1996). However, this balance could be altered to an abnormal flora (i.e. the balance in favour of harmful bacteria) by a number of endogenous and exogenous factors (Mitsuoka & Emeritus, 1992; Mitsuoka, 1996). A predominance of harmful bacteria in GIT microflora may predispose an individual to a number of clinical disorders, including cancer, inflammatory diseases, and ulcerative colitis, while making

the host more susceptible to infections by transient enteropathogens from the environment (Fooks *et al.*, 1999).

It has been suggested that the normal gut microflora can be re-established, when influences causing the microflora imbalance are eliminated, and this re-establishment can be accelerated by the administration of *exogenous* viable beneficial bacterial cells or non-digestible substrates that selectively stimulate the growth of *endogenous* beneficial bacteria strains within the colon (Gibson & Roberfroid, 1995; Holzapfel *et al.*, 1998). The former are called '*probiotics*'; the latter are named '*prebiotics*'.

Based on the understanding of the probiotic concept and the possible mechanisms by which probiotics operate, a working definition of probiotics has been defined as "preparations consisting of viable microorganisms and/or their components or metabolites, that protect or otherwise benefit a host either directly or by augmenting natural physiological or defence mechanisms" (Hamilton-Miller *et al.*, 1999). During the last two to three decades, many attempts have been made to improve the health status of hosts by modulating the indigenous intestinal microflora by probiotics. Strains of the genera *Lactobacillus* and *Bifidobacterium* are the most commonly used probiotic preparations (Goldin *et al.*, 1980; Alm, 1991; Fuller, 1992). The major perceived probiotic effects of probiotic LAB strains include: control of viral- and bacterial-induced intestinal infections, modulation of immune responses, normalisation of the intestinal motility, improvement of lactose maldigestion, and inhibition of the metabolic events in the gut lumen which may promote colonic carcinogenesis (Gilland, 1989; Sanders, 1993a; Salminen *et al.*, 1996; Macfarlane & Cummings, 1999; Gill, *et al.*, 2000; Kailasapathy & Chin, 2000; Rolfe, 2000).

In accordance with these health benefits, many probiotic preparations and probiotic-containing food products, ranging from dairy foods (yoghurt, cheese, sour milk), beverages, cereals, to infant formulae, are marketed worldwide (Robinson, 1989; Fonden, 1995; Lee & Salminen, 1995; Mann, 1997; Hasler, 1998; Klaenhammer, 2000).

However, considerable controversies regarding the applications of probiotics have been associated with the lack of substantial scientific evidence for the probiotic concept (Havenaar & Huis in't Veld 1992); uncertainty of the mechanisms of proposed health benefit(s); lack of well-designed, controlled clinical trials to demonstrate the claimed

probiotic effects (Atlas, 1999); and the questionable viability of probiotic strains in the final product before use (Cangella *et al.*, 1997; Hamilton-Miller *et al.*, 1999; Larkin, 1999). Therefore, the credibility of some probiotics has been questioned (Hamilton-Miller *et al.*, 1996; Larkin, 1999). As a result, new and more specific probiotic organisms with proven health-promoting attributes are required and being increasingly introduced into food products.

As a food ingredient, whatever health benefit(s) it may confer, probiotic strains should be as safe as ordinary foods. Although LAB have been consumed for centuries without significant adverse effects on human health, different systemic or local infections such as *Lactobacillus*-endocarditis, bacteraemia, organ abscesses, etc., caused by LAB strains that are taxonomically very close to current commercially available probiotic LAB strains, have been occasionally reported (Gallemore *et al.*, 1995; Ha *et al.*, 1999; Mackay *et al.*, 1999). The likely association between the consumption of a beverage containing a commercial human probiotic strain (*Lb. rhamnosus* strain GG) in large amounts and a case of liver abscess (Rautio *et al.*, 1999), and the consumption of large amounts of yoghurt with the progression of autoimmune liver disease (Chaiken, 1994) have been reported recently. As a result, the safety issue of probiotics has become a serious concern (Rodricks, 1996; Guarner & Schaafsma, 1998; Salminen *et al.*, 1998a,b; Wagner & Balish, 1998; Adams, 1999). The safety status of probiotics has been recommended and used as the most important criterion to select probiotic organisms (Salminen *et al.*, 1998a; Ouwehand *et al.*, 1999).

Studies within the Milk and Health Research Centre of Massey University (New Zealand) have recently identified and characterised some probiotic LAB strains (*Lactobacillus rhamnosus* HN001), *Lb. acidophilus* HN017, *Bifidobacterium lactis* HN019 and *Lb. rhamnosus* HN067) (Prasad *et al.*, 1999) with immunomodulatory effects in both animals (Gill, 1998; Gill *et al.*, 2000) and human subjects (Arunachalam *et al.*, 2000; Tannock *et al.*, 2000; Gill & Rutherford, 2001b; Gill *et al.*, 2001a,b,c). Although these LAB strains (HN001, HN017, and HN019) originated from the current food chain (cheese or yoghurt) or human GIT (HN067) (Section 3.1, Table 3.1), they have no history of consumption by human beings in the large doses required for a probiotic preparation. Hence, the safety status of these probiotic LAB strains has to be investigated before introducing them into the food market.

The overall aim of this study was to investigate the safety profiles of HN001, HN017, HN019, and/or HN067.

As viable bacterial cells, being consumed in large amounts, LAB may in theory pose some potential biological risks or hazards. These risks include opportunistic systemic infections; adverse metabolic effects on gut microflora; over-stimulation of the immune system; and unexpected gene transfer (e.g. antibiotic resistance) (Salminen *et al.*, 1998a). Therefore, the safety of these probiotic strains was evaluated from the following different aspects.

- The potential pathogenicity and adverse metabolic effects of HN001, HN017, and HN019 on hosts were evaluated by *in vitro* mucin degradation (**Chapter 4**) and platelet aggregation tests (**Chapter 6**).
- The opportunistic infectivity of test strains (HN001, HN017 and HN019) were investigated in immunocompromised mice (**Chapter 9**).
- Acute oral toxicity (**Chapter 7**) and dose-response (**Chapter 8**) experiments were conducted to evaluate the overall influences of test strains (HN001, HN017 and HN019) on the health of animals.
- The genetic stability of HN001, HN017, HN019, and HN067 were investigated with respect to their antibiotic susceptibility and plasmid profiles (**Chapter 5**).
- Finally, the potential adverse impact(s) of test strains (HN001 and HN019) on immune system were investigated using an autoimmune animal model (CBA/CaH mice) (**Chapter 10**).

2 Literature review

The nutritional and health benefits of fermented foods have been realised by humans since ancient times (Campbell-Platt, 1994; Saavedra, 1995; Milner, 1999). However, it was Elie Metchnikoff (1910) at the turn of the last century who first hypothesised that ingestion of viable lactobacilli (in yoghurt) had a positive effect on the colon microflora and thereby improved human health and longevity. Since then, vigorous attempts have been made, especially during recent decades, to improve the health status of humans or animals by modulating the intestinal microflora using live microbial adjuncts, now called ‘probiotics’. Of these live microbials, strains of the genera *Lactobacillus* and *Bifidobacterium* are the most commonly used probiotic preparations (Fuller, 1992). Some results from scientific studies have established a variety of preventive and/or therapeutic health benefits for some probiotic organisms, such as the efficacy of *Lb. GG* against some intestinal disorders (Salminen *et al.*, 1998a). However, controversies still exist due to the lack of solid evidence for some claimed probiotic effects (Havenaar & Huis in’t Veld, 1992). Further careful investigation on the scientific basis of probiotics and their functional properties is required to reinforce the probiotic concept and to endorse their credibility (Clydesdale, 1997). The emphasis of this review is on the concept of probiotics including the definition, characterisation, and health benefits; on the basic concept of intestinal microflora; and safety aspects of probiotics. Methods, which have been used to evaluate the safety of probiotic LAB strains, will also be discussed.

2.1 Probiotics

The term ‘probiotic’ is derived from the Latin word ‘pro’ (for) and Greek word ‘bios’ (life), thereby meaning ‘for life’. It was first used by Lilly and Stillwell (1965) to describe a ‘substance’ produced by one microorganism (LAB) that could stimulate the growth of another microorganism. Because the word ‘substance’ may evoke something

that is dead rather than alive, or even include some antibiotics, Fuller (1989) re-defined 'probiotics' as 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance'. Later, Havenaar & Huis in't Veld (1992) expanded this definition to include food and non-food use and the use of mono and mixed cultures. The host specific origin of living microorganisms which is addressed in Fond'en's (1995) definition. A more specific definition including the application path and dosage requirement was used by Guarner & Schaafsam (1998), who described probiotics as "living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition". Naidu and colleagues (1999) described probiotics as "a microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract". This definition expanded the mechanisms of probiotic effects into immunomodulation and improvement of nutritional status, in addition to the modulation of intestinal microflora of the host. But, the microbial viability requirement was deleted from this definition. Recently, a working definition of probiotics as "preparations consisting of viable microorganisms and/or their components or metabolites, that protect or otherwise benefit a host either directly or by augmenting natural physiological or defence mechanisms" has been proposed (Hamilton-Miller, 1999). According to this definition, probiotic preparations could be the components or metabolites of microbials, hence the viability of microbials are not necessarily required as long as it can beneficially affect the host's health by any mechanism (s). As a preparation, it may be food, or non-food such as supplement; it may contain one or more different microorganisms and could be consumed orally or applied by other different means. So far this may be the most complete explanation of probiotics.

2.2 Characterisation of probiotic strains

In light of the proposed mechanisms of probiotic effects, probiotic strains have to persist in the gastrointestinal tract (GIT) to prevent their rapid removal by intestinal peristalsis (Naidu *et al.*, 1999). In other words, colonisation or at least temporary colonisation is necessary for most probiotic organisms to exert their probiotic effects. It has been suggested that a potentially successful human probiotic strain will have the following desirable properties: be of human origin, survive the passage of the GIT, have certain

colonisation abilities, adhere to particular intestinal cells, have sustained health benefits, and lastly but most importantly, be safe for prolonged human consumption. Some basic requirements have been suggested to select probiotic strains (Table 2.1).

Table 2.1 Selection criteria for probiotic microorganisms

Desired properties	Remarks
1. Human / food origin	Important for species-specific health effects, may be a key factor for colonisation in the GIT
2. Acid and bile tolerance	Pre-requisite for passage survival or establishment in GIT (oral administration)
3. Safe for human consumption	With safety documentation, no risk for causing opportunistic infections, good tolerance in hosts with abnormal immune responses, genetically stable
4. Adhesion to human intestinal mucosal surface	Key factor for immunostimulation, competitive exclusion of pathogens, transient colonisation
5. Survive the passage of the GIT	Basic requirement to exert health benefits
6. Validated health benefits	e.g. Modulation of immune response, production of antimicrobial substances, improvement of lactose intolerance, alleviation of diarrhoeal diseases or other GIT disorders, adjustment of cholesterol metabolism, production of vitamins or beneficial enzymes. With dose-response data for minimum effective dosage in different products
7. Good technical properties	Culturable on a large scale, survive the processing and storage process, no negative effect on product quality

Adapted from Salminen et al., 1998b; Mattila-Sandholm et al., 1999; Ouwehand et al., 1999a.

2.2.1 Host specificity

Human origin has been advocated as an important attribute of probiotic strains by the majority of researchers. In theory, several important bacterial intrinsic properties such

as adherence to the gut mucosal surface, colonisation of GIT, and immune modulation effects on host animals, are reported to be species-specific (Tannock *et al.*, 1982; Bengmark, 1998). The intestines of different species vary in chemical composition and nutrient availability (Tannock, 1990), host specificity can be achieved by specific adhesins and receptors on bacterial and host cells, and/or by nutritional or physiological adaptation to different cell types or gut environments. It can be assumed that the human mucosa will tolerate colonisation only with bacteria with which it has had a symbiotic coexistence, whereas other bacteria will be quickly rejected (Bengmark, 1998). The species specificity of adherence and colonisation of LAB may be the major determinants for the host specificity of health promoting effects of probiotic organisms. This may therefore explain why many currently successful human probiotic strains such as *Lb. casei* Shirota, *Lb. rhamnosus* GG, and *Lb. johnsoni* (LA-1), are indicated to be of human origin (Salminen *et al.*, 1998a).

However, some dairy strains of LAB show moderate to good adhesion properties to human cell lines (Lehto & Salminen, 1997; Prasad *et al.*, 1999), and some common commercial dairy strains (*Lb. bulgaricus* and *Lb. acidophilus*) may exert positive effects without adhering (Marteau *et al.*, 1995; Jiang *et al.*, 1996; Vesa *et al.*, 1996; Arunachalam *et al.*, 2000). On the other hand, human origin refers the strain isolated from healthy human subject. In this case, the strain isolated from one subject does not necessarily colonise the GIT of another individual. Furthermore, the strain isolated from a specific human subject may be a transit organism originated from the dairy food product which was consumed by this individual prior to the collection of samples. If this situation is true, some so called human origin strains may have low potential to colonise human GIT. Therefore, whether or not a probiotic destined for consumption by humans must be of human origin is a question still under (Salminen *et al.*, 1998a).

2.2.2 Viability

It is largely believed that the presence of a certain number of viable probiotic organisms in the GIT (especially in the colonic microflora) is key to the success of their preventive or therapeutic health effects (Salminen *et al.*, 1996; Kailasapathy & Rybka, 1997; Ouwehand *et al.*, 2000). 10^{6-7} viable probiotic cells/g of intestinal contents is proposed to be the physiologically active concentration *in vivo* (Sanders, 1993b; Charteris *et al.*,

1997; Kailasapathy & Rybka, 1997). A concentration of 10^5 cfu/g or ml of the final product is suggested as the therapeutic minimum dosage (Naidu *et al.*, 1999). The number of viable bacterial cells in a probiotic product is used as one of the important indicators of product quality ($>10^7$ cfu/ml bifidobacteria cells should be present in fresh probiotic dairy products in Japan; 'Live and Active Culture' logo indicates the product contains more than $1 \times 10^{7-8}$ cfu/g of LAB in USA) (Kailasapathy & Rybka, 1997).

The components of some bacteria may have positive health effects (immune stimulation), and several studies have shown that non-viable probiotics are also able to positively affect the host's health (Kaila *et al.*, 1995; Ouwehand *et al.*, 1999a). Heat-treated probiotics have also been observed to inhibit the adhesion and invasion of diarrhoea causing bacteria (Coconnier *et al.*, 1992). The immunomodulatory effect of non-viable probiotic LAB strains has also been demonstrated recently (Portier *et al.*, 1993; Yasui *et al.*, 1989; 1999). However, most authors believe that viable probiotic microorganisms lead to a more pronounced immune response and improvements in clinical symptoms than those that have been heat-inactivated (Majamma *et al.*, 1995; Kaila *et al.*, 1995). From the safety point of view, inactivated cells may be more reasonable if they can deliver similar health effects with that of viable cells. However, so far, most probiotic effects in human beings are derived from the consumption of viable probiotic strains; data regarding the effect of non-viable probiotics is limited.

2.2.3 Adherence of LAB to GIT mucosal surface

Adherence refers to the ability of a bacterial cell to stick to a solid surface. Mechanisms of adherence are diverse and complicated. Generally the retention of bacteria in the human intestine can result from specific adherence to epithelial cells, from non-specific adherence to other intestinal bacteria, or from entanglement in mucus (Sanders, 1993a). As mentioned previously, the intestines of different species vary in chemical composition, these characteristics and the specific interaction between bacterial cell adhesins and host cell receptors confer the properties of host and tissue specificity to individual bacterial strains. Therefore, the observation of host-specificity of adherence is expected.

In spite of the species-specificity, other factors such as the physiological condition of the host, the viability of bacteria strains, and the co-existence of other bacterial strains

including probiotic strains and normal gut microflora, may also affect the adherence of specific probiotic strains. It has been observed that the adhesion of bifidobacteria to intestinal mucus isolated from elderly persons was especially low (Ouweland *et al.*, 1999b). This may be one explanation for the lower *Bifidobacterium* numbers in the elderly and suggests that special probiotic strains are required for this particular sub-population. Certain combinations of probiotic strains may have synergistic adhesion effects.

Currently, the ability of bacterial strains to attach to the intestinal mucosa is one of the main selection criteria for probiotic microorganisms. To become established as either a permanent or temporary inhabitant of the gut, a probiotic strain needs to have the capacity to adhere to the intestinal epithelial cells in order to avoid removal by the normal gut-cleansing mechanisms (O'Sullivan *et al.*, 1992; Sanders, 1993a; Salminen *et al.*, 1998a). An adherent strain may have a greater chance of colonising or staying longer in the gut than a strain that cannot adhere. Some correlation appears to exist between adhesion and colonisation of bifidobacteria (Ouweland *et al.*, 1999b). It has been shown that an adherent probiotic strain can persist on colonic mucosa several days after it has disappeared from faecal samples (Alander *et al.*, 1999).

Adhesive strains of probiotic LAB may be more efficient at delivering health benefits than non-adherent strains. For example, the adherent strains *Lb. rhamnosus* GG, and *Lb. reuteri* have been found to significantly shorten the duration of diarrhoea, while the non-adhesive strain *Lb. delbrueckii* spp. *bulgaricus* did not (Ouweland *et al.*, 1999b). This could be related to the competitive advantages of adhesive strains, i.e. these strains have a greater chance of competing for binding sites and nutrients on the mucosal surface with pathogenic strains. Also by adhering to the mucosal surface probiotic strains producing antimicrobial substances are in much closer proximity to pathogens. Other studies have also indicated that a correlation exists between the ability to adhere to the gut mucosa and the titres of specific antibody against tested *Lactobacillus* strains in the serum of volunteers who received these probiotic strains (O'Halloran *et al.*, 1998).

Although adherence has been advocated as an important strain selection criterion, so far, only a few *Lactobacillus* strains [*Lb.* GG (Elo *et al.*, 1991; Saxelin, 1997); *Lb. acidophilus* LA-1 (Bernet *et al.*, 1994); and *Lb. rhamnosus* HN001, *Lb. acidophilus*

HN017, and *B. lactis* HN019 (Gopal *et al.*, 2001)] are reported as able to adhere to human Caco-2 or HT-29 cell lines in *in vitro* studies. Common commercial dairy strains such as *Lb. bulgaricus* and *Lb. acidophilus* are neither adhesive *in vitro* to Caco-2 cells nor *in vivo* in humans. The bifidobacterial strains tested so far are either not adhesive or only slightly adhesive (Bengmark, 1998).

2.3 Ecology of intestinal microflora

The GIT microflora represent an ecosystem of the highest complexity and our understanding of this system and its interactions is still limited (Berg, 1992b).

2.3.1 Development of intestinal microflora

The foetus exists in a sterile environment until birth (Mitsuoka & Emeritus, 1992; Ballongue, 1998). After birth the digestive tract is rapidly colonised by bacteria comprised mainly from the mother's cervical and vaginal microflora and bacteria from the environment (Hill, 1990; Alm, 1991; Tannock, 1994b, 1995). Within 48 hours of birth, the colon of neonates fed with breast milk and supplementary formula contains $10^9 - 10^{10}$ bacteria/gram of faeces, consisting mainly of enterobacteria, staphylococci, and streptococci (Mitsuoka *et al.*, 1984; Ballongue, 1998). Bifidobacteria appear in the stools of breast-fed babies from 2 to 5 days after birth and become the predominant ($10^{10} - 10^{11}$ bacteria/gram of faeces) microorganisms of the faecal flora (rising to 99%) by the end of the first week (Alm, 1991). In contrast, the level of other bacteria (clostridia, bacteroides, enterobacteriaceae, streptococci, and staphylococci) decrease by this time (Mitsuoka, 1996; Ballongue, 1998). Thus, nearly 100% of all bacteria cultured from stools of breast-fed infants were bifidobacteria (Mitsuoka, 1984). Traditionally, it has been believed that bifidobacteria are found exclusively in the faeces of breast-fed infants, whereas in bottle-fed infants *Lb. acidophilus* is the most commonly found organism. Recent comparative studies have revealed that the number and species of bifidobacteria in the faeces of breast- and bottle-fed infants are not significantly different, i.e. bifidobacteria are the predominant faecal microorganisms in both groups of infants (Mitsuoka, 1984). The only difference between the two groups is that the bottle-fed infants have other microorganisms such as bacteroides, eubacteria, peptostreptococci, clostridia, enterobacteria, streptococci, and bacilli at a greater

frequency and at higher levels than breast-fed infants (Mitsuoka, 1984; Mitsuoka & Emeritus, 1992).

During weaning, when an adult diet is consumed, the stools of infants shift to the Gram-negative bacillary flora of adults: bifidobacteria decrease by 1 log, the numbers of bacteroidaceae, eubacteria, peptococcaceae, and usually clostridia outnumber bifidobacteria, which constitute 5 to 10 % of the total flora. The counts of enterobacteriaceae and streptococci decrease to less than 10^8 cfu/gram faeces; lactobacilli, megasphaerae, and veillonellae are often found in adult faeces, but the counts are usually less than 10^7 cfu/gram faeces. The species and biovars of bifidobacteria alter from infant-type (*B. infantis* and *B. breve*) to adult-type (*B. adolescentis* and *B. longum*) (Mitsuoka, 1996).

With the advancing of age, the composition of faecal flora changes dramatically. Elderly people (>60 years) exhibit a decrease in *Bifidobacterium* population and a significant increase in the population of clostridia including *Clostridium perfringens*. The numbers of lactobacilli, streptococci and enterobacteriaceae also increase. This phenomenon is considered to be a result of ageing, but it might accelerate senescence (Mitsuoka, 1984; 1992).

2.3.2 Functions of the resident intestinal microflora

It is becoming increasingly accepted that the colonic microflora may play an important role in the maintenance of the host's health (Gibson & Roberfroid, 1995; Mitsuoka, 1996; Holzapfel *et al.*, 1998). The healthy human GIT, particularly the large intestine, as described above, contains a very large and diverse population of microorganisms that can be categorized into potentially pathogenic or health-promoting groups (Figure 2.1) (Gibson & Roberfroid, 1995; Gibson *et al.*, 1997). The bacteria from the former group may be involved in the onset of localised or systemic infections, intestinal putrefaction, toxin formation and production of mutagenic and carcinogenic substances. On the other hand, the organisms from the 'health-promoting' group, such as lactobacilli and bifidobacteria, may confer general health-promoting benefits to a number of physiological functions such as resistance to colonisation by pathogens (Hentges, 1992) or inhibition of growth of harmful bacteria; immunomodulation (Hamann *et al.*, 1998;

Blum *et al.*, 1999; Hansson *et al.*, 1999); production of certain essential nutrients like vitamins (Liem *et al.*, 1977; Shane *et al.*, 1983) and short chain fatty acids (SCFA)

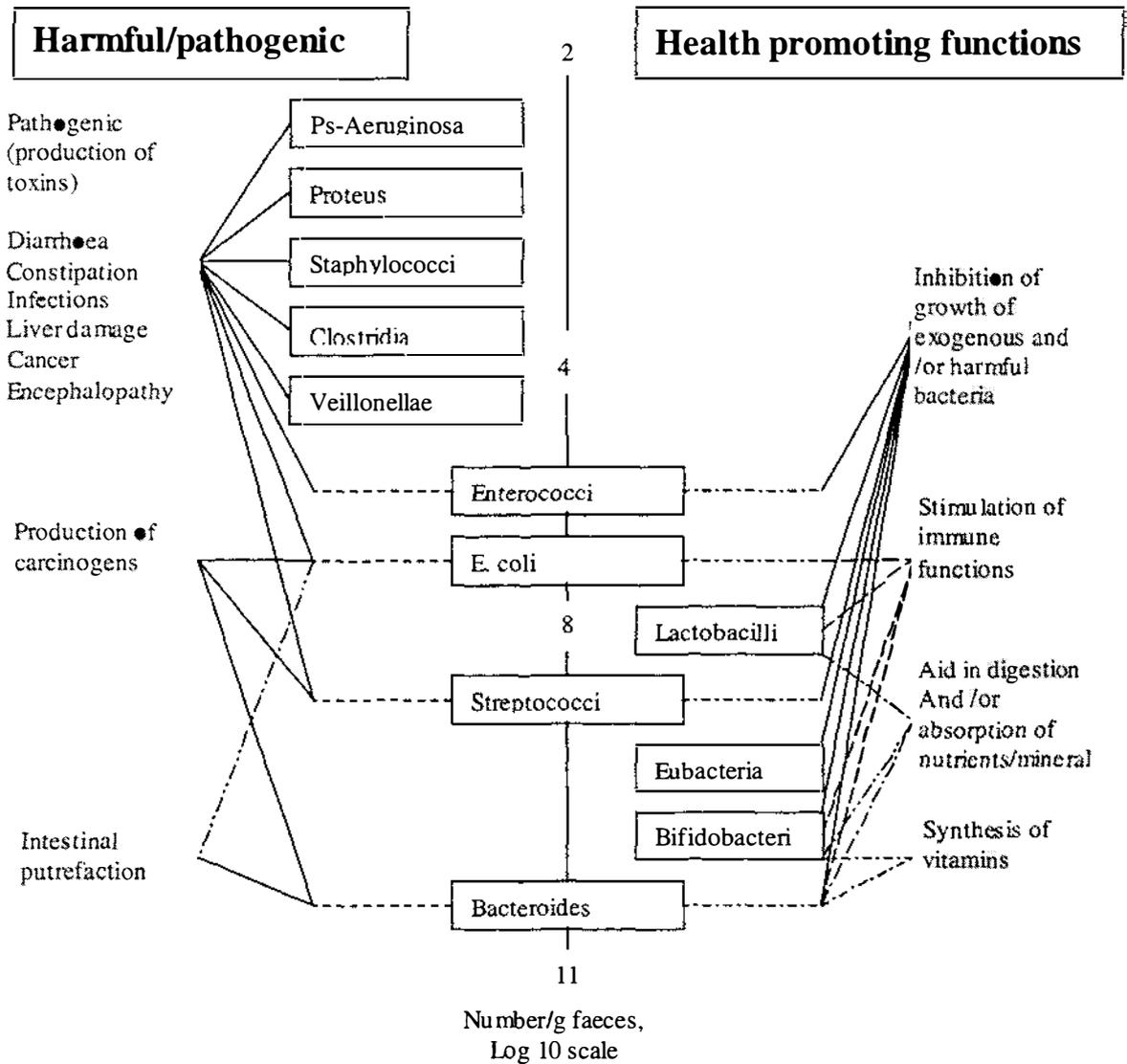


Figure 2.1 General scheme of the composition and health effects of predominant human faecal bacteria. The figure shows approximate numbers of the different genera. The bacteria are generally split into those groups that have harmful or pathogenic influences on human health, those that have beneficial effects, and those that may have both. *Adapted from Gibson & Roberfroid (1995).*

(Siigur *et al.*, 1994) which are absorbed from the lumen and utilised by gut epithelium and vital organs such as the liver. Some bacteria are classified into groups in which some species may have health-promoting effects, but other species may have harmful effects on their hosts (Gibson & Roberfroid, 1995). In healthy subjects, these two

groups of microorganisms are well balanced and beneficial bacteria dominate (Mitsuoka, 1996).

Although the composition of the intestinal microflora is rather stable in healthy individuals, it can be altered to an abnormal flora (i.e. the balance in favour of harmful bacteria) by a number of endogenous and exogenous factors, such as peristalsis disorders, cancer, surgical operations, liver or kidney diseases, pernicious anaemia, radiation or antibiotic therapies, emotional stress, ageing, immune disorders, and poor diet structures etc. (Mitsuoka, 1992; 1996). A predominance of harmful bacteria in GIT microflora may predispose an individual to a number of clinical disorders, including cancer, inflammatory diseases, ulcerative colitis, while making the host more susceptible to infections by transient enteropathogens from the environment (Fooks *et al.*, 1999).

2.3.3 Modulation of intestinal microflora with probiotics

Theoretically, the normal gut microflora can be re-established, when influences causing the microflora imbalance are eliminated, and this re-establishment may be accelerated by the oral administration of beneficial bacterial species such as lactobacilli and bifidobacteria (Metchnikoff, 1910; Goldin *et al.*, 1980; Alm, 1991), or the administration of dietary substrates which selectively stimulate the growth of beneficial bacterial species in the GIT. These special dietary substrates (such as non-digestive fibres or oligosaccharides) are called prebiotics (Gibson & Roberfroid, 1995); these beneficial lactobacilli or bifidobacteria, as described previously (Section 2.1), are named as probiotics. Prophylactic use of probiotics or prebiotics in hosts with unfavourable conditions should be able to reduce or prevent the shifting of GIT microflora towards the 'harmful' species. However in practice it is not easy to prove this hypothesis.

The use of LAB preparations to improve the gut microflora has been practiced for nearly 100 years, and many positive findings have been obtained. However, some debate regarding the colonisation ability of exogenous bacterial species within the GIT still exist (Tannock, 1995).

In a study using a simulator of the human intestinal microbial ecosystem (SHIME), Alander and colleagues (1999) demonstrated that the introduction of probiotic LAB strains (lactobacilli and bifidobacteria) into the SHIME reactor had a remarkable impact on the composition of microflora in this system and its metabolic activities (production of lactic acid). A marked, but temporary, increase in the number of LAB including bifidobacteria, and decrease in the numbers of enterobacteriaceae and *C. spp* was observed. This result indicates that the introduction of probiotic LAB strains may help to maintain the balance of GIT microflora.

The establishment of lactobacilli and bifidobacteria in the GIT of germ free mice has been demonstrated (Tannock *et al.*, 1982; Norin *et al.*, 1994). After 4 weeks of inoculation, lactobacilli were present throughout the GIT (10^{8-9} cfu/g of gut content), whereas, bifidobacteria were present in numbers $>10^8$ cfu/g of gut content in the caecum and colon (Norin *et al.*, 1994).

The modulatory effect of probiotics on the human GIT microflora has also been observed in several studies. Johansson and colleagues (1993) found administration of *Lactobacillus* strains in fermented food significantly increased the *Lactobacillus* counts in the jejunum mucosa of human individuals, and high levels remained 11 days after the termination of administration. The levels of streptococci increased by 10- to 100-fold and the levels of sulphite-reducing clostridia in the jejunum decreased by 10- to 100-fold 1 day after administration was terminated. In the rectum, the anaerobic bacterial counts and the Gram-negative anaerobic bacterial counts decreased significantly by the end of administration. Meanwhile, a 1,000-fold decrease in the number of *Enterobacteriaceae* was observed in the rectal mucosa of some subjects. Similar results have been reported by other workers (Lidbeck *et al.*, 1987; Sepp *et al.*, 1993; Alander *et al.*, 1997; Spanhaak *et al.*, 1998).

However, evidence of true GIT colonisation and continued excretion without continued ingestion of LAB is lacking. A persistent colonisation of any part of the GIT by an exogenous, non-pathogenic microorganism has never been observed in man (Marteau & Rambaud, 1993). All of the published results so far indicate that the colonisation of the human GIT by LAB strains is temporary, or a transitory colonisation (Saavedra, 1995). The duration of the inoculated LAB strains appearing in the GIT or faeces, or the clinical or experimentally determined benefits, may be only one or two weeks (Lidbeck

et al., 1987; 1993). *Lb. GG* is one of the few exceptional cases in which the inoculated *Lb. GG* was detected in the faeces 4 weeks after the termination of oral administration (Saxelin, 1997).

The lack of colonisation of the colon could be attributed to the bacteriostatic barrier effect due to the endogenous microflora (Bouhnik *et al.*, 1992; Tannock, 1995; Salminen *et al.*, 1998a). Difficulty in colonising the digestive tract of adults by probiotic bacteria is expected even when strains of an intestinal origin are utilised. Adult animals are already colonised by an established microflora in which each species occupies its own ecological niche and will prevent the settlement of introduced strains; this ability of the indigenous GIT microflora is termed 'colonisation resistance' (van der Waaij & Berghyis-der Vries, 1974). Thus, the inoculation of neonatal animals with probiotic bacteria might be a more successful means of establishing a resident 'probiotic' bacterial population (Tannock, 1995).

Any new coloniser, after overcoming the chemical and physiological host defence systems, must be able to compete successfully with the established microbial species for suitable nutrients, the required atmospheric conditions, and specified attachment sites (see detail below). To overcome all these potential difficulties, the probiotic strains should have certain intrinsic characteristics to survive passage, and further colonise (at least temporarily) the GIT.

2.4 Established health effects and issues to be addressed

A number of health-related effects of probiotics are considered well established and clinically well documented (Rowland, 1999). These reported health effects are usually strain specific. Other health benefits have not been fully substantiated and some claims for the potential health benefits of probiotics remain unproven (Macfarlane & Cummings, 1999; Ouwehand *et al.*, 1999a).

2.4.1 Improvement in lactose utilisation

Lactose maldigestion is due to insufficient activity of lactase or lactose hydrolysis enzyme in the human small intestine. This can be due to either a congenital enzyme (β -

galactosidase) deficiency or reduction in enzyme activity during intestinal disorders such as gastroenteritis (O'Sullivan *et al.*, 1992).

Individuals with lactose maldigestion may suffer from various degrees of abdominal discomfort and/or diarrhoea following the ingestion of unfermented fresh milk or other dairy products due to the formation of hydrogen gas by colonic microbial action on the undigested lactose in the gut (Gilliland, 1989; O'Sullivan *et al.*, 1992). Some yoghurt bacteria and probiotic microorganisms have been shown to produce β -D-galactosidase, which may improve tolerance to lactose maldigestion (Gilliland & Kim, 1984; Martini *et al.*, 1987). It has been repeatedly demonstrated in different clinical studies that the breath hydrogen output and/or symptoms of lactose maldigesters were significantly reduced or improved following consumption of yoghurt containing viable LAB strains (Gilliland & Kim, 1984; Marteau *et al.*, 1990; Montes *et al.*, 1995). The viable LAB cultures may induce lactase synthesis in the gut mucosa (O'Sullivan *et al.*, 1992); semisolid fermented milk products (yoghurt) may increase the gastric transit time (Vesa *et al.*, 1996), these two mechanisms may also contribute to the alleviation of lactose intolerance. But, the efficacy of LAB on lactose maldigestion is not always documented (Newcomer *et al.*, 1978; Payne *et al.*, 1981; 1983; Hove *et al.*, 1999). Inadequate β -galactosidase activity and lower numbers of viable bacteria cells presented in the products used, and the misdiagnosis or over-reported intolerance in those studies has been suggested as possible reasons for these negative results (Gilliland, 1989; Marteau & Salminen, 1997).

2.4.2 Preventive/therapeutic effect on diarrhoeal diseases

Different probiotic preparations containing lactobacilli, enterococci and bifidobacteria have been reported to prevent, alleviate or cure diarrhoea caused by viral or bacterial (or antibiotic associated *C. difficile*) infections. The effects of probiotics on enteric infections are more strain specific. Probiotic strains *Enterococcus faecium* (Wunderlich *et al.*, 1989), *Lb. reuteri* (Shornikova *et al.*, 1997), *Lb. GG* (Isolauri *et al.*, 1991; Roza *et al.*, 1995; Pedone *et al.*, 1999; Guandalini *et al.*, 2000), as well as the combination of *B. bifidum* and *Streptococcus thermophilus* (Saavedra *et al.*, 1994) have been used to prevent and/or improve the recovery from acute infectious diarrhoea in infants or children. The improvement of diseases was demonstrated by the reduction in incidence,

duration, and/or rotavirus shedding. The positive effect of *Lb. GG* in preventing or altering the course of antibiotic associated diarrhoea (AAD) (*C. difficile* associated diarrhoea) has also been reported (Arvola *et al.*, 1999; Biller *et al.*, 1995; Pochapin, 2000). Furthermore, probiotics have been reported to be effective in the prevention of traveller's diarrhoea (Scarpignato & Rampal, 1995; Hilton *et al.*, 1996), although the effectiveness of treatment varies with the destination of the travellers (Clements *et al.*, 1981, 1983; Oksanen *et al.*, 1990). The differing outcomes seen within the same large-scale probiotic studies suggest that the efficacy of a probiotic is highly dependent on the type of patient/subject selected for treatment (Saavedra, 2000).

The potential mechanisms by which probiotic agents might exert their protective or therapeutic effects against acute infectious diarrhoea include competition with pathogens for nutrients; acidification of luminal contents which inhibits growth of certain enteropathogens; production of antimicrobials or inhibitory substances (e.g. bacteriocins) against pathogens; competition for receptors or adhesion to the intestinal mucosa (i.e. colonisation resistance); and immunomodulation (Saavedra, 1995).

2.4.3 Immunomodulatory effects

Probiotic lactobacilli and bifidobacteria have been shown to influence different aspects of immune functions ranging from humoral, cellular, to non-specific immunity (Gill *et al.*, 2000). Several reports recently described the effects of probiotics on sIgA in both rodents and humans (Majamaa *et al.*, 1995; Perdigon *et al.*, 1995; Fukushima *et al.*, 1998; Yasui *et al.*, 1999). Although the specific results varied, generally an enhanced sIgA production was observed during probiotic consumption. Similarly, feeding of heat-killed *Lb. casei* Shirota has been reported to inhibit the production of serum IgE, or *in vitro* production of IgE by spleen cells (Matsuzaki *et al.*, 1998).

Probiotic bacteria have been shown to influence immune responses non-specifically by enhancing phagocytosis of pathogens as well as modifying cytokine production. An enhancement in the phagocytic activity of granulocyte and monocyte populations in the blood of human volunteers after consumption of *Lb. acidophilus* and *B. bifidum* (Schiffirin *et al.*, 1995) or *B. lactis* HN019 (Arunachalam *et al.*, 2000) has been documented. The enhancing effect on phagocytic cell activity by *B. lactis* HN019 and *Lb. rhamnosus* HN001 has also been shown in animals and humans (Gill *et al.*, 2000;

Gill & Rutherford, 2001a,b; Gill *et al.*, 2001a, b, c). Because phagocytic activity is associated with natural immunity and phagocytes are involved in antibody immune responses as antigen-presenting cells, it is possible that the stimulation of intestinal IgA responses induced by LAB may be explained partly by an effect on phagocytic cell function (Salminen *et al.*, 1998b).

De Simone *et al.* (1993) reported that yoghurt or heated yoghurt administered to mice increased the percentage of B lymphocytes in the Peyer's patches, and the proliferative responses of spleen cells to mitogens. Gill *et al.* (2000) also reported that *Lb. rhamnosus* HN001 and *B. lactis* HN019 have similar immunoenhancing properties in mice. Perdigon *et al.* (1999) reported that *Lb. casei* and *Lb. plantarum* were able to stimulate an increase of CD4⁺ cells in Peyer's patches of mice after oral feeding. But, in a human study, Schiffrin *et al.* (1995) did not detect any modification of lymphocyte subpopulations in the blood of subjects who received fermented milk supplemented with a *B. lactis* strain Bb 12 or *Lb. acidophilus* strain LA1 for 3 weeks.

Several studies have shown that cytokine production by immune cells can be altered by probiotic use. Administration of different probiotic strains such as lactobacilli or bifidobacteria have been shown to significantly increase the production of tumour necrosis factor (TNF- α), interleukins (IL-6, IL-2 and IL-5), and interferon gamma (IFN- γ) either *in vitro* (Marin *et al.*, 1998) or *in vivo* (Kishi *et al.*, 1996; Matsuzaki, 1998; Arunachalam *et al.*, 2000). The effect of probiotic treatment on cytokine production was found to be dose- and strain-dependent; heat-killed *S. thermophilus* has also been reported as being effective in stimulating cytokine production (Marin *et al.*, 1998).

Probiotics appear to have some effect on ameliorating intestinal allergy. They may reduce translocation of foreign proteins by tightening the mucosal barrier or by affecting the induction of tolerance (Sutas *et al.*, 1996). In a clinical study, *Lb. GG* has been used to manage cow's milk allergy and atopic eczema in infants and children, resulting in a significant improvement in a clinical allergy score compared to a placebo (Majamaa & Isolauri, 1997; Isolauri, 2000). Faecal TNF- α , which is associated with intestinal inflammation, also decreases following *Lb. GG* administration.

Collectively, it appears that probiotic bacteria may have a selective influence on components of non-specific immunity, i.e. immunostimulatory responses, in healthy

subjects, but down-regulatory effects in milk-hypersensitive individuals. However, the mechanisms by which this occurs remain to be elucidated.

2.4.4 Antimutagenic activities

Probiotics have been suggested as a means of reducing the risk of the development of cancer. Participation of intestinal microflora in toxic events resulting in activation or detoxification of mutagens, carcinogens, and tumour promoters has been documented (Bengmark, 1996). Inhibition of bacterial enzymes involved in the synthesis of colonic carcinogens may be a beneficial mechanism. Enzymes such as glucuronidase, azoreductase, and nitroreductase could be inhibited by reduction in pH values in colon lumen (Vanderhoof & Young, 1998). Some intestinal flora themselves may be carcinogenic, and simple modification of the flora may be helpful. In general, species of *Bifidobacterium* and *Lactobacillus* have low levels of enzyme activity (which are associated with carcinogenesis substances) in the gut in comparison with that of other anaerobes, such as bacteriodes and clostridia (Gibson & Roberfroid, 1995). On the other hand, some LAB have been shown to be able to absorb mutagens derived from food or gut contents into their carbohydrate polymers in the cell wall, and this property of LAB is independent of their viability (Zhang & Ohta, 1991; 1993). Therefore, in theory the antitumour action of probiotics may be operated by the following mechanisms: 1) inhibition of carcinogens and/or procarcinogens; 2) inhibition of bacteria that convert procarcinogens to carcinogens; 3) activation of the host's immune system; 4) reduction of intestinal pH to reduce harmful microbial activity; and 5) alteration of colonic motility and transit time to reduce the retention of carcinogens or procarcinogens within the lumen (McIntosh, 1996).

Animal studies have confirmed that yoghurt and fermented milks containing probiotic bacteria inhibit tumour formation and proliferation (Kato *et al.*, 1994). Co-administration of lactulose and *B. longum* to rats injected with the carcinogen azoxymethane reduced intestinal aberrant crypt foci (a pre-neoplastic marker) (Rowland & Grasso, 1975). *Lb. GG* can also protect against the formation of a dimethylhydrazine (DMH)-induced colon cancer in rats (Goldin *et al.*, 1996). The preventive and prophylactic effects of a *Lb. casei* preparation (BLP, Yakult Honsha Co. Ltd, Japan) on recurrence of superficial bladder cancer have been reported (Aso *et al.*, 1992; 1995).

Most of the reported anti-cancer effects of probiotics were observed in a small number of animal studies. Indirect results from human epidemiological studies (Rafter, 1995) or faecal enzyme profiles (Goldin *et al.*, 1980; McIntosh, 1996) have not always been consistent. Thus, extensive investigation must be conducted before probiotics can be considered for cancer prevention.

2.4.5 Other health benefits

Several studies have been performed on the cholesterol lowering effect of probiotics. The results have been rather conflicting, and so far, no consistent picture has emerged (Walker & Gilliland, 1993; Marshall & Taylor, 1995; Lichtenstein & Goldin, 1998). The evidence that supplementation of probiotics-containing diet has a beneficial hypocholesterolaemic effect in humans is insufficient (O'Sullivan *et al.*, 1992; Kailasapathy & Rybka, 1997).

Selective probiotic LAB strains (such as *Lb. GG*) have also been reported to be effective in preventing the recurrence of *Candida vaginitis* (Reid *et al.*, 1990; Hilton *et al.*, 1992; 1995), treatment of irritable bowel syndrome (Newcomer *et al.*, 1983; Young & Vanderhoof, 1997); diabetes (Matsuzaki *et al.*, 1997a, 1997b), and relieving of hypertension (Furushiro *et al.*, 1990; Hata *et al.*, 1996). Some strains (such as *L. casei* and *S. faecalis*) were also associated with the metabolism of folic acid and the synthesis of vitamin B12 (Shane *et al.*, 1983). Unfortunately to date, there is no convincing evidence to support these applications and the possible mechanisms for these perceived health effects are unclear.

2.4.6 Issues

The wide range of claimed health benefits of probiotics has stimulated the rapid expansion of the market of probiotic-containing product worldwide (Robinson, 1989; Hugh & Hoover, 1991; Fond'en, 1995; Mann, 1997; Shortt, 1998). In addition to their application to dairy products, probiotic bacteria are now being applied to more new food products including fermented cereals, infant formulae, beverages, and some therapeutic foods (Hugh & Hoover, 1991; Lee & Salminen, 1995). The commercial and industrial interests in probiotics further stimulate and encourage the efforts from the scientific

community. As a result, more and more novel probiotic strains purported to have health effects have been identified and introduced into both food and dietary supplement markets (Lee & Salminen, 1995; Donohue *et al.*, 1998).

On the other hand, there is also considerable controversy regarding the application of probiotics, and the credibility of some probiotic products has been questioned (Atlas, 1999; Larkin, 1999).

- Firstly, the health claims associated with some of the probiotic preparations often lack solid clinical supporting data (von Wright & Salminen, 1999). There are too few properly controlled human trials to demonstrate the claimed health benefits of probiotics (Sanders, 1993; Atlas, 1999; Larkin, 1999). Most claims for the potential health effects of probiotics remain unproven in humans (Macfarlane & Cumming, 1999). Furthermore, the value of using probiotics in healthy adults, which is the main market for probiotic-containing products, is unknown (Larkin, 1999).
- Secondly, with the exception of the action of bacterial β -galactosidases in the alleviation of lactose maldigestion, the mechanisms of most claimed probiotic effects are not fully understood (von Wright & Salminen, 1999). Modulation of GIT microflora is believed to be the main mechanism by which most probiotic organisms exert their health benefits; but knowledge about the GIT ecosystem is limited.
- Thirdly, safety aspects of excessive consumption of viable bacterial cells have been actively discussed recently (van der Kamp, 1996; Saxelin *et al.*, 1996; Mattila-Sandholm *et al.*, 1999). Mainly this is because of the occasionally occurring clinical infections associated with lactobacilli or bifidobacteria (Sussman *et al.*, 1986; Aguirre & Collins, 1993; Gasser, 1994) that are taxonomically very close to currently commercially available probiotic LAB strains (Sherman *et al.*, 1987; Gallemore *et al.*, 1995; Ha *et al.*, 1999). In particular, a probiotic *Lactobacillus* strain was found associated with liver abscesses (Rautio *et al.*, 1999).
- Finally, some probiotic products do not contain the numbers of bacterial cells that is essential to promote health; some products do not contain the bacterial species which are expected or claimed on the product labels (Iwana *et al.*, 1993; Hamilton-Miller *et al.*, 1996; Canganella *et al.*, 1997; Hamilton-Miller *et al.*, 1999).

However, this situation is rapidly changing. Well-designed clinical studies are accumulating, showing that specific probiotic microbes, mainly lactobacilli and bifidobacteria, can alleviate or prevent diverse intestinal disorders. Some indication of the mechanisms of action can also be deduced from the data available; while rapidly developing molecular techniques offer new tools to verify the survival of the probiotics in the gut and the subsequent adhesion to mucosa (von Wright & Salminen, 1999). Nevertheless, future studies need to address the characterisation and safety assessment of new probiotic strains, the possible mechanisms of claimed health effects, and the justification of the probiotic effects in well-designed clinical trials.

2.5 Safety of probiotics

2.5.1 History of safe use

Traditional dairy strains of LAB have a long history of safe use (Salminen *et al.*, 1998a,b). Since antiquity, LAB have been used in a large number of food fermentations to preserve fresh food from putrefaction and to improve the taste and texture of the final product (Campbell-Platt, 1994; Hammes *et al.*, 1994; Lee, 1997). The history of their safe use in traditional fermented foods such as fish, meat, cereals, fruits, vegetables, legumes and dairy foods, means that LAB, isolated from these sources, are regarded as non-toxic, food-grade microorganisms (van der Kamp, 1996). In addition, LAB are common commensals of the human intestinal tract, and are abundant inhabitants of practically all the non-sterile sites of the human body except for the skin (Pot *et al.*, 1993). Bifidobacteria and lactobacilli both constitute major groups of the normal GIT microflora in healthy humans (Section 2.3)(Biavati *et al.*, 1992; Hammes *et al.*, 1992). The long history of human exposure and consumption of LAB without any apparent public health problems and their commensal status has led most LAB strains particularly *Lactococcus* and *Lactobacillus* to be classified as 'Generally Recognised As Safe' (GRAS)(Table 2.2)(Salminen *et al.*, 1998a).

2.5.2 Safety concerns about probiotics

Although the health benefits of probiotics have drawn considerable attentions from scientific researchers, food producers, and consumers, the safety of probiotics (particularly the novel strains with no established safety documentation) has been

questioned (Section 2.2.6). As viable microorganisms, naturally occurring (rather than genetically modified) probiotic LAB theoretically have potential to involve in four different types of detrimental effects on the host: systemic infections, risk of deleterious metabolic activities, risk of adjuvant side-effects on the immune system, and risk of gene transfer. Of all of these potential side effects, opportunistic systemic infections are the most important concern.

Table 2.2 Classifications of probiotic organisms and their safety status*

Organisms	Infection potential
<i>Lactobacillus</i>	Mainly non-pathogens, some opportunistic infections (usually in immunocompromised patients)
<i>Lactococcus</i>	Mainly non-pathogens
<i>Leuconostoc</i>	Mainly non-pathogens, some isolated cases of infections
<i>Streptococcus</i>	Oral streptococci (including <i>Streptococcus thermophilus</i>) mainly non-pathogens; some opportunistic infections
<i>Enterococcus</i>	Some strains are opportunistic pathogens with haemolytic activity and antibiotic resistance
<i>Bifidobacterium</i>	Mainly non-pathogens, some isolated cases of infections
<i>Saccharomyces</i>	Mainly non-pathogens, Some isolated cases of infection

* Adapted from Donohue & Salminen, 1996.

2.5.2.1 Pathogenic potential of probiotics

Infection is defined as the colonisation of the body by a microorganism that is capable of causing disease (Salyers & Whitt, 1994). To initiate an infection process, a bacterium has to gain access to the host in sufficient numbers, find a niche and multiply or at least survive there, and avoid the host's defence mechanisms.

Under normal conditions, the host is protected by a set of effective constitutive (skin and mucosal surfaces; transferrin, phagocytes, complement) and specific defence

(antibodies and cytotoxic T cells) mechanisms. The action of antimicrobial substances and body secretions on the intact skin and mucosal surfaces helps to prevent contact of the human body with a pathogen. The mucosal surface of the GIT, respiratory tract, and urogenital tract are covered by a thick mucus layer which traps bacteria before they reach the epithelium and inhibit the growth of trapped bacteria (Figure 4.1) by non-specific inhibitory substances (lysozyme, lactoferrin, lactoperoxidase). The constant sloughing of epithelial cells (carrying any adhering bacteria with them) and sIgA secreted by mucosal associated lymph tissues (which helps to trap bacteria in the mucus and prevent the binding of bacteria or their toxic products to mucosal cells) is another important mucosal defence mechanism. All these mechanisms prevent the infection-causing bacteria from adhering and further invading the human body. If a few bacteria escape the host defence mechanisms described above, they could be cleared by the second defence system of the body. Within tissues and the blood, transferrin binds iron so that it is not available to the invading bacteria; the phagocytes then engulf and destroy the invaders. The activation of complement attracts and aids phagocytes to attack bacteria; specific antibodies bind to the specific bacteria cells, which activates the complement system and other immune responses to destroy the bacteria; the specific antibodies also neutralise the bacterial toxins; cytotoxic T cells recognise and kill host cells infected by bacteria (Salyer & Whitt, 1994).

In order to complete an infection process, bacterial cells have to equip themselves with certain virulence factors that could promote their colonisation, invasion, and escape the host's complexity defence system (Falkow, 1992). A variety of virulence factors of infecting bacteria have been well described by Salyer & Whitt (1994). For example, an intestinal pathogenic bacterium with the ability to excessively degrade mucin or release sIgA proteases, with certain motility and chemotaxis, and with well developed adhesion properties, may escape the trapping action of the mucin layer and gain access to the mucosal surface. Adherence to host cells is an important bacterial virulence factor (although it has been advocated as a good attribute to select probiotic strains, see Section 2.4.3), particularly in areas that are washed with fluids. Other different virulence factors such as capsules or lipopolysaccharides (e.g. O antigens of *Enterobacteriaceae*) protect the bacteria from the action of phagocytes or the complement system of the blood (Hacker & Ott, 1992). The ability of various

organisms to change the composition of certain surface molecules during infection also helps these bacteria (e.g. streptococci) to evade specific humoral immune responses.

In the healthy human body, bacteria without or with only a limited virulence capacity have no ability to invade host. Members of the normal microflora stay at their natural resident sites and do not have the ability to enter into tissues or the bloodstream. If there are any viable microorganisms present in tissues or the bloodstream for some reason (e.g. lesions of skin or mucosae, surgical procedures), these bacterial cells will be quickly killed and removed by host's defence mechanisms, so no infection will occur.

However, the potential of any viable microorganism to cause an infection cannot be totally excluded (von Wright & Salminen, 1999). Under certain circumstances such as immunosuppression, some bacteria belonging to the normal human microflora may cause serious diseases if they carry a large number of putative virulence factors; these bacteria are termed as facultative pathogens or opportunists, such as certain species of *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *S. sanguis*, and certain yeast species (Hacker & Ott, 1992).

2.5.2.2 Specific infections associated with LAB

As mentioned previously (Section 2.3.1), the genera *Lactobacillus* and *Bifidobacterium* are the major inhabitants of human GIT, where they co-exist harmlessly with their host and other members of the GIT microflora. Due to the lack of virulence factors, infections caused by lactobacilli or bifidobacteria have never been reported in healthy humans. However, in the compromised host otherwise harmless organisms may prove pathogenic. In general no heterotrophic organism can really be regarded as harmless under all circumstances and the LAB are no exception to this rule (Adams, 1999). Sometimes bacteria without virulence factors can result in serious diseases such as bacterial infective endocarditis (IE), an infection of the heart valves caused by members of the host's commensal microflora, which enter the bloodstream and then adhere to and colonise the damaged heart valves. Any transient bacteraemia has the potential to result in IE. Therefore, any viable probiotic LAB strain may act as opportunists to cause infections in immunocompromised people or hosts with a damaged defence system.

Rare cases of local or systemic infections including IE, caused by 'non-pathogenic' LAB especially lactobacilli or bifidobacteria have been reported (Bayer *et al.*, 1978; Friendland *et al.*, 1990; Boulanger & Lee, 1991; Harty *et al.*, 1994). The numbers of LAB-caused bacteraemia are very low in comparison with other bacteraemia cases (incidence of enterococci 5-15%; lactobacilli 0.1%, leuconostocs < 0.01%) (Aquirre & Collins, 1993; Gasser, 1994; Saxelin *et al.*, 1996). Most cases of IE are caused by members of the host's commensal microflora such as α -haemolytic streptococci or viridians streptococci (50%), staphylococci (25%), enterococci (5-15%). Lactobacilli have only rarely been isolated from IE cases (0.05% to 0.4%)(Gasser, 1994). The majority of the LAB strains linked to clinical cases belong to the species *E. faecium* and *E. faecalis*, but a few belong to *Lb. rhamnosus*, *Lb. casei* (or *Lb. paracasei*), and *Lb. platarum* (Gasser, 1994). Some other species of lactobacilli such as *Lb. acidophilus* and *Lb. jenseii* have also been implicated in clinical infections (Aquirre & Collins, 1993). Oral manipulation or uterine infections are the major predisposing factors that lead to infection of abnormal cardiac valves with non-pathogenic *Lactobacillus* (Sussman *et al.*, 1986; Gallemore *et al.*, 1995), which indicates that the source of contamination was clearly endogenous. Furthermore, most cases of local sepsis caused by LAB with or without blood stream infection was proximal to a site that may have provided massive contamination (Brook & Frazier, 1993). Until recently, *E. spp* were considered to be innocuous commensals of the intestinal tract of humans, but recent evidence has revealed that enterococci are serious opportunistic pathogens of immunodeficient hosts (Wagner & Balish, 1998).

In a few cases of clinical infections, the possible implication of consumption of milk or dairy products with or without probiotic cultures has been discussed, but no definite 'cause-effect' relationship has been established. A few cases of blood stream infections caused by fungi, all of which occurred in patients with a catheter, have been reported in humans treated with the probiotic yeast *Saccharomyces boulardii* (Zunic *et al.*, 1991; Salminen *et al.*, 1998a). Translocation is a prerequisite for pathogenicity for most opportunistic gut pathogens (Ford *et al.*, 1996). Normal gut microflora, particularly the species *Bifidobacterium* and *Lactobacillus*, are believed to play an important role in inhibiting the translocation of pathogenic organisms via their colonisation resistance mechanisms. Conversely, probiotic bacteria may also be able to translocate in immunodeficient hosts, this hypothesis has been proven by Link *et al.* (1995) and

Wagner *et al.* (1997). *Lb. acidophilus* and *Lb. GG* has been isolated from the liver, spleen, and mesenteric lymph nodes of gnotobiotic mice fed with these strains. The translocation rate of *Lb. acidophilus* and *B. animalis* in immunodeficient bg/bg-nu/nu mice was reported to be 50% (Wagner *et al.*, 1997). Although the translocation of LAB from the gut to extra-gut tissues, especially the mucosal associated lymph tissues (MALT) (where they are killed by the host's immune defence system *in situ*) (Owens & Berg, 1980), has been suggested as a normal and beneficial physiological process associated with immune stimulation and the effects of alerting the local immune defence (Bengmark & Jeppsson, 1995), it is possible that when the host's immune system is damaged these translocated non-pathogenic bacteria could survive, multiply, and even disseminate into the bloodstream resulting in infections. Therefore, translocation may be an important indicator of potential bacterial pathogenicity. The clinical infections described above may be evidence of this postulate.

2.5.2.3 Adverse metabolic activities

The potential side-effects of probiotic LAB strains on the metabolism of the human body may include their effect on the composition and metabolic activities of GIT microflora; bile salt metabolism within the gut; degradation of intestinal mucin, and the activities of enzymes that are implicated with the pathogenesis of IE.

a) Influence on intestinal microflora: It is difficult to study the role of probiotics in the modification of the intestinal microflora because of the lack of knowledge about this complex ecosystem and the lack of appropriate techniques to sample or identify changes in the endogenous microflora (Holzapfel *et al.*, 1998). Currently, most results in this area are based on faecal samples, which obviously are a poor description of the status in the terminal ileum or the ascending colon.

b) Bile salt deconjugation: Many LAB possess the enzymes necessary for bile salt deconjugation or for dehydroxylation (Eyssen, 1973; Gilliland & Speck, 1977; Walker & Gilliland, 1993). Strains of *Lb. acidophilus* and *B. spp.* ingested as fermented dairy products are able to transform conjugated primary bile salts into toxic free secondary bile salts by deconjugation in the small intestine (Marteau *et al.*, 1995; Walker & Gilliland, 1993). Physiologically, the bacterial metabolism of bile salts does not occur in the upper small bowel, but only in the distal ileum and colon. Deconjugation of bile

salts in the distal small intestine and colon may be important in controlling serum cholesterol concentrations because deconjugated bile salts lead to less absorption of lipids from the gut (Eyssen, 1973). However, extensive bacterial deconjugation or dehydroxylation of bile salts in the upper small intestine is detrimental in several ways. First, free bile salts are more rapidly absorbed in the jejunum by passive diffusion, which can lead to insufficient concentrations of total bile salts presenting in the distal small bowel and may result in lipid maldigestion or diarrhoea. Secondly, the dehydroxylated bile salts are cytotoxic and co-carcinogenic, so excessive dehydroxylation of bile salts may induce cellular lesions in the small intestine or increase the chance of cancer developing (Marteau *et al.*, 1995). Therefore, caution has to be taken if strains with extensive bile salt deconjugation or dehydroxylation activities are used as probiotics. Overall however, the biological effect of probiotics on bile salt deconjugation is believed to be minimal and is generally not considered as a dangerous side effect of the tested product (Salminen *et al.*, 1998a; Marteau & Salminen, 1999).

c) Mucin degradation: The proposed primary function of the mucus layer coating on the surface of gastrointestinal tract, respiratory tract and urogenital tract is to protect the delicate underlying epithelial cells from attack by corrosive acid and pepsin of the gastric juice (Allen *et al.*, 1984); the shear forces generated by the digestive processes (Corfield *et al.*, 1988); and invasion by pathogens or potential pathogens (Cohen & Laux, 1995; Colina *et al.*, 1996). Any changes in the mucus content and structure will compromise the mucosal defence barrier functions contributed by the mucus layer (Cohen & Laux, 1995; Smith *et al.*, 1995). It has been suggested that elimination of the mucus layer could result in an increase in the populations of bacteria adhering to the enteric surfaces, and also possibly facilitate the translocation of normal intestinal bacteria (Ruseler-van Embden *et al.*, 1989). Therefore, excessive degradation of the intestinal mucus layer by probiotics may theoretically be detrimental (Salminen *et al.*, 1998a). Strains that do not degrade mucin are thought to be non- or less-invasive; strains that adhere but do not degrade mucin are also thought to be therapeutic in the treatment of mucosal diseases such as pouchitis, ulcerative colitis and Crohn's disease (Donohue *et al.*, 1998). Some endogenous bacteria such as strains of bacteroides, and a few strains of lactobacilli and bifidobacteria, may have the ability to degrade mucus (Ruseler-van Embden *et al.*, 1989; Hoskins, 1981; Macfarlane & Gibson, 1991).

d) Platelet aggregation: Platelet activation and aggregation is largely believed to contribute to the pathogenesis of IE, and aggregation of platelets by lactobacilli is thought to be an important contributory factor in the progression of *Lactobacillus* endocarditis (Adams, 1999). Herzberg and coworkers (1992) reported that inoculation of New Zealand White rabbits with the bacterial strain which can cause platelet aggregation *in vitro* (Agg⁺ strains), consistently caused IE with significantly larger vegetation, more gross lesions in major organs, and greater mortality than inoculation with the Agg⁻ strains (strains unable to induce platelet aggregation *in vitro*). It has also been reported that the isolates of bacteria from patients with IE uniformly induced irreversible platelet aggregation (Kessler *et al.*, 1987; Harty *et al.*, 1993; 1994). Further experiments showed that most oral lactobacilli have the ability to aggregate platelets *in vitro* (Harty *et al.*, 1993). Therefore, a lack of platelet aggregating potential has been listed as one of the most important criteria for probiotic selection (Harty *et al.*, 1993; Kirjavainen *et al.*, 1999).

2.5.2.4 Adverse effects on immune system

Consumption of probiotic LAB has been associated with the enhancement of both innate and acquired immune responses (Section 2.2.3)(Gill, 1998; Matsuzaki & Chin, 2000). Some LAB strains have shown significant adjuvant activity in animal models (Classen *et al.*, 1995; Pouwels *et al.*, 1996; Chin *et al.*, 2000), so *Lactobacillus*-based live vector vaccines have been proposed (Pouwels *et al.*, 1996). Immunostimulation may be helpful to an immunodeficient host if the probiotic activates other host defences against pathogens. But, unrestricted stimulation of the immune system by probiotics could be detrimental for patients suffering from autoimmune diseases (Guarner & Schaafsma, 1998). Autoimmunity or autoimmune diseases are thought to be multi-aetiological immune dysfunctions, and the penetration of the gut by bacterial antigens (e.g. heat-shock proteins) is one of the suggested initial factors leading to a loss of tolerance towards self-components in genetically predisposed individuals (Salyer & Whitt, 1994; Hamilton *et al.*, 1998). Stimulation of inflammation and autoimmunity by bacteria has been described in immunodeficient mice (Rosen, 1994; Sartor, 1997). There is growing evidence that the composition of the endogenous intestinal microflora may have an important role in the expression of systemic autoimmunity in both humans and animal models (Famularo, *et al.*, 1997). The association between bacterial antigen

and autoimmune responses, and the adjuvant activity of LAB strains has led to the suggestion that LAB may be involved in the pathogenesis of some models of autoimmunity in experimental animals and possibly in humans. The possible association between the consumption of large amounts of yoghurt with the progression of autoimmune liver disease has been reported recently (Chaiken, 1994). Therefore, from a safety point of view, further research must be conducted to determine if probiotic bacteria or their products can induce inflammatory or autoimmune diseases in immunocompetent and especially in immunodeficient patients, who may be more susceptible to such diseases because of aberrant immune responses to microbes or microbial products (Kyriakis *et al.*, 1992; Wagner & Balish, 1998).

2.5.2.5 Transmission of antibiotic resistance genes

Resistance to antibiotics is a common characteristic of many LAB strains (Rinckel & Savage, 1990; Charteris *et al.*, 1998). Some antibiotic resistance attributes of LAB are known to be plasmid-linked (Rinckel & Savage, 1990; Tannock *et al.*, 1994a; Wang & Lee, 1997). Among the antibiotic resistance attributes, transmissible vancomycin resistance is particularly noteworthy because vancomycin is one of the last effective antibiotics effective against infections caused by multidrug-resistant pathogens (Eliopoulos *et al.*, 1994; Morelli & Wright, 1997). Several LAB, including species of enterococci (Quintiliani *et al.*, 1993; Morelli, 1996); *Leuconostoc* spp. (Nicas *et al.*, 1989); and several species of lactobacilli and pediococci are known to be vancomycin resistant (Leclercq & Derlot, 1988; Swenson *et al.*, 1990; Leclercq & Courvalin, 1997).

Plasmid-associated traits including antibiotic resistance have the potential to spread to other strains, species, and even to other genera of bacteria (Wang & Lee, 1991; Thompson & Collins, 1997). LAB themselves may also act as a recipient to plasmid-linked attributes (e.g. virulence factors or new antibiotic resistance) from other strains (Thompson & Collins, 1997). Therefore, any plasmid-associated antibiotic resistance of dietary LAB strains could have the potential to pass into cells of harmful pathogenic microbes and present a public health risk (Eliopoulos *et al.*, 1994). It has been proposed that strains harbouring antibiotic resistance plasmids should not be used either as human or animal probiotics (Stokes *et al.*, 1993). Checking the ability of a proposed probiotic

strain to act as a donor of conjugative antibiotic resistance genes may therefore be a prudent precaution (Stokes *et al.*, 1993).

Furthermore, antibiotic-resistant probiotic bacteria could pose a danger to immunodeficient hosts by secreting enzymes that are able to degrade antibiotics (Gold & Moellering, 1996; Wagner & Balish, 1998). It has been shown that β -lactamase is produced by commensal bacterial species in the upper respiratory tract; this enzyme is able to protect *S. pyogenes* against penicillin (Brook & Gober, 1995).

Therefore, routine antibiotic susceptibility testing has been advocated as an essential selection criterion for probiotic cultures to ensure that they are unlikely to participate in undesirable gene transfer cascades *in vivo* (Charteris *et al.*, 1998).

2.5.3 Published probiotic safety studies

In response to the different concerns regarding the safety of probiotics, several studies including *in vitro* virulence factor assessment, *in vivo* toxicity tests in experimental animals, human clinical trials, and epidemiological surveillances, have been conducted.

Due to the important role of mucin in the mucosal barrier system, a mucin degradation test is considered the first toxicity marker in probiotic safety assessment (Marteau & Salminen, 1997; Donohue *et al.*, 1998). Norin *et al.* (1994) inoculated germ free mice with *Lb. acidophilus* A10 and *B. bifidum* B11 to observe any potential adverse metabolic effects on the host animals. The parameters observed include degradation of mucin, gut enzyme activities, deconjugation of bile salts, and conversion of cholesterol to coprostanol. There was not any adverse effects detected based on these indicators. In a similar study by Ruseler van Embden *et al.* (1995), the *in vitro* and *in vivo* (in rats) mucin degrading activities of the commercial probiotic strains *Lb. rhamnosus* GG, *Lb. acidophilus*, and *B. bifidum* were studied using hog gastric mucin (HGM) and human intestinal glycoproteins (HIG) as substrates. The changes in protein and carbohydrate concentrations, and the loss of blood group antigens (A and H) in mucin glycoproteins, were used as indicators of mucin degradation. No mucin degrading activities were detected in this study. Colina *et al.* (1996) studied the mucinolytic activity of *Candida albicans* using a petri dish assay; this may be an alternative method to demonstrate objectively the mucin degrading activity of probiotic LAB strains.

Considering the potential implication of LAB with platelet aggregation activity and I \bar{E} , Korpela and coworkers (1997) investigated the ability of the probiotic strain *Lb. GG* to induce human platelet aggregation *in vitro*. The result of their experiments showed that the test *Lactobacillus* strains had no influence on either spontaneous or physiological triggers (agonists) inducing platelet aggregation.

Investigation of the transferable antibiotic resistance of LAB has been recommended as an important parameter when evaluating the safety of probiotics (Morelli & Wright, 1997; Salminen *et al.*, 1998a). However, there are few studies being conducted to demonstrate the safety of probiotics from this aspect. A study by Tynkkynen *et al.* (1998) suggested that the vancomycin resistance in *Lb. GG* is unlikely to be transmissible. To date, this may be the only report investigating the genetic stability of probiotic LAB strains.

The fate (survival passage from GIT, translocation, or colonisation) of probiotics and their active components following oral administration needs to be determined to predict not only positive probiotic effects but also potential side-effects (Salminen *et al.*, 1998a). The survival of ingested probiotics at different regions of the GIT differs between strains (Marteau & Rambaud, 1993). Some strains cannot survive passage through the acidic condition found in the stomach or tolerate bile salts; while other strains such as bifidobacteria or *Lb. acidophilus* can pass through the entire gut in very high concentrations (Marteau Rambaud, 1993). In general, most currently available probiotic strains may survive passage through the entire GIT, but can only transiently colonise the gut environment.

Very few translocation studies investigating the safety of probiotics have been published. It has been reported that *Lb. GG* and *Lb. acidophilus* can translocate from the GIT into the liver and mesenteric lymph nodes of germ-free mice (Link *et al.*, 1995). *Lb. GG* and another probiotic strain *Lb. reuteri* have also been reported to be able to translocate into internal organs (spleen, liver, and kidney) in 50% of immunodeficient gnotobiotic beige-athymic mice (*bg/bg-nu/nu*) (Wagner *et al.*, 1997). But results from Dong *et al.* (1987) showed that *Lb. GG* did not translocate from the GIT following oral feeding of mice that had received lethal dose irradiation; in fact, treatment with *Lb. GG* prolonged the survival time of the mice. Other studies have also demonstrated that

administration of probiotic strains (*Lb. acidophilus*, *Lb. rhamnosus* GG, *Lb. reuteri*) in immunodeficient animals reduced the infection rate and increased the immune responses of the host animals, and no adverse effects were associated with the feeding of these probiotic strains (Naaber *et al.*, 1998; Alak *et al.*, 1999). No probiotic translocation in conventional animals has been observed although some other indigenous LAB strains have been isolated from the tissues of healthy animals (Berg & Garllington, 1979; Steffen & Berg, 1983; Ma *et al.*, 1990; Swank & Deitch, 1996; Naaber *et al.*, 2000).

Most toxicity studies have been conducted in experimental animals. In 1979, Momose and colleagues investigated the chronic and acute toxicity of a probiotic strain *B. longum* BB-536 in mice and rats. For the acute toxicity test, 4-week old mice were orally fed freeze-dried test strains at a dose rate of 10^{12} cfu/mouse within 24 hours, and animals were killed 6 days later. In the chronic toxicity test, the test LAB strains were mixed with the animal feeds at a rate of 0.5g/100g diet. Rats were fed this diet *ad lib* for one year. The animals' body weight change, feed intake, blood biochemical parameters, and organ histology were examined. It was believed that if the probiotic strains had any toxicity, then diarrhoea or systemic infections would probably be the most common consequence following administration of these viable strains. Chronic toxicity may present as malnutrition, growth retardation, damage or dysfunction of the internal organs (liver, heart, and kidney), and abnormality in metabolic activities. No acute or chronic oral toxicity was detected in this study. The LD₅₀ (50% lethal dose) value in this group of rats was reported as more than 50g/kg body weight for oral administration. In another study by Donohue *et al.* (1993), the acute oral toxicity of *Lb. rhamnosus* GG, *S. thermophilus*, and *Lb. helveticus* were investigated in Swiss mice. Again, the animals' general health status, feed intake and growth rate were used as toxicity indicators. The authors did not report any treatment-associated toxicity or dose-related adverse effects. The LD₅₀ value in this study was >6g/kg body weight.

Recently, an experimental endocarditis animal model, in which the vegetations on the heart valves induced by placing a polyethylene catheter in the heart cavity and following intravenous injection of the test strains, was used to study the virulence of LAB (Pelletier *et al.*, 1996). Owing to the non-physiological conditions and the large

number of bacteria injected, the relevance of this experimental model has been questioned (Morelli & Salminen, 1997).

The safety of selected probiotics has also been studied in several short-term clinical trials using healthy human volunteers (Lidbeck *et al.*, 1987; Salminen & Donohue, 1996; Saxelin, 1997; Wolf *et al.*, 1995; 1998; Arunachalam *et al.*, 2000). The presence or absence of gastrointestinal disorders was studied in some trials. Most of these studies did not detect adverse effects of tested probiotics on human health in comparison with that of control group subjects who received placebo only. In Arunachalam and coworkers' study (2000), no obvious adverse effects on the immune status of human individuals were identified following a 3-week administration of the probiotic strain *B. lactis* HN019. Wolf and coworkers (1998) observed the serum chemistry, haematology, immune profiles, urinalysis, physical examination, gastrointestinal tolerance and faecal microflora data in a group of HIV infected subjects following consumption of *Lb. reuteri* for 35 days. They did not detect any clinically significant changes in any of the safety parameters measured.

Oral administration of probiotic *Lactobacillus* or *Bifidobacterium* strains to new born babies (Bennet *et al.*, 1992), premature or full-term infants (Millar *et al.*, 1993; Langhendries *et al.*, 1995; Guerin-Danan *et al.*, 1997), healthy children (Sheen *et al.*, 1995) or children with diseases such as diarrhoea (Isolauri *et al.*, 1994) and lactose intolerance (Montes *et al.*, 1995) have also been proven to be safe. In all of the studies carried out in infants or children, no detectable adverse effects were associated with the probiotic feedings. It was concluded in an extensive review (Naidu *et al.*, 1999) that "all probiotic LAB strains administered in the 143 human trials were well tolerated, and not a single adverse effect or event was ever reported in any of the 7526 subjects involved in these studies spanning a time line of four decades."

The long history of safe use of probiotic LAB strains is still the best proof of their safety. If the potential risk of a probiotic is nil or very low, the best approach to assess its safety is probably to analyse retrospectively in epidemiological studies and prospectively using pharmacovigilance methods (Morelli & Salminen, 1997). Saxelin *et al.* (1996) using this method studied the safety of different commercial LAB strains in Finland. 16s rRNA gene PCR amplification and carbohydrate fermentation profiles (API diagnostic systems) were used to identify isolates from clinical samples, and these

were compared with the reference commercial strains. Results showed that no clinical isolate was identical to isolates originating from commercial dairy products or from the pharmaceutical products studied. It was concluded that the origin of *Lactobacillus* bacteraemia isolates in Southern Finland during 1989 - 1994 was not related to the consumption of fermented dairy foods.

To date, there have been no published epidemiological studies regarding the safety of the probiotic *Bifidobacterium*. This may be related to the relative low prevalence of clinical infections or bacteraemia associated with this species.

2.5.4 Safety status of current commercial probiotics

A number of extensive reviews on the safety of probiotic LAB strains have been published recently (Gasser, 1994; Saxelin *et al.*, 1996; Marteau & Salminen, 1997; Salminen *et al.*, 1998a; Saarela, 2000). Several specialist workshops (e.g. 'The Safety of Lactic Acid Bacteria', 11/1994; 'Lactic Acid Bacteria as Probiotics', 11/1995; 'Functional Food Science-FAIR programme; and 'PROBDEMO Workshop on Probiotic Safety and Selection Criteria', 11/1996) have been organised to discuss the safety issues of probiotic organisms. The purpose of these meetings was to establish a clearly written consensus document on the safety and probiotic properties of LAB (van der Kamp, 1996; Salminen *et al.*, 1998a). From these discussions, the following conclusions have been drawn:

- All cases of LAB-associated clinical infections published to date were observed in people with underlying abnormal conditions. No case of infection with LAB has been observed in healthy subjects, pregnant women or extreme age subjects (young children or the elderly).
- No case of LAB-infection has been observed in people working with LAB.
- No case of infection has been linked to the consumption of fermented foods or probiotic LAB strains except a single report (Rautio *et al.*, 1999) in which the endogenous origin of the isolated LAB could not be excluded.
- All lactobacilli should be classified as group 1 (no risk), but that strains of the species *Lb. rhamnosus* warrant further careful surveillance (Gasser, 1994; Adams, 1995; van der Kamp, 1996).

- Enterococci are much more frequently associated with clinical infections than other LAB, therefore the use of this species as probiotics should be based on sufficient knowledge about their safety and demonstrated health benefits.
- No deleterious metabolic effects of probiotics have been reported.
- To ensure the maximum safety for consumers, all probiotic candidates, especially novel strains or traditional strains without thorough safety documentation, should be carefully reviewed and evaluated before being incorporated into food or pharmaceutical products.

2.5.5 Recommendations for probiotic safety assessment

To date no general guidelines for the safety assessment of probiotics from official authorities or legislative bodies have been suggested. However, different recommendations from scientific or academic fields have been published recently (Donohue & Salminen, 1996; van der Kamp, 1996; de Vos *et al.*, 1997; Salminen *et al.*, 1998a; Adams, 1999). The following studies were recommended by one or more of these publications to be carried out on probiotic strains to ensure their safety for human /animal consumption:

2.5.5.1 Intrinsic properties

- Antibiotic resistance and plasmid profiles; transferability of antibiotic resistance
- Detrimental enzyme activities (enzymes involving in bile salt deconjugation and dehydroxylation; amine metabolism; platelet aggregation)
- Platelet aggregating ability
- Virulence factors (mucin degradation; adhesion and binding properties)
- Infectivity studies (cell lines; immunocompromised animal models; bacterial translocation, and lymphoid organ weight)

2.5.5.2 Pharmacokinetics

- *In vivo* studies on GIT survival passage; colonisation at different levels of the GIT
- Interference with the GIT microbial ecology

2.5.5.3 Acute or sub-acute oral toxicity studies

- The general health and well-being, feed intake, growth, gut mucosa and internal organ histology or the presence of infections following ingestion of test strains
- The haematology and major blood biochemistry (e.g. plasma levels of glucose, protein, cholesterol, or metabolic enzymes)

2.5.5.4 Dose-response studies

- The minimum and maximum doses required for the purported health benefits or potential detrimental effects on hosts
- The balance between the efficacy and minimum adverse effects

2.5.5.5 Adverse effects on the immune system

- Immunocompromised (deficiency or autoimmunity) animal models
- Studies in human volunteers with autoimmune disease or disorders involving autoimmune responses such as Crohn's disease, juvenile rheumatism, systemic lupus erythematosus

2.5.5.6 Studies in human volunteers

- Studies on the tolerance of human subjects to test probiotics. These studies should be based on the knowledge of maximum safety and sufficient efficacy data obtained from different animal models.

2.5.5.7 Epidemiological surveillance

- Following the introduction of food products containing probiotic strains, the prevalence of infections caused by probiotic-related bacteria in a large population who are consuming or have consumed the new probiotic-food product needs to be surveyed. This may be the most important safety data among this recommendation list.
- Identify the LAB isolates from clinical infections and compare with probiotic strains used in food products that have been consumed by certain populations.

2.5.6 Principles of safety studies

According to the conclusions from the PROBDEMO project (European Commission FAIR CT96-1028 demonstration project, 1996-1997) (de Vos *et al.*, 1997), the following principles should be followed in considering the safety of traditional or novel probiotic LAB strains:

- 1) It is the sole responsibility of food manufacturers and producers to provide a safe food. Probiotic foods should be as safe as other conventional foods.
- 2) When the probiotic strain turns out to be a novel strain it must be subjected to the appropriate legal approval (e.g. EU directive for novel foods). The safety testing recommended above should be undertaken.
- 3) 'State-of-the-art' taxonomy is required to fully describe a probiotic strain. This currently includes DNA-DNA hybridisation and rRNA sequence determination. Strains should also be deposited in an internationally recognised culture collection.
- 4) The best test for food safety should be a well-documented history of safe human consumption.
- 5) Strains that will result in novel foods include:
 - A strain that belongs to species for which no pathogenic strains are known but which does not have a long history of safe use.
 - A new strain that belongs to species for which pathogenic strains are known.
 - A strain that has been genetically modified.
- 6) Strains that will not result in novel foods include:
 - A strain that has a long history of safe use, it will be safe as a probiotic strain.
 - A strain that belongs to a species for which no strains are known to be pathogenic, and for which other strains have a long history of safe use, making it likely to be safe as probiotic strain.
- 7) Strains that should not be marketed include:
 - Strains that carry transferable antibiotic resistance genes.
 - Strains that have not been properly taxonomically described using the approaches recommended above.

2.5.7 Food/health authorities' views on the safety of probiotics

Lactobacillus: Official views on the safety of proposed probiotic lactobacilli are sometimes contradictory between countries. In 1993, after considering the available evidence on the probiotic strain *Lb. rhamnosus* GG, the UK Government's Advisory Committee on Novel Foods and Processes (ACNFP) approved the incorporation of *Lb. GG* into food products. Whereas, under a German classification scheme, *Lb. rhamnosus* was excluded from the 'no risk' category, Group 1. On the information available, the LABIP (Lactic Acid Bacteria Industrial Platform) workshop (1994) concluded that all lactobacilli should be included in Group 1; with the exception of *Lb. rhamnosus*, which they felt, required further surveillance (Adams & Marteau, 1995; van der Kamp, 1996).

Enterococcus: Foods containing enterococci have a long history of safe use, but in view of their more frequent association with human infections, the LABIP workshop recommended that any new food products containing enterococci should be demonstrated to pose no health risks using the available model systems. The use of *E. faecium* strain K77D as a starter culture is permitted in Denmark after 4 years of the safe marketing of foods containing this strain. Although approval for this strain is not strictly necessary under the EU regulations, approval was obtained from the ACNFP (UK) in 1995 based on evidence of the strain's stability, lack of pathogenicity, and the absence of a transmissible gene encoding vancomycin resistance.

Health claims: Any medicinal and health claim for cultured milk products containing probiotics have to comply with existing EU and UK legislation on such matters.

2.6 Summary

Decades of work have meant that significant progress has been made in our understanding of the impact of probiotics on human health. It can be readily concluded that probiotics have great potential in several fields. Possible uses of probiotics include prevention and therapy of infections, reduction of malignancies, immunomodulation, alleviation of lactose maldigestions, improvement of different diarrhoeal diseases, blood-lipid lowering, and potentially, promotion or maintenance of human health. Many of the specific effects purported to be due to the ingestion of probiotics, however,

remain scientifically unsubstantiated, and it is rare that specific health claims can be made.

The viability of probiotics in commercial food products (e.g. yoghurts), the gut colonisation ability, and the safety of probiotic organisms, is the subject of much debate. Due to a lack of understanding of the gut microflora, the real effect of probiotics on this ecosystem is not clear. Where is the target region for probiotics to colonise? How do they deliver their health benefits? To date, there are no clear answers to these questions.

It is largely agreed that probiotics should be shown to be as safe as ordinary foods before being incorporated into food or pharmaceutical products. The safety of any probiotic strains, especially the newly identified strains, has to be clearly demonstrated based on substantial scientific evidence. This could be done using a series of *in vitro* and *in vivo* safety experiments such as the mucin degradation test, platelet aggregation assays, antibiotic resistance and plasmid profiles, opportunistic infections in immunocompromised animals, tolerance of animals with abnormal immune responses (immunodeficiency or autoimmune response), and animal trials to detect any potential acute or sub-acute oral toxicity of test strains.

The safety of probiotics also has to be assessed in human volunteers before they are marketed. Epidemiological studies following the introduction of probiotics into food products can provide more solid safety evidence. So far, there are no serious safety issues regarding the use of currently available probiotic preparations. Future safety assessment may include studies using subjects with sub-optimal immune responses to ensure the maximal safety for all consumers. More fundamental studies towards the understanding of human gut microflora and the interactions between this ecosystem and probiotics, the mechanisms of probiotic effects, and the relevance of immunomodulation for promotion of health in healthy human subjects, need to be conducted. The purported health benefits of probiotic organisms also need to be demonstrated in well-controlled clinical trials to defend the credibility of probiotics.

3

Materials and methods

Some general materials and methods used in the experiments of this thesis are described in this chapter. The specific experimental methods and materials for each particular experiment will be introduced in the respective chapters.

3.1 Bacterial strains

Bacterial strains used in this study are listed in Table 3.1.

3.2 Water supplies and sterilisation

The water (MQ-H₂O) used in the preparation of all solutions, unless otherwise stated, was purified by a Milli-Q Plus Ultra-Pure Water System (Molsheim, France). Sterilisation of solutions and equipment was at 121°C for 25 min in a commercial autoclave (TOMY SS-325 Autoclave, ALPHATECH, Auckland, New Zealand). Some solutions as indicated in specific chapters were filter-sterilised using a 0.2µm Sterile Acrodisc® (Gelman Sciences, Pall Corporation, Ann Arbor, MI. Cat: 4184). Glassware used for the preparation of solutions for work with DNA was acid washed and sterilised. All labware, pipette tips and microcentrifuge tubes used for PCR and other DNA manipulations from stocks were handled with clean disposable gloves.

3.3 Media preparation and storage

Sterilised solid media was stored at room temperature, except MRS agar and broth which were stored at 4°C; sterilised broth, cell culture media, and plates were stored at 4°C. Media supplements were added into the sterilised liquid broth or agar when the media cooled to 50 ~ 55 °C after autoclaving. The pH of media, unless otherwise stated, was adjusted with 1 M HCl or 1 M NaOH. Autoclaved agar media was cooled to

50~55°C in a water bath before being poured into petri dishes. The formulae of specific media are described in Appendix 1.

Table 3.1 Bacterial strains used in this study

Genera	Origin	Strain ID	Source
<i>Lactobacillus</i> species			
<i>Lb. rhamnosus</i>	Cheese	HN001 (DR20™)	NZDRI ^a
<i>Lb. rhamnosus</i>	Human	53103 (GG)	ATCC
<i>Lb. rhamnosus</i>	Human	HN067	Uni Otago ^b
<i>Lb. acidophilus</i>	Yoghurt	HN017	NZDRI
<i>Lb. acidophilus</i> LA-1	Yoghurt LC-1	LA-1	Nestle
<i>Lb. plantarum</i> 45		HN045	NZDRI
<i>Lactococcus</i> species			
<i>Lact. lactis</i> (subsp <i>lactis</i>)		MG 1363	NZDRI
<i>Bifidobacterium</i> species			
<i>B. lactis</i>	Yoghurt	HN019 (DR10™)	NZDRI
<i>B. lactis</i>		Bb12	
<i>B. lactis</i>	Human	HN049	NZDRI
<i>B. lactis</i>	Human	HN098	NZDRI
<i>Escherichia</i> species			
<i>E. coli</i>	Human	25922	ATCC
<i>E. coli</i>	Human	V517	ATCC
<i>Staphylococcus</i> species			
<i>S. aureus</i>	Human	25923	ATCC
<i>S. aureus</i>	Human	6571	NCTC
<i>Salmonella typhimurium</i>	Rodents	14028	ATCC

a: Culture Collections of New Zealand Dairy Research Institute, Palmerston North, New Zealand

b: Culture Collections of University of Otago, Dunedin, New Zealand.

3.4 Common buffers and solutions

The common buffers and reagents used in this study are described in Appendix 2.

3.5 Growth, maintenance, and storage of cultures

3.5.1 Anaerobic conditions

The BBL Gas Pack Anaerobic System (Becton Dickinson, Cockeysville, MD, USA), including BBL anaerobic jar and disposable hydrogen plus carbon dioxide generator envelope, was used for the incubation of LAB strains. BBL[®] Dry Anaerobic Indicator Strips (Becton Dickinson Microbiology Systems, Merck, Germany) were used to monitor the anaerobic conditions.

3.5.2 Bacterial cultivations

a) **Pure LAB cultures** were obtained by streaking for single colonies on MRS or MRS supplemented with 0.05% cysteine HCl (Sigma, C-1276) (MRS-C, for *B. lactis* HN019) agar plates and incubated at 37°C under anaerobic conditions for 48hrs. Single colonies were selected and sub-cultured in MRS or MRS-C broth for 18~20hrs. The sub-cultured cells were concentrated by centrifugation and resuspended in MRS or MRS-C broth, then stored at 4°C with regular sub-culturing (once a week).

b) **Pure non-LAB cultures** (*Staphylococcus*, *Salmonella*, and *E.coli* cultures) were maintained by the same method as described above but different media were used and aerobic incubation conditions applied. The incubation time was 24 hrs rather than 48hrs for agar plates. BHI agar plates and broth were used to cultivate *Staphylococcus* and *Escherichia coli* (*E. coli*); *Salmonella* was inoculated in BHI broth and on coliform agar plates.

3.5.3 Storage of bacterial cultures

For longer term storage of bacterial cultures, 10ml of the concentrated sub-cultures of each bacterial strain was mixed with 10ml of autoclaved 30% glycerol and then dispensed into 2ml sterilised eppendorf tubes which were kept at -80°C.

3.6 Experimental equipment

Experimental equipment used in this study is listed in Appendix 3.

3.7 Mucin purification

Since the partially purified hog gastric mucin (HGM, Lot 47H7085, Type III, Sigma Chemical Co. USA) containing large amount of gut content residues including lumen bacteria, it was further purified using a modification of method reported elsewhere (Miller & Hoskins, 1981). Briefly, 10 gram of HGM was stirred for 24hrs at room temperature in 500ml of 0.1 M NaCl containing 20 mM phosphate buffer (pH 7.8), and a few drops of toluene (BDH). The pH of this suspension was readjusted to 7.2 with 2 M NaOH one hour later. After centrifugation at 10,000 x g (RC 5C, Rotor SS-34, Sorvall[®], 4°C, 30min), the supernatant was cooled to 2~4 °C and pre-chilled 99.99% ethanol was added to a final concentration of 60% (v/v). The resulting precipitate was collected by centrifugation (4,000 x g, 4°C, 10min) and dissolved in 0.1 M NaCl, which was precipitated with ethanol two more times. The final mucin precipitate pellet was washed once with ethanol and then dissolved in and dialysed against distilled water (4 L) for 24hrs at room temperature with four changes. The dialysed mucin solution was then lyophilised and used as a source of purified mucin in all further experimentation. The mucin substrate incorporated in culture medium was autoclaved before use.

3.8 SDS-PAGE

The mini-gel slab method was used and a standard protocol (Gallagher & Smith, 1995) was followed. Different polyacrylamide gel concentrations (7.5 ~ 12.5%) were used; sodium dodecyl sulphate (SDS) concentration was 0.1% (w/v). The gel buffer (1.5M Tris-HCl, pH 8.8), stacking gel buffer (0.5 M Tris-HCl, pH 6.8), and running buffer (5x concentration: 0.125 M Tris-HCl, 1.0 M glycine, 0.5% SDS, pH 8.3) were stored at 4°C before use. The ratio of sample to sample buffer (1.25ml of stacking buffer, 2 ml of 10% SDS, 5 ml of H₂O, 1.0ml of β -mercaptoethanol, 0.25ml of 0.1% w/v bromophenol blue) was 1:3. The mixture of H₂O, acrylamide/Bis (30%/2.67%), and gel buffer was degassed 15 min (stirring at RT) before adding remaining reagents. Ammonium persulphate (10mg per 100 μ l H₂O) was made fresh for every gel preparation. Samples

were boiled for 2 min in sample buffer before applying to gels. 2 μ l of Rainbow standards was applied. The gel was run at constant current (50mA) for 2.5 to 3 hrs. Gels were stained with silver staining, or Coomassie blue and Periodic acid-Schiff (PAS) (Carlsson, 1993) staining methods were used to detect the change of molecular size and the loss of carbohydrate residues from the glycoproteins.

3.9 Staining of polyacrylamide gels

3.9.1 PAS staining

A standard method described by Carlsson (1993) was followed. Briefly, the gel was incubated in fixation solution (acetic acid:methanol:H₂O =10:35:55) for 2hrs and then changed to periodate solution [0.7g periodic acid in 100ml of 5% (v/v) acetic acid] and further incubated for 1hr. After rinsing briefly in H₂O, 50ml of meta-bisulfite solution [0.2g sodium meta-bisulfite in 100ml of 5% (v/v) acetic acid] was added and incubated for 5-10min, then changed to fresh meta-bisulfite solution and incubated for another 5-10 min. The gel was rinsed with H₂O briefly and then placed in Schiff's reagent (BDH), which was incubated until red bands appeared (30 min to 1 hr); the gel was then destained with fixation solution until clear background appeared.

3.9.2 Coomassie blue staining

The gels were stained for 30 min to 1 hr in Coomassie blue stain (Coomassie brilliant blue R-250 0.2g, H₂O 250 ml, methanol 200ml, acetic acid 50ml) with gently shaking, and then destained in destain (methanol 40%, acetic acid 10%, H₂O 50%) overnight. The destain was changed several times within the first 1 to 2 hrs.

3.9.3 Silver staining

Silver staining was carried out with a BIORAD kit and the manufacturer's protocols were followed. Briefly, the gels were incubated in fixative 1 (40% methanol/10%acetic acid/50% H₂O) and fixative 2 (10% ethanol/5% acetic acid/85% H₂O) for 30min, 2x 15min respectively before incubation in oxidizer (3 min). The gels were then incubated in deionised water several times (2 to 3 min each time) until the yellow colour was removed. After incubation in silver reagent for 15 min, the gels were incubated in

developer solution several times (5 min each time) until the brown colour developed. Lastly, the gels were incubated in 5% acetic acid for 5 min before being rinsed with deionised water.

3.10 DNA extractions

The total genomic DNA of LAB cultures was isolated using the method described by Cocconcelli *et al.* (1995) and Ward *et al.* (1995). Briefly, 200µl of overnight broth cultures was pelleted by centrifugation (Biofuge, 4000xg, 10 min at RT) and then washed twice in 1ml of sterile MQ-H₂O. The cells were re-suspended in 100µl sterile MQ-H₂O in an eppendorf tube, which was boiled for 10 min and then immediately put on ice. After incubation on ice for 10 min, the cell debris was pelleted by centrifugation (10 min at 10,000 x g). The supernatant was collected and used for PCR reactions. The DNA concentration in the supernatant was determined by the absorbance at 260nm (A₂₆₀) and 230nm (A₂₃₀) according to the formula:

$$\text{DNA concentration } (\mu\text{g/ml}) = (A_{260} - A_{230}) \times 50. \text{ (Stam \& Stulp, 1988)}$$

3.11 PCR

PCR was conducted using either PHC or Perkin Elmer equipment (A3.6) using the following protocols.

The reaction system included: 45µl PCR Supermix; 2 µl primer (as stated in each specific chapter); 1µl magnesium chloride (MgCl₂); 2 µl sample DNA (50ng). All reagents in this system except sample DNA were purchased from GIBCO BRL Life Technologies. The PCR reactions were completed using different thermal cycles as stated in the specific chapters.

3.12 PCR product purification

The High PureTM PCR Product Purification Kit (Boehringer Mannheim Corp., Indianapolis, USA) was used to purify PCR products following the manufacturer's instructions.

3.13 PCR product visualisation

After electrophoresis under the conditions as stated in each specific chapter, the agarose gel was stained in ethidium bromide (0.5 µg/ml in 0.5 x TBE buffer) for 10 min and washed in MQ-H₂O for 30 min. The gels were then photographed under UV light with Polaroid film (Type 667, ISO 3000/36, PID, UK).

3.14 Southern blotting & DNA hybridisation

The southern blotting procedures recommended by ECL DNA Direct Labelling and Detection Systems (RPN 3005, 3004, Amersham Life Science, UK) were modified slightly. Briefly, the gel was depurinated in 0.125M HCl (gently agitated) for 10-20min and then the DNA in this gel was denaturated in denaturising buffer (NaCl 87.66g, NaOH 20g, water 1000ml) for 30 min. The gels were then placed in neutralisation buffer (NaCl 87.66g, Trizma base 60.5g, water 1000ml, pH 7.5) and agitated gently for 30 min. A stack of 3 MM papers (more than 10 pieces) with the same size as the bottom of the glass tray was prepared. A small amount of DNA transfer buffer (20x SSC: Tri-sodium citrate 88.23g, NaCl 175.32g, water 1000ml, pH 7.8) was added into the glass tray and the prepared 3 MM papers were layered on the bottom of the tray one by one. The 20x SSC buffer was added as required and the amount of 20x SSC buffer was adjusted so that there was no extra solution on the top of the 3 MM papers. The whole glass tray was covered by one layer of glad-wrap film so that the film lay flat across the 3 MM paper stack. A window of size 5-7 mm smaller than the gel was cut in the middle of the glad film. The prepared gel was then put on the 3 MM papers at the position of the glad film window. A piece of Hybond-N membrane (slightly larger than the gel) soaked in water and 20x SSC, was then put on the top of the gel. Three layers of 3 MM papers wetted with 20x SSC were put on the top surface of the Hybond-N membrane. A stack of tissue towels (at least 5 cm high) was put on the top of the 3 MM papers. Finally, a plastic tray was placed on the top of the towel stack. The tissue towels were changed when they were completely wetted. The transfer was allowed to proceed overnight. After blotting, the nucleic acid was fixed to the Hybond-N membrane by radiating the membrane with UV light (1 min). The fixed membrane was wrapped with foil paper and kept in vacuum condition at RT.

The blot was put into pre-heated (65°C) hybridisation buffer [5x SSC, 5x Denhardt's solution (BSA 2g, Ficoll 400 2g, Polyvinylpyrrolidone 2g, H₂O 100ml), 0.5% (w/v) SDS] and prehybridised for 1 hr with constant agitation before the labelled probe was added. The hybridisation preceded overnight at 65°C with gentle agitation. The blot was then washed with 1x SSC, 0.1% SDS for 15 min at 65°C, followed by washing in 0.1x SSC, 0.1% SDS for 15 min at 65°C. Lastly, the blot was removed from the washing solution, wrapped in glad film, and then exposed to X-ray film.

3.15 Preparation of probiotic cultures for inoculation in mice

Stock cultures of lactobacilli and bifidobacteria were propagated at 37°C in MRS or MRS-C (for bifidobacteria) broth for 48 hrs, and then further sub-cultured several times. A 18-20hr fresh culture was concentrated by centrifugation (4000 x g, 10 min). The cell pellet was re-suspended in 10% skim milk at a desired concentration after washing 3 times in cold PBS. Cell counts were determined by both plate counting and culture absorbance (OD at 600 nm, Hitachi U-2001) methods. Cell suspensions and skim milk based control diets were prepared daily.

3.16 Animals

Male BALB/c mice aged 6 to 8 weeks, bred at the Small Animal Production Unit, Massey University, were housed individually in stainless steel cages with a 12 hr light-dark cycle (8am-8pm), in a controlled atmosphere (temperature 22 ± 2°C; humidity 55 ± 2%). The animals were fed a skim milk powder (SMP) based diet (Appendix 4) and fresh water *ad libitum*.

For experimental purposes, the animals' general health status was scored daily on a 5-point based scale (general health scores-GHS, Table 3.2). Animals showing any signs of illness were observed twice a day; feed and water intake, and body weight gain was monitored once a week.

3.17 Feeding of probiotic cultures in mice

Freshly prepared bacterial cultures in 10% (w/v) skim milk were fed to mice orally (30 to 50 µl) with a Gilson Pipette using sterile disposable tips as indicated in specific

chapters. Animal were manually restrained and no anaesthesia was given. The bacterial culture solutions were directly applied into the mouth cavity of the mice which were released after the cultures were completely swallowed.

Table 3.2 Mouse general health score scales

Scores	Description
1	mouse bright-eyed and alert, smooth coat with a sheen, responds to stimulus, shows interest in its environment
2	fur slightly ruffled, a loss of sheen to the coat, remains alert and active
3	fur noticeably ruffled, parts of coat forms clumps, not alert or active, less interested in environment outside of cage
4	mouse hunched over and lethargic, little interest shown in environment, fur clumped
5	mouse non-reactive to stimulus, fur has a “bottle brush” appearance, i.e., standing on end, mouse hunched over preferring to sleep than react to environment, mouse and paws are cold to touch, signs of hyperventilation when handled

3.18 Collection of blood and tissue samples

At the end of each experiment, animals were humanely euthanased by isoflurane overdose. Blood samples were obtained by cardiac puncture using a 1-ml syringe and 23-gauge needle. The blood was collected into different test tubes (EDTA/heparin/non-additives) according to the requirement of specific assays. Blood samples were processed within 30 min, except in the case of serum that was separated by centrifugation 2 hrs after bleeding. All whole blood samples were kept at RT and sera were kept at -20°C before use. Before excising tissue samples, the surface of the viscera was swiped with a sterile swab that was then cultured in BHI media to test for contamination of the viscera. The mesenteric lymph nodes (MLN), spleen, kidney and a sample of liver tissues were excised aseptically. A small piece (0.5x0.5 cm) of ileum (2 cm away from the caecum), caecum (middle portion), and colon (2 cm away from the caecum) were excised, fixed in Bouins Fluid (A2.12) for 8-12hrs and then transferred to 70% ethanol.

3.19 Bacterial translocation (BT) assay

For the BT assay, tissue samples were collected into a set of pre-weighed sterile 1.5ml eppendorf tubes containing 0.2ml of BHI broth and the tissue weight was recorded. The whole MLN, spleen, and a portion of liver were homogenised with a tissue grinder (Duell[®] 20, Kontes Glass co. or plastic tissue grinders, BEL Art Products), or a 1-ml syringe by sucking up and pushing down in the eppendorf tubes. 100µl of tissue suspensions or blood samples were plated separately on MRS, MRS-C, BHI, or coliform agar plates respectively as stated in the specific chapters. BHI and coliform agar plates were incubated aerobically; MRS and MRS-C plates were incubated anaerobically (Section 3.5.1) at 37°C for 72hrs. The growth of bacterial cells on agar plates was observed and the number of colonies was counted using a colony counter (A3.10). The positive cultures were identified by colony and cell morphology, oxidase and gram stain reactions, indole test, carbohydrate fermentation patterns (BBL CRYSTAL[™], Becton Dickinson, Maryland, USA), and/or RAPD finger printing techniques as stated in each specific chapter.

3.20 Haematology

Blood samples were collected into EDTA treated tubes. A blood smear was made for each sample and stained with Diff-Quik[®] Stain Set (A2.15). At least 300 leucocytes were counted under low power on a microscope to determine the differential percentages of white blood cells (lymphocytes, neutrophils, monocytes, and eosinophils). Total leucocytes (WBC), red blood cells (RBC), and platelet counts (PLC), and mean corpuscular volume (MCV), haemoglobin concentration (HB), haematocrit (HT, or packed cell volume PCV), corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were determined on a blood analyser (A3.11).

3.21 Histology

Intestinal tissue samples were processed on an automatic tissue processor (Shardon Elliott, Camberly, Surrey, England) and were embedded in paraffin wax. Tissue sections were cut at 6 µm thickness and stained with haematoxylin and eosin (H & E). The epithelial cell height, villous height, crypt depth, and mucosal thickness were

measured under a light microscope (Olympus BH2-RFCA) using a microscale. The longitudinally-oriented villi and crypts were measured according to the reported method by Sharma (1995). Briefly, villus height was measured from the tip to the base of each villus; crypt depth was measured from the bottom to the opening of each crypt; and the mucosal thickness (maximum) was measured from the tip of the villus, or the surface of the mucosa (for colon and caecum), to the interface of the lamina propria with the muscular mucosa. Ten measurements were taken at random from each sample for each parameter and the mean of these measurements was used for statistical analysis.

Spleen weight index (SWI) was expressed as the actual spleen weight (mg) divided by the body weight (g).

3.22 Measurement of protein concentration

The levels of total protein in samples (plasma or mucin suspensions) were determined with a BCA Protein Assay Kit (Pierce, USA).

The levels of plasma albumin were measured using the BCP Albumin Reagent (Sigma, 631-M) and a protein standard solution (Sigma, 540-10). The absorbance of reactions at the wavelength of 562nm was measured in a spectrophotometer (A3.2).

3.23 Measurement of carbohydrate concentration

Total plasma glucose concentration was determined using a Glucose 100 Trinder kit (Sigma Diagnostic Co. USA). The total carbohydrate concentration of mucin suspensions was measured by the phenol-sulphuric acid assay method (Chaplin, 1986). The absorbance of reactions at the wavelength of 490nm was measured in a spectrophotometer (A3.2).

3.24 Measurement of total plasma cholesterol

The levels of plasma total cholesterol were measured using the Cholesterol 500 reagents (Sigma, St. Louis, USA). The absorbance of reactions at the wavelength of 500nm was measured in a spectrophotometer (A3.2). The concentration of cholesterol (mg/dl) in samples was calculated with the formula: $[(ABS_{test} - ABS_{blank}) / (ABS_{calibrator} - ABS_{blank})] \times calibrator$ (mg/dl).

3.25 Preparation of spleen cell suspensions

Single spleen cell suspensions were prepared using the following procedures, which were carried out within a class II biohazard cabinet (Clyde Apac, BH 2000). Each spleen was washed twice individually in 2ml of complete RPMI media (A1.9) in a 12-well tissue culture plate (Costar) and then transferred into a fresh well containing 2ml RPMI media. The washed spleen was then cut into small pieces and dispersed using a 1-ml syringe by sucking up and pushing down until no visible particles were apparent. The cell suspensions were then transferred into 15ml sterile centrifuge tubes containing 5 ml RPMI media and centrifuged for 10 min at 1000rpm (Megafuge 1.0R, rotor 2252). After discarding the supernatant, the cells were resuspended in 5 ml sterile ACK lysis buffer (A2.13) and incubated at RT for 5 min with occasional mixing. 5ml of RPMI media was added to each tube, mixed and then centrifuged for 10 min at 1000rpm. The cells were washed twice in 5 ml of RPMI media by centrifugation and resuspended in 4ml of RPMI media. Five hundred microlitres of 5-fold diluted cell suspensions were transferred into a flow cytometer tube containing 2 μ l of propidium iodide (which was used to detect the dead cells) and the cell population was counted using a flow cytometer (A3.8). The population of viable cells was adjusted to the desired levels as indicated in each specific chapter.

3.26 Preparation of peritoneal macrophages

The abdominal skin of euthanased mice was aseptically opened and reflected. Eight to 10 ml of RPMI media was then injected into the peritoneal cavity using a 10ml syringe. The peritoneum was gently massaged several times before the media was withdrawn and collected into a 10ml vacutainer tube which was kept on ice inside a class II biohazard cabinet. The harvested cells were washed 3 times with 10ml of RPMI media by centrifugation (Megafuge 1.0R, 1000rpm, 10 min, 4°C) and then suspended in 4 ml of RPMI media. The number of cells in the suspension was determined by flow cytometry and then adjusted to the desired concentration as stated in each specific chapter (e.g. 1 x 10⁶ cells/ml).

3.27 ELISA for antibody assessment

Alkaline phosphatase (AKP) conjugated antibodies, were purchased from SeroTec (UK).

Serum antibody levels were measured using a standard ELISA method (Bhatia *et al.*, 1996). The wells of a 96-well microtitre plate (Nunc-Immuno™ plate, MaxiSorp™ surface, Nalge Nunc International, Denmark) were coated with 200 µl of coating buffer (A2.2) containing antigen specific to the antibody being detected, by incubation at RT or 4 °C (as stated in each specific chapter) overnight. The wells were washed three times with PBS-T buffer (A2.3) using a plate washer (A3.5). The uncovered sites of the wells were blocked with 200 µl of 1% BSA or 5% FCS by incubation at RT or 37°C (as stated in each specific chapter) for 2 hrs. The wells were again washed three times with PBS-T. 100µl of samples, controls, and standards diluted in PBS were added to triplicate wells and the plate was incubated at RT (2 hrs) or 4°C (overnight) as indicated in specific chapters. The plate was washed with PBS-T three times and rinsed with PBS once. 100µl of AKP-conjugated anti-mouse antibodies (affinity antibody, specific to the antibody in serum to be detected) was added to each well and the plate was incubated at 37°C for 1 hr. Following three washes in PBS-T, 50µl of AKP substrate solution (A2.14) was added into each well and the plate was incubated at RT for 30min to 1 hr until a clear colour had developed. 25µl of 3M NaOH or 1M H₂SO₄ was added to each well to terminate the reactions. The reaction absorbance at 405nm was read on an ELISA reader (A3.4).

3.28 Lymphocyte phenotyping

Flow cytometric analysis was used for monitoring the expression of CD4⁺, CD8⁺, CD3⁺, CD25⁺, and CD40⁺ antigens on peripheral blood leucocytes (PBL). The staining of PBL was performed using the method of Lloyd *et al* (1995) with some modifications. Briefly, 100µl of EDTA treated whole blood was incubated with 5µl of fluorescein-isothiocyanate (FITC) conjugated monoclonal antibodies (MoAbs) to CD4, CD8, or CD3 (Serotec) or phycoerythrin (PE) conjugated MoAbs to CD25 or CD40 (Serotec) on ice for 20 min. The cells were then washed twice in PBS by centrifugation (Biofuge,

4000 rpm, 10 min). The PBL cells were fixed with 100µl of 8% formaldehyde for 1 min and the erythrocytes were lysed by the addition of 1 ml sterile MQ-H₂O. After washing twice in PBS, the PBL were analysed on a FACSCalibur flow cytometer (A3.8) using an excitation wavelength of 488nm and an emission wavelength of 525nm for FITC-conjugated antibodies or 575 nm for PE-conjugated antibodies. The lymphocyte population was gated according to the light scatter profiles of PBL. Platelets and lysed RBC were excluded from the lymphocyte gate position according to their smaller forward scatters; granulocytes were excluded according to their side scatter and relatively larger forward scatter. The lymphocyte phenotype was expressed as the percentage of mononuclear leucocytes expressing particular molecular markers.

3.29 Spleen cell proliferation assay

In vitro proliferative responses of spleen cells to mitogens were determined using a commercial cell proliferation kit (Cell Proliferation ELISA kit, BrdU colorimetric kits, cat: 1647229, Boehringer Mannheim Corp., Indianapolis, USA) as previously described (Cross & Gill, 1999). Briefly, 50 µl of spleen cells in complete RPMI-1640 medium (2×10^6 cells/ml) prepared using the method described in Section 3.20 were aliquoted in triplicate into the wells of a 96-well flat bottom tissue culture plate (Nunclon™ Surface, DELTA, Nunc™, Nalge Nunc International, Denmark), and cultured in the presence or absence of T- and B-cell mitogens. Mitogens (50 µl/well), concanavalin A (ConA, 2.5µg/ml, Sigma), lipopolysaccharide (LPS, 5µg/ml, Sigma), phytohaemagglutinin (PHA, 1/75 dilution of the stock, or 10µg/ml, Sigma), pokeweed mitogen (PWM, 1/50 dilution of the stock, or 2.5µg/ml, Sigma), or MTg (4µg/ml) as an antigen were added to wells at a predetermined optimal concentration. Fifty microlitre of complete RPMI-1640 medium was added to the control wells. The cells were incubated at 37°C, in a humidified CO₂-air (5:95, v/v) atmosphere for 72 hrs or 96 hrs prior to the addition of 10µl of BrdU labelling reagent (1:100 dilution of stock). The cells were cultured at 37°C for another 16hrs after labelling and then centrifuged to remove the labelling medium. The cell proliferation over the final 16hrs of culture was determined by measuring the incorporation of 5-bromo-2'-deoxyuridine (BrdU labelling reagent) using peroxidase-conjugated anti-bromodeoxyuridine antibodies (anti-BrdU-POD) and a peroxidase substrate system. The absorbance of each well was read at 450nm using an ELISA plate reader (A3.4) and the results were expressed as mean absorbance values

with their standard errors for triplicate samples. The cell proliferation index was expressed as “Absorbance of mitogen positive wells / Absorbance of mitogen negative wells”.

3.30 Blood leucocyte and peritoneal macrophage phagocytosis

Assessment of the phagocytic activity of PBL or peritoneal macrophages by flow cytometry was based on the method of Wan *et al.* (1993). Briefly, 100µl of EDTA-treated whole blood or peritoneal macrophages (10^6 /ml cells) was incubated with 10µl of FITC-labelled bacteria (7.5×10^6 cfu/assay) (*E. coli* K12, FITC-conjugated *Escherichia coli* BioParticles, E-2861, Molecular Probes, Eugene, OR) at 37°C for 20 min (for macrophages) or 30 min (for PBL). The PBL were fixed with 100µl of 8% formaldehyde for 1 min and 1ml of ice-cold sterile MQ-H₂O was added to lyse the erythrocytes. The samples were then centrifuged for 10min at 4000rpm (Biofuge) and the cell pellets were resuspended in 0.5ml PBS, which was then transferred to flow cytometer tubes (FACS) containing 50µl of trypan blue (3 mg/ml). Peritoneal macrophage phagocytic cell activity was determined using the same method as that used for PBL, except that the fixation step was omitted. The level of phagocytic activity was detected on a flow cytometer (A3.8). The position of the phagocytic cells of PBL and peritoneal macrophages was gated according to the light scatter (forward scatter and 90° side scatter) of these cells. Ten thousand phagocytic cells or macrophages were counted per sample. The cells with phagocytic activity were defined as the cells which had taken up the FITC-labelled bacterial cells. The fluorescence of extracellular bacteria was quenched by trypan blue since the dead cells (FITC-*E. coli*) were selectively labelled by this dye. Thus only the intracellular FITC particles were measured. The phagocytic cell activity was expressed as the percentage of FITC-positive phagocytic cells out of the total number of phagocytic cells or macrophages in each sample.

3.31 Statistical analysis

Data were analysed using SAS software. For numerical data such as feed intake, body weight gain, carbohydrate or protein concentrations, the differences in means were

analysed by analysis of variance using GLM (General Linear Models) model, i.e. ANOVA analysis. The data were expressed as mean \pm SEM (standard error of mean).

For some data, such as growth rate and differences between treatment groups, correlation analysis was carried out using the linear correlation model.

For categorical (qualitative) data, such as the incidence of bacterial translocation or EAT, the Fisher's Exact Test (2-tail) was used to compare the statistical significance of differences between treatment groups. Fisher's Exact Test was used to correct the statistic error which may occur when chi-square test is used to analyse data in which one or more of the expected values are less than 5 (Hays, 1994; Freund & Simon, 1997; Lewis & Trail, 1999).

Statistical significance is indicated as follows, unless otherwise stated in the specific chapters:

NS = no statistical significance ($p > 0.05$)

* = $P < 0.05$

** = $P < 0.01$

In all figures, means with the same letter are not significantly different.

4 Probiotics and mucin degradation

Abstract

The mucus layer (mucin) coating the surface of the gastrointestinal tract plays an important role in the mucosal barrier system. Any damage or disturbance of this layer will compromise the host's mucosal defence function. In this study, the ability of HN001, HN017, and HN019 to degrade mucin *in vitro* was evaluated, in order to assess their potential pathogenicity and local toxicity. The LAB strains were incubated in medium containing hog gastric mucin (HGM, 0.2%) at 37°C for 48 hrs, following which any decrease in carbohydrate and protein concentration in the ethanol precipitated portion of the culture medium was determined, using phenol-sulphuric acid and BCA protein assays respectively. The change in molecular weight of mucin glycoproteins, following incubation with the test strains, was monitored by SDS-PAGE. To exam the ability of the test strains to degrade mucin visually and more directly, the test strains were also cultured on agarose containing 0.3% HGM, and incubated anaerobically for 72 hrs at 37°C. No significant change in the carbohydrate or protein concentration in mucin substrates was found following incubation with the test strains. No *de novo* mucin fragments were derived from the mucin suspension incubated with test strains, and no mucinolysis zone was found on agarose. These results demonstrate that the probiotic LAB strains tested here were unable to degrade gastrointestinal mucin *in vitro*, which suggests that HN001, HN017, and HN019 are likely to be non-invasive and non-toxic at the mucosal interface.

4.1 Introduction

Most probiotics including the LAB strains evaluated in this study are intended to be consumed orally. They deliver their health benefits through colonising or at least temporarily colonising the gastrointestinal tract (GIT). Therefore, the GIT is the first and probably the only site where probiotic organisms come into contact with the host. The primary local toxicity of these strains on the GIT can be detected by investigating their adverse effects on the mucosal surface.

The gut mucosal surface is coated with a gel-like mucus layer which consists primarily of a high molecular weight ($\sim 10^3$ kDa) mucus glycoprotein (mucin, Figure 4.1) (Smith & Podolsky, 1986; Smith *et al.*, 1995). The proposed primary function of this gel layer is to protect the delicate underlying epithelial cells from attack by corrosive acid and pepsin of the gastric juices from the stomach and duodenum, i.e. autodigestion (Allen *et al.*, 1984); shear forces generated by digestive processes (Corfield *et al.*, 1988; Smith *et al.*, 1995); and invasion by pathogens or potential pathogens (Cohen & Laux, 1995; Colina *et al.*, 1996). Gut bacterial pathogens must traverse this mucus layer before they adhere, colonise, and subsequently invade the epithelial cells. Any changes in the mucus content and structure will compromise the mucosal defence barrier functions contributed by the mucus layer (Cohen & Laux, 1995; Smith *et al.*, 1995).

It has been suggested that elimination of the mucus layer could result in an increase in the populations of bacteria adhering to the enteric surfaces, and also possibly facilitate the dissemination of normal intestinal bacteria into extra-intestinal tissues (Ruseler-van Embden *et al.*, 1989). The gut mucosal barrier, in addition to these primary roles, also prevents the uptake of un-degraded antigens and regulates the development of potentially harmful local and systemic immune responses (Reynolds, 1996). Therefore, an ability to degrade mucin is considered an important indicator of pathogenicity and local toxicity of lumen bacteria. Consequently mucin degradation test has been recommended as one of the important toxicity markers in the safety assessment of probiotic strains (Donohue & Salminen, 1996; Salminen & Donohue, 1996).

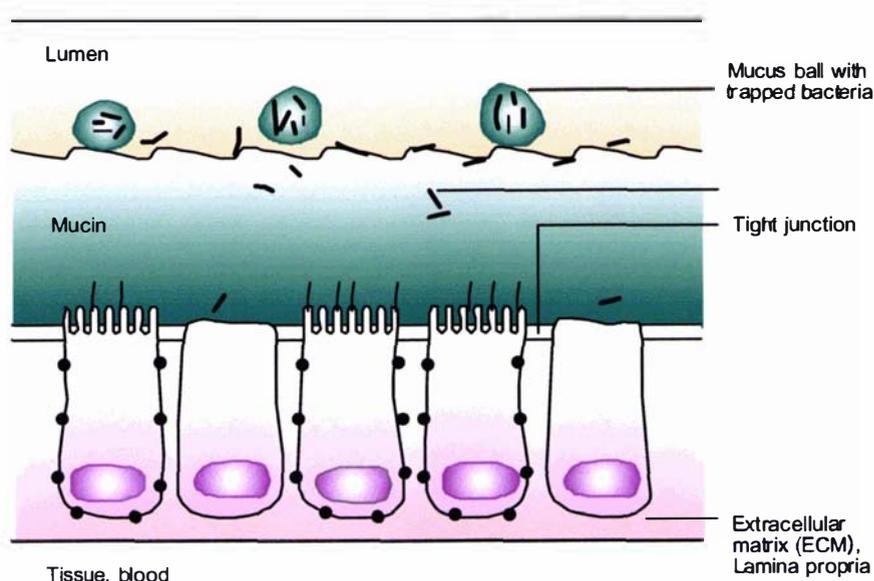


Figure 4.1 The defence function of the mucus layer on the surface of GIT—a simplified representation. Mucus layer and mucus balls within the lumen prevent most of the lumen bacterial cells from approaching the GIT epithelial cells.

4.2 Materials and methods

4.2.1 Bacteria strains

Lb. rhamnosus HN001, *Lb. acidophilus* HN017, and *B. lactis* HN019 were used as test strains; *Lb. acidophilus* LA-1 was used as the reference strain in this experiment. The mucinolytic positive control strains were isolated from a fresh faecal sample of a healthy adult male via the following procedure: 2~3 grams of the fresh faecal sample was suspended in 20 ml of sterile distilled water and then centrifuged at 1500 x g (Megfuge 1.0R) for 15 min. The supernatant was collected and kept at 0~4°C prior to use, while a portion was stored at -20°C for later use. The purified test and reference LAB cultures were subcultured in MRS or MRS-C broth for 24hrs prior to use. Two hundred microlitres of faecal microflora were inoculated into 10 ml BHI broth (A1.2) at 37°C for 24 hrs. These 24 hrs-cultures were used in later analysis and 2 ml of these cultures were autoclaved for 20 min at 121°C and then used as negative controls.

4.2.2 Mucin degradation in broth medium

Two hundred microlitres of the viable and the autoclaved bacterial cultures were incubated at 37°C for 48 hrs with 10 ml of medium B containing 0.2% (w/v) HGM,

with or without 3% (w/v) glucose. Each sample was assayed in triplicate, and growth was monitored by changes in the turbidity (OD_{610nm}) of the cultures. At the end of the incubation period, the culture suspensions were centrifuged at $10,000 \times g$ for 30 min and the 'bacteria-free' supernatants were collected into another set of centrifuge tubes, which were then heated at $100^{\circ}C$ for 5 min to inactivate any mucinolytic enzymes. Mucin glycoproteins were precipitated by the addition of cold 99.99% ethanol (the final ethanol concentration was 60% v/v). After centrifugation, the supernatants were aspirated and stored at $0-4^{\circ}C$, while the pellets were washed with 70% (v/v) ethanol and re-suspended in 0.5 ml 10 mM Tris-HCl buffer ($pH 8.0 \pm 0.1$). These ethanol-precipitated mucin suspensions, and the supernatants separated from it, were used to determine carbohydrate and protein concentration and for SDS-PAGE analysis.

4.2.3 Measurement of total carbohydrate and protein concentrations

Total carbohydrate and protein concentration of the culture supernatants and the mucin pellet suspensions were determined using the methods described in Section 3.22 and Section 3.23. 0.05% galactose (BDH) was used as a standard solution for carbohydrate analysis because galactose is the major carbohydrate component on mucin glycoprotein molecules (Piller & Piller, 1993).

4.2.4 Calculation of mucin degradation

The decrease of total carbohydrate and protein content in ethanol-precipitated mucin residues was used as an indicator of the degradation of oligosaccharide chains and protein back bones, respectively. Percent degradation of mucin carbohydrate and protein in each culture was determined using the formula: % degradation = $[1 - (\text{concentration in test samples} / \text{concentration in control sample})] \times 100\%$. The samples of autoclaved cultures were used as negative controls for specific strains. Positive mucin degradation was defined according to Miller and Hoskins's (1981) method. The coefficients of variation (CV) of the recovery rate of mucin carbohydrate and protein in autoclaved samples were determined (16% and 8.5% respectively). The lower recovery limit of carbohydrate and protein in cultures, where no mucin degradation occurred, was expected to be 68% and 83% respectively. Any more than 32% decrease in carbohydrate concentration, and /or more than 17% decrease in protein concentration in mucin samples, was defined as positive mucin degradation (Miller & Hoskins, 1981).

4.2.5 SDS-PAGE

The mini-gel slab method was used following a standard protocol (Gallagher & Smith, 1995) as described in Section 3.8. Different polyacrylamide gel concentrations (7.5 ~ 12.5%) were used and both silver staining (BIORAD kit), Coomassie blue (R-250) and Periodic acid-Schiff (PAS) staining methods (Section 3.9) were used to detect the change of molecular size and the loss of carbohydrate residues from the glycoproteins. For gels stained with multi-colour staining method, PAS staining was applied prior to stain with Coomassie blue. Any *de novo* band with a smaller molecular weight compared to the pattern of native glycoproteins (control group), or the loss of PAS positive stain of the mucin bands, was defined as positive mucin degradation.

4.2.6 Assessment of mucin degradation by petri dish assay

HGM and agarose (type-I-A, Sigma) were incorporated with medium B at concentrations of 0.5% (w/v) and 1.5% (w/v) respectively, with or without 3% (w/v) glucose. Agarose plates were made with autoclaved medium B-containing mucin. Ten microlitres of bacterial cultures were inoculated onto the surface of the agarose medium. The plates were incubated at 37°C anaerobically (BBL, Gas Pack Anaerobic System) for 72 hrs and subsequently stained with a reported method (Colina *et al.*, 1996). Briefly, 0.1% amido black in 3.5 M acetic acid was added into the plate and incubated at room temperature for 30 min. The plates were then destained with 1.2 M acetic acid until the mucin lysis zone (discoloured halo) around the colony of positive control cultures (faecal flora) appeared. The mucin degradation activity was defined as the size (diameter) of the mucin lysis zone.

4.3 Results

4.3.1 Bacterial growth

No bacterial growth was detected in the blank control (medium B inoculated HGM only, without cultures) or the heat-treated cultures (medium B inoculated with HGM and heat-killed bacterial cells). A negligible growth was observed in the test (HN001, HN017 or HN019) and reference cultures (LA-1) in medium containing no glucose, while the faecal floral cultures showed substantial growth in this medium. All of the

viable bacterial cultures, including test and reference strains, grew well in medium containing 3% glucose (Table 4.1).

Table 4.1 Growth of LAB or faecal microflora in different culture media

Cultures	Medium B		Medium B with 3% Glucose		Medium B with 0.3% HGM		Medium B with 3% Glucose, 0.3% HGM	
	OD [®]	pH	OD [®]	pH	OD [®]	pH	OD [®]	pH
HN001	0.139	7.1	1.512	3.5	0.269	7.1	1.489	3.5
HN017	0.114	7.1	1.477	3.5	0.252	7.1	1.303	3.5
HN019	0.100	7.1	1.724	3.5	0.356	7.1	1.728	3.5
LA-1	0.113	7.1	1.851	3.5	0.376	7.1	1.386	4.0
FF [#]	1.485	7.0	1.795	4.5	1.894	6.5	1.772	4.5
H-FF [§]	-0.029	7.1	-0.007	7.1	0.160	7.1	-0.017	7.1
Blank [*]	-0.021	7.1	0.009	7.1	0.121	7.1	0.005	7.1

[®] Absorbance (OD_{600nm}) changes of cultures after incubation at 37°C for 48 hrs

[#] Faecal flora

[§] Heat-killed faecal flora

^{*} No inoculums

Values in this table represent means of triplicate cultures.

4.3.2 Changes in total mucin carbohydrate and protein concentrations

No significant changes occurred in the total mucin carbohydrate (CHO) and protein (PRO) content in ethanol precipitated mucin pellets of medium containing 3% glucose, following incubation with different cultures. Compared with that of blank control and heat-treated cultures, the recovery percentage of mucin CHO and PRO in samples containing no glucose, and treated with faecal flora, dropped to a low level (Table 4.2), while the content of CHO and PRO in mucin samples treated with test and reference strains had only a slight change. Referring to the definition of mucin degradation described in Section 4.2.4, no significant mucin CHO and PRO degradation occurred in mucin samples treated with test strains (HN001, HN017, or HN019), or the reference strain (LA-1), while faecal flora demonstrated a dramatic mucinolytic activity.

4.3.3 SDS-PAGE analysis

The mucin residues of all the cultures from medium containing glucose showed nearly identical electrophoresis patterns following SDS-PAGE. Figures 4.2 and 4.3 demonstrate the distinct SDS-PAGE patterns of mucin samples incubated with

Table 4.2 Degradation of mucin carbohydrates and proteins

Treatment	% Recovery [#]		% Degradation ^{\$}	
	CHO*	PRO*	CHO*	PRO*
HN001	133.6	107.3	-33.9	-7.3
HN017	108.3	95.0	-8.3	5.0
HN019	96.7	144.7	3.3	-44.7
LA-1	95.3	96.1	4.7	3.9
Faeces	21.9	51.3	78.1	48.7

The values included in this table represent the means of triplicate tests.

[#] % Recovery was calculated by the formula: (concentration of test/concentration of control) X 100%.

^{\$} % Degradation was calculated by the formula: (1- (concentration of test/concentration of control)) x 100%

* CHO = carbohydrate; PRO = protein

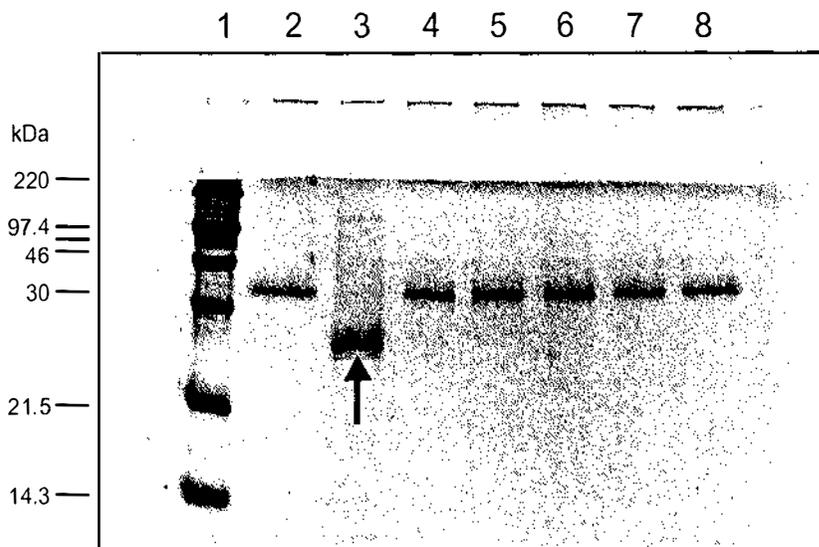


Figure 4.2 SDS-12.5% polyacrylamide gel analysis of mucin glycoproteins (I). The gel was silver stained. L1: Rainbow protein standards; L2: heat-treated faecal flora; L3: viable faecal flora; L4: reference strain LA-1; L5~L7: HN019, HN017; HN001; L8: blank control. A small *de novo* fragment of 25 kD molecular weight (indicated with an arrow) was detected in L3.

faecal flora, in comparison to those samples treated with test and reference strains. Test and reference cultures had identical SDS-PAGE patterns to that of the blank control samples. All native glycoprotein components were stained by PAS stain, the electrophoretic patterns of these samples presented as a diffused smear rather than a clear band (Figure 4.2). The PAS positive stain (pink) of the carbohydrate components

of the sample which was treated with faecal microflora faded, and a smaller *de novo* fragment was detected from this sample (L3 in Figure 4.3). Samples treated with test strains had identical electrophoretic patterns and PAS staining reactions to that of control (non-inoculated, i.e. no bacteria) samples.

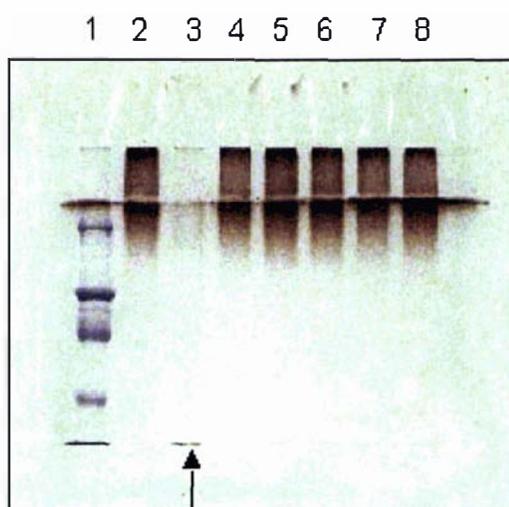


Figure 4.3 SDS-7.5% polyacrylamide gel analysis of mucin glycoproteins (II). The gel was stained with Coomassie blue and PAS. L1: protein standard ladder; L2: heat-treated faecal flora; L3: viable faecal flora; L4: reference strain LA-1; L5 ~ L7: HN019, HN017; HN001; L8: blank control. Faecal flora-treated samples lost their carbohydrate residue (PAS positive component) and gained a smaller *de novo* Coomassie blue-positive fragment (arrowed).

4.3.4 Assessment of mucin degradation in petri dish

No mucinolysis activity was detected in cultures on medium containing glucose, whereas in medium in which HGM was the only energy source (no glucose added), faecal flora clearly exhibited mucinolytic activity. In comparison, no mucinolytic zone appeared around the colonies of the test strains (Figure 4.4).

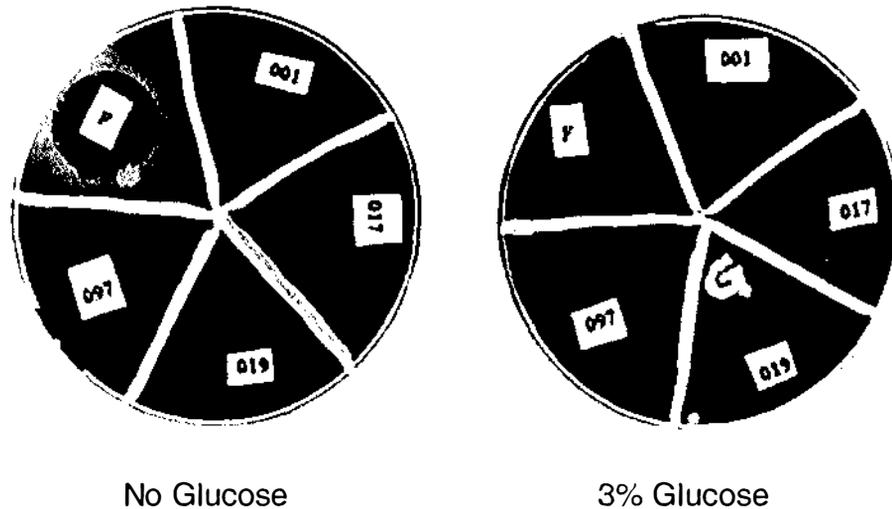


Figure 4.4 Mucin degradation assay in agarose petri dish. 001 = HN001; 017 = HN017; 019 = HN019; 097 = LA-1. The discoloured zone (halo) around colonies indicates mucinolytic activity of specific strains. Only faecal flora (F) exhibited mucin degradation activity in medium without glucose.

4.4 Discussion

The production of mucin-degrading enzymes has been suggested as a virulence determinant for a number of enteropathogens, including *Vibrio cholerae*, *Bacteroides fragilis*, *Shigella* spp, *Helicobacter pylori*, and *Yersinia enterocolitica* (Colina *et al.*, 1996). It is possible that an organism with extensive mucin-degrading activity not only has the potential to invade the host but can also facilitate mucosal penetration by other pathogens and toxic agents; conversely an organism that cannot degrade mucin is unlikely to have the potential to invade (Ruseler-van Embden *et al.*, 1995). Therefore, the mucin degrading activity of probiotic LAB strains HN001, HN017, and HN019 was investigated.

Commercially available hog gastric mucin (HGM) was used to assess the mucinolytic properties of the probiotic strains as it is similar in structure and chemical properties to human gastric mucin (Allen *et al.*, 1984). The ability of normal human faecal flora (1% of the total viable faecal flora) to degrade gastric mucin has been demonstrated in previous studies (Hoskins *et al.*, 1985; Macfarlane & Gibson, 1991). Our results confirmed this observation, and therefore faecal microflora were used as the positive mucin degrading control culture.

The ability of a strain to grow in an environment where a specific nutrient is the only energy source is an indicator of whether the test strain can utilise this nutrient. Test strains inoculated in a medium where mucin was the only carbohydrate source had a negligible growth (Table 4.1), whereas the positive control strains (faecal microflora mixture) grew reasonably well in this medium. The addition of glucose into medium B resulted in normal growth for all strains. This result indicated that the test strains (HN001, HN017, HN019) may lack enzymes required to metabolise the components of mucin glycoprotein, therefore they could not utilise this large complex glycoprotein substrate as the carbohydrate source for growth. The growth of faecal microflora in medium without glucose failed to induce a decrease in pH similar to that observed in the presence of glucose. This suggests that a decrease in pH in the presence of 3% glucose was most likely due to the growth of faecal LAB species; and that the bacterial growth as seen in faecal cultures containing HGM was most probably due to the growth of non-acid producing bacteria. In other words, most of the gut LAB are unlikely to degrade mucin.

The degradation of mucin would undoubtedly reduce the molecular size of glycoproteins. For the mucin glycoprotein, where carbohydrate residues constitute most of the molecular mass (85% w/w) (Smith & Podolsky, 1986; Ruseler-van Embden *et al.*, 1995), the amount of change in carbohydrate residues on the protein backbone can be detected by SDS-PAGE (Carlsson, 1993; Corfield & Warren, 1996). Mucin glycoproteins used in these experiments were purified by the ethanol-precipitation method, where the native mucin components were precipitated by ethanol. Thus, theoretically, the mucin degrading activity of bacteria can be revealed by changes in the carbohydrate, and/or protein content of the ethanol precipitated mucin portion. While there was no change in the overall carbohydrate or protein concentration in the supernatants of mucin samples incubated with faecal flora, obvious mucinolytic activity was demonstrated in other assays employed in the present study. This result may indicate that the degraded mucin components were utilised by enteric bacterial strains as an energy source, rather than just simply being released into the medium. Thus the carbohydrate and protein concentration, as well as the glycoprotein molecular weight changes, in the ethanol-precipitated portion of the mucin samples, were used as indicators of mucin degradation.

SDS-PAGE analysis revealed that mucin samples treated with test and reference LAB strains had identical electrophoretic patterns to that of the control (i.e. no bacteria), and heat-treated faecal samples (Figures 4.2, 4.3). In comparison, mucin samples incubated with viable faecal flora exhibited a distinct electrophoretic pattern in which the native mucin component (heavier band) disappeared, and was replaced by a smaller *de novo* fragment. Furthermore, the PAS positive staining carbohydrate components, which have been widely used to detect mucin carbohydrate (Corfield & Warren, 1996), almost disappeared from the faecal flora treated sample, while the *de novo* smaller fragment was devoid of carbohydrate residues (i.e. PAS stain negative, Coomassie blue stain positive). The inability of test strains to degrade mucin was also demonstrated by this assay.

To confirm the results of these two experiments, and to demonstrate the mucinolytic activity visually, a petri dish assay was conducted. This method has been used by Colina *et al.* (1996) to detect the mucin degradation activity of *Candida albicans*. The results presented in Figure 4.4 showed that the test strains did not produce a mucinolytic zone around their colonies, while there was a large mucinolytic zone around the colony of faecal flora. None of the strains exhibited mucin degrading activity in agarose medium containing glucose, which correlates to the result of the mucin degradation test in broth medium. These findings demonstrate that the mucin degrading ability of faecal flora is nutrient-dependent, so that in an environment where carbohydrate is readily available (e.g. glucose), faecal flora had no mucinolytic activity. In contrast, in the medium where mucin was the only carbohydrate source, faecal flora exhibited extensive mucinolytic activity. In other words, glucose is used preferably by faecal microflora. This indicates that the composition of the medium in which the bacterial strains are inoculated is critical for detecting mucinolytic activity of specific strains.

From these experimental results it can be concluded that the mucin degrading activity of specific microorganisms in faecal microflora are inducible, i.e. within an environment where ready-for-use nutrients are available (such as the GIT of healthy human subjects), the bacterial cells with mucinolytic abilities may not produce or secrete the mucinolytic enzymes, and do not degrade mucin. However in the situation in which a nutrient stress presents, and there are not enough ready-for use nutrients, the selective microorganisms with mucinolytic abilities will produce or secrete mucinolytic enzymes and utilise

gastric mucin to survive. In other words, most microorganisms from GIT microflora do not degrade mucin under normal condition, but when the host is under stress (such as gastrointestinal disorders, starving, bacterial overgrowth in small intestine), some bacterial cells may exhibit their mucin degrading activities.

In summary, these experiments have demonstrated that the test probiotic strains HN001, HN017, and HN019 have no ability to degrade mucin *in vitro*, while in the same assays faecal flora exhibited obvious mucin degrading activity. These results are consistent with the findings of previous studies (Vercellotti *et al.*, 1977; Norin *et al.*, 1994; Ruseler-van Embden *et al.*, 1995). Our findings also demonstrated that the mucin degradation ability of faecal flora is nutrient-dependent, the presence or absence of glucose in medium has a decisive effect on the expression of mucin degrading activity of test cultures. Test LAB strains (HN001, HN017, or HN019) did not express mucin degrading activity in medium either with or without ready-for-use nutrients (e.g. glucose), so they may have no ability to degrade mucin *in vivo*.

Based on these results, we can conclude that the probiotic strains HN001, HN017, and HN019 do not degrade gastric mucin. They are likely to be non-toxic, and devoid of invasive pathogenicity in the GIT mucosal defence system of host animals, and therefore likely to be safe for consumption.

5

Effect of probiotic strains HN001 and HN019 on platelet aggregation *in vitro*

Abstract

The effect of probiotic strains HN001 and HN019 on the activation and aggregation activities of human blood platelets was investigated using flow cytometry. Whole blood samples from 6 healthy individuals were incubated with HN001 or HN019 *in vitro* and subsequently labelled with platelet specific monoclonal antibodies (MoAbs), fluorescein isothiocyanate (FITC) conjugated anti-CD41a (which reacts with both resting and activated platelets) and phycoerythrin (PE)-streptavidin conjugated anti-CD62p (which reacts specifically with activated platelets), before analysis by flow cytometry. Platelet rich plasma (PRP) was used to assist the gating of the platelet cluster. Adenosine 5'-diphosphate (ADP) and epinephrine were used as the physiological agonists. The mean fluorescence intensity (MFI) of PE and percentage of platelets expressing the CD62p marker were used to assess the degree of platelet activation. The percentage of CD62p positive platelets together with the light scatter profiles of the agonist-activated platelets were used to identify the occurrence or degree of platelet aggregation. Results from this work showed that HN001 and HN019 were unable to induce spontaneous platelet activation and aggregation; they also failed to exacerbate the platelet aggregation activity induced by ADP and epinephrine. These findings suggest that the test probiotic strains *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* HN019 are unlikely to participate in the pathogenesis of infective endocarditis (IE) or other thrombotic disorders.

5.1 Introduction

The interaction between platelets and blood-borne bacteria is likely to be part of the pathophysiological mechanism of septicaemia and disseminated intravascular coagulation, as well as bacterial infective endocarditis (IE). IE is one of the most frequently encountered problems associated with opportunistic infections caused by lactic acid bacteria (LAB), particularly lactobacilli (Gallemore *et al.*, 1995; Adams & Marteau, 1995; Pelletier *et al.*, 1996). Platelet activation and aggregation are largely believed to contribute to the pathogenesis of IE, and aggregation of platelets by lactobacilli is thought to be an important contributory factor in the progression of *Lactobacillus* endocarditis (Herzberg *et al.*, 1992; Harty *et al.*, 1993; 1994; Gasser, 1994; Adams, 1999).

It has been reported that platelets in plasma, activate and aggregate in response to incubation with selected Gram-positive bacteria including LAB strains (Herzberg & Brintzenhofe, 1983; Herzberg *et al.*, 1983; Kurpiewski *et al.*, 1983). In a later study Herzberg and coworkers (1992) found that inoculation of New Zealand white rabbits intravenously with a bacterial strain that can cause platelet aggregation *in vitro* (i.e. Agg⁺ phenotype, such as *Streptococcus sanguis* 133-79), consistently caused IE with significantly larger vegetation (the colonisation of a platelet-fibrin clot on the endothelial surface of heart by bacteria), more gross lesions in major organs, and greater mortality than inoculation with Agg⁻ phenotype strains (i.e. strains unable to induce aggregation). It has been also reported that the isolates of bacteria including *Lb. rhamnosus* from patients with IE uniformly induced irreversible platelet aggregation *in vitro* (Kessler *et al.*, 1987; Harty *et al.*, 1993; 1994).

The specific mechanisms of the interaction between bacteria and platelets have been suggested in previous studies (Clawson & White, 1971; Durack & Beeson, 1972; Sullam *et al.*, 1987). It has been found that some lactobacilli can produce enzymes (e.g. glycosidases and proteases) that enable the breakdown of human glycoproteins and the synthesis and lysis of human fibrin clots, these processes could aid the colonisation and survival of bacteria initiating an endocarditis vegetation (Oakey *et al.*, 1995; Pelletier *et al.*, 1996; Herzberg, 1996). Furthermore, researchers have found that most oral lactobacilli are commonly able to induce platelet aggregation *in vitro* and could thus

potentially cause IE *in vivo* if they came into contact with platelets in blood (Harty *et al.*, 1993; Salminen *et al.*, 1998a; Adams, 1999).

LAB from the gastrointestinal tract or oral cavity may be introduced into the bloodstream by poor dental hygiene, gastrointestinal lesions or surgical procedures, so they may contribute to the pathogenesis of IE and other pathological process associated with platelet aggregation (Aguirre & Collins, 1993). Therefore a lack of platelet aggregation potential has been listed as one of the important criteria for probiotic selection (Harty *et al.*, 1993; Donohue & Salminen, 1996; Kirjavainen *et al.*, 1999). Investigation of the platelet aggregation ability or production of IE-associated enzymes has been recommended to evaluate the intrinsic properties of probiotic strains (Pelletier *et al.*, 1996; Korpela *et al.*, 1997; Marteau & Salminen, 1997). This study examined the potential of HN001 and HN019 to induce or enhance human blood platelet activation and aggregation *in vitro*.

5.2 Materials and methods

5.2.1 Preparation of probiotics HN001, HN019, and *Lb. GG*

Stock cultures of test strains *Lb. rhamnosus* HN001, *B. lactis* HN019, and a commercial probiotic strain *Lb. rhamnosus* GG (*Lb. GG*) used as reference strain or negative control, were propagated using the method described in Section 3.5 before being subjected to testing. The cells were washed 3 times in cold PBS and resuspended to an OD₆₁₀ of 1.0±0.02 and then concentrated 4-fold to give a suspension containing ~ 2x10⁹ cfu /ml (Harty *et al.*, 1993; Korpela *et al.*, 1997).

5.2.2 Chemicals and monoclonal antibodies

Chemicals: Adenosine 5'-diphosphate (ADP) reagent (Cat: 885-3), epinephrine reagent (Cat: 885-5) and prostaglandin E₁ (PGE₁ Cat: P5515) were purchased from Sigma Diagnostics®(USA). Working solutions (ADP 0.2mM/L, epinephrine 0.1mM/L, and PGE₁ 3µM/L) were prepared freshly before use. ADP and epinephrine were used as physiological agonists to induce platelet activation and aggregation, PGE₁ was used as an antagonist to block thrombin-induced activation.

Monoclonal antibodies: Fluorescein isothiocyanate (FITC) conjugated anti-CD41a (Cat 31084X, Lot M037162) and phycoerythrin (PE)-streptavidin conjugated anti-CD62p (Cat 31795X, Lot M03785) were purchased from PharMingen International (Australia).

5.2.3 Collection and preparation of blood samples for platelet aggregation assays

Blood samples were collected from 6 normal healthy individuals (aged 24 ~ 42 years) who were having a blood test prior to receiving a hepatitis B vaccination at the Massey University Student Health Service. The written consent from these blood donors was obtained before the blood collection. These individuals were nonsmokers, had had no alcohol consumption within the last 48hrs, and were drug-free for at least 2 weeks. A light standard breakfast was allowed before collecting the blood (at 10:00 ~ 10:30am). Two millilitres of blood was collected into a vacutainer tube (heparin anticoagulant). Twenty microlitre samples of whole blood were immediately transferred into a set of 2-ml eppendorf tubes containing Tyrode's buffer (pH 7.4, Section A 2.16) and different reagents or bacterial cells as indicated in Table 5.1.

Table 5.1 Reaction system for platelet aggregation test

Tubes	LAB [®]	Tyrode's buffer	Fixatives [#]	EPN [*] ADP	PGE ₁	Blood
1		100µl				20µl
2		50µl	50µl			20µl
3		50µl		25µl each		20µl
4		50µl			50µl	20µl
5	5µl	95µl				20µl
6	5µl	45µl	50µl			20µl
7	5µl	45µl		25µl each		20µl
8	5µl	45µl			50µl	20µl

[®]LAB Either HN001, HN019 or *Lb. GG*
[#]Fixatives 8% formaldehyde in PBS, pH 7.4
^{*}EPN Epinephrine

In these reaction systems, the ratio of bacterial cells to platelets was approximately 1:1. The samples were incubated at room temperature (RT) for 30 min before adding 100µl of 8% formaldehyde (pH 7.4) to stop the reactions. After a 1 min incubation at RT, 1 ml of ACK lysis buffer (Section A 2.13) was added into each tube and incubated for 10 min at RT. The fixed platelets were then harvested by centrifugation (4,000 x g, RT, 6 min). Fifty microlitres of Tyrode's buffer and 5µl each of FITC-CD41a and PE-CD62p MoAbs were added into each tube, which were then incubated at RT in the dark for 20 min. One millilitre of PBS was added to each sample before flow cytometry analysis.

5.2.4 Flow cytometry analysis

Blood samples prepared as above were analysed by flow cytometry on a Becton Dickinson FACSCalibur (Section A3.8) 30min after labelling with MoAbs. Light scatter and fluorescence data were obtained with gain settings (FITC-CD41a: 520 voltage; PE-CD62P: 566 voltage) in the logarithmic mode (Shattil *et al.*, 1987; Laffi *et al.*, 1996). The platelets were primarily distinguished from erythrocytes and leucocytes on the basis of their forward- and side-light scatter profiles and were further confirmed by running a platelet rich plasma (PRP) sample which was prepared following a standard method (Ault & Mitchell, 1994). According to their much smaller scatter profiles, debris or "machine noise" was excluded from the platelet gate position. By setting up the appropriate forward- and side-scatter threshold, a gate was set around the platelets. Ten thousand cells within the 'platelet' gate (gate 1, i.e. R1) were analysed and the cells expressing FITC-CD41a (a platelet specific marker) were selected as "gate 2" (R2) for further analysis. A new density plot (side-scatter *versus* FITC fluorescence intensity) of cells from "gate 2" was plotted to set up the real platelet gate position, "gate 3" (R3). The expression of PE-CD62p (the activated platelet specific marker) was displayed in a quadrant dot plot (PE-CD62p fluorescence intensity *versus* FITC-CD41a fluorescence intensity). The mean fluorescence intensity (MFI), percentage of PE-CD62p positive cells from "gate 3", and the light scatter profiles of cells from "gate 2" or "gate 3" were used to evaluate the degrees of platelet activation or aggregation. Referring to the fluorescence profiles of non-labelled platelets in PRP and samples treated with fixative or PGE₁ before labelling with CD62p, the antibody positive platelets were defined as those cells with fluorescence intensity higher than Log 10¹.

5.2.5 Haematology

Total red blood cell (RBC) and platelet counts, haemoglobin concentration (HB), haematocrit (HT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were determined on a Roche Cobas Minos (ABX, Levallois, France).

5.3 Results

All the haematology parameters (RBC, HB, HT, MCV, MCH, MCHC) of blood samples were in the normal range of the human population. In combining the use of PRP and MoAb CD41a, the platelet cluster was located and accurately gated (Figure 5.1a~c). CD41a positive cells can be easily distinguished from negative cells on the histogram plot (Figure 5.1b).

5.3.1 Platelet activation

The platelets incubated with the test probiotic LAB strains HN001, HN019, or reference strain *Lb. GG* had a similar CD62p expression percentage and MFI with that of resting platelets (4.6 ~ 8.8% and 107 ~ 220 respectively). ADP and epinephrine treated platelets had much higher levels of CD62p positive cells (64.25%) and totally different fluorescence profiles (MFI 2910.3) compared to the resting, or probiotic-treated platelets (Table 5.2, Figures 5.2 and 5.3). The CD62p positive platelet peak on the histogram plot of samples treated with ADP and epinephrine was obviously right shifted (i.e. fluorescence increased) compared to that of resting platelets (Figure 5.2). The activation of platelets induced by physiological agonists was clearly demonstrated in Figure 5.3; in contrast, resting (PGE₁ treated) or probiotic-treated platelets had negligible CD62p expression. Results displayed in Figure 5.3 also show that incorporation of probiotic LAB strains HN001, HN019, or *Lb. GG* did not further increase the expression of CD62p in samples treated with ADP and epinephrine. Following incubation with test probiotic strains, the percentage and MFI of CD62p positive platelets treated with agonists were slightly decreased compared to the samples treated with ADP and epinephrine only, although the only statistically significant difference was with the *Lb. GG* treated samples (Table 5.2).

Table 5.2 Platelet activation by probiotic strains

Treatment	Platelet Fluorescence (PE- CD62p)	
	Percentage positive [*]	Fluorescence intensity ^{##}
PGE1	5.185 ± 0.9616 ^c	101.50 ± 17.820 ^c
HN019	8.864 ± 0.9227 ^c	220.80 ± 35.580 ^c
HN001	4.658 ± 0.8046 ^c	107.70 ± 20.230 ^c
<i>Lb.</i> GG	5.820 ± 1.2516 ^c	145.40 ± 37.740 ^c
ADP+ENP [@]	64.25 ± 4.2960 ^a	2910.3 ± 372.23 ^a
HN019 +ADP+ENP	64.27 ± 11.060 ^a	2596.3 ± 935.12 ^{ab}
HN001 +ADP+ENP	59.09 ± 10.440 ^{ab}	2249.5 ± 748.64 ^{ab}
<i>Lb.</i> GG+ADP+ENP	50.38 ± 11.103 ^b	1755.7 ± 593.73 ^b

@ Epinephrine; # The values of MFI multiplied by the percentage of CD62p positive cells

* Statistical significance (p<0.001); Means with same letters have no significant statistical difference.

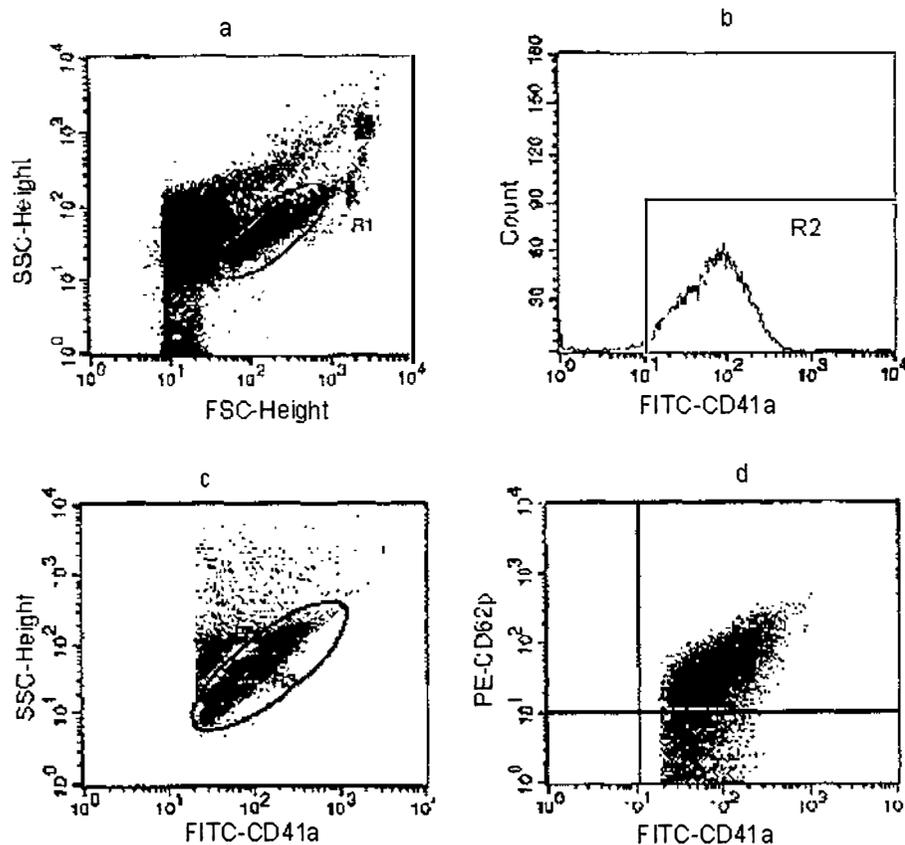


Figure 5.1 Platelet gating and analysis by flow cytometry. The platelet cluster was located according to the lighter scatter profiles of PRP, and confirmed using MoAb FITC-CD41a (a). The fluorescence profiles (FITC-CD41a) of cells within the platelet cluster (R1) are displayed on graph (b); CD41a positive cells were re-selected as (R2) according to their light scatter to exclude the leucocyte-platelet conjugates displayed in graph (c). The quadrant dot plot graph (d) demonstrates the percentage of doubly labelled platelet cells (both CD62p and CD41a positive cells).

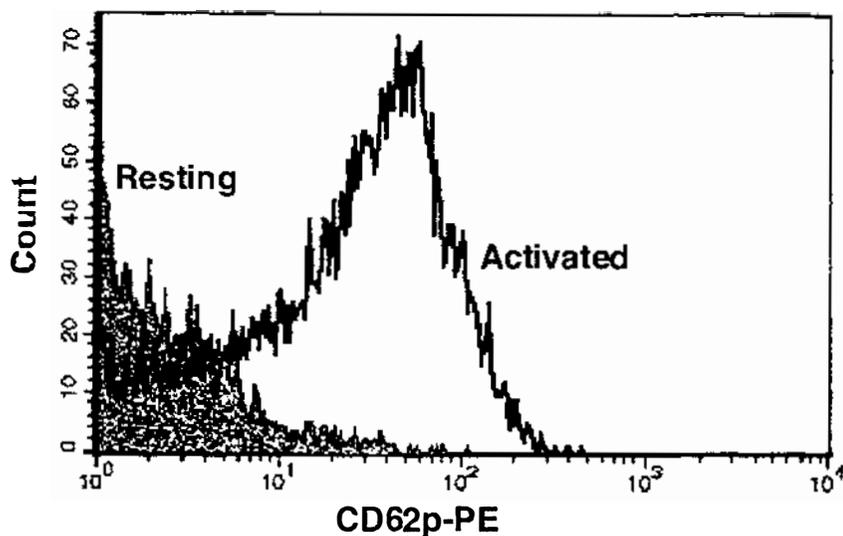


Figure 5.2 Changes in platelet fluorescence profile following activation. Platelets were incubated with PGE1 (resting) or ADP and epinephrine (activated). The flow cytometry analysis showed that activated platelets expressed a much stronger PE-CD62p fluorescence intensity than that of resting platelets.

5.3.2 Platelet aggregation

As displayed in Figure 5.4, resting platelets had a much lower forward scatter height (Figure 5.4a) than that of ADP and epinephrine-treated platelets (Figure 5.4b), the cluster of activated platelets was obviously right-up shifted, which reflects the increment of the expression of platelet activation markers (CD62p) and the size of platelet particles. Blood samples incubated with probiotic strains had very similar light scatter profiles to that of resting platelets. Incorporation of probiotics in the reaction systems of platelets treated with ADP and epinephrine did not change the light scatter profiles of the activated platelets. This data demonstrate that HN001, HN019 and *Lb.* GG did not induce, or exacerbate platelet aggregation.

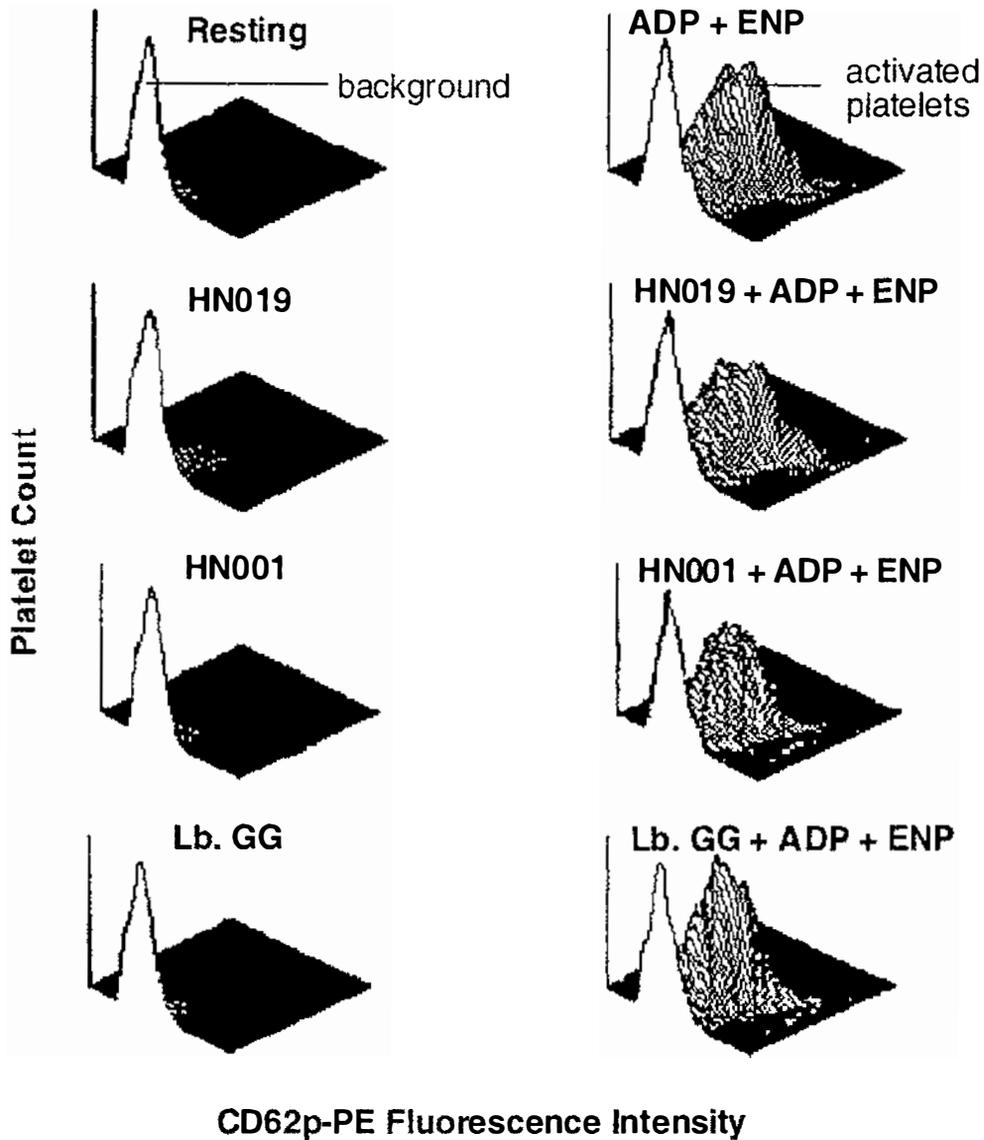


Figure 5.3 Fluorescence profiles of platelets incubated with test LAB strains. Resting platelets (left column) have easily distinguishable fluorescence (PE-CD62p) profiles from that of activated platelets (right column). HN001, HN019, or *Lb. GG* did not change the fluorescence patterns of platelets compared to the resting platelets; nor did these LAB strains affect the fluorescence profiles of activated platelets that were treated with ADP (adenosine 5'-diphosphate and ENP (epinephrine).

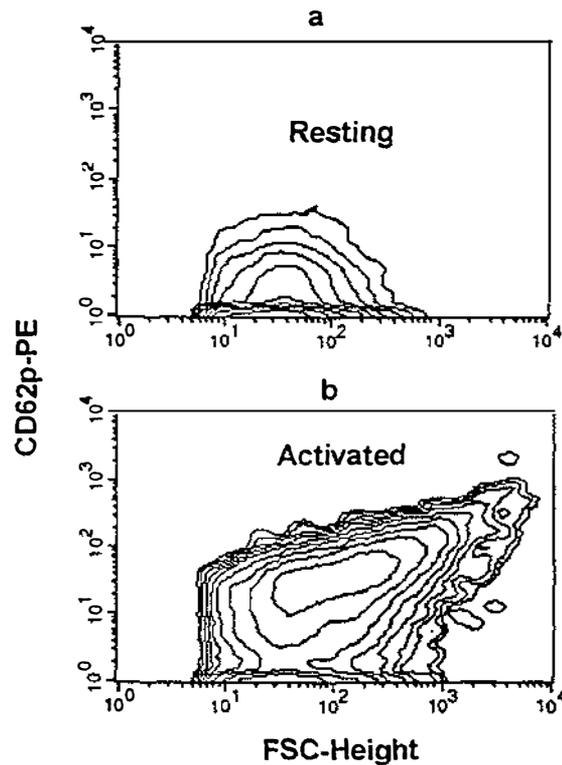


Figure 5.4 Changes in light scattering profiles of activated platelets. Resting platelets (a) have lower CD62p expression rate (fluorescence intensity) and forward scatter height than that of activated platelets (b). Both fluorescence intensity and forward scatter height of platelets increased one log scale after activation. Forward scatter increments indicate the occurrence of platelet aggregation.

5.4 Discussion

Lb. rhamnosus HN001 and *B. lactis* HN019 have been isolated from our current food chain (cheese and yoghurt respectively), and have thus probably been consumed by humans for centuries without any safety concerns. The experiments conducted in the previous chapter (Chapter 4) have demonstrated that test strains HN001 and HN019 have no ability to degrade gastric mucin. In a study by Arunachalam and coworkers (2000), no obvious adverse effects on elder individuals' health was identified following 3-weeks consumption of HN019 in low fat milk. In a more recent study, Tannock *et al.* (2000) reported that consumption of HN001 is safe for humans. All of these factors indicate that the probiotic strains tested here are highly unlikely to be pathogenic. However, bacteria from the gut or oral cavity have the chance to be passively introduced into blood stream by surgical or other invasive procedures, hence these 'non-pathogenic' LAB still may have the potential to cause infections or participate in the

pathogenesis of some diseases including IE. Platelet aggregation is the initiating key event in the thrombosis process and has an important contribution in the progression of IE. In this case, a strain with platelet aggregating properties may be more virulent than other non-aggregating strains. In other words, strains that do not aggregate platelets should be 'safer' for human consumption than the aggregating strains. This study was therefore carried out to verify the safety of HN001 and HN019 on this aspect.

Lb. GG was included as a reference strain as it possesses the most comprehensive safety data of all of the commercial probiotic strains (Saxelin, 1997) and has been shown to have no ability to induce platelet aggregation *in vitro* (Korpela *et al.*, 1997). To assess the normal functions of tested platelets and observe any potentially exacerbate effect of test LAB on platelet aggregation under physiological conditions, ADP and epinephrine were used as the physiological triggers of platelet aggregation. Both PGE₁ (an antagonist) and fixatives were used to achieve minimum activation of platelets that were used as the negative control. A ratio of approximately 1:1 of platelets to bacterial cells, which was reported as the optimal ratio to induce platelet aggregation, was applied in this study (Kurpiewski *et al.*, 1983; Kessler *et al.*, 1987; Harty *et al.*, 1993). Flow cytometry using platelet-specific MoAbs, used in this study, is the most sensitive and specific technique for studying platelet function (Abram *et al.*, 1990; Ault & Mitchell, 1994). Previously, it has been reported that there is inter-individual variation in the degree of augmentation of platelet aggregation (Harty *et al.*, 1993; Korpela *et al.*, 1997). Thus, 6 whole blood samples were included in this study to reduce the artificial effects caused by individual variation. In this study, no obvious inter-individual variation in the degree of augmentation of platelet activation and aggregation was observed; this may be related to the differences in experimental methods between current and previous studies.

Under these experimental conditions, ADP and epinephrine induced extensive platelet activation and aggregation (Figures 5.2 and 5.3, Table 5.2) in all blood samples, but the test LAB strains HN001, HN019, or *Lb. GG* failed to induce any spontaneous, or to enhance agonist-induced, platelet activation or aggregation. HN001 and *Lb. GG* belong to the same species (*Lb. rhamnosus*), so may share some common characteristics. According to Gasser (1994) and Adams (1999), the overwhelming majority of non-enterococcal LAB endocarditis cases reported have been caused by *Lactobacillus*

species particularly *Lb. rhamnosus* and *Lb. casei* or *Lb. paracasei*. However, the results obtained here demonstrated that HN001 is unlikely to be involved in the pathogenesis of IE. Furthermore, in a total of 53 cases of endocarditis reported over 55 years (1938 ~ 1993), no cases have been associated with *B. spp.* (Gasser, 1994). Therefore, there are no reports documenting the platelet aggregation properties of bifidobacteria.

According to Kirjavainen *et al.* (1999), the incidence of LAB caused bacteraemia is quite low (0.24%, 8 out of 3317 blood culture isolates) and in most cases patients with positive LAB cultures had a severe underlying disease that predisposed them to bacteraemic complications. Furthermore, *Lactobacillus* is only a rare cause of endocarditis (Sussman *et al.*, 1986), being reported at a rate of less than 1 case per year (Gasser, 1994). Nevertheless, the ability to aggregate human platelets is found to be a normal attribute of the lactobacilli in the oral cavity of normal human subjects (Harty *et al.*, 1993). Therefore, LAB strains with no platelet aggregating activity might have a limited possibility to participate in the pathogenesis of IE.

In conclusion, the probiotic LAB strains HN001 and HN019 are unable to induce spontaneous platelet activation, and do not enhance agonist-induced platelet aggregation processes. Therefore, they are not likely to have the potential to participate in the pathogenesis of infective endocarditis, and from that perspective, are unlikely to contribute to vascular/coronary heart disease.

6

Antibiotic susceptibility and plasmid profiles of HN001, HN017, HN019 and HN067

Abstract

The antimicrobial susceptibility and plasmid profiles of HN001, HN017, HN019, and HN067 were investigated in this study. Eighteen commonly used antibiotics were examined by disk diffusion method. Southern blotting and DNA hybridisation techniques were applied to verify the identity of DNA bands isolated from the LAB. The strains had very similar antibiotic susceptibility profiles to reference commercial probiotic LAB strains. Most of the strains investigated were sensitive to Gram-positive spectrum antibiotics (erythromycin, novobiocin), β -Lactams (penicillin, ampicillin, cephalothin) and broad-spectrum antibiotics (rifampin, spectinomycin, tetracycline and chloramphenicol). In contrast, they were resistant to most of the Gram-negative spectrum antibiotics (fusidic acid, nalidixic acid, polymyxin B) and aminoglycosides (neomycin, gentamicin, kanamycin and streptomycin). Three *Lb. rhamnosus* strains HN001, HN067 and *Lb. GG* and two other LAB strains (*Bifidobacterium lactis* Bb12, *Lb. plantarum* HN045) were resistant to vancomycin, although they were highly susceptible to 8 other commonly-used antibiotics. Plasmid screening experiments demonstrated that none of the LAB strains tested carried plasmids, which indicates that the antibiotic resistance attributes are not associated with plasmids.

6.1 Introduction

Bacterial antibiotic resistance is one of the major health care threats worldwide (Huovinen, 1999) and the increasing difficulties in treating infectious diseases caused by antibiotic resistant pathogens, has even been described as a global health crisis (Tollefson *et al.*, 1999; Skold, 2000). Although the misuse (or clinical overuse) of antibiotics is attributed as one of the major causative factors for the development of antibiotic resistant strains, the spread of resistant strains from person to person and from one ecosystem to another (e.g. animal to human) is also an important consideration (Khachatourians, 1998; Williams, 2000).

Lactic acid bacteria (LAB) are resistant to some antibiotics. Normally the resistance is intrinsic and not transmissible (Lim *et al.*, 1993; Charteris *et al.*, 1998a,b). Therefore no particular safety concern is associated with the intrinsic antibiotic resistance. However, some antibiotic resistance attributes of LAB strains are known to be plasmid-linked, such as resistance to chloramphenicol (*Lactobacillus plantarum*)(Ahn *et al.*, 1992), erythromycin (*Lb. reuteri*) (Axelsson *et al.*, 1988), tetracycline (*Lb. fermentum*)(Ishiwa & Iwata, 1980), gentamicin (*Lb. acidophilus*, *Lb. reuteri*)(Vescovo *et al.*, 1982), and vancomycin (*Lb. acidophilus*, *Enterococcus faecalis*, *E. faecium*)(Johnson *et al.*, 1990). Plasmid-associated traits, including antibiotic resistance, have the potential to spread to other strains, species, and even to other genera of bacteria (Thompson & Collins, 1989; Wang & Lee, 1997). Among the antibiotic resistance attributes, transmissible vancomycin resistance is particularly noteworthy because vancomycin is one of the last effective antibiotics that is known to be broadly efficacious against clinical infections caused by multidrug-resistant pathogens (Eliopoulos *et al.*, 1994; Rowe, 1996; Morelli & Wright, 1997). Several LAB, including species of enterococci (Quintiliani *et al.*, 1993; Adams & Marteau, 1995; Morelli, 1996); *Leuconostoc* spp. (Nicas *et al.*, 1989); and several species of lactobacilli and pediococci are known to be vancomycin resistant (Leclercq & Derlot, 1988; Ruoff *et al.*, 1988; Johnson *et al.*, 1990; Swenson *et al.*, 1990). Conjugative antibiotic resistance plasmids, like pAM β 1 are common in enterococci, and the transfer of this plasmid has been shown to occur *in vivo* to an *Enterococcus faecalis* recipient strain in mice (McConnell *et al.*, 1991; Morelli, 1996).

In light of this situation, the question of whether antibiotic resistance genes can be transferred by probiotic LAB strains to the endogenous flora or to pathogens, and what impact the transfer may have on subsequent antimicrobial therapy, has arisen (Marteau & Salminen, 1997). It has been claimed that any plasmid-associated antibiotic resistance of dietary LAB strains could have the potential to pass into cells of pathogenic microbes (Eliopoulos *et al.*, 1994), and present a public health risk. On other hand, the administration of antimicrobial substances can alter the intestinal microflora balance and suppress certain beneficial groups, including lactobacilli and bifidobacteria. As discussed in Chapter 2, the altered microflora balance may result in intestinal disorders. The susceptibility of probiotic LAB to various antimicrobial agents are of interest in understanding the alteration of normal intestinal microflora when antimicrobial agents were taken (Yazid *et al.*, 2000). As a result, “routine antibiotic susceptibility testing has been advocated as an essential selection criterion for probiotic cultures to ensure that they are unlikely to participate in undesirable gene transfer cascades *in vivo*” (Charteris *et al.*, 1998a, b). It is proposed that strains harbouring antibiotic resistance plasmids should not be used either as human or animal probiotics (Stokes *et al.*, 1993). Checking the ability of a proposed probiotic strain to act as a donor of conjugative antibiotic resistance genes may therefore be a prudent precaution (Stokes *et al.*, 1993).

The objective of this study was to evaluate the safety of four 4 probiotic LAB strains with respect to their antibiotic resistance attributes.

6.2 Materials and methods

6.2.1 Probiotic strains HN001, HN017, HN019, and HN067

In addition to the LAB strains HN001, HN017, HN019, *Lb.* GG, LA-1, a newly identified probiotic strain *Lb. rhamnosus* HN067, three commercial probiotic strains *B. lactis* Bb12, *B. lactis* HN049 and *B. lactis* HN098 were also examined. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 (Ahn *et al.*, 1992) and *Sta. aureus* NCTC 6571 were used as quality control strains in antibiotic susceptibility tests (Stokes *et al.*, 1993; Hindler *et al.*, 1994). *E. coli* strain v517 (Rochelle *et al.*, 1985) and *Lb. plantarum* HN045 were used as a plasmid size marker or plasmid positive

control strains (Ahn *et al.*, 1992). The plasmid-free strain *Lactococcus lactis* (subsp *lactis*) MG 1363 (M) was used as a negative control in plasmid screening tests (Coolbear *et al.*, 1994). All of these strains were obtained from the New Zealand Dairy Research Institute Culture Collection (NZDRI, Palmerston North, NZ), with the exception of *E. coli* ATCC 25922 and *Sta. aureus* ATCC 25923 which were kindly provided by Mr. T. Langford (MedLab Central, Palmerston North, NZ). The LAB strains HN001, HN017, HN019, HN067, LA-1, and *Lb.* GG used in this study came from the working stock cultures of another research project within NZDRI, so these microorganisms had been subcultured for more than 10 generations before subject to investigation in this study.

6.2.2 Media and antibiotics

MRS or MRS-C broth and agar (A1.5-1.8) were used to cultivate lactobacilli and bifidobacteria respectively. BHI medium was used to culture *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and *S. aureus* NCTC 6571. M17 broth (Difco, USA) plus 1%

Table 6.1 Antibiotics used in experiments

Antibiotics	µg/disk	Antibiotics	µg/disk
β-Lactams		Broad Spectrum	
Penicillin G	10	Chloramphenicol.	30
Ampicillin	10	Rifampin	5
Cephalothin	30	Spectinomycin	100
Cloxacillin	1	Tetracycline	30
Gram+ve Spectrum		Aminoglycosides	
Erythromycin	15	Kanamycin	30
Novobiocin	5	Gentamicin	10
Vancomycin	30	Neomycin	30
		Streptomycin	10
Gram -ve Spectrum			
Fusidic acid	10		
Nalidixic acid	30		
Polymyxin B	300		

glucose was used to cultivate *Lact. lactis* subsp *lactis* MG1363. The antibiotics used in this experiment are listed in Table 6.1. All antibiotic powders, were purchased from Sigma Chemical Company (USA), except rifampin, neomycin, and novobiocin, which

were obtained from GIBCO (Life Technologies, USA). The stock solutions were filter sterilised (0.2µm filter) and kept at -20°C. Working solutions were prepared to a specific concentration at which equal volumes of solutions could be loaded onto the antibiotic carrier disks (3MM Whatman® filter paper, diameter 6.35mm, 3MM Chr, England). Vancomycin disks (VA30) were purchased from OXOID (England).

6.2.3 Antibiotic susceptibility tests

a) *Preparation of cultures*

One colony with typical morphology was selected from each of the purified sub-cultured agar plates and inoculated into the corresponding broth. The inoculations were incubated at 37°C for 12hrs. The cell density of the cultures was adjusted to $1-2 \times 10^8$ cfu/ml according to the absorbance at 660nm (0.08-0.1) (Hitach U-2000 Spectrophotometer, Japan).

b) *Disk diffusion test*

The Bauer method (Bauer *et al.*.,1966) was modified as follows: 200µl of fresh cultures were added into bottles containing 100ml autoclaved melted agar (50°C) and mixed well gently. The agar was then equally poured into four petri dishes. Four sterilised filter paper disks were placed on the agar surface aseptically after the agar had solidified and 5 µl of four different antibiotic solutions were immediately applied to the different disks. Agar plates with antibiotic disks were incubated at 37°C anaerobically (LAB plates) or aerobically (*E. coli* and *Staphylococcus* plates) for 24hrs. The diameters of the inhibition zones were measured (Taurus 300 F5C ruler , ±1mm) under a colony reading light apparatus (Gallenhamp Colony Counter, England). The results (average of 5 readings) were expressed as sensitive (S), intermediate (I) and resistant (R) according to the reported interpretative standards (Woods & Washington, 1995).

6.2.4 Plasmid isolation

Plasmid DNA was isolated based on the methods described by Frere (1994) and Anderson & McKay (1983) with minor modifications. One colony with typical

morphology was picked from agar plates and inoculated in 10 ml of broth which was incubated at 37°C for 8 - 12hrs. The growth of cultures was monitored with a spectrophotometer. When the absorbance (OD₆₆₀) of cultures reached 1-1.5, they were removed from 37°C incubator and centrifuged at 5000 x g for 10 min (4°C). Cell pellets were washed with 5ml of ice-cold TES buffer (A 2.9, pH 8.0). Washed cell pellets were re-suspended in 300µl of TES buffer containing RNase (100µg/ml), lysozyme (20mg/ml), and mutanolysin (40µg/ml), and incubated on ice for 1 hr. Then 250-300 mg of acid washed glass beads (<106 µm, Sigma) were added into the incubated cultures and the mixtures were vortexed. DNA was extracted from the cells using the chloroform-isoamyl extraction method, and the fragments were analysed on a 0.75% (w/v) agarose gel (resolution was 3.5V /cm for 5hrs). Gels were stained with ethidium bromide (0.5µg/ml) and photographs were taken under UV light with Polaroid film (Type 667, ISO 3000/36, PID, UK).

6.2.5 Southern blotting and DNA hybridisation

a) *Preparation of probe*

Total DNA was isolated from bacterial cells using the reported method (Luchansky *et al.*, 1991). Chromosomal DNA probe fragments were amplified by PCR using total DNA as template and two oligo-nucleotide primers Y1 (5'-TGGCTCAGAACGAACGCTGGCGGC-3') and Y2 (5'-CCCACTGCTGCCTCCCGTAGGAGT -3 ') which cover the conserved region of 16S rRNA (Young *et al.*, 1991; Ward *et al.*, 1995). The PCR was performed using the protocol described in Section 3.11 with the following temperature profile: 94°C, 3 min for one cycle; 94°C, 45 seconds, 45°C, one min, 72°C, 2 min for 40 cycles; 45°C, 7 min, 72°C, 4 min for one cycle. The PCR products were then pooled together and purified using the method described in Section 3.12. The purified PCR product of chromosomal DNA from LAB strains was then labelled using ECL DNA Direct Labelling and Detection Systems (RPN 3005, 3004, Amersham Life Science, UK) following the supplier's protocols and was used as probe to hybridise with the DNA fragments that were transferred onto a membrane from an agarose gel. Briefly, purified PCR product was diluted to a concentration of 10ng/µl using the water supplied by company. 10µl of diluted DNA in a microcentrifuge tube was denatured by heating 5 min in a boiling water bath. The

denatured DNA was immediately cooled on ice for 5 min and then centrifuged briefly (1,000 x g, 4°C) to collect the DNA samples at the bottom of the tube. 10µl of DNA labelling reagent was then added to the cooled DNA. After gentle and thoroughly mixing, 10µl of the glutaraldehyde solution was added and mixed thoroughly. The DNA and labelling solution mixtures were incubated for 10 min at 37°C and were immediately used for DNA hybridisation.

b) Southern blotting & DNA hybridisation

Southern transfer, DNA-DNA hybridisation, DNA detection, as well as the developing of autoradiography film were all conducted according to the protocols recommended by ECL DNA Direct Labelling and Detection Systems (RPN 3005, 3004, Amersham Life Science, UK). The southern blotting protocols were slightly modified and described in Chapter 3 (Section 3.14).

6.3 Results

6.3.1 Antibiotic susceptibility

The results of disk diffusion tests are shown in Table 6.2. All test and reference strains were susceptible to β-Lactams (penicillin, ampicillin, cephalothin), Gram-positive spectrum antibiotics (erythromycin, novobiocin), and broad-spectrum antibiotics (chloramphenicol, rifampin, spectinomycin and tetracycline). Almost all of the strains were resistant to Gram-negative spectrum antibiotics (fusidic acid, nalidixic acid and polymyxin B) and aminoglycosides (gentamicin, kanamycin, neomycin, and streptomycin). Compared with other β-Lactams, cloxacillin was much less effective in inhibiting test and reference strains, with only HN001 being sensitive to it. Strains HN001, HN067, *B. lactis* Bb12, HN045 and *Lb.* GG were resistant to vancomycin, while HN017, HN019, HN049, LA-1 and HN098 were susceptible. The general antibiotic susceptibility patterns of test and reference strains are summarized in Table 6.3.

Table 6.2 Antibiotic susceptibility profiles of test probiotics

	HN001	HN017	HN019	HN067	<i>Lb.</i> GG	LA-1	Bb1-12	HN045	HN049	HN098
Ampicillin										
Cephalothin										
Penicillin										
Cloxacillin		±	R	±	R	±	R	R	R	R
Erythromycin										
Novobiocin										
Vancomycin	R			R	R		R	R		
Spectinomycin										
Chloramphenicol										
Rifampicin										
Tetracycline										
Gentamicin	R		R	±	R	R	R	R	R	R
Kanamycin	R	R	R	R	R	R	R	R	R	R
Neomycin	R		R	R	R	R	R	R	R	R
Streptomycin	R	R	R	±	R	R	R	R	R	R
Fusidic acid	R	R		R	R	R	R	R		R
Nalidixic acid	R	R	R	R	R	R	R	R	R	R
Polymyxin B	R	R	R	R	R	R	R	R	R	R

R: resistant; ± marginally (intermediate) susceptible; not indicated: susceptible.

Table 6.3 Susceptibility status of various antibiotics to probiotics

Sensitive	Variable	Resistant
Ampicillin	Fusidic acid ^a	Kanamycin
Cephalothin	Gentamicin ^b	Nalidixic acid
Penicillin	Neomycin ^c	Polymyxin B
Erythromycin	Cloxacillin ^d	Streptomycin
Novobiocin	Vancomycin ^e	
Chloramphenicol		
Spectinomycin		
Rifampin		
Tetracycline		

a HN019 and HN049 were susceptible, others were resistant

b HN017 was susceptible, HN067 was intermediate, others were resistant

c HN017 was susceptible, others were resistant

d HN001 was susceptible, HN017, HN019, and *Lb. GG* were intermediate, others were resistant

e HN001, HN067, *B. lactis* Bb12, *Lb. GG*, and HN045 were resistant, others were susceptible.

6.3.2 Plasmid profiles

The plasmid profiles of test and reference strains are shown in Figure 6.1. Only one DNA fragment (from test strains) with the same molecular weight as chromosomal DNA of *E. coli* v517 was detected. Eleven putative plasmid bands were identified from *E. coli* v517, 9 plasmid bands were detected from *Lb. plantarum* HN045. No plasmid DNA was detected in the plasmid-free strain *Lact. MG1363* (Figure 6.2a).

DNA hybridisation tests (Figure 6.2b) showed that the single point fragments detected in the test and plasmid-free strains hybridised with a chromosomal DNA probe at the expected position for genomic DNA. In contrast, the multiple fragments of the putative plasmid-positive strain HN045 and supercoiled DNA did not hybridise (Figure 6.2a).

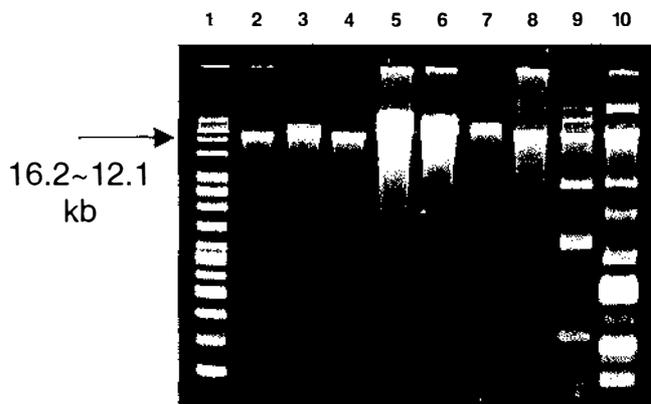


Figure 6.1 Agarose gel electrophoresis of “plasmid” DNA. Lane 1: Supercoiled DNA ladder; Lane 2: HN001; Lane 3: HN017; Lane 4: HN019; Lane 5: HN067; lane 6: LA-1; Lane 7: *Lb. GG*; Lane 8: HN049; Lane 9: HN045; Lane 10: *E. coli.v517*. No plasmids were detected in strains from Lanes 2 ~ 8.

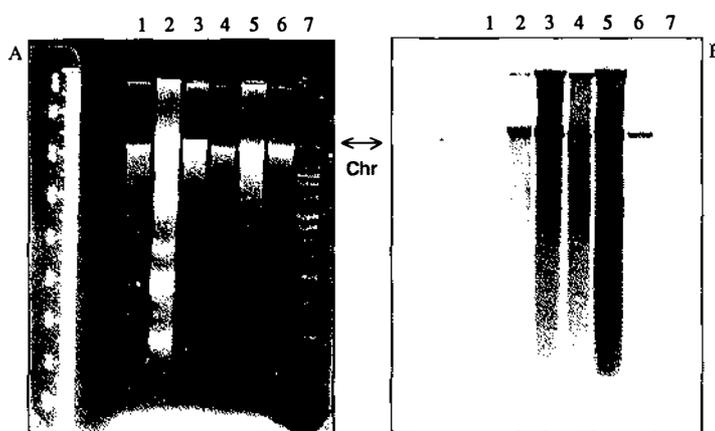


Figure 6.2 Southern blotting and chromosomal DNA-DNA hybridisation

Lane 1: plasmid-free strain MG 1363; Lane 2: plasmid positive strain HN045; Lane 3: HN067; Lane 4: HN019; Lane 5: HN017; Lane 6: HN001; Lane 7: supercoiled DNA ladder.

- (A) *Agarose gel electrophoresis of DNA before southern blotting.* One identical molecular weight fragment was detected from strains in Lanes 1, 3 ~ 6. Several putative plasmid bands were detected in the positive control strains in Lane 2.
- (B) *DNA-DNA hybridisation.* A purified PCR product of chromosomal DNA from LAB strains was used as probe (Figure 6.3) to hybridise with the DNA fragments that were transferred onto a membrane from an agarose gel (A). The DNA fragments from strains in Lanes 1 and 3-6 as well as the “chromosomal” DNA band of Lane 2 hybridised with the chromosomal DNA probe; supercoiled DNA (Lane 7) and plasmids of HN045 (Lane 2) did not hybridise with the probe other than at the expected position –marked ‘Chr’.

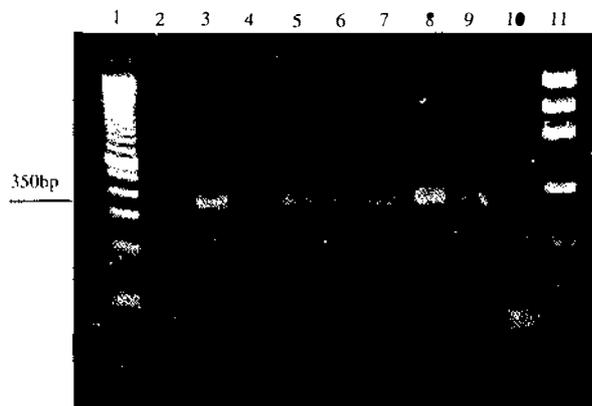


Figure 6.3 Agarose gel electrophoresis of PCR product of chromosomal DNA probe. L1: 100bp DNA ladder; L2: no DNA loading; L3: HN049; L4 and L10: LA-1; L5: *Lb. GG*; L6: HN067; L7: HN019; L8: HN017; L9: HN001; L11: low mass (100~2000bp) DNA ladder.

6.4 Discussion

There have been several studies on the antibiotic sensitivity and resistance of dairy starter bacteria, which were triggered by the problems caused by antibiotic residues in milk causing starter failures in milk acidification (Morelli & von Wright, 1997). The investigation of antibiotic susceptibility in LAB strains from the safety assessment point of view is a relatively recent area of study (Charteris *et al.*, 1998a,b; Tynkkynen *et al.*, 1998).

The results of this study (Table 6.2) add further proof to these findings. All 10 LAB strains tested in the current study, whatever their sources (human origin or dairy food, Section 3.1, Table 3.1), had quite similar antimicrobial susceptibility profiles. They were all sensitive to penicillin and resistant to streptomycin, except HN067 which was only marginally susceptible to streptomycin. All tested lactobacilli and bifidobacteria were susceptible to the majority of tested β -Lactams (3/4), Gram-positive spectrum antibiotics (2~3/3), and broad spectrum antibiotics (4/4). In contrast, almost all of the tested lactobacilli and bifidobacteria were resistant to aminoglycosides (3~4/4) and Gram-negative spectrum antibiotics (2~3/3). These results are essentially in accordance with previous reports (Sutter & Finegold, 1976; Lim *et al.*, 1993; Charteris *et al.*, 1998a,b). It was also found that cloxacillin was an unusual β -Lactam agent, in that only one strain (HN001) was sensitive to this antibiotic and the other 9 tested LAB strains

were resistant (6/10) or marginally susceptible (3/10). Olukoya and co-workers' study (1993) also found 80% of the *Lb. spp* that they tested (including *Lb. plantarum*, *Lb. acidophilus*, and other species) were resistant to cloxacillin.

Considering the vancomycin resistance attributes of LAB strains, the results of the present study demonstrated that 3 out of 4 tested bifidobacteria strains including HN019, and 2 of the tested *Lb. acidophilus* (HN017 and LA-1) were susceptible to vancomycin, whereas all 3 tested *Lb. rhamnosus* strains (HN001, HN067, and *Lb. GG*) were resistant to this antibiotic. These results are similar to the findings of Lim *et al.* (1993) and Tynkkynen *et al.* (1998); but differ from Charteris and co-worker's observations (1998a,b), in which vancomycin resistance was found to be a general characteristic of bifidobacteria. The difference in bifidobacterial sensitivity to vancomycin between different studies may be due to differences in the assay methodology, since vancomycin is reported to diffuse poorly in agar media. Some species of LAB, including *Lb. casei*, *Lb. rhamnosus*, *Lb. plantarum* and other species, which are commonly used or exist in food products are intrinsically resistant to vancomycin (Tynkkynen *et al.*, 1998).

Compared with reference strains (LA-1, *Lb. GG*, HN049, HN098, and *B. lactis* Bb12) and other LAB strains investigated in previous studies, test strains (HN001, HN017, HN019, and HN067) had no atypical antibiotic susceptibility patterns. As with other LAB, the probiotic LAB strains tested here are resistant to several antibiotics, however, they are also susceptible to many commonly used clinical antimicrobials such as penicillin, ampicillin, cephalothin, erythromycin, chloramphenicol, rifampicin, novobiocin, spectinomycin and even tetracycline. Dietary LAB strains and related organisms (with the exception of enterococci) are seldom associated with infections especially in healthy hosts (Morelli & von Wright, 1997). In the case of subjects with sub-optimal immune functions or surgical procedures, LAB of indigenous or dietary origin may have the chance of penetrating their inhabitant site (the gut) and causing opportunistic infections (Aguirre & Collins, 1993; Gasser, 1994). Nevertheless, with so many antibiotics available that are effective against the LAB strains tested here, there should be no serious consequence even if a rarely LAB-associated opportunistic infection did happen.

However, if the antibiotic resistance attributes of LAB were transferable, then these resistance genes could be disseminated to clinically significant strains, such as the multi-drug resistance pathogens, resulting in serious public health hazards (Eliopoulos *et al.*, 1994). Currently, it is not clear how antibiotic resistance genes might be transferred to pathogenic bacteria. However, several mechanisms by which the resistance genes can be transferred from one bacterium to another, such as transformation, conjugation, and transduction, exist. Conjugation, mediated by plasmids or conjugative transposons, is currently the most well understood of these mechanisms (Olsen, 1999). Thus, antibiotic resistance plasmids of probiotic organisms are particularly interesting from the safety point of view. Nevertheless, relatively few studies in this area have been carried out (Tynkkynen *et al.*, 1998) and no genetic studies on bifidobacterial antibiotic resistance appear to have been published (Morelli & von Wright, 1997).

The results from plasmid screening experiments in this study showed that there were no detectable plasmids in the DNA samples isolated from test strains HN001, HN017, HN019, or HN067, and reference strains *Lb.* GG and LA-1. The plasmid-free status of *Lb.* GG has been also reported by others (Tynkkynen *et al.*, 1998). The electrophoretic patterns of DNA isolated using plasmid isolation methods from test LAB strains did not show any apparent plasmids, and the single DNA fragment from each test strain had the same molecular weight as chromosomal DNA of *E. coli* v517 (Figure 6.1 and Figure 6.2a). This result indicates that test and reference probiotic LAB strains that were isolated for this study did not carry any plasmids. To further prove the plasmid-free status of test strains, the identity of DNA fragments isolated from these strains was examined using DNA-DNA hybridisation techniques.

In DNA hybridisation tests, the probe used for hybridisation was a chromosomal DNA fragment (350bp) developed by PCR reactions using Y1 and Y2 as primers (Figure 6.3), which have complementary sequences to 16s rRNA conserved regions (Young *et al.*, 1991; Ward *et al.*, 1995); total genomic DNA extracted from LAB cultures was used as the target. Since hybridisation occurred at the position where genomic DNA would be expected, this result confirms that the single-point DNA fragment detected from the cultures of tested strains was not of plasmid origin. In other words, the LAB strains tested here are plasmid-free. From this result we can conclude that the antibiotic

resistance attributes of the tested probiotic LAB strains are unlikely to be plasmid-linked. However, further studies in our research programme have detected 2 plasmids (one 9 kb and one 40 kb in size) from the DNA samples of HN001 (Dr. C. J. Pillidge, NZDRI, personal communication). This disparity may have been caused by the spontaneous loss of plasmid from the strain of HN001 used in this study during repeated cultivation of this strain, since both plasmids were absent in HN001 isolated from milk powder samples (Dr. C. J. Pillidge, NZDRI, personal communication). Nevertheless, the vancomycin and other antibiotic resistance properties in HN001 in this study showed that these resistance are chromosomally determined.

Plasmid-linked resistance of LAB to the antibiotics vancomycin (Orberg & Sandine, 1984), chloramphenicol (Morelli *et al.*, 1988), erythromycin (Ishiwa & Iwata, 1980), tetracycline (Wang & Lee, 1997), and gentamicin (Vescovo *et al.*, 1982) have been commonly reported among food-borne bacteria. In contrast, the lactobacilli and bifidobacteria strains tested here were each susceptible to most of these particular antimicrobials with the exception of vancomycin (variable between strains, i.e. HN017 and HN019 were sensitive; HN001 and HN067 were resistant) and gentamicin (resistant). Although plasmid-linked antibiotic resistance genes have been identified in some LAB, the antibiotic resistance including vancomycin resistance in most LAB strains (particularly lactobacilli) is generally thought to be constitutive or chromosomal in origin and not inducible or transferable (Orberg & Sandine, 1984; Nicas *et al.*, 1989; Swenson *et al.*, 1990; Handwerger *et al.*, 1994; Morelli & von Wright, 1997). The transfer of vancomycin resistance from *Lactobacillus* has not been observed (Nicas *et al.*, 1989; Tynkknen *et al.*, 1998).

Generally, the spread of drug-resistance between different microorganisms mainly occurs in the plasmid-associated resistance attribute (Halbert, 1988; Morelli *et al.*, 1988; Khachatourians, 1998). The intrinsic or constitutive (chromosomal) antibiotic resistance is non-transmissible (Morelli & von Wright, 1997), so no particular safety concerns are associated with this sort of drug resistance in dietary LAB which have otherwise been demonstrated as safe. The four probiotic LAB strains (HN001, HN017, HN019, and HN067) tested here are plasmid-free, their antibiotic resistance attributes are therefore intrinsic or constitutive and thus are not transferable. On the other hand, there are many safe antibiotics which are effective against the LAB strains tested in this

study, so in the unlikely event of opportunistic infections caused by these LAB strains, effective medical treatment should not be difficult. Regarding the safety concerns, these probiotic organisms should be safe for human and animal consumption.

7

Acute oral toxicity of probiotic strains HN001, HN017, and HN019

Abstract

The *in vivo* safety of probiotic LAB strains *Lb. rhamnosus* HN001, *Lb. acidophilus* HN017 and *B. lactis* HN019 was evaluated by investigating the acute oral toxicity, bacterial translocation, and gut mucosal pathology following consumption of these strains in conventional mice. Male adult (6-8 weeks old) BALB/c mice were fed with HN001, HN017 or HN019 for 8 consecutive days at a high dose of 10^{11} cfu/mouse/day. Their feed and water intake, live body weight gain, morbidity and mortality were monitored. At the end of feeding, blood and tissue suspensions of liver, spleen, and mesenteric lymph nodes (MLN) of animals were cultured, and their intestinal mucosal architecture was examined microscopically. Results showed that these probiotic strains had no adverse effect on any of the parameters mentioned above and no bacterial translocation was detected. Based on these findings, the oral LD₅₀ (50% lethal dose) of HN001, HN017 and HN019 is likely to be greater than 50g/kg/day for mice, and the acceptable daily intake (ADI) value is 35g dry bacteria per day for a 70kg person. This suggests that these three probiotic LAB strains are non-pathogenic and likely to be safe for human consumption.

7.1 Introduction

Safety assessment has been listed as an important criterion for selecting probiotic strains (Conway, 1996; Nousiainen & Setälä, 1998) and is regarded as the first and most important step prior to incorporation of these strains into food products (Boyd, 1993; Hull, 1995). But, there are still no general guidelines or specific policy requirements on this issue. Acute oral toxicity has been advocated as a fundamental test for assessing safety (Stine & Brown, 1996; Willem *et al.*, 1997; Donohue *et al.*, 1998) and has been applied previously in several safety assessment studies (Momose *et al.*, 1979; Donohue *et al.*, 1993). Bacterial translocation is another recommended indicator for probiotic safety assessment (van der Kamp, 1996; Marteau & Salminen, 1997) as it is the first step in the pathogenesis process for many opportunistic indigenous lumen strains (Berg, 1983; Steffen & Berg, 1983)

The probiotic strains evaluated in this study are intended to be consumed orally by humans and animals, and their translocation ability (if there is any) is expected to be a good indicator of their potential infectivity. The local pathogenicity of bacteria on their primary administration and colonisation sites should be examined as well (Havenaar & Huis in't Veld, 1992). Therefore, in this study, the safety of HN001, HN017 and HN019 were evaluated on the basis of an oral toxicity test, and furthermore, intestinal mucosal histology and bacterial translocation of mice inoculated with test strains were also investigated.

7.2 Materials and methods

7.2.1 Probiotic strains

LAB strains, *Lb. rhamnosus* HN001, *Lb. acidophilus* HN017, and *B. lactis* HN019 were used as test strains. Two commercial strains, *Lb. rhamnosus* GG (*Lb.* GG) and *Lb. acidophilus* LA-1 (*Lb.* LA-1) were used as reference strains. The source, propagation, purification, and preservation of these strains are described in Section 3.1 and 3.5. The preparation of LAB cell cultures for inoculation of mice is described in Section 3.15. The cell pellets were re-suspended in 10% skim milk at a concentration of 10^{12} cfu/ml which was used for inoculation. In most of the previous studies in mice to investigate the immunomodulating effects of LAB, 10^9 cfu/day/mouse was used (Perdigon *et al.*,

1990, 1999). The dosage used in this study (10^{11} cfu/mouse/day) is therefore a significantly higher dose.

7.2.2 Animals

Forty-eight male BALB/c mice were housed individually in stainless steel cages. The housing conditions, access to diet and water are described in Section 3.16.

7.2.3 Experimental design

After 7 days of acclimatisation to experimental conditions, the mice were randomly assigned into 1 of the 6 groups, and were orally administered HN001, HN017, HN019, *Lb.* GG, LA-1, or 10% skim milk only. Fifty microliters of cell suspensions (10^{12} cfu/ml in skim milk) or the control diet (10% skim milk) were fed orally to each mouse using a sterile pipette twice a day (10^{11} cfu/mouse/day) for 8 days. The interval between the two feedings was 4 hrs. Throughout the experiment, the animals' general health status was scored daily on a 5-scale based standard (general health scores-GHS) described in Section 3.16 (Table 3.2). Signs of illness were observed twice a day; feed intake (FI) and live weight were measured once a week, and the specific growth rate (SGR) was expressed as the daily weight gain, i.e. $SGR = (\text{weight } x - \text{weight } y) / (\text{time } x - \text{time } y)$.

7.2.4 Bacterial translocation

After feeding with test strains for 8 days, animals were humanely euthanased by isofluorane overdose. Blood samples were obtained by cardiac puncture. Before excising tissue samples, the viscera surface was wiped with a sterile swab that was cultured in BHI media to test for artificial contamination of the viscera. The mesenteric lymph nodes (MLN), spleen and a sample of liver tissues were excised, processed, and cultured using the method described in Section 3.19. Each sample was divided into two portions, one was plated on MRS or MRS-C (A1.5-1.8) agar plate, another portion was plated on BHI agar plate.

7.2.5 Identification of translocated organisms

Randomly amplified polymorphic DNA (RAPD) finger-printing method (Cocconcelli *et al.*, 1995; Johansson *et al.*, 1995) was used to identify the organisms detected in MLN, spleen, and liver tissues. The organisms grown on MRS or MRS-C agar plates were cultured in MRS or MRS-C broth for 48hrs and then checked under a microscope. Microorganisms possessing attributes similar to the LAB strains used in this study were selected according to the appearance of colonies, cell morphology, growth characteristics in liquid media, mobility and Gram reaction, and were then subjected to RAPD finger printing assays. Total genomic DNA of suspected cultures and purified test strains were isolated by methods reported elsewhere (Cocconcelli *et al.*, 1995; Ward *et al.*, 1995) and modified as described in Section 3.10. The DNA fragments isolated were then amplified using the RAPD technique (Cocconcelli *et al.*, 1995). The following primers with arbitrary nucleotide sequences (CG content >50%, GIBcoBRL Life Technologies) were used: LO1 (GCATGACCT, for HN001); AP2 (AGTCAGCCAC, for HN017 and *Lb.* LA-1); J17 (GTCCCGTGGT) together with LO1 (for HN019). PCR was performed on a Teche PHC-3 Thermal Cycler (John Morris Scientific Ltd, Auckland, New Zealand) with the following temperature profile: 94°C, 3 min for one cycle; 94°C, 45 seconds, 45°C, one min, 72°C, 2 min for 40 cycles; 45°C, 7 min, 72°C, 4 min for one cycle. The PCR was then terminated and the samples were cooled to 4 °C. The products from the PCR were purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim, USA). The purified RAPD products were visualised by ethidium bromide staining on agarose gels (2%, type 1-A, low EEO, Sigma Chemical co. USA) following 3 hours resolution in 0.5x TBE buffer at 55V (Section 3.13). The RAPD patterns of suspected strains were compared with those of the test strains.

7.2.6 Histology

Tissue samples (0.5x0.5 cm) from ileum, caecum, and colon were excised, and fixed in Bouins fluid (Section A2.12). The samples were then processed, examined, and evaluated using the method described in Section 3.21.

7.3 Results

7.3.1 General health status

Throughout the experimental period, no noticeable behavioural or activity changes were observed in the mice, and no treatment-related illness or death occurred. There was no observable difference in animals' hair lustre between treatment and control groups.

7.3.2 Feed intake and growth rate

Oral administration of HN001, HN017, and HN019 had no adverse effect on the animals' feed intake (FI) (Figure 7.1), or specific growth rate (SGR, g/day)(Figure 7.2). Statistical analysis (SAS software, GLM) revealed that there was no significant difference in FI and SGR between different groups ($P>0.05$). There was also no correlation between SGR of mice and the treatment they received ($r=0.07$, $p>0.05$).

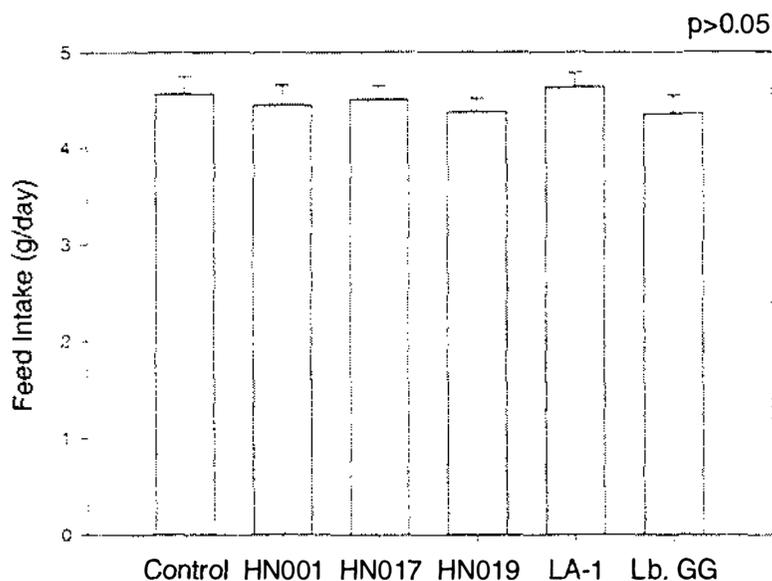


Figure 7.1 Daily feed intake (FI) of mice fed with probiotic LAB strains (Mean + SEM).

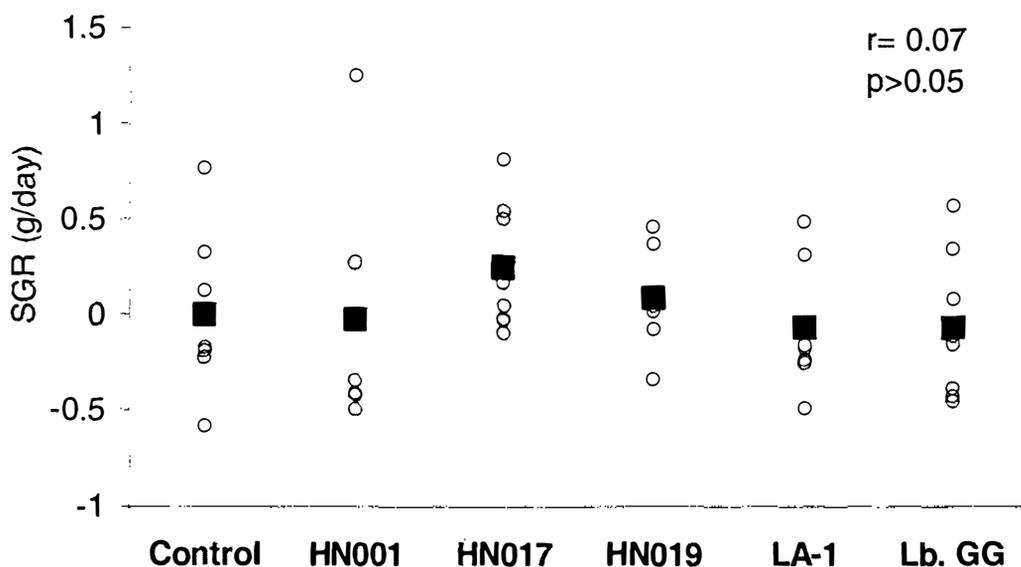


Figure 7.2 Growth rate of mice fed with probiotic strains. The values included are daily weight gain (gram) of each mouse in each specific group. The correlation analysis was made between treatment and specific growth rate. Black squares refer to group mean values.

7.3.3 Histological changes

Macroscopic examination did not reveal any obvious differences in the size or appearance of visceral organs between each group. No hepatomegaly or splenomegaly occurred, and no caecal enlargement was observed. The spleen weight index (SWI, the ratio of spleen weight to mouse body weight in gram) of the mice is shown in Figure 7.3. There was no significant difference in the SWI between test and control groups of mice, although the mice receiving HN019 had relatively higher SWI than that of LA-1 treated mice ($p > 0.05$). Microscopic observations did not find any signs of inflammation, degeneration or necrosis of the intestinal mucosa, and there was no remarkable difference in the arrangement of epithelial cells between test and control groups. Tables 7.1 and 7.2 demonstrate the results of mucosal structure measurements. LAB fed mice (particularly the mice receiving HN017 and HN019) had higher ileum crypt depth than that of control mice ($p = 0.019$). No significant difference was detected in villus height, epithelial cell height, or mucosa thickness between the groups ($p > 0.05$). Although HN017 and HN001 fed mice had relatively higher colon and caecum mucosal thickness respectively compared to that of other groups, this difference was not statistically significant ($P > 0.05$).

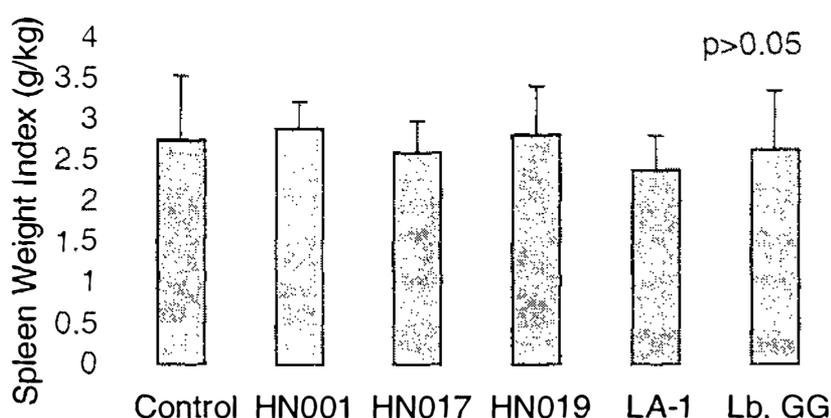


Figure 7.3 Spleen weight index of mice fed with probiotic LAB strains. The values included in this figure are the spleen weight (mg) / body weight (g) (Mean + SEM).

Table 7.1 Changes in ileum mucosal architecture

Treatment	Villus Height (μm)		Crypt Depth (μm)		Mucosal Thickness (μm)		Epithelial Cell Height (μm)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
HN001	186.85	12.93	54.37	3.69	243.92	15.46	16.14	0.84
HN017	212.36	11.38	63.67*	4.32	285.05	17.12	15.64	0.80
HN019	198.20	15.08	69.55*	1.56	269.25	14.69	15.92	0.43
LA-1	181.98	9.13	53.64	5.57	238.31	13.21	15.42	0.72
Lb. GG	186.13	12.36	53.12	3.47	243.50	14.89	15.62	0.88
Control	202.73	19.23	48.49	4.69	280.12	15.39	15.31	1.04
P values	0.605		0.019		0.124		0.968	

SAS (GLM) analysis. * $P < 0.05$ (HN017 or HN019 vs Control)

7.3.4 Bacterial translocation

There was no bacterial growth in the visceral swab cultures, which indicates that the visceral surface was not contaminated. Positive growth on agar plates was defined by the presence of any microorganism (even a single colony). The incidence of translocation of bacteria from the gut into different tissues is shown in Table 7.3. No bacteraemia was detected in any of the groups. There was also no statistically significant difference in the general translocation incidence between test and control

groups, although the mice receiving HN001 or *Lb.* GG had relatively higher MLN translocation rates than that of other groups.

Table 7.2 Changes in colon and caecum mucosal architecture

Treatment	Colon				Caecum			
	Mucosal Thickness (μm)		Epithelial Cell Height (μm)		Mucosal Thickness (μm)		Epithelial Cell Height (μm)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
HN001	136.33	10.79	15.93	0.68	110.37	7.18	13.94	0.67
HN017	158.56	12.06	14.62	0.37	89.64	15.59	15.11	0.75
HN019	125.74	9.95	16.35	0.69	97.94	6.23	15.27	0.63
LA-1	127.99	10.11	15.13	0.88	103.78	6.79	14.04	0.77
<i>Lb.</i> GG	146.82	20.84	15.62	0.71	105.33	12.68	14.86	0.49
Control	133.14	11.55	15.87	0.65	86.83	6.51	13.43	0.47
P value	0.565		0.744		0.312		0.399	

SAS (GLM) analysis

Table 7.3 Bacterial translocation to different tissues

Treatment	MLN	Liver	Spleen	Blood	Total
HN001	6/8	0/8	1/8	0/8	7/32
HN017	2/8	1/8	1/8	0/8	4/32
HN019	2/8	1/8	2/8	0/8	5/32
LA-1	1/8	1/8	1/8	0/8	3/32
<i>Lb.</i> GG	4/8	2/8	2/8	0/8	8/32
Control	2/8	2/8	1/8	0/8	5/32

Data represent: No. of positive cultures / No. of tested tissues; Fisher's Exact Test: $P > 0.05$.

The RAPD electrophoresis patterns of suspected LAB strains isolated from tissues are illustrated in Figures 7.4 to 7.6. No RAPD pattern from suspected strains matched the patterns of finger-print DNA derived from pure stock cultures of known strains, which indicates that none of the viable bacterial cells recovered from tissues were derived from the feeding test strains.

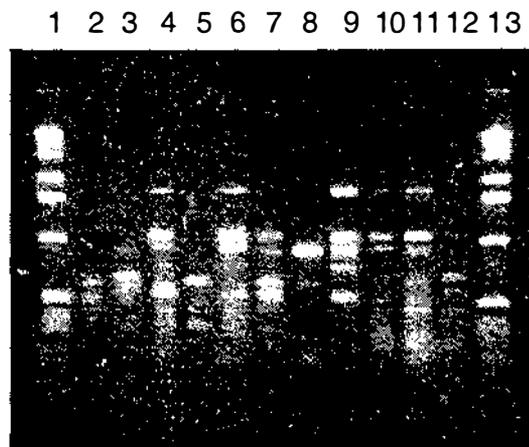


Figure 7.4 RAPD patterns of strains recovered from animals fed with HN001. Lanes: 1 & 13: 1 kb DNA ladders; Lanes 2-11: strains isolated from tissues; Lane 12: purified HN001. No pattern from suspected strains matched with the pattern of HN001.

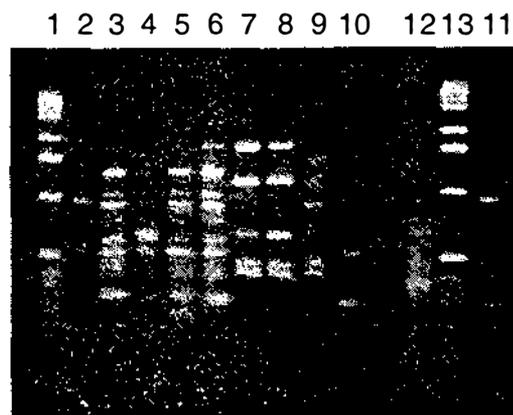


Figure 7.5 RAPD patterns of strains recovered from animals fed with HN017 or LA-1. Lanes: 1 & 13: 1 kb DNA ladders; Lanes 2-8: recovered strains from animals fed with LA-1; Lane 9: purified LA-1; Lanes 10-11: recovered strains from animals fed with HN017; Lane 12, purified HN017. No pattern from suspected strains matched with the patterns of HN017 and LA-1.

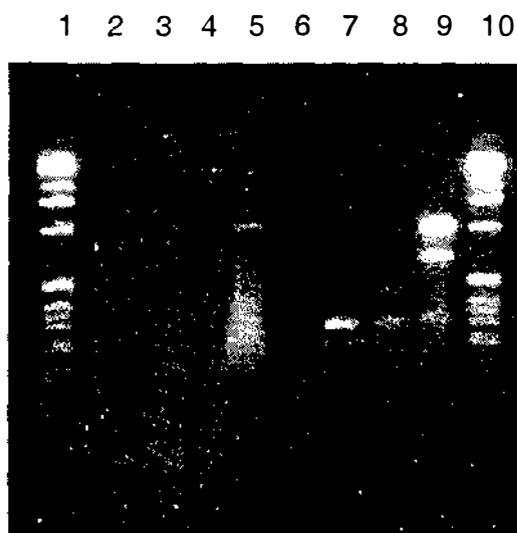


Figure 7.6 RAPD patterns of strains recovered from animals fed with HN019. Lanes: 1 & 10: 1 kb DNA ladders; Lanes 2~8: strains isolated from tissues; Lane 9: purified HN019. No pattern from suspected strains matched with that of HN019.

7.4 Discussion

According to OECD Guidelines (1987), acute oral toxicity is the adverse effects occurring within a short time of oral administration of a single dose of a substance or multiple doses given within 24hrs. In the assessment and evaluation of the toxic characteristics of a substance, determination of acute oral toxicity is usually an initial step. It provides information on health hazards likely to arise from short-term exposure by the oral route.

Appetite, activity and live weight gain are the most general and sensitive indicators of health status for animals. In accordance with this, the feed intake (FI), specific growth rate (SGR), and the behaviour and condition of the mice were used in this study to evaluate the acute toxicity of test strains. All animals were healthy and survived the inoculation after 8 days. No adverse effects of the test strains on FI or SGR was found, indicating that the probiotic strains HN001, HN017, and HN019 do not exhibit gross acute oral toxicity effects on the experimental animals' general health status, growth and development.

The integrity of the gut mucosa is important in host defences (Fuller, 1992). The ability to disturb this physical barrier is an indicator of potential pathogenicity for most

obligate and facultative pathogens. Ma and co-workers (1990) have demonstrated an association between the pathological alterations in mucosal structure and high translocation rate of *Lb. murinus* in mice. Takeuchi & Sprinz (1967) also reported changes in the structure of the intestinal mucosa caused by invasion of *Salmonella typhimurium*. Histological examination of intestinal tissues from mice fed with the different LAB in this study showed no obvious morphological changes. In general, the integrity of the mucosa, orientation and arrangement of mucosal epithelial cells were normal in all animals, and no differences in structural measurements were observed between test and control group animals although there were some differences between different test strains. The relative higher ileum crypt depth in LAB-fed mice suggest that oral feeding of probiotic LAB strains may enhance the mucosal secretion activities, but this hypothesis needs to be verified in future studies. This suggests that the three probiotic LAB strains used here have no harmful effects on gut mucosa, which is in accordance with previous work (Ma *et al.*, 1990).

As mentioned above, translocation is a prerequisite for pathogenicity for most opportunistic gut pathogens (Ford *et al.*, 1996). However, it has been suggested that the occasional translocation of LAB from gut to extra-gut tissues, especially the gut-associated lymphoid tissues (GALT), is a normal and beneficial physiological process associated with immune stimulation and the effects of alerting the local immune defence (Bengmark & Jeppsson, 1995). The translocation of normal indigenous intestinal bacteria in healthy mice has been reported by several groups (Hale & Hill, 1973; Berg, 1983; Ma *et al.*, 1990). Gordon *et al.* (1955) reported that 50% of normal mice contained bacteria in their MLN, liver, and spleen, or even occasionally bacteraemia. It has also been proposed that a small number of indigenous gut bacteria translocate continuously into lymphoid tissues outside the intestine, where they are killed by the host's immune defence system *in situ* (Owens & Berg, 1980), while their contents act to stimulate the immune system. In this study, a positive culture was defined as the appearance of any colony (even one) on agar plates, although the presence of at least 10 or 50 colonies has been used previously as the criterion for positive growth by other workers (Carter & Collins, 1974; Berg, 1992a). In this study a few bacterial cells were recovered from extra-intestinal tissues, predominately the MLN. However, the general translocation incidence of bacteria in this study was similar between the different strains of LAB and the control group. This indicates that translocation was not associated with

the feeding of LAB. The significance of the relatively higher MLN translocation rate in mice receiving *Lb. rhamnosus* HN001 or *Lb. GG* needs to be further investigated.

To further confirm the inability of probiotic LAB strains to translocate, RAPD analysis was conducted. This form of analysis is recognised as a reliable and efficient method for identifying strains in many situations (Ramos & Harlander, 1990; Cocconcelli *et al.* 1995; Johansson *et al.*, 1995), and for identifying the recovered microorganisms from tissues. The results of RAPD analysis showed that LAB strains recovered from extra-intestinal tissues had apparently different RAPD patterns to those of test LAB strains used for feeding. From this it can be concluded that organisms detected in different systemic tissues are likely to be indigenous, rather than derived from dietary sources (i.e. test strains). In other words the three test strains HN001, HN017, and HN019 either had no ability to translocate from gut to systemic tissues, or if they did translocate to GALT and other extra-intestinal tissues they were killed by the host's immune mechanisms. The evidence that there was no change in spleen weight (Figure 7.3) in treated animals also indicates that the test LAB strains did not cause or induce systemic infections in mice. This suggests that the strains used were non-invasive and are likely to be safe for human use.

The inoculation dose used in this study is probably the highest used in LAB toxicity studies to date. Based on the results of this work, the acute oral LD₅₀ (50% lethal dose) of test strains is greater than 50g/kg/day. Considering the safety factor of 100 (i.e. for human use, the dose should be 20 fold less than the minimal dose to cause toxicity in animals, plus another 5-fold less for individual variation in responses to specific substance in human populations) (Rodricks, 1996), the acceptable daily intake (ADI) level of test strains will be 35g freeze-dried bacteria per day for a 70kg individual, which is 700 times the amount of LAB normally consumed by humans (Donohue *et al.*, 1993; Kailasapathy & Rybka, 1997). In addition, HN001, HN017 and HN019 share similar safety status with the commercial probiotic organisms, *Lb. GG* and LA-1, which have well documented safety profiles (Saxelin, 1997; Salminen *et al.*, 1998a) and have been safely used in dairy foods for several years. It therefore follows that consumption of the probiotic strains HN001, HN017, and HN019 at a normal dose should be safe for humans.

8

Tolerance of mice to four week feeding with probiotic strains HN001, HN017, and HN019

Abstract

The tolerance of animals to 4-week probiotic feeding were investigated in groups of BALB/c mice which were fed with test LAB strains HN001, HN017, or HN019 at doses of 5×10^7 , 1×10^9 or 5×10^{10} cfu /mouse/day. Animals' feed intake, water intake, and live body weight were monitored once a week. At the end of the 4-week observation period, samples of blood, liver, spleen, kidney, mesenteric lymph nodes, and gut tissues (ileum, caecum, and colon) were collected to determine: haematological parameters (red blood cell and platelet counts, haemoglobin concentration, mean corpuscular volume, mean corpuscular haemoglobin, and mean corpuscular haemoglobin concentration); differential leucocyte counts; blood biochemistry (plasma total protein, albumin, cholesterol, and glucose); mucosal histology (epithelial cell height, mucosal thickness, and villus height); and bacterial translocation to extra-gut tissues (blood, liver, spleen, kidney and mesenteric lymph nodes). DNA fingerprinting techniques (RAPD) were used to identify any viable bacterial strains recovered from these tissues. The results showed that four weeks consumption of these LAB strains had no significant adverse effect on the animals' general health status, haematology, blood biochemistry, gut mucosal histology parameters, or the incidence of bacterial translocation. A few viable LAB cells were recovered from the tissues of animals in both control and test groups, but DNA fingerprinting clearly showed that none of these were the inoculated strains. No dose-dependent effects were detected for any of the observed indicators. The results obtained in this study suggest that the probiotic LAB strains HN001, HN017, and HN019 are non-toxic for mice at high dose and are therefore likely to be safe for human use as probiotics.

8.1 Introduction

The studies conducted in previous sections showed that probiotic LAB strains HN001, HN017, and HN019 were devoid of acute oral toxicity in mice (Chapter 7), i.e. short term consumption of these probiotic LAB strains at a high dose rate did not adversely affect the animals' health. Most probiotics, including the strains currently being evaluated, are more likely to be incorporated into food products rather than being used as therapeutics. Therefore, they are more likely to be consumed on a daily basis, for long periods of time. As a food ingredient, an essential requirement for the product is that it is safe for consumers (Rodricks, 1996; Lindgren, 1999). To assess the hazardous nature of a substance, it is important to consider the likely exposure of the consumer to the substance (Klijn *et al.*, 1995). Lack of acute oral toxicity of a substance does not necessarily equate to an absence of harmful effects if the substance is administered chronically.

A LAB strain which is non-pathogenic for healthy hosts during short term exposure may become pathogenic in hosts with sub-optimal immune functions when it is consumed at a high dose for long periods of time. A recently published report (Rautio *et al.*, 1999) regarding the possible implication of a commercial probiotic strain *Lb. rhamnosus* GG in a case of severe clinical infection provides evidence of this possibility. Therefore it is necessary to investigate the consequence of longer term consumption of these probiotic organisms on host health. To date, there has been only a limited number of reports examining the chronic toxicity of probiotic bacteria (Momose *et al.*, 1979). In the current study, the possible health hazards likely to arise from repeated exposure to probiotic LAB strains HN001, HN017, and HN019 over four weeks, was conducted in accordance with the 1995 OECD guidelines for 'repeated dose 28-day oral toxicity study in rodents'. The effects of dietary consumption of these strains at different doses on the animals' health, growth, haematology and blood chemistry, as well as the potential infectivity and pathogenicity (translocation and mucosal histology) of these organisms were observed.

8.2 Materials and methods

8.2.1 Animals

One hundred male BALB/c mice (aged 6-8 weeks) were housed in the same conditions described in Section 3.16.

8.2.2 Probiotic strains

The same bacterial strains (HN001, HN017, and HN019) as those stated in Section 7.2.1 were tested in this experiment. *Lb. acidophilus* (LA-1) was included as a reference strain for comparative purposes. The source, propagation, purification, storage of these strains are described in Section 3.1 and 3.5. The preparation of LAB cell cultures for inoculation of mice is described in Section 3.15. The cell pellets were re-suspended in 10% skim milk at three different concentrations (1×10^9 , 2×10^{10} , and 1×10^{12} cfu/ml).

8.2.3 Experimental design

After acclimatisation to experimental conditions for two weeks, 78 mice with relative optimal growth (highest live body weight gain) were selected and randomly assigned into five different groups. With a sterile pipette, mice in four of the groups were orally inoculated with one of the four different LAB strains at three different doses, while the fifth group (control) was orally administered 10% SMP only. Fifty microlitres of cell suspensions, or 10% SMP, was administered at each inoculation so that each mouse (average body weight 20 g) in the treatment groups received 5×10^7 , 1×10^9 , or 5×10^{10} cfu LAB /day. The treatments lasted for 28 days, during which time, the activity, behaviour, and hair lustre of each mouse were observed and recorded daily, while water intake (WI), feed intake (FI), and body weight were measured weekly. The specific growth rate (SGR) was expressed as the average weight gain (g) in one week. On day 29, all animals were humanely euthanased by isofluorane overdose and their blood and tissue samples were collected aseptically for further laboratory analysis.

8.2.4 Haematology

Blood samples were obtained and analysed using the method described in Section 3.18. Total and differential leucocyte counts, total red blood cell (RBC) and platelet counts, haemoglobin concentration (HB), mean corpuscular volume (MCV), mean corpuscular

haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were determined.

8.2.5 Blood biochemistry

The levels of total plasma protein (BCA Protein Assay Kit, Pierce, Illinois, USA), albumin (BCG Albumin Reagent, 631-M, Sigma, St. Louis, USA), total plasma glucose (Glucose 100 Trinder, Sigma, St. Louis, USA), total plasma cholesterol (Cholesterol 500, Sigma, St. Louis, USA), were determined using commercial diagnostic kits and reagents (Sections 3.22-3.24).

8.2.6 Bacterial translocation

Blood and tissue samples [spleen, liver, and mesenteric lymph nodes (MLN)] were cultured using the methods described in Section 3.19. The bacterial strains recovered from tissues were identified using RAPD fingerprinting techniques (Section 7.2.5). Analysis of these samples was conducted at the same time as the samples from Chapter 7.

8.2.7 Histology

The gross anatomy of the visceral organs of each mouse was checked and recorded. Spleen weight index (SWI) was expressed as the actual spleen weight (mg) divided by body weight (g). Intestinal tissue samples were collected and processed using the method described in Section 3.21.

8.3 Results

Throughout the experimental period, there was no noticeable change in activity, behaviour, or hair lustre in any of the groups of mice. No diarrhoea or other treatment-related sickness or death was recorded. At the end of the experimental feeding period, all animals were alive and healthy.

8.3.1 Feed and water intake

There was no significant difference in daily feed (FI) and water intake (WI) among animals fed with the different probiotic strains, the reference strain or the control diet.

No significant difference was detected in FI and WI among different dose groups (Figures 8.1 and 8.2).

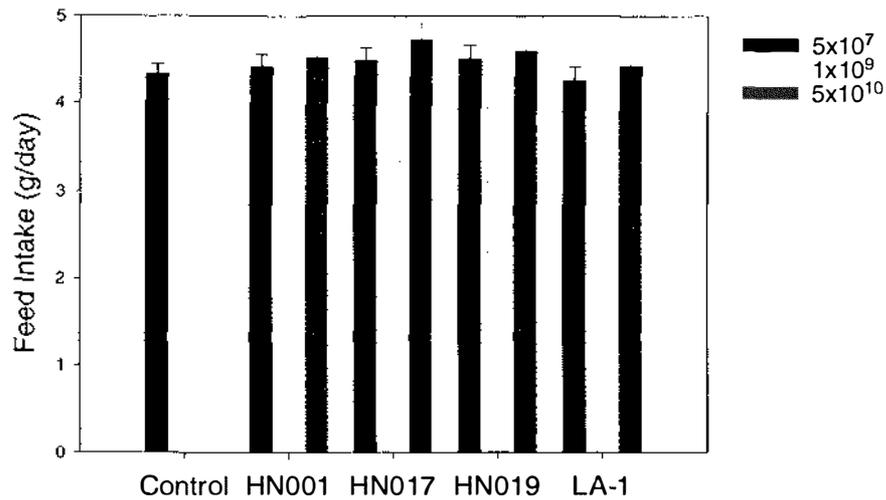


Figure 8.1 Daily feed intake of mice fed with probiotics for four weeks. Data included are the group Means + SEM. Doses: cfu/mouse/day. Control group did not receive LAB.

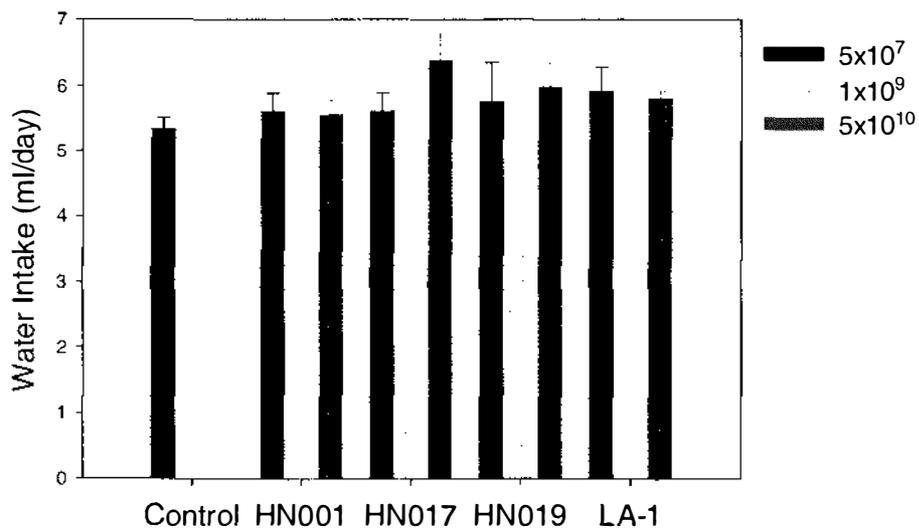


Figure 8.2 Daily water intake of mice fed with probiotics for four weeks. Data included are the group Mean + SEM. Doses: cfu/mouse/day. Control group did not receive LAB.

8.3.2 Growth

Data on live body weight gain are shown in Figure 8.3. There was no significant difference in growth rate between the treatment groups and the control group ($P > 0.05$). Growth rate of mice receiving HN001, HN017, or HN019 exhibited dose-associated

fluctuation, but no significant statistical difference between treatments ($p>0.05$). The mice given probiotics had a similar total body weight increase to that of control group mice during the experiment period.

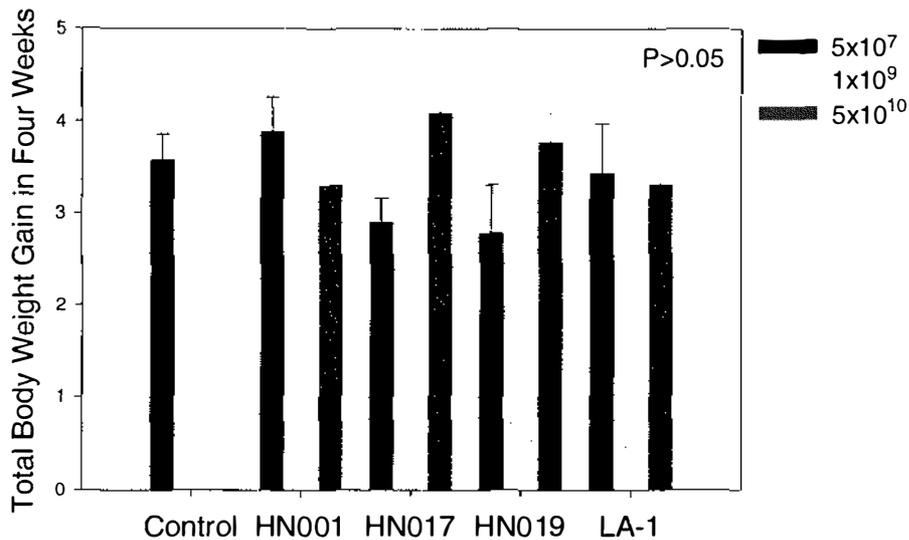


Figure 8.3 Overall body weight gain of mice fed with probiotics for four weeks. Data shown are group means + SEM of weight gain. Control group mice did not receive any LAB. Doses: cfu/mouse/day. Control group did not receive LAB.

8.3.3 Haematology

The effects of feeding different LAB strains on haematological parameters is shown in Table 8.1. No statistical significant differences in total and differential leucocyte counts were detected among the treatment groups at any of the different doses, although the number of neutrophils in animals fed with HN001, and the total leucocyte count in mice treated with LA-1, were relatively higher than other groups. There was no significant difference in RBC, HB, HT, MCV, MCH, MCHC or platelet counts among the mice in the different groups.

8.3.4 Blood biochemistry

The levels of total plasma protein, albumin, glucose and total plasma cholesterol are shown in Table 8.1. Animals fed with the probiotic strains had similar blood biochemistry profiles to those of the control (non LAB-fed) animals.

Table 8.1 Effect of four week probiotic feeding on the haematology and biochemical parameters of mice (Mean \pm SEM)

	Control	HN001	HN017	HN019	LA-1
HB (g/l)	150.4 \pm 2.08	150.0 \pm 1.88	150.1 \pm 1.86	149.9 \pm 1.68	148.2 \pm 2.40
RBC ($\times 10^{12}$ /l)	9.64 \pm 0.18	9.62 \pm 0.10	9.62 \pm 0.10	9.76 \pm 0.09	9.40 \pm 0.10
Platelet ($\times 10^9$ /l)	531.2 \pm 51.6	603.9 \pm 36.4	583.9 \pm 42.8	585.5 \pm 21.9	552.9 \pm 36.8
HT (l/l)	0.45 \pm 0.07	0.47 \pm 0.004	0.45 \pm 0.004	0.46 \pm 0.25	0.45 \pm 0.004
MCV (fl)	47.00 \pm 0.32	47.33 \pm 0.16	47.21 \pm 0.21	47.15 \pm 0.25	47.58 \pm 0.31
MCH (pg)	15.56 \pm 0.27	15.54 \pm 0.13	15.56 \pm 0.17	15.32 \pm 0.21	15.60 \pm 0.15
MCHC (g/l)	331.0 \pm 4.18	328.6 \pm 2.81	330.5 \pm 3.25	325.2 \pm 3.28	328.9 \pm 3.71
WBC ($\times 10^3$ /l)	3.72 \pm 0.78	3.24 \pm 0.33	3.09 \pm 0.42	2.88 \pm 0.47	3.87 \pm 0.60
Lymphocytes (%)	60.33 \pm 3.06	56.20 \pm 3.64	65.00 \pm 2.71	60.30 \pm 2.79	65.27 \pm 3.77
Neutrophils (%)	37.83 \pm 2.90	40.60 \pm 4.07	31.50 \pm .52	34.23 \pm 3.12	31.18 \pm 3.63
Plasma protein (g/dl)	6.40 \pm 0.67	6.91 \pm 0.31	6.37 \pm 0.13	6.83 \pm 0.30	6.30 \pm 0.17
Albumin (g/dl)	3.92 \pm 0.23	3.79 \pm 0.17	4.07 \pm 0.18	3.89 \pm 0.18	3.93 \pm 0.15
Glucose (mg/dl)	128.4 \pm 13.3	118 \pm 6.30	122.5 \pm 6.70	120.0 \pm 7.40	124.6 \pm 7.0
Cholesterol (mg/dl)	120.8 \pm 4.45	126.5 \pm 4.33	122 \pm 4.69	120.8 \pm 3.28	125.9 \pm .61

All values $P > 0.05$ (control vs probiotic-fed animals).

8.3.5 Histological changes

Macroscopic examination did not reveal any obvious differences in the size and appearance of visceral organs between groups, and there was no noticeable hepatomegaly, splenomegaly, or caecal enlargement. There was no significant difference in SWI among different groups of mice (Table 8.2). Microscopic observations found no signs of inflammation, degeneration or necrosis of the intestinal mucosa. There were no significant differences in the arrangement of epithelial cells between the test and control groups. Table 8.3 shows the results of mucosal structural measurements, where no significant differences were detected in VH, EH, or MT among the different groups; animals receiving LA-1 had relatively higher caecal mucosal thickness than that of animals fed with HN017 ($p > 0.05$).

Table 8.2 Effect of four week probiotic feeding on spleen weight index of mice

Treatment	Mean	SEM
HN001 (n = 18)	3.36	0.13
HN017 (n = 18)	3.40	0.16
HN019 (n = 18)	3.27	0.10
LA-1 (n = 18)	3.77	0.22
Control (n = 6)	3.38	0.16

The data included in this table are the total values of three different dose groups within each probiotic group. These values were derived from the formula "spleen weight (mg)/mouse body weight (g)". All values P>0.05.

Table 8.3 Gut mucosal histology of mice fed with probiotics for four weeks

	HN001	HN017	HN019	LA-1	Control	P values
IVH	239.7 ± 6.63	231.9 ± 10.7	216.8 ± 15.3	242.5 ± 10.6	216.6 ± 5.32	0.31
IMT	310.6 ± 11.1	296.0 ± 9.22	311.9 ± 29.3	307.1 ± 13.6	288.4 ± 7.05	0.81
IECH	25.43 ± 1.99	28.40 ± 2.32	27.58 ± 1.13	24.16 ± 1.75	24.44 ± 2.68	0.50
CAMT	122.57 ± 9.6	109.1 ± 5.10	131.4 ± 13.4	142.0 ± 9.08	131.3 ± 2.75	0.15
CAECH	25.24 ± 1.48	24.27 ± 1.52	23.76 ± 0.90	23.09 ± 1.26	25.80 ± 1.16	0.84
COMT	207.6 ± 16.6	244.8 ± 35.1	200.1 ± 12.5	220.3 ± 9.60	208.4 ± 14.8	0.41
COECH	27.74 ± 2.27	28.46 ± 0.65	26.21 ± 1.86	29.94 ± 1.73	27.92 ± 1.21	0.64

The values (μm) included in this table are the means of animals in the highest dose (5×10^{10} cfu/mouse/day) groups. Ten measurements from each sample were taken and the mean was used to calculate the group mean for specific parameters. IVH: ileum villus height; IMT: ileum mucosal thickness; IECH: ileum epithelial cell height; CAMT: caecal mucosal thickness; CAECH: caecal epithelial cell height; COMT: colon mucosal thickness; COECH: colon epithelial cell height.

8.3.6 Bacterial translocation

A translocation-positive animal was defined as an animal that had at least one tissue sample (including blood) containing one or more viable bacterial cells. A positive translocation tissue was defined as a tissue from which at least one viable bacterial cell was recovered (one colony). The number of positive animals and positive tissue samples in each treatment group are shown in Table 8.4 and Table 8.5 respectively.

Statistical analysis (Fisher's Exact Test) did not reveal any significant differences in the proportion of translocation positive animals and positive tissues between the different groups ($p > 0.05$). The identification of bacterial cells recovered from tissues was carried out together with the samples from the experiment described in Chapter 7, therefore the RAPD fingerprinting results from this experiment are included in Figures 7.4 to 7.6 (Chapter 7). No RAPD pattern from the tissue samples matched that of any of the test LAB strains fed to the mice.

Table 8.4 Incidence of bacterial translocation in mice fed with probiotics for four weeks

Dose*	Control	HN001	HN017	HN019	LA-1
5×10^7	-	0/6	0/6	1/6	1/6
1×10^9	-	1/6	1/6	2/6	3/6
5×10^{10}	-	4/6	0/6	0/6	1/6
Total	2/6	5/18	1/18	3/18	5/18

The numbers included in this table represent the number of positive animals / total number of examined animals). * Dose: cfu of LAB/mouse/day. Fisher's Exact Test: $P > 0.05$ (between different treatment groups or different dose groups).

Table 8.5 Incidence of bacterial translocation to different tissues

Treatment	MLN	Spleen	Liver	Kidney	Blood
Control	1/6	0/6	1/6	0/6	0/6
HN001	5/18	0/18	0/18	0/18	0/18
HN017	0/18	0/18	0/18	1/18	0/18
HN019	3/18	0/18	0/18	0/18	0/18
LA-1	3/18	1/18	2/18	2/18	0/18
P values*	0.165	1.00	0.106	0.693	NA

The numbers included in this table represent the number of positive tissues/total number of examined tissues). * Fisher's Exact Test.

8.4 Discussion

In this study, animals were fed with $5 \times 10^{7-10}$ cfu of tested LAB/mouse/day, which is a more physiologically relevant dose range than 10^{11} cfu/mouse/day which was used in the acute oral toxicity study (Chapter 7).

All of the animals in this experiment were healthy prior to sampling. No noticeable abnormal behaviour, change in activity, or decline in hair lustre was detected in mice

after four weeks of feeding with the probiotic strains HN001, HN017, and HN019. The feed intake (FI) and water intake (WI) of the mice fed with LAB showed no significant changes during the experimental period compared with that of the control animals. These results indicate that the test strains have no adverse effect on the general health status of mice when administered orally for four weeks. Growth rate (body weight gain) was not affected by the administration of the test LAB strains even in the high dose groups. No dose-dependent effect was observed in FI, WI or body weight gain. This is consistent with the findings of Donohue *et al.* (1993) and Momose *et al.* (1979) who examined the toxicity of the same reference commercial LAB species assessed in this study.

Infectivity and pathogenicity are two important components of safety studies on probiotic bacteria. Increases in peripheral blood total leucocytes or neutrophils are useful indicators of bacterial infection. In this study, the total leucocyte counts and proportion of neutrophils in both control and probiotic-fed mice were relatively lower than previously reported values ($4-14 \times 10^3/\mu\text{l}$ and 65:50) (Loeb *et al.*, 1978). This may be related to the variation between different animal species and experimental conditions. It has been reported that mice housed alone (as in this study) or in small groups had the lowest peripheral leucocyte count (Loeb *et al.*, 1978). Splenomegaly and hepatomegaly are indirect indicators of infection, and again in this study there were no macroscopic changes in the spleen or liver morphology in animals administered with test strains. Furthermore, animals in the test groups had similar spleen weight index to those of control mice. These results suggest that the mice did not experience any noticeable physiological changes in the blood or visceral tissues resulting from the 4-week feeding with probiotic strains HN001, HN017, or HN019.

In feeding studies, bacteraemia or translocation of bacteria indicates the degree of bacterial infectivity and pathogenicity of the strains being tested (Ford *et al.*, 1996; Urao *et al.*, 1996). Since no LAB strains used for feeding in this study were detected in the blood or extra-gut tissue samples, it can be concluded that none of these strains exhibit invasive properties. The ability of indigenous gut flora to translocate into extra-intestinal tissues has been demonstrated in several published studies (Hale & Hill, 1973; Berg & Garllington, 1979; Berg, 1983; Steffen & Berg, 1983; Ma *et al.*, 1990; Swank & Deitch, 1996), thus the few isolated microorganisms found in the tissue samples here

may have been derived from the indigenous gut flora rather than from the animals' dietary intake. This is further supported by the results of the RAPD fingerprinting assay, where no RAPD patterns identical to the test strains were detected.

The importance of the intestinal mucosa in the gut barrier system has been discussed in Chapter 4 and Chapter 7. In this study, oral inoculation with the test strains had no adverse effects on the integrity of the gut mucosa, providing further evidence that these strains are non-toxic and non-pathogenic. Momose *et al.* (1979) had also documented a similar result in a toxicity study on dietary bifidobacteria strains (*Bifidobacterium longum* BB-536).

Biochemical assays can be used for detecting moderate to mild deficiencies of nutrients or an imbalance in nutrient metabolism (Swendseid, 1987). These deficiencies are usually apparent before any clinical symptoms or changes in body weight. To detect adverse sub-clinical effects of test strains, a range of haematological and general blood biochemical parameters was monitored. It was noted that the test strains had no adverse effects on RBC, HB, HT, platelet count, or other haematological parameters. The total plasma protein, albumin, glucose, and total plasma cholesterol levels of animals treated with test strains were not significantly different from those of control mice, with the values of all these parameters being within the normal range of the control mice. This suggests that colonisation with test strains over a period of four weeks has no adverse effect on mouse haematology and blood biochemistry. The metabolism of protein, carbohydrate, and lipid (reflected by the plasma concentration of these substances) was not affected by feeding with the test strains. Tortuero *et al.* (1995) have also found that administration of *Streptococcus faecacium*, *S. thermophilus* spp, or *Lactobacillus bulgaricus* spp had no effect on plasma glucose, total protein, and albumin levels.

In summary, feeding mice with probiotic strains *Lb. rhamnosus* HN001, *Lb. acidophilus* HN017 or *B. lactis* HN019 at doses of $5 \times 10^7 \sim 5 \times 10^{10}$ cfu/mouse/day for four weeks had no adverse effect on general health status, growth, haematology, blood biochemistry and histology parameters examined in this study. The test strains did not cause infection and did not translocate from the original colonisation site (gut) after feeding. This indicates that chronic consumption of test LAB strains has no harmful effects on animal health, and it is therefore unlikely to result in any adverse effect in humans.

9

Safety assessment of probiotic strains HN001 and HN019 in immunocompromised mice

Abstract

Although the use of probiotic LAB strains are believed to be safe for healthy animals and human subjects, the possible risk of opportunistic infections by LAB in immunodeficient hosts remain a serious concern. This study aimed to evaluate any potentially adverse effects of dietary supplementation of two probiotic LAB strains, HN001 and HN019, in immunocompromised animal model. Immunosuppression was induced 24 hrs prior to probiotic feeding by the administration of subcutaneous injections of dexamethasone (200µg/mouse/48hrs). Freshly prepared HN001 or HN019 were fed to animals at a dose rate of $1.5 \sim 2.5 \times 10^7$ cfu/mouse/day for 7 days. A pathogenic bacterial strain *Salmonella typhimurium*, which was fed to one group of mice in a single dose (1.5×10^6 cfu/mouse), was included as a positive control in a bacterial translocation assay. The general health status, feed consumption, and changes in live body weight of animals were monitored during this experimental period. At the end of the experiment, the animals' blood and tissue samples were collected to determine the haematological parameters, total and differential leucocyte counts, and bacterial translocation. Results showed that no significant changes were noted in any of the safety parameters measured among the LAB-fed mice. Dietary supplementation of probiotic organisms had no detrimental effect on the animals' general health, feed intake, or growth. No translocation or systemic infection caused by HN001 or HN019 was detected. These findings suggest that probiotic strains *Lb. rhamnosus* HN001 and *B. lactis* HN019 are well tolerated in immunocompromised mice without any significant safety concerns.

9.1 Introduction

Probiotic lactic acid bacteria (LAB) are largely believed to be innocuous in immunocompetent hosts (Gasser, 1994; Salminen *et al.* 1998a; Naidu *et al.* 1999), and the experiments described in the previous two chapters also demonstrated this. However, some LAB or Gram-positive bacteria closely related to probiotic LAB species, such as *Enterococcus*, *Streptococcus*, *Lactobacillus*, and bifidobacteria, have been associated with infections in patients with sub-optimal immune functions (Aguirre & Collins, 1993; Gasser, 1994; Rautio *et al.* 1999). The patients with compromised immune functions may be attacked by various saprophytic organisms and a number of commensal organisms, even those which are considered to be non-virulent (Peloux, 1985; Adams, 1999). This kind of opportunistic infection is the major cause of morbidity and mortality in patients with compromised immune responses that occur in conditions such as cancer, AIDS or HIV infections, and individuals receiving irradiation or immunosuppressive chemotherapy (Greenberg & Cohen, 1977; Esteban *et al.*, 1998; Morra *et al.*, 1999; Chokeychai *et al.*, 2000).

The causative relationship between immunosuppression and opportunistic infection has been confirmed by a large number of animal experiments (Owens & Berg, 1980; Kmet *et al.*, 1995; Ohsugi *et al.*, 1996; Wagner *et al.*, 1997). Although the infections caused by LAB are usually attributed to the endogenous flora, the infectivity of some particular probiotic strains in immunocompromised hosts has been reported on occasion (Link *et al.*, 1995; Wagner *et al.*, 1997; Rautio *et al.* 1999). The possible risk of opportunistic infections by LAB strains raises obvious concerns about the safety of probiotic organisms in immunodeficient hosts (Wagner *et al.*, 1997; Guarner & Schaafsma, 1998). Therefore, the tolerance of probiotic strains in immunocompromised hosts has been recommended as one of the key safety indicators for probiotic organisms (van der Kamp, 1996; Donohue *et al.*, 1998; Duffy, 2000). This study aimed to investigate the safety or tolerance of probiotic candidates LAB strains *Lb. rhamnosus* HN001 and *B. lactis* HN019 in immunocompromised BALB/c mice.

9.2 Materials and methods

9.2.1 Experimental design

a) Animals and immunosuppression

Male BALB/c mice aged 6-8 weeks with an average body weight of 20 grams were randomly allocated into different treatment groups (Table 9.1) after 2 weeks acclimatisation to experimental conditions as described in Section 3.16.

Table 9.1 Experimental design

Groups	No. of mice	Saline	DEX	Probiotics	Salmonella
1	8	✓			
2	8		✓		
3	8		✓	✓ HN019	
4	8		✓	✓ HN001	
5	8		✓	✓ Lb. GG	
6	8		✓		✓

Dexamethasone (DEX, DBL dexamethasone sodium sulfate injection, U.S.P., F.H. Faulding & Co., Victoria, Australia) was injected subcutaneously (s.c.) to animals from groups 2 - 6 at alternative sites of the thighs at a dose of 200 µg/mouse/48 hrs during the entire experimental period. Mice in the control group (group 1) were given sterile saline (0.9%) injections using the same method as the DEX injections. The animals' general health status was scored daily on a 5-scale based standard, i.e. general health scores (GHS) as described in Section 3.16 (Table 3.2). The animals were observed twice a day for potential signs of illness; their feed and water intake, body weight were monitored once a week.

b) Feeding

Twenty-four hours after the first DEX injection, mice in test groups (groups 3-5) received daily oral inoculation of 50 µl freshly prepared probiotic cultures (i.e. 1.5 ~ 2.5 x 10⁷ cfu/mouse/day), and the animals in the negative control group (group 1) were fed with the same volume of a 10% skim milk powder based diet. The feeding of probiotics

or control diet was continued for 7 days. The animals in the positive control group (group 6) were orally challenged with a single dose (1.5×10^6 cfu/mouse) of a *S. typhimurium* suspension.

9.2.2 Probiotic strains

LAB cultures of *Lb. rhamnosus* HN001, *B. lactis* HN019 and a commercial strain *Lb. rhamnosus* GG (*Lb.* GG) were cultivated, purified, and maintained using the methods described in Section 3.5. An enteric pathogenic strain *S. typhimurium* (*S. typhimurium* ATCC 14028) was obtained from the New Zealand Reference Culture Collection (Medical Section, ESR Health, Wellington, New Zealand) and used as the positive control in the translocation assays. The stock cultures of *S. typhimurium* were sub-cultured for 18-24 hrs in BHI broth (Section A1.2) and then purified on coliform agar plates (Section A1.3) using the methods described in Section 3.5.2 b. The purified cultures were washed 3 times in sterile cold phosphate buffered saline (PBS) and the cell concentrations were adjusted to the desired concentrations (LAB 3 ~ 5×10^8 cfu/ml; *S. typhimurium* : 3×10^7 cfu/ml). The cells were then centrifuged and re-suspended in 10% (w/v) skim milk.

9.2.3 Bacterial translocation

At the end of the experiment, animals were humanely euthanased by isoflurane overdose. The blood and tissue samples [liver, spleen, kidney, mesenteric lymph nodes (MLN)] were collected and homogenized aseptically using the methods described in Section 3.19. One hundred microlitre of blood or tissue suspensions were inoculated on MRS or MRS-C, BHI, or coliform agar plates, which were incubated anaerobically (MRS and MRS-C agar plates) or aerobically (BHI and coliform agar plates) at 37°C for 48 hrs. The number of colonies on the agar plates was recorded and the positive cultures on MRS, MRS-C, or coliform agar plates were identified using the method described in Section 3.19. The cells recovered from tissues or blood were identified to species level.

9.2.4 Haematology

Total and differential percentages of white blood cells (lymphocytes, neutrophils, monocytes, and eosinophils), red blood cells (RBC) and platelet counts (PLC), mean

corpuscular volume (MCV), and haematocrit (HT) were determined using the methods as described in Section 3.20.

9.2.5 Immune responses

The phagocytic cell activities (phagocytosis) of peripheral blood leucocytes from mice which received LAB were measured using the method described in Section 3.30.

9.3 Results

9.3.1 General health status

Throughout the entire period of the experiment no noticeable behavioural or activity changes were observed in immunosuppressed mice that were fed with probiotic LAB strains, the mice in these groups remained healthy during the experimental period. In contrast, most of the *S. typhimurium* challenged mice (6 out of 8) exhibited signs of infection or developed diarrhoea 4 to 5 days after the challenge. One mouse from this group died on day 6. The average general health score (GHS) values in this group was 3.6 ± 0.7 (mean \pm SEM), whereas mice in normal control group and probiotic-fed groups had GHS values of 1.0 ~ 1.5 (score 1: normal healthy animal; score 5: severely sick animal).

9.3.2 Feed and water intakes and live weight changes

There was no significant difference in the feed intake between DEX-treated mice which received probiotic LAB strains and the normal mice (control) or animals received DEX injections only (group 2). *S. typhimurium* challenged mice had significantly lower feed and water intake compared with the mice in other groups (Figures 9.1 & 9.2). The mice which received DEX treatment lost body weight over the experimental period. *S. typhimurium* challenged mice had greater weight loss than the other groups ($P < 0.001$). There was no significant difference in the changes in body weight between mice which received DEX only and the mice which were treated with DEX plus probiotic feedings (Fig 9.3).

9.3.3 Haematological changes

Animals which received probiotic dietary supplementation had similar haematological parameters to those of the control group mice. In contrast, mice which received oral *S. typhimurium* challenge had significantly lower PLT, higher RBC and HT than the other groups (Fig 9.4).

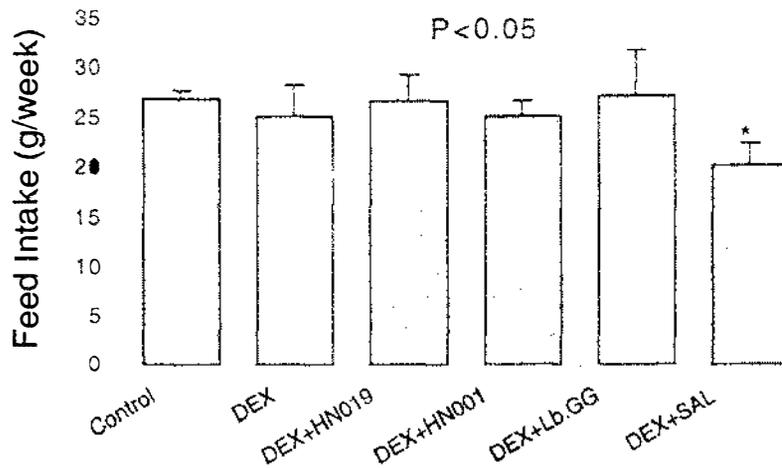


Figure 9.1 Total feed intakes of mice receiving DEX and probiotics (Mean + SEM). SAL: *S. typhimurium*; Bars with asterisk (*) are significantly different to the other groups.

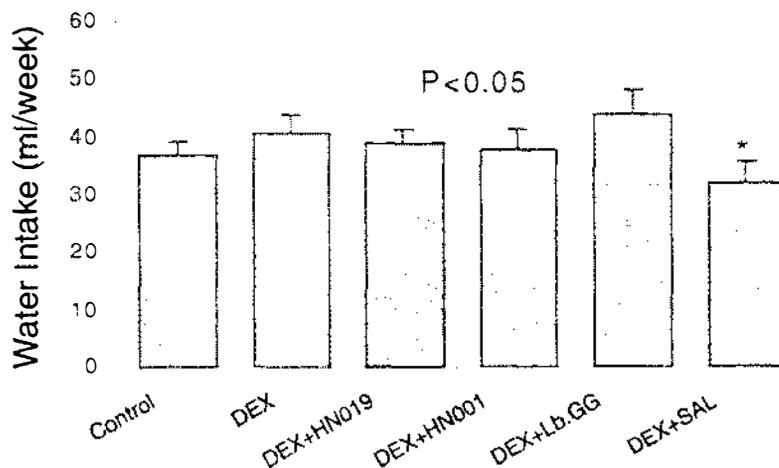


Figure 9.2 Total water intakes of mice receiving DEX and probiotics (Mean + SEM). SAL: *S. typhimurium*; Bars with asterisk (*) are significantly different to the mean of DEX + Lb. GG group.

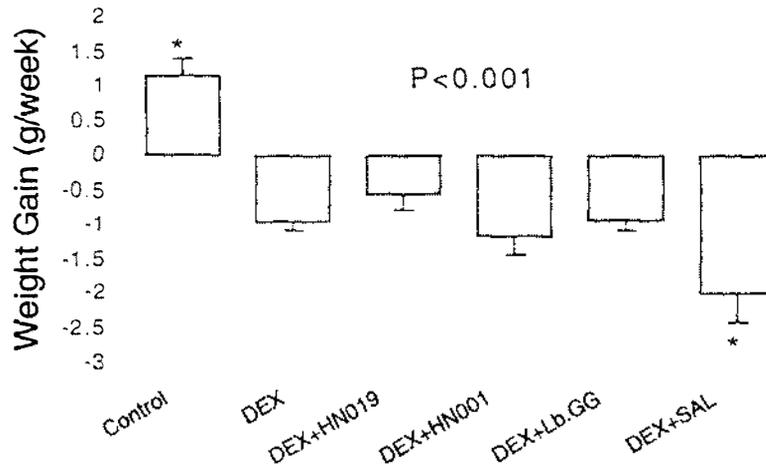


Figure 9.3 Changes in body weight of mice administered DEX and probiotics (Mean + SEM). SAL: *S. typhimurium*; Bars with asterisk (*) are significantly different to other groups.

9.3.4 Effect on health

Animals treated with DEX had a significant decline in their peripheral blood lymphocyte count (reduced by more than 50%, Figure 9.5), spleen weight (reduced by more than 50%, Figure 9.6), and blood leucocyte phagocytic cell activity (reduced by more than 30%) (Figure 9.7). Mice which received probiotic feeding and DEX-treatment had similar WBC counts, percentages of neutrophils and lymphocytes, and spleen weight indices compared to animals which received DEX only. HN001 and HN019 treated mice which received DEX-treatment had comparable leucocyte phagocytic activities to those of normal control mice which did not received DEX-treatment. In contrast, *S. typhimurium*-challenged mice which received DEX-treatment had significantly higher WBC and spleen weight index than other groups (Figure 9.6).

9.3.5 Bacterial translocation

No viable isolates of HN001, HN019, or *Lb. GG* were detected from any of the blood or tissue samples (Table 9.1) from immunocompromised mice. There were only a few viable cells recovered from either the spleen, liver, kidney or MLN of animals in the test and control groups on BHI agar plates, but no bacteria were cultured on *Lactobacillus* MRS or MRS-C agar. *Salmonella* challenged mice all became infected with *S. typhimurium*; the majority of the tissue samples from these animals were culture-positive on coliform agar plates. The cells recovered on BHI agar plates from the

tissues from LAB-fed mice were all identified as species other than that with which the mice were inoculated. A pure culture identified as *S. spp* was obtained from all *Salmonella* infected mice. There were no significant differences in the general bacterial translocation rate between probiotic LAB-fed mice and control group mice. Some animals from the *Salmonella*-challenged group exhibited spleen and MLN enlargement

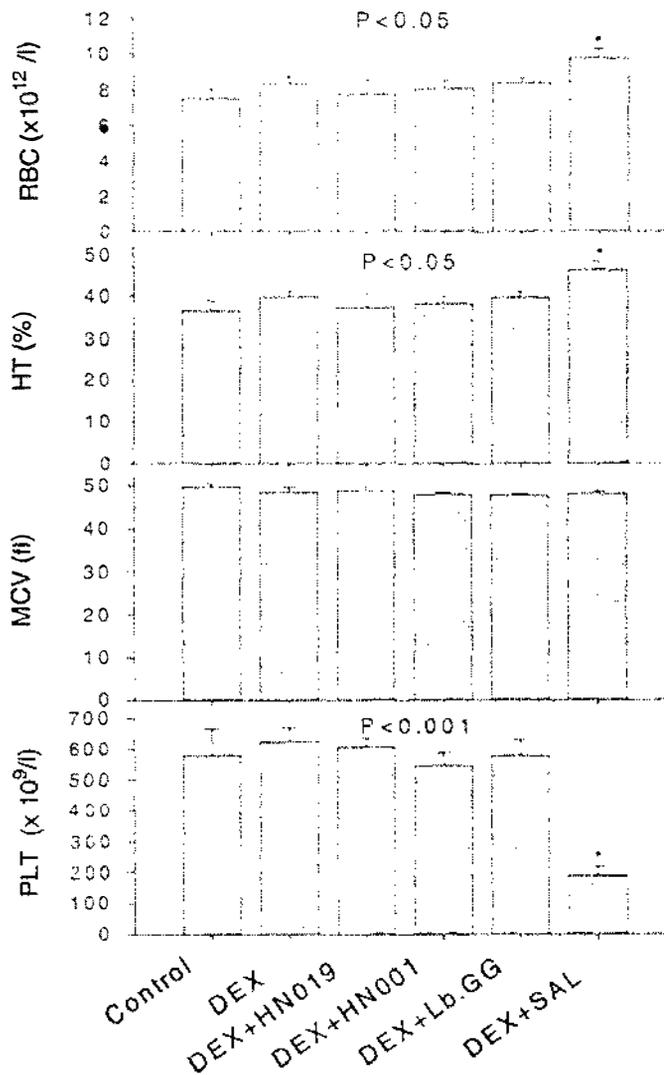


Figure 9.4 Haematological changes in mice administered DEX and probiotics. SAL: *S. typhimurium*; Bars with asterisks (*) are significantly different to other groups (RBC: DEX+SAL vs Control; HT: DEX+SAL vs Control, DEX+HN019, and DEX+HN001). Blood samples were collected and analysed at the end of the experiment (day 7).

which was reflected by the spleen and MLN weight, and there were some grey spots (tissue necrosis) on the liver surface from animals in this group (in 3-5 out of 8 animals).

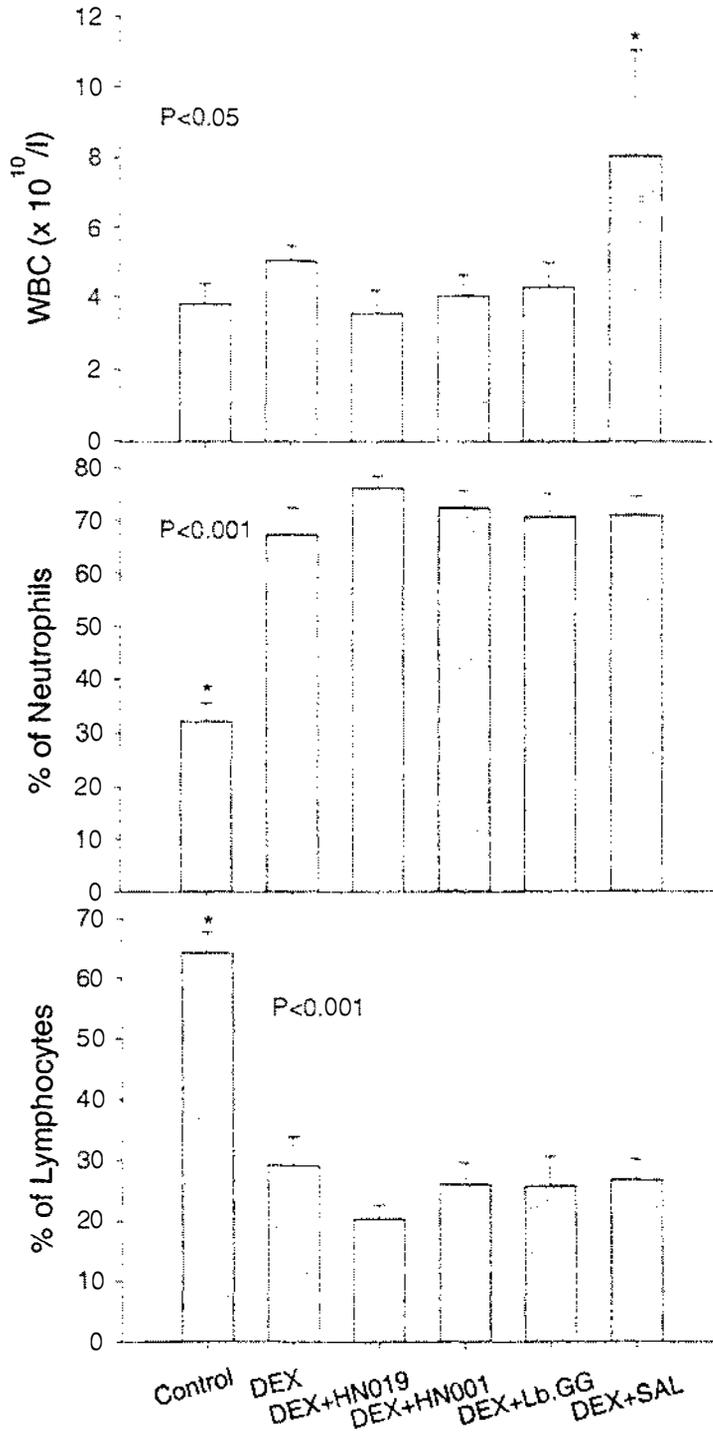


Figure 9.5 Total and differential blood leucocyte counts in mice administered DEX and probiotics. SAL: *S. typhimurium*; Bars with an asterisk (*) are significantly different to other groups. Blood samples were collected and analysed at the end of the experiment (day 7).

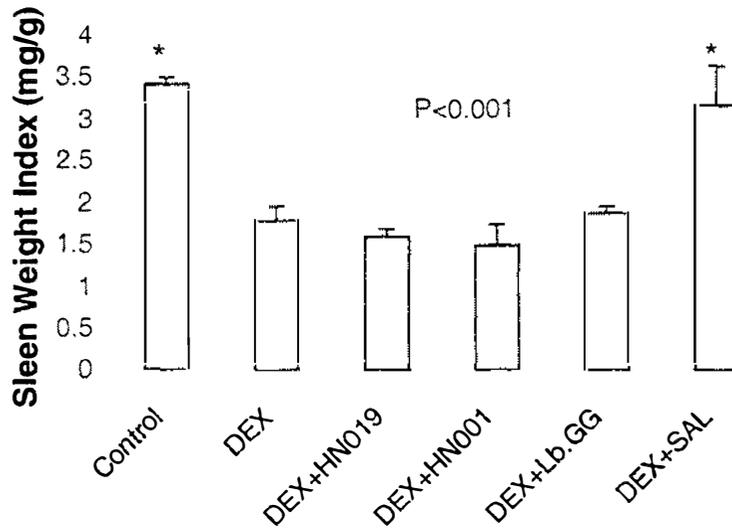


Figure 9.6 Spleen weight index in mice administered DEX and probiotics. SAL: *S. typhimurium*; Bars with an asterisk (*) are significantly different to other groups. The spleens were collected and weighed at the end of experiment; spleen weight index was calculated according to the final body weight (at day 7).

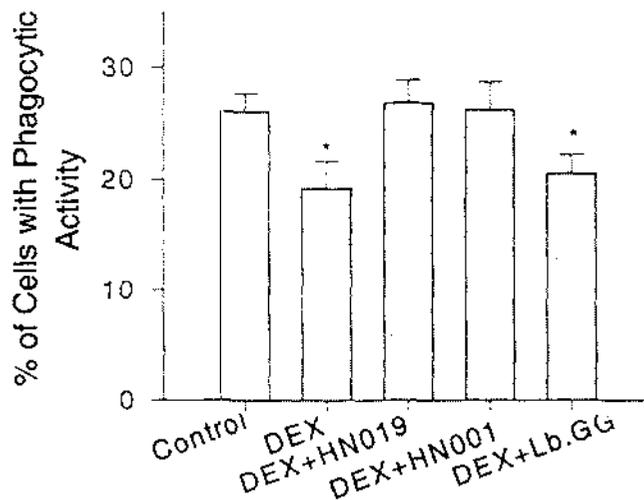


Figure 9.7 Phagocytic cell activity of peripheral blood leucocytes from mice receiving DEX and probiotics. Bars with (*) are significantly different to other groups. The blood samples were collected at the end of the experiment (day 7). Blood samples were not available from 4 mice in Group 6 (salmonella challenged mice, one was dead on day 6, others were severely sick); the quality of the available blood samples from this group was therefore not suitable for this analysis.

Table 9.2 Bacterial translocation in mice receiving DEX and probiotics or *S. typhimurium*.

Treatment	Tissues										Animals ^{\$}	
	Blood		Spleen		Liver		Kidney		MLN			
	Total [@]	LAB/SAL [#]										
Control	0/8	0/8	1/8	0/8	1/8	0/8	0/8	0/8	0/8	1/8	0/8	2/8
DEX	0/8	0/8	1/8	0/8	0/8	0/8	0/8	0/8	0/8	1/8	0/8	1/8
DEX+HN019	0/8	0/8	2/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	2/8
DEX+HN001	0/8	0/8	1/8	0/8	2/8	0/8	2/8	0/8	1/8	0/8	0/8	3/8
DEX+Lb.GG	0/8	0/8	2/8	0/8	0/8	0/8	0/8	0/8	1/8	0/8	0/8	2/8
DEX+SAL	2/4	1/4 (sal)	6/8	6/8 (sal)	5/8	5/8 (sal)	5/8	4/8 (sal)	2/8	2/8 (sal)	2/8 (sal)	8/8

The values in this table are : (No. of positive cultures or animals / No. of tissues or animals tested); SAL: *S. typhimurium*

@: the general bacterial translocation incidence; #: incidence of LAB or *S. typhimurium* translocation; \$: general BT positive animals.

Probiotic LAB supplementation did not induce bacterial translocation in immunocompromised mice. Fisher's Exact Test: P>0.05 (LAB groups vs control and DEX group); P<0.001 (SAL groups vs other groups).

9.4 Discussion

Immunosuppression can be experimentally established in conventional animals using immunosuppressive agents, such as cyclophosphamide (Nakayama *et al.*, 1997), corticosteroids (Fauci, 1979; Cohn, 1991), or anti-metabolites (Altmann, 1974) and exposure to X-Irradiation (Dong *et al.*, 1987). Cyclophosphamide often induces delayed toxicity in rodents (Rehg, 1988) in spite of its immunosuppressive effect. X-Irradiation not only compromises the immune system but also damages the intestinal mucosal architecture (Gordon *et al.*, 1955; Wells *et al.*, 1988a). Both of these two methods have serious bone marrow inhibition side effects (Anton, 1987). In contrast, glucocorticosteroids (GCC) particularly dexamethasone (DEX), a potent synthetic GCC with the ability to regulate virtually all components of the immune system, have relatively fewer adverse effects on other systems with the exception of their anti-inflammatory properties (Wira *et al.*, 1990; Minton *et al.*, 1991; Kunicka *et al.*, 1993; Nonnecke *et al.*, 1997). Therefore, DEX has been frequently used as an immunosuppressant to establish an immunocompromised animal model in rodents (Rehg *et al.*, 1988; Rasmussen & Healey, 1992; Sukura *et al.*, 1995; Yang *et al.*, 1993) and other animal species (Wesley *et al.*, 1989; Isobe & Lillehoj, 1993; Lindenstrom & Buchmann, 1998). The DEX administration dosage and frequency used in this study were based on a primary pilot study. Among the immunosuppressive effects of GCC, the changes in leucocyte kinetics and functions are the most profound and consistent response (Fauci, 1979; Cohn, 1991). Lymphocytopaenia and neutrophilia are the classic components of the GCC-induced stress profile (Fauci, 1975; 1979; Cohn, 1991). Likewise, total and differential leucocyte counts, and blood leucocyte phagocytic cell activity were used as indicators of immunosuppression in this study, and the results agree with the findings in the reports described above. The profound lymphocytopaenia and neutrophilia (Figure 9.5) and decrease in phagocytic cell activity (Figure 9.7) of mice in response to DEX injections demonstrated that the animals' immune responses were suppressed efficiently. The dramatic decrease in spleen weight in DEX-treated mice (Figure 9.6) also indicated some immunosuppression in these animals.

Opportunistic infection is the major concern regarding the safety of probiotic LAB strains in immunocompromised hosts (Gasser, 1994; Wagner *et al.*, 1997; Guarner & Schaafsma, 1998; Adams, 1999). Most opportunistic pathogens originate from the

gastrointestinal tract (GIT) (Wells *et al.*, 1988b; Stechmiller *et al.*, 1997; Duffy, 2000) via translocation – the passage of viable or nonviable microbes and their by-products (including endotoxins) across an intact GIT to other extraintestinal sites, such as the MLN, spleen, liver, kidney, and bloodstream (Ma *et al.*, 1990; Ford *et al.*, 1996). In this regard, the possibility of translocation of the viable probiotic LAB strains HN001 and HN019 was investigated to evaluate their opportunism (potential infectivity). *Salmonella* species are known to initiate infection of mammalian hosts by penetrating the intestinal epithelium of the small bowel (Jones *et al.*, 1994). The high translocation rate of *S. typhimurium* in rodents has been reported in previous studies (Gautreaux *et al.*, 1994; Nichterlein *et al.*, 1996; Shu *et al.*, 2000). Thus, a *S. typhimurium* strain (ATCC 14028) was included as a BT positive control in this study. Results obtained here demonstrate that HN001 or HN019 could not be detected from the tissue samples or bloodstream of immunosuppressed mice which were inoculated daily with these two probiotic strains for 7 days, i.e. they have no ability to penetrate GIT and translocate into extra-gut tissues in this model of immunosuppression. In contrast, animals (with DEX treatment) challenged with a single dose of *S. typhimurium* had a 100% translocation rate, which involved tissues ranging from MLN, liver, spleen, to kidney and even blood (Table 9.2). Signs of systemic infections were also demonstrated by marked increases in spleen weight indices and WBC counts in animals which received *S. typhimurium*. The decrease in platelet count (PLT) in this group of animals may be associated with the systemic infections.

Corresponding to the translocation assay result, DEX-treated animals which received probiotic supplementation had similar total and differential leucocyte counts (Figure 9.5), and spleen weight indices (Figure 9.6) compared with the animals which received DEX only. All these findings indicate that HN001, HN019, and *Lb. GG* did not induce opportunistic infection in mice with compromised immune functions. This result is in accordance with the findings reported by Dong *et al.* (1987), Naaber *et al.* (1998) and Wolf *et al.* (1998). Regarding the translocation ability of *Lb. GG*, the difference between the result from this study and some of the previous reports (Link *et al.*, 1995; Wagner *et al.*, 1997) could be explained by the different animal strains which were used in different studies, germ-free OF1 mice and NIH mice rather than conventional BALB/c mice, were used in previous studies.

There are several reports documenting the application of probiotics in immunocompromised hosts to stimulate the immune responses, and protect hosts against infections by other pathogenic microbes (Berg *et al.*, 1993; Petrino *et al.*, 1995; Naaber *et al.*, 1998; Alak *et al.*, 1999; Bonet *et al.*, 1999). In those studies, *Lb. spp* or other probiotic organisms (*Sacharomyces boulardii*) inhibited the translocation by other gut flora or inoculated pathogens, but the probiotics themselves could not be detected in the extra-gut tissues (Dong *et al.*, 1987; Berg *et al.*, 1993; Naaber *et al.*, 1998). In other words, probiotics investigated in those studies helped to control infections produced by other opportunistic microorganisms, rather than negatively damaging the host's health or causing opportunistic infections themselves. In the present study, HN001 and HN019 reversed/reduced the immunosuppressive effects of DEX on leucocyte phagocytic activities of mice which received DEX-treatment (Figure 9.7). DEX-treated mice which received HN001 or HN019 had comparable leucocyte phagocytic activity to that of control group mice which did not receive DEX-treatment. This result suggests that HN001 and HN019 supplementation has certain counter-active effects against the immunosuppression caused by DEX in mice. Similar immune enhancing and anti-infection properties of HN019 or/and HN001 have been demonstrated in previous studies in conventional mice (Gill *et al.*, 2000; Shu *et al.*, 2000). Interestingly, the commercial probiotic LAB strain *Lb. GG* did not reduce the suppressive effects of DEX on phagocytic cell activity, although it has been reported in the literature to stimulate lymphocyte and antibody responses; there are no reports on the effect of *Lb. GG* on phagocytic cell functions.

In general, BALB/c mice with compromised immune functions tolerated daily feeding of probiotic strains HN001, HN019 or *Lb. GG* at doses of $1.5\sim 2.5 \times 10^7$ cfu/mouse/day for 7 days. There was no detectable difference in the animals' general health status between DEX and DEX plus probiotic LAB-treated mice. All of the LAB-fed mice were ostensibly healthy at the end of this experimental feeding period. The feeding of HN001, HN019, or *Lb. GG* did not affect the feed and water intake, changes in body weight, or haematological parameters measured here. These results suggest that dietary supplementation of test strains HN001, HN019 or *Lb. GG* have no adverse effect on the physiological health of immunodeficient mice and these observations are in consistent with previous studies on the safety of probiotics in immunocompromised hosts (Dong *et al.*, 1987; Wolf *et al.*, 1998). In contrast, mice which received *S. typhimurium* exhibited

obvious signs of sickness or infection 4 - 5 days post challenge. The *Salmonella*-challenged mice also had significantly lower feed and water intake compared to other groups.

All of the immunocompromised mice lost body weight with the *Salmonella*-challenged mice showing the highest decrease. However, no synergistic interaction between LAB feeding and weight loss was observed. The immunosuppression and catabolic effects of DEX may contribute to the loss of body weight (Distelhorst, 1988; Haynes, 1990). DEX-treated mice had an increased urine output (reflected by the obvious wet beddings compared to control mice which did not receive DEX treatment), whereas, they had comparable water intake to the animals which did not receive DEX-treatment (Figure 9.2). This negative water balance (relative mild-dehydration) may be another reason for the body weight loss in these mice.

In summary, oral administration of probiotic LAB strains *Lb. rhamnosus* HN001 or *B. lactis* HN019 in immunocompromised BALB/c mice had no significant impact on the animals' health and growth. No systemic infection or translocation was detected following the inoculation of HN001, HN019, or *Lb. GG*. These results suggest that HN001 or HN019 can be fed to immunocompromised animals without any significant detrimental effects.

10

Effect of feeding probiotic strains HN001 and HN019 on experimental autoimmune thyroiditis

Abstract

The possibility of intestinal microflora contributing to the pathogenesis of autoimmune diseases has raised issues regarding the safety of probiotic organisms in individuals with such immune dysfunctions. In this study, the effect of consumption of probiotic LAB strains *Bifidobacterium lactis* HN019 and *Lactobacillus rhamnosus* HN001 on the induction and progression of experimental autoimmune thyroiditis (EAT) was investigated in CBA/CaH (H-2^k) mice. Probiotic LAB strains HN001 or HN019 in skim milk were fed to mice daily ($1 \sim 1.5 \times 10^8$ cfu/mouse/day) for 5 to 9 weeks. EAT was induced by subcutaneous injection of mouse thyroglobulin (MTg) plus Freund's adjuvant (complete and incomplete, CFA and IFA) or lipopolysaccharide (LPS). *In vitro* spleen lymphocyte proliferative responses to stimulation with MTg were determined. The presence of, and degrees of mononuclear cell infiltration in thyroid gland tissues were examined to determine the development and severity of EAT. Results showed that 8 weeks after immunisation, 16.67% - 50% of the mice developed EAT, with mild lymphocyte infiltration in the thyroid glands. Probiotic-feeding had no significant effect on the incidence and severity of EAT in comparison with the control group. It can be concluded that dietary supplementation of HN001 and HN019 has no detrimental effect on the development and progression of EAT in mice.

10.1 Introduction

Autoimmunity or autoimmune diseases are thought to be multi-aetiological immune dysfunctions. The penetration of gut bacterial antigens into lymphoid tissues is one of the suggested initial factors leading to a loss of tolerance towards self-components in genetically predisposed individuals (Famularo *et al.*, 1997; Hamilton *et al.*, 1998). There is growing evidence that the composition of the endogenous intestinal microflora may have an important role in the expression of systemic autoimmunity in both humans and animal models. Decreased susceptibility to autoimmunity in specific pathogen free (SPF) or germ free (GF) animals compared with conventionally housed animals has been reported in models of autoimmune thyroiditis (Penhale & Young, 1988), haemolytic anaemia (Murakami *et al.*, 1994), arthritis (Kool *et al.*, 1992; Thompson & Elson, 1993), systemic lupus erythematosus (SLE) (Terada *et al.*, 1991; Apperloo-Renkema *et al.*, 1994), and inflammatory bowel disease including Crohn's disease and ulcerative colitis (Jewell & Patel, 1985; Rutgeerts *et al.*, 1991; Balfour, 1997; MacDonald *et al.*, 2000).

The ability of probiotic bacteria to modulate immune responses has been demonstrated in a large number of studies (Gill, 2000; Matsuzaki & Chin, 2000). Consumption of probiotic LAB has been associated with the enhancement of both innate and acquired immune responses. Some LAB strains have significant adjuvanticity in animal models (Claassen *et al.*, 1995; Pouwels *et al.*, 1996; Chin *et al.*, 2000; Gill *et al.*, 2000), therefore a *Lactobacillus*-based live vector vaccine has been proposed (Pouwels *et al.*, 1996). The beneficial effects of consumption of immune-enhancing LAB strains to immunocompetent hosts are obvious, but the consequence of over-activation of the immune system by these organisms in hosts with immune dysfunctions, such as individuals genetically predisposed to autoimmunity, has raised some concerns (Guarner & Schaafsma, 1998; Wagner & Balish, 1998). For example, it has been demonstrated experimentally that *Lb. casei* cell wall components (given intraperitoneally) are able to induce cardioangitis (an autoimmunity associated heart disease) in mice (Okitsu-Negishi *et al.*, 1996).

It has been reported in several studies that some LAB strains can translocate from the gut into tissues (liver, spleen, and kidney) or the blood stream in immunodeficient hosts

(Sussman *et al.*, 1986; Link *et al.*, 1995; Wagner *et al.*, 1997; Ha *et al.*, 1999). With respect to the association between bacterial antigens and autoimmune responses, and the adjuvant activity of LAB strains, the possibility of LAB being involved in the pathogenesis of some models of autoimmunity in experimental animals and possibly in humans has been suggested (Famularo *et al.*, 1997). Therefore, from a safety point of view, the potential of probiotic bacteria (especially the immuno-stimulating strains), to induce destructive inflammation or autoimmunity needs to be investigated (Wagner & Balish, 1998).

Murine experimental autoimmune thyroiditis (EAT) mimics the main cellular and pathological manifestations of Hashimoto's thyroiditis (a human organ-specific autoimmune disease), and has been frequently used as a model to study human autoimmunity (Simon *et al.*, 1985; Via & Shearer, 1988; Alimi *et al.*, 1998). The present study aimed to investigate the effect of oral consumption of probiotic strains *Lb. rhamnosus* HN001 and *B. lactis* HN019 on the development and progression of EAT in order to evaluate the safety of these two organisms in hosts with immune dysfunction. EAT can be induced with thyroglobulin (Tg), a known thyroid autoantigen that is common to both mouse and humans (Kong, 1996). Inbred mice with the H-2^k haplotype such as CBA/j mice have been reported to be the most susceptible animal models for the induction of EAT (Esquivel *et al.*, 1977; Nicoletti *et al.*, 1994; Bhatia *et al.*, 1996; Kong, 1996). CBA/CaH mice have very similar genetic phenotype to CBA/j mice, and also have the H-2^k haplotype, therefore this animal model was used in this study due to the unavailability of CBA/j mice.

10.2 Materials and methods

10.2.1 Preparation of mouse thyroglobulin

Thyroglobulin (Tg) is the main protein synthesised in thyroid follicle cells and accounts for up to 75% of the total thyroid protein (Verschueren *et al.*, 1991; Kong, 1996). It has been reported that Tg from different species (bovine, rodents, pig, porcine, human) is similar with regard to physical, biochemical, and molecular properties. Tg is a glycoprotein containing about 10% carbohydrate. Native Tg is composed of 2 identical subunits (MW ~330,000) giving a protein of ~660,000 molecular weight (Andreoli *et al.*, 1969).

Mouse thyroglobulin (MTg) was prepared using the following protocol. Frozen thyroids from 300 inbred mice (CBA/CaH or BALB/c, mixed sex, aged 15 weeks and over) were thawed at 4°C and then transferred into a chilled 15ml heavy-duty centrifuge tube. The thyroids were homogenised using an Ultra-Turrax T25 homogeniser (Janke & Kunkel, IKA® Labortechnik, Germany) at maximum speed (scale 6 or 25,000/min work load) for 1 min with intervals of 10 seconds on ice. The homogenate was ultra

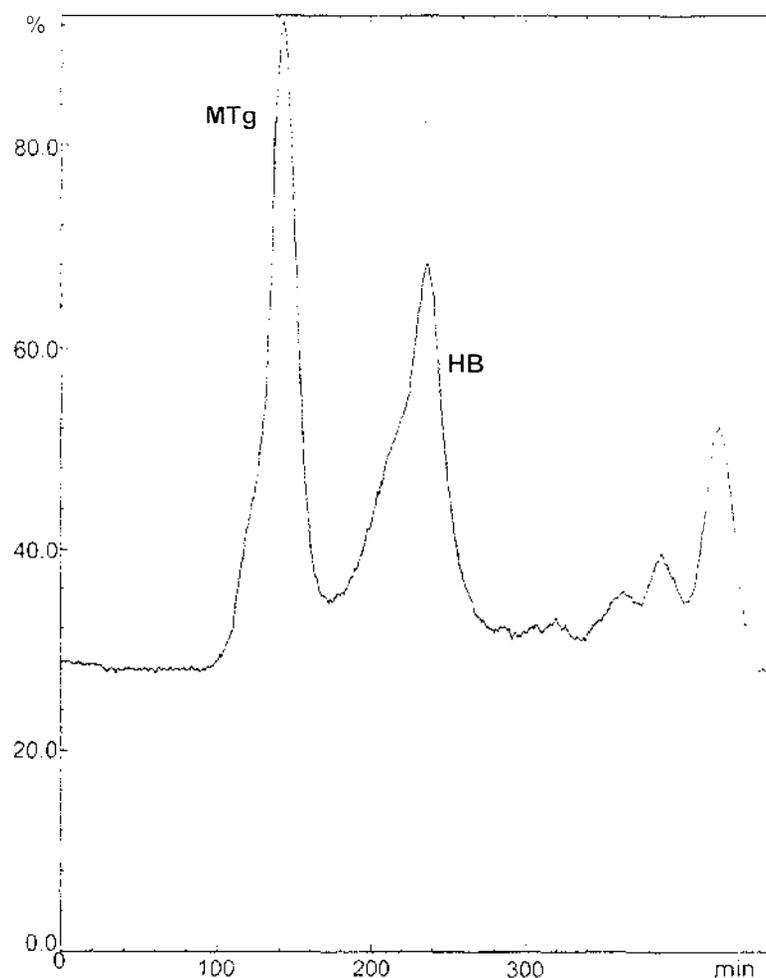


Figure 10.1 Separation of MTg from thyroid extracts. 2ml of the thyroid extracts prepared by the method described above (Section 10.2.1) was loaded onto a Sephacryl-300 gel column. The gel filtration was controlled by an FPLC system. The column was washed with PBS (pH 7.4) at a flow rate of 0.3ml/min. The fractions from 95 min to 180 min were collected (2ml/fraction) using a computer controlled LCC-500 plus fractionation system. Protein concentration was monitored using UV absorbance (280nm). 10 fractions within the first major peak (MTg) with the highest protein concentration were pooled together and used for further MTg analysis. MTg: mouse thyroglobulin; HB: haemoglobin. Y-axes represents the relative protein concentration, x-axes represents the elution time (min).

centrifuged in a Beckman ultracentrifuge (NVTi 90 rotor, 100,000 x g or 35,000 rpm) at 4 °C for 1hr. The clarified supernatants (free of membranous debris) were collected and separated on a sephacryl-300 gel column (A3.12) on a fast protein liquid chromatography (FPLC) system (A3.13). The protein fractions and concentration were monitored at 280nm. The first major protein peak containing MTg which appeared at about 120 to 160 min (just before the haemoglobin peak) (Figure 10.1), was collected in 2 ml fractions in glass tubes using a computer controlled automatic fractionation system (Liquid Chromatography Controller LCC-500 plus, Pharmacia Biotech). Fractions within the range of the MTg peak were pooled together and the protein concentration was determined using a BCA Protein Assay Kit (Section 3.22). The protein (MTg) in the pooled fractions was concentrated to greater than 800µg/ml using a protein concentration unit (Centriplus™ Concentrators, Amicon, Inc, MA, USA). The purity of the thyroglobulin was checked by SDS-PAGE analysis and compared with a commercially purified bovine thyroglobulin preparation (Lot: 29F8170, Cat: T-1001, Sigma Chemical Co., MA, USA) (Figure 10.2). The purified thyroglobulin suspension (in PBS) was dispensed into 2ml tubes and stored at -70°C before use.

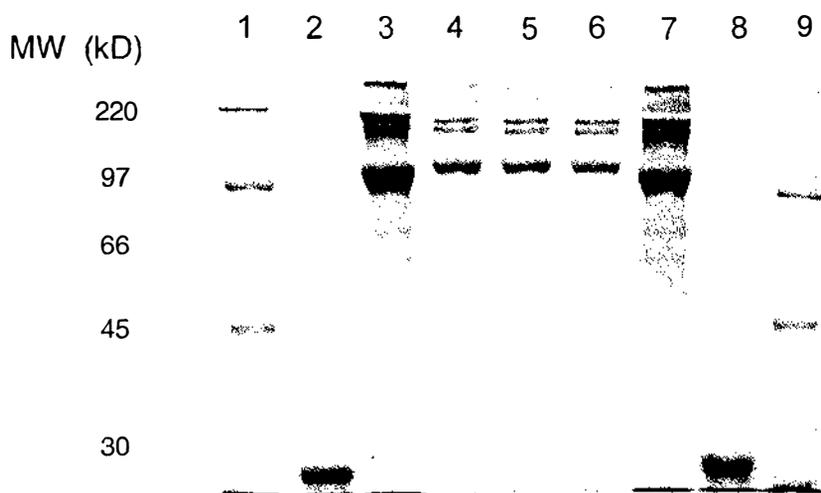


Figure 10.2 SDS-7.5% polyacrylamide gel of MTg purified from mouse thyroid extracts. Lanes 1 and 9: high range rainbow molecular weight markers (Amersham Pharmacia Biotech, RPN 756); lanes 2 and 8: amylase (Sigma, A-8781); lanes 3 and 7: commercial bovine thyroglobulin (Sigma); lanes 4 to 6: MTg samples. Samples were run on a 7.5% polyacrylamide mini slab gel with constant current (50mA) for 2 hrs. The gel was then stained with Coomassie blue. The banding patterns for bovine Tg and MTg were similar, showing 3 major bands with MW of 100-200kD. It is possible that the lower MW band is the result of degradation by proteases naturally occurring in the thyroid gland as no protease inhibitors were used during the purification procedure.

10.2.2 Animals

Female CBA/CAH (H-2^k) mice (Festing, 1979) aged 4-6 weeks were purchased from the Animal Resources Centre, Perth, Western Australia, and acclimatised to experimental conditions (Section 3.16) for two weeks before the commencement of treatment. Fresh water and standard mouse chow (Unifeeds, Palmerston North, New Zealand) were supplied *ad libitum*. During the experimental period, the animals' health status was observed daily; the body temperature (anus temperature) was taken every other day using an electronic thermometer. The body weight of the mice was measured once a week.

10.2.3 Feeding of probiotics

Stock probiotic strains *Lb rhamnosus* HN001, *B. lactis* HN019, and a commercial probiotic strain *Lb. rhamnosus* GG (*Lb. GG*) were prepared using the method described in Section 3.15. The cell populations were adjusted to $2\sim 5 \times 10^9$ cfu/ml. Thirty microlitres of bacterial suspension were fed orally to each mouse on a daily basis using a sterile pipette. Feeding of probiotics commenced one week prior to the beginning of MTg injections, and was sustained until the end of the experiment.

10.2.4 Induction of EAT

a) *Experiment 1: MTg+CFA and IFA-induced EAT*

MTg (800µg/ml in PBS) was emulsified (1:1) with complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA) using 2ml Luer-lock syringes. One hundred microlitres of MTg emulsion (400µg/ml in CFA) was injected subcutaneously at multiple sites on the inside of the back legs of the mice on day 0. After 7 days, a booster MTg (400µg/ml in IFA) injection was given to each mouse.

b) *Experiment 2: MTg+LPS-induced EAT*

One hundred microlitre of MTg (400µg/ml, in PBS, pH 7.4) was injected into the mice using the same procedure as described above. Three hours following each MTg injection, 100µl of lipopolysaccharide (LPS, 200µg/ml, TCA-precipitated from *E. coli* 055:B5, cat: L6529, Sigma) was injected into each mouse (in the alternative leg to that

of MTg injections). A third dose of MTg (100 μ l, 400 μ g/ml in PBS) and LPS (20 μ g in 100 μ l PBS) was injected into each mouse 5 weeks after the first MTg injection. Animals were humanely euthanased at 4, 7, or 8 weeks after the first MTg injection to collect blood, spleen, and thyroid glands. Lymphocytic infiltration in the thyroids and spleen cell proliferative responses to MTg were assessed as indicators of autoimmune responses.

10.2.5 Autoantibody detection

The levels of mouse serum anti-MTg IgG1 and IgG2a antibodies were determined by ELISA to confirm the establishment of an autoimmune reaction (Section 3.27). Antibody levels of individual samples were presented as the OD value (absorbance at 405nm) corresponding to the dilution of sera as indicated in each case.

10.2.6 Spleen lymphocyte proliferative responses to MTg

Spleen lymphocyte proliferative responses to MTg stimulation *in vitro* were determined using the method described in Sections 3.25 and 3.29. Results were expressed as the proliferation index [the ratio of the absorbance (OD value at 450nm) of MTg⁺ wells to that of MTg⁻ wells].

10.2.7 Histology

Thyroid glands attached to the larynx and trachea were excised carefully and immediately immersed in Bouin's fluid (A2.12). The thyroids were transferred to 70% ethanol within 8 to 12 hours and processed for hematoxylin and eosin (H & E) staining. Six sections from each thyroid (6 μ m thick) were evenly cut and mounted on glass slides, so that 12 sections were made for each sample. The pathological changes were evaluated according to the extent of mononuclear cell infiltration in the thyroid tissues. A scaling system of 0-4 (Table 10.1) as described previously by Kong (1996), and Peterson & Braley-Mullen (1995) was used to evaluate the pathological index (PI). Histological changes of mice were used as an indicator of the development and progression of EAT. Animals with at least one section with mononuclear cell infiltration in thyroid tissues with a PI of equal or greater than 0.5 was defined as EAT positive.

Table 10.1 Thyroid pathological index

Pathological index	Histological Changes
0	No infiltration, normal gland showing intact follicles with epithelial cell layer
0.5	Low levels of mononuclear cell infiltration without clear foci, mononuclear cells distributed perivascularly
1	Definite mononuclear cell infiltration with clear foci, 10 to 20% tissue affected with follicular destruction
2	Infiltration involving 25 to 50% of tissues
3	Infiltration involving 50 to 75% of tissues
4	Infiltration involving more than 80% of tissues

10.3 Results

10.3.1 Clinical observations

All of the mice were ostensibly healthy and survived to the end of the experiment. No body temperature or body weight changes (Figure 10.3) associated with the probiotic feedings or MTg injections were detected. The animals receiving MTg injections did not exhibit signs of hypothyroidism.

10.3.2 Immune responses

Thyroglobulin-immunised mice produced measurable titres of IgG1 and IgG2a class antibodies against MTg (Table 10.2), confirmed the onset of an autoimmune response. Animals treated with MTg had significantly higher spleen weight indices than control mice (Figure 10.4); probiotic feeding had no effect on this parameter.

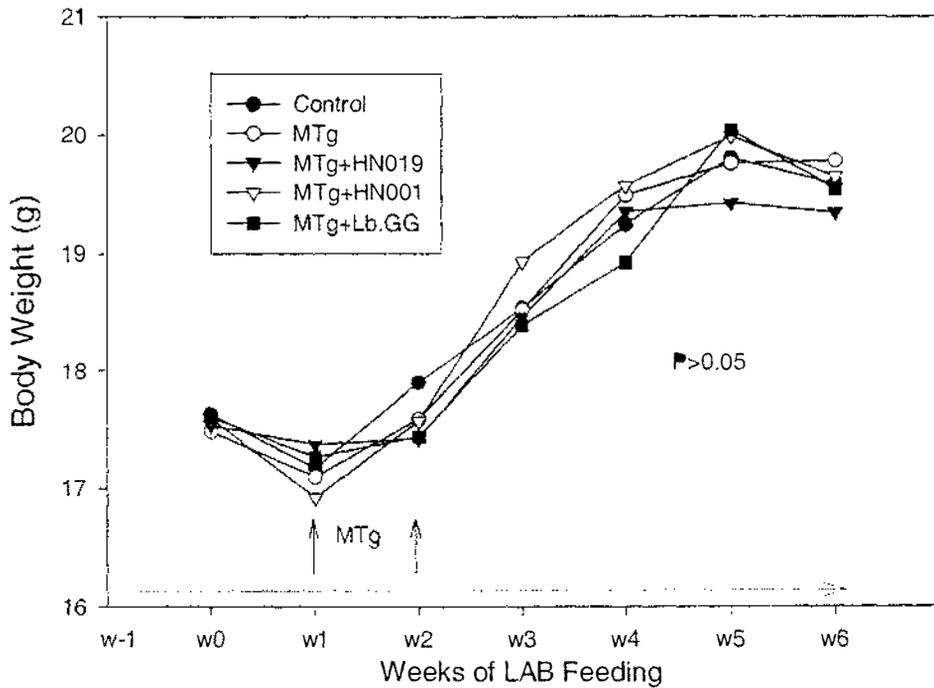


Figure 10.3 Changes in body weight during the period of probiotic feeding. Data represents the average body weight of mice in each group from experiment 1. Control: normal mice receiving saline only; MTg: mice treated with MTg only; MTg+HN019, HN001, or Lb. GG: mice treated with MTg and HN019, HN001, or Lb. GG.

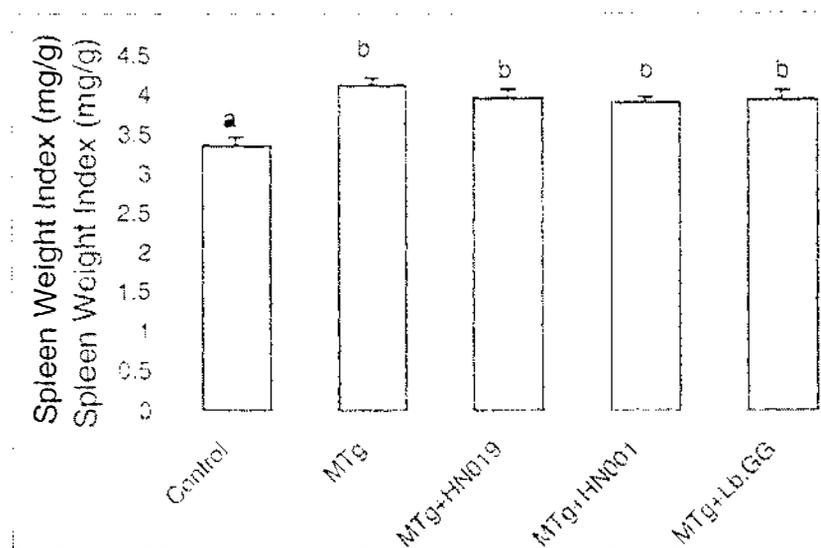


Figure 10.4 Spleen weight indices (Mean+SEM) of mice fed with probiotics for 7 weeks. Data represents the overall results from Experiment 1 and 2. Spleen weight index was expressed as spleen weight (mg)/body weight (g). Means with different letters are significantly different from each other ($p < 0.05$).

Probiotic feeding had no effect on MTg-induced lymphocyte proliferation; spleen cell proliferation indices of probiotic fed mice receiving MTg injections were similar to that of mice which received MTg only (Figure 10.5) ($P>0.05$).

Table 10.2 Serum anti-MTg antibody levels (Delta OD, Mean±SEM)

	Control (n=12)	MTg (n=12)	P values
IgG1	0.0037±0.0004	2.1302±0.23	<0.0001
IgG2a	0.0094±0.0011	0.5497±0.096	<0.0001

Samples were diluted to 1:5000.

10.3.3 Thyroid histological changes

MTg injections induced only mild thyroid histological changes in a small proportion of animals (less than 25% in experiment 1). The pathological index (PI) was around 0.5 to 1.0; i.e. mononuclear cell infiltration was not remarkable. As indicated in Table 10.3, the average PI in each group was less than 0.15 (experiment 1) or 0.5 (experiment 2). Tissue samples from two mice that were fed with HN019 (in experiment 1) or *Lb. GG* (in experiment 2) had infiltration of different types of cells including neutrophils, eosinophils, and lymphocytes. There was no significant difference in the incidence of

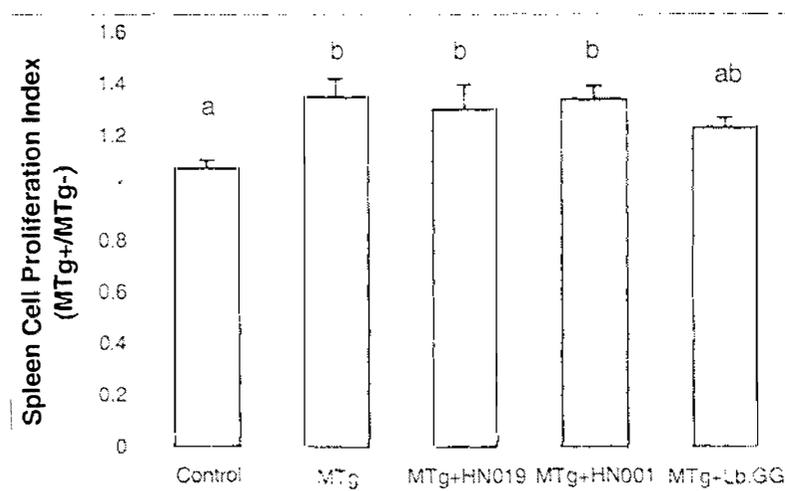


Figure 10.5 Spleen cell proliferative responses to MTg (Experiment 2). Means with different letters are significantly different from each other.

Table 10.3 Incidence of EAT and histological changes in the thyroids of mice receiving probiotics

	Group	Number of mice	PI [#] Mean±SEM	EAT [@] No. (%)
Experiment 1 (2 MTg injections; sampling at week 4)	Control	12	0	0
	MTg*	12	0.01±0.01	2 (18.18)
	MTg + HN019	12	0.01±0.07	3 (25.00)
	MTg + HN001	12	0.01±0.01	2 (16.67)
	MTg + Lb. GG	11 [§]	0.02±0.02	2 (18.18)
	Total	59		8 (17.02)
Experiment 2 (3 MTg injection; LPS was used; sampling at weeks 4, 7, and 8)	Control	12	0	0
	MTg*	12	0.12±0.07	6 (50)
	MTg + HN019	12	0.17±0.04	5 (41.67)
	MTg + HN001	12	0.25±0.13	6 (50)
	MTg + Lb. GG	6	0.34±0.21	3 (50)
	Total	54		20 (47.62)

[#] Pathological index=total score /No. of sections examined; the average PI of mice in control group (did not receive MTg or LAB) was used as baseline. P>0.05 by ANOVA test (SAS-GLM).

* Mice treated with MTg only (no LAB supplementation).

[§] One sample was damaged during processing so it was excluded.

[@] No. of mice with at least one thyroid section with histological changes characteristic of EAT (PI >=0.5). Overall EAT incidence: P>0.05 (LAB groups vs MTg); P<0.05 (Control vs MTg and LAB groups).

EAT between treatment and control mice which received MTg only ($p=0.739$ by Fisher's Exact test) although the mice fed LAB had relatively higher PI ($p<0.05$) in experiment 2. Approximately 50% of the animals in experiment 2 developed EAT following MTg injections (Table 10.3).

10.4 Discussion

In previous experimental autoimmune thyroiditis (EAT) studies, different animal models, adjuvants, and immunisation protocols have been used. All of these factors may affect the incidence and severity of EAT induction. To date no reports on the sensitivity of CBA/CaH mice to EAT induction by MTg have been made, although it has a similar genetic phenotype with the EAT sensitive mouse model CBA/j mice. Mice and the immunisation regimen used in experiment 1 failed to induce a significant level of EAT, therefore, a different immunisation protocol and adjuvant was used for experiment 2.

Complete and incomplete Freund's adjuvants (CFA and IFA) are commonly used to facilitate the induction of EAT (Kong, 1996). Bacterial lipopolysaccharide (LPS) is another widely used adjuvant which assists in the induction of EAT in susceptible mice (Simon *et al.*, 1985, Kong, 1996). In experiment 1 of this study, CFA and IFA were used as the adjuvant with MTg to induce EAT. The results from this experiment showed that there was only mild lymphocyte infiltration in thyroids from a few animals (Table 10.3). The incidence and severity of EAT induced by MTg in this experiment was not significant enough to assess the effect of probiotic feeding on the development and progression of autoimmune disease. This could have been due to the mouse strain CBA/CaH not being profoundly susceptible to EAT or the antigen dose/adjuvant/MTg cocktail used. To verify the susceptibility of CBA/CaH mice to EAT, and examine the possible effect of the adjuvant on EAT induction in this mouse model, LPS instead of CFA and IFA, was used as the adjuvant in experiment 2. It has been reported previously that administration of LPS can lead to the abrogation of self-tolerance to MTg even in poor responder mice, and the use of LPS as an adjuvant has been reported to result in more uniform disease induction in experimental models of autoimmunity (Esquivel *et al.*, 1977; Kong, 1996).

In a previous study by Kong (1996), 4 weeks after the first MTg injection, almost 100% of mice (CBA/j) developed EAT with 20 to 60% thyroid involvement. Other studies found that thyroid infiltration was most extensive on days 14 and 21 (Okayasu *et al.*, 1981; Alimi *et al.*, 1998) or by day 49 (Esquivel *et al.*, 1977) after the primary immunisation. These results were not reproduced in experiment 1 although the autoimmune responses were remarkable in MTg treated mice (Table 10.2). No obvious thyroiditis was observed 4 weeks after the first MTg injection. In experiment 2, the detectable mononuclear cell infiltration in the thyroids of these mice was observed only by 7 or 8 weeks after the first MTg injection, when LPS rather than CFA and IFA was used as the adjuvant, and three MTg injections were given. In general, the histological changes induced by MTg in the present study were much less severe than those reported in other rodent species (Nicoletti *et al.*, 1994; Kong, 1996; Damotte *et al.*, 1997). The average pathological indices of thyroids in MTg-treated mice here were less than 0.5 in most groups and the EAT incidence was only around 50% in experiment 2 and 17–25% in experiment 1. However, in some previous studies, a similar EAT incidence to that observed in the present study has been reported (Rose *et al.*, 1971; Bhatia *et al.*, 1996).

The difference in EAT incidence between the two experiments in the present study, as well as the studies carried out by others, may be due to the differences in immunisation schedules, the animal strains, and the types of adjuvant used.

The major purpose of this work was to evaluate the effect of probiotic feeding on the development and progression (or severity) of EAT. Normally, spleen or lymph node cell proliferative responses to autoantigen (e.g. MTg) *in vitro* and the degrees of mononuclear cell infiltration in thyroid tissues are used to determine the effect of treatment on the development and progression of EAT (Okayasu *et al.*, 1981; Nicoletti *et al.*, 1994; Peterson & Braley-Mullen, 1995; Kong, 1996).

The probiotic feeding was commenced one week before the MTg immunisation and sustained until the end of this experiment, i.e. these test probiotic strains and their products (antigens) had contacted the gastrointestinal tract of mice prior to the MTg and adjuvant being administered. If the antigens from these probiotic strains could participate in the pathogenesis of EAT through activation of the immune system, there should be some effect on either EAT incidence or severity associated with the feeding of the specific LAB strains. The results from the histological evaluation (Table 10.3) and spleen cell proliferation tests (Figure 10.5) suggest that oral supplementation of probiotic strains HN001 and HN019 had no adverse effect on the development and progression of EAT. This result is in accordance with previous studies regarding the effects of non-pathogenic LAB strains on autoimmunity, where several reports have documented the possible beneficial protective effects of LAB on a range of autoimmune diseases such as colitis (Malin *et al.*, 1996; Madsen *et al.*, 1999; Verdu *et al.*, 2000), and arthritis (Peltonen *et al.*, 1994; Nenonen *et al.*, 1998). The mechanisms by which probiotic-feeding might contribute to lowering the severity or control of autoimmunity-associated disorders are unknown.

The experiments conducted here indicated that the long term daily dietary supplementation of living probiotic organisms *B. lactis* HN019 and *Lb. rhamnosus* HN001 had no adverse effect on the induction or progression of MTg induced experimental autoimmune thyroiditis in CBA/CaH mice. However, because the incidence and severity of EAT was lower than expected, this study did not unequivocally demonstrate the effect of test probiotic strains HN001 and HN019 on the development and progression of autoimmune diseases. Further studies in a proven

susceptible animal model (e.g. CBA/j mice) may be helpful to clarify the effect of test probiotic strains on autoimmunity, and conclusively demonstrated that the consumption of these two strains is unlikely to cause serious safety concerns in hosts with, or predisposed to, autoimmune diseases.

11 General discussion

11.1 Overview

Lactic acid bacteria (LAB) have been consumed by humans via fermented foods for a long time primarily for their properties in prolonging the shelf life of food products (e.g. yoghurt), in improving food palatability and digestibility, as well as host's health status (Huis in't Veld *et al.*, 1998; Caplice & Fitzgerald, 1999). The accumulation of knowledge about the physiology of gastrointestinal microflora and their importance in association with human and animal health has led to the development of the 'probiotic' concept (Lilly & Stillwell, 1965; Fuller, 1989; Gibson *et al.*, 1997; Holzapfel *et al.*, 1998). LAB were the first, and are currently the most commonly used probiotic microorganisms in food products (Naidu *et al.*, 1999). A wide range of preventive or therapeutic effects of selected probiotic LAB strains have been documented based on vigorous scientific studies (Gilland, 1989, Sanders, 1993a; Salminen *et al.*, 1996; Kasper, 1998; Macfarlane & Cummings, 1999; Gill, *et al.*, 2000; Kailasapathy & Chin, 2000; Rolfe, 2000). There is a rapidly expanding market worldwide for probiotic-containing products ranging from dairy foods (yoghurt, cheese, sour milk), beverages and cereals, to infant formulae (Robinson, 1989; Fuller, 1997; Fond'en, 1995; Lee & Salminen, 1995; Hasler, 1998). The development of probiotic-containing food products is recognised as a niche opportunity by the food industry (Hugh & Hoover, 1991; Mann, 1997; Shortt, 1998). However, the controversy regarding the application of probiotics still exists; particularly the safety of probiotic strains has caused some serious concerns (Sanders, 1993b; Rodricks, 1996; Hamilton-Miller *et al.*, 1999). Demonstration of the safety of probiotic microorganisms has been proposed as a compulsory criterion for the selection of probiotic candidates (Sanders, 1993b; Gasser, 1994; van der Kamp, 1996; Salminen *et al.*, 1998a; Adams, 1999).

11.2 Aim

Lactobacillus rhamnosus HN001, *Lb. acidophilus* HN017, and *Bifidobacterium lactis* HN019 are three newly identified and characterised probiotic candidates with immune-enhancing and anti-infection properties (Gill, 1998; Prasad *et al.*, 1999; Arunachalam *et al.*, 2000; Gill *et al.*, 2000; Shu *et al.*, 2000; Tannock *et al.*, 2000). Other health promoting effects of these strains are currently under investigation within the Milk and Health Research Centre at Massey University. Although these LAB strains were isolated from the current food chain (cheese and yoghurt), they have no safety records when consumed as probiotics at relatively high doses (10^6 – 10^9 cells per day) (Lee & Salminen, 1995). Therefore, the safety status of these probiotic organisms needs to be demonstrated with scientific evidence before these strains can be introduced into food or nutraceutical products.

This study was aimed to investigate any potential adverse effects of the consumption of HN001, HN017, and HN019 to hosts, via a series of *in vitro* and *in vivo* experiments. The results of this study have established the basis for further human clinical trials and should be used as one of the important criteria for consideration by official food regulatory authorities and relevant organisations to commercialise these probiotic strains.

11.3 Safety of HN001, HN017, and HN019

As a living microorganism, a probiotic organism theoretically may exert four types of side effects, i.e.: systemic infections; deleterious metabolic activities; side effects of immunomodulation; and gene transfer (Marteau & Salminen, 1999). There are no general guidelines for the safety assessment of probiotics from official regulatory authorities or legislative parties so far, but several different versions of recommendations or outlines from the scientific community have been published (van der Kamp, 1996; Salminen *et al.*, 1998a; Adams, 1999). Generally, these recommendations include studies on: the intrinsic properties of probiotic organisms; the pharmacokinetics of ingested probiotic strains; the virulence factors of probiotic strains; the acute or sub-acute oral toxicity of probiotic strains; the dose-response of ingested probiotics in experimental animals; the potential infectivity of probiotics; the

consequence of consumption of probiotics on the immune system of the host; potential minor adverse effects on human volunteers; and epidemiological surveillance of populations. The safety experiments conducted in this study were designed based on the theoretical adverse effects of probiotics and the recommended studies listed above (with the exception of human trials).

11.3.1 Potential pathogenicity

11.3.1.1 Opportunistic infections

The potential of HN001, HN017, and HN019 to cause systemic infections was investigated in both healthy and immunocompromised animal models via the observation of bacterial translocation (include bacteraemia), total and differential blood leucocyte counts, spleen weight index, and signs of illness. Bacterial translocation into internal organs (such as liver, spleen, or kidneys) or the blood stream is direct evidence of bacterial invasion, which indicates the degree of bacterial pathogenicity (Ford *et al.*, 1996; Urao *et al.*, 1996). Increases in peripheral blood polymorphonuclear cells and splenomegaly are indirect indicators of bacterial infection. Some LAB strains have been implicated in clinical infections in subjects with sub-optimal immune systems (which predispose them to infections) (Aguirre & Collins, 1993; Gasser, 1994; Harty *et al.*, 1994), and a possible association between the consumption of certain probiotic preparations (e.g. *Lb.* GG) with the occurrence of severe clinical infections (liver abscesses) has been discussed recently in the literature (Rautio *et al.*, 1999). However, the results of the present study showed that during the experimental period no direct or indirect signs of systemic infections were detected in normal or immunocompromised mice which received oral feeding of the probiotic LAB strains HN001, HN017, or HN019 at doses from $10^7 - 10^{11}$ cfu/mouse/day for 8 to 28 days. This result is in accordance with the available reports regarding the safety status of current commercial probiotic LAB strains (Saxelin *et al.*, 1996; Adams, 1999). According to these reports, the numbers of local or systemic infections caused by LAB are very low (0.1-0.2%) in comparison with other bacteraemia cases (Aguirre & Collins, 1993; Gasser, 1994) and none of the isolates from the systemic infections corresponded to any of the commercial *Lactobacillus* strains (Saxelin *et al.*, 1996).

The existence of digestive tract lesions and immunodeficiency are speculated as being two factors that contribute to the translocation of endogenous bacteria from the gut lumen (Marteau & Salminen, 1999). Immunosuppression has been convincingly proven to be a trigger of bacterial translocation (Berg, 1983; Ohsugi *et al.*, 1996; Nakayama *et al.*, 1997). The translocation of normal gut flora including species of lactobacilli and bifidobacteria has been reported in immune deficient hosts (Berg & Garllington, 1979; Ma *et al.*, 1990; Kmet *et al.*, 1995; Naaber *et al.*, 2000). The results from the present study demonstrated that none of the immunodeficient mice exhibited signs of translocation when fed on these probiotic LAB (HN001, HN017, or HN019). Other researchers have reported studies in which probiotic bacteria inhibited the translocation of gut bacteria or pathogens, while the probiotics themselves did not translocate out of the gut lumen (Dong *et al.*, 1987; Naaber *et al.*, 1998; Shu *et al.*, 2000). It has also been reported that the consumption of probiotic *Lactobacillus* strains by patients with AIDS, Crohn's disease, or premature infants, did not induce any infections or side effects (Malin *et al.*, 1996; Naaber *et al.*, 1998; Wolf *et al.*, 1998).

In addition, animals receiving test probiotics showed no significant differences in their total and differential blood leucocyte counts, spleen weight index, and general health status compared to that of control group mice. These results are in accordance with the findings from bacterial translocation experiments, i.e. test probiotic LAB strains HN001, HN017, and HN019 have no potential to cause infections under the experimental conditions used in this study.

The inability of probiotic LAB including the strains tested in this study, to cause infection may be related to their intrinsic characteristics. Most LAB are generally considered as non-pathogenic saprophytes and so far no virulence factors associated with LAB have been defined (Naidu *et al.*, 1999). Some probiotic LAB strains have been shown to be able to stabilise the intestinal mucosal barrier system by reducing the permeability and improving mucosal immune responses (Salminen *et al.*, 1996; Saxelin, 1997; Gill *et al.*, 2000).

11.3.1.2 Potential adverse metabolic activities

To investigate the potential pathogenicity of test LAB strains, their possible deleterious metabolic activities were studied including mucin-degrading activities *in vitro*, their

potential adverse effect on mucosal surfaces *in vivo*, and their potential to participate in platelet aggregation processes. Excessive degradation of the intestinal mucous layer (mucin) may theoretically be detrimental. Elimination of the mucus layer on the GIT epithelial surface could result in an increase in the populations of bacteria adhering to the enteric surfaces, which is the initial step for bacterial invasion, and could also possibly facilitate the dissemination of normal intestinal bacteria into extra-intestinal tissues (Ruseler-van Embden *et al.*, 1989). Therefore, an ability to degrade mucin is considered a valuable indicator of pathogenicity and local toxicity of lumen bacteria (Donohue & Salminen, 1996; Salminen & Donohue, 1996). In addition, the intestinal mucosal architecture of mice which received test probiotic LAB strains were quantitatively measured to detect any detrimental effect of test strains on the mucosal surface. The results from this study demonstrated that test strains HN001, HN017, and HN019 had no ability to degrade gastric mucin *in vitro* and had no adverse effects on intestinal mucosal structures following *in vivo* dietary delivery to mice. This suggests that test probiotic LAB strains are unlikely to induce detrimental effects on the gut mucosal surface. A similar result to this study was also reported by Ruseler-van Embden *et al.* (1995), who found that *Lb. GG*, *Lb. acidophilus*, *B. bifidum* were unable to degrade intestinal mucus glycoproteins.

Infective endocarditis (IE) is one of the most frequently encountered infections associated with LAB particularly enterococci, although the involvement of lactobacilli is at a relatively lower frequency (Gallemore *et al.*, 1995; Adams & Marteau, 1995; Pelletier, 1996). Platelet activation and aggregation is largely believed to contribute to the pathogenesis of IE, and aggregation of platelets by lactobacilli is thought to be an important contributory factor in the progression of *Lactobacillus* endocarditis (Herzberg, 1992; Harty *et al.*, 1993; 1994; Gasser, 1994; Adams, 1999). Non-pathogenic LAB from the gastrointestinal tract or oral cavity may be introduced into the blood stream by poor dental hygiene, gastrointestinal lesions or surgical procedures, so they may contribute to the pathogenesis of IE and other pathological processes associated with platelet aggregation (Aguirre & Collins, 1993). Therefore the lack of any platelet aggregating potential has been listed as an important criterion for probiotic selection (Harty *et al.*, 1993; Donohue & Salminen, 1996; Kirjavainen *et al.*, 1999). Accordingly, the potential of HN001 and HN019 to induce or enhance human blood platelet activation and aggregation *in vitro* was investigated to evaluate their

pathogenicity or deleterious metabolic activity from another angle. The results showed that HN001 and HN019 did not induce or enhance the platelet aggregation process. The results obtained here further indicate that test LAB HN001 and HN019 are unlikely to participate in the pathogenesis processes of IE. This result is in accordance with previous reports regarding the platelet aggregating ability of other *Lactobacillus* strains (Harty *et al.*, 1993; Korpela *et al.*, 1997; Kirjavanen *et al.*, 1999).

All these results suggest that probiotic LAB strains HN001, HN017, and HN019 have no adverse metabolic activity or potential pathogenicity with respect to the parameters investigated here. According to Marteau & Salminen (1999), “clinical diseases due to deleterious metabolic effects of probiotics have never been reported”. These characteristics of probiotic LAB strains further demonstrate their commensal nature.

11.3.2 Effect on autoimmunity

It has been reported that when administered parenterally, bacterial cell wall components such as peptidoglycan polysaccharides from lactobacilli can induce arthritis, cardioangitis, or other autoimmune diseases (Blancuzzi *et al.*, 1993; Schwab, 1993; Okitsu-Negishi *et al.*, 1996). A systemic uptake of bacterial cell wall polymers from the intestinal lumen has been observed in rats (Sator *et al.*, 1988). Therefore, from a safety point of view, the potential of probiotic bacteria or their by-products (especially the immuno-stimulating strains) to induce destructive inflammation or autoimmunity needs to be investigated (Guarner & Schaafsma, 1998; Wanger & Balish, 1998). Until now, there have been no reported immunological side effects following oral consumption of probiotics, except for one case of autoimmune hepatitis that may have been enhanced by ingestion of large doses of yoghurt over a prolonged period (Chaiken, 1994; Marteau & Salminen, 1999). The major physiological benefits (identified so far) of the probiotic LAB strains HN001 and HN019 are their immune-enhancing attributes. Whether or not they have adverse effects on hosts with autoimmune diseases or hosts susceptible to the induction of autoimmune responses needs to be clarified. The effect of long-term (5 to 9 weeks) oral consumption of these two strains on the development and progression of experimental autoimmune thyroiditis (EAT) was therefore investigated in CBA/CaH (H^{2k}) mice, which are supposed to be a strain sensitive to the induction of autoimmune thyroiditis (Kong, 1996). Results from this experiment

showed that consumption of HN001 and HN019 had no adverse effects on the incidence or severity of EAT in mice. This result indicates that test LAB strains have no immunological side effects in hosts with this autoimmune disease. Perdigon and co-workers (1991) also found oral administration of high doses of LAB did not induce immunological side effects in mice. In another study, Pelto *et al.* (1998) observed that probiotic bacterium *Lb. rhamnosus* GG down-regulated the milk-induced inflammatory response in milk-hypersensitive subjects but had an immunostimulatory effect in healthy subjects. Some authors have even suggested using probiotic LAB for controlling autoimmune responses (Weeler *et al.*, 1997; Madsen *et al.*, 1999; Matsuzaki & Chin, 2000; Verdu *et al.*, 2000).

11.3.3 Genetic stability

Another theoretical side effect of probiotics is gene transfer. It is well known that resistance to antibiotics is a common characteristic of many LAB species (Lim *et al.*, 1993; Charteris *et al.*, 1998). Some antibiotic resistance genes, especially those encoded by plasmids, can be transferred between microorganisms (Thompson & Collins, 1989; Wang & Lee, 1997). It has been shown that the plasmid (pAM β 1) encoded macrolide (e.g. erythromycin)-lincosamide-streptogramin type B (MLS) resistance could be transferred from *Lb. reuteri* to *E. faecium*, and from *E. faecium* to *E. faecalis* in the mouse GIT (McConnel *et al.*, 1991). In light of this situation, the question of whether probiotic bacteria can transfer resistance genes to endogenous flora or to pathogens, and what impact the transfer may have on subsequent antimicrobial therapy, has been raised (Marteau & Salminen, 1997). Several species of lactobacilli and pediococci are known to be vancomycin resistant (Leclercq & Derlot, 1988; Swenson *et al.*, 1990). If the vancomycin resistance gene is transferred from probiotics to pathogenic bacteria, a serious problem will arise since vancomycin is one of the last effective antibiotic agent that is known to be broadly effective against clinical infections caused by multi-drug-resistant pathogens (Eliopoulos *et al.*, 1994; Rowe, 1996; Morelli & Wright, 1997). It is proposed that strains harbouring antibiotic resistance plasmids should not be used either as human or animal probiotics (Stokes *et al.*, 1993). Therefore, the antibiotic resistance/susceptibility and plasmid profiles of LAB strains HN001, HN017, HN019, and HN067 were examined in this study to clarify their genetic stability. The results obtained here showed that the probiotic LAB strains tested

in this study did not carry any plasmids. Therefore, the antibiotic resistance attributes of HN001, HN017, HN019, and HN067 are intrinsic and not linked with plasmids, thus their antibiotic resistance attributes are not likely to be transferred to other microorganisms. In addition, the strains tested in this study had no unusual antibiotic resistance patterns compared to the reference commercial probiotic strains tested here and other LAB. There are many safe antibiotics which are effective against the LAB strains tested in this study, so in the unlikely event of opportunistic infections caused by these LAB strains, treatment should not be difficult. From a safety point of view, these probiotic strains are extremely unlikely to present a public health risk.

11.3.4 Toxicity / tolerance

In addition to experiments conducted to investigate the potential theoretical side effects of probiotics, different animal trials such as acute toxicity testing and longer-term feeding (dose-response) trials, were carried out to observe any potential adverse effect of probiotics on animal health. Acute oral toxicity tests in animal models have been advocated as a fundamental means of assessing safety (Stine & Brown, 1996; Willem *et al.*, 1997); these have been applied previously in several safety assessment studies (Momose *et al.*, 1979; Donohue *et al.*, 1993). Test probiotic LAB strains are meant to be consumed via food products, so the longer-term effect of consumption of these strains on animal health needs to be examined. Excessive intake of nutrients, including biologically active components in functional foods have been documented to elicit adverse effects (Milner, 1999). It has been suggested that large numbers of 'harmless' bacteria may damage cells in tissue culture (Lelieveld *et al.*, 1995). Hence, dose-response studies are always recommended in safety assessment recommendation lists to find out the minimal dose for probiotic effects and the maximal tolerance dose (Salminen *et al.*, 1998a; Milner, 1999). In this study, the animals' food and water intake, live body weight gain, haematology and blood biochemistry during or following administration of test probiotic strains HN001, HN017, or HN019, were monitored. Results from these *in vivo* trials showed administration of test LAB strains at a fixed high dose (10^{11} cfu/mouse/day) for one week or at different doses (10^7 – 10^{10} cfu/mouse/day) for four weeks had no adverse effect on any of the health parameters observed in normal healthy or immunocompromised mice. This finding suggests that HN001, HN017, and HN019 have no acute toxicity or harmful effects on animals'

health when consumed at high doses for a short time, or consumed for long periods of time.

11.4 Further studies

A large number of experiments have been recommended to assess the safety of probiotics. It is impractical and impossible to carry out all of these recommended tests to evaluate the safety of a particular probiotic strain. Some of these experiments have little relevance to the host's health status (such as the bile salt deconjugation activity of LAB). Therefore, it is important to select and conduct the most useful and relevant assays from the long list of available tests. There are four theoretical adverse effects that viable probiotic organisms may confer (Section 2. 5). The best method to determine the safety of new probiotic strains, and perhaps it should be a fundamental requirement, may be to conduct at least one or two experiments covering each aspect of those theoretical adverse effects. The model systems which were used in this study (i.e. *in vitro* experiments including mucin degradation, platelet aggregation, antibiotic resistance and plasmid screening; *in vivo* experiments including acute toxicity and dose-response test, safety in immunocompromised animals, or in animals with experimental autoimmune thyroiditis) could be used in future safety investigations for other probiotic strains. The autoimmune animal model (CBA/CAH H^{2k} mice) used in this study was not sensitive enough for induction of autoimmune disease (EAT) as expected. So, other animal models such as CBA/J H^{2k} with proven sensitivity to EAT should be used in future studies. Regarding the plasmid status of *Lb. rhamnosus* HN001, further studies need to be conducted to investigate the association of antibiotic resistance with the plasmids which were detected from the mother strains (C.J. Pillidge, NZDRI, personal communication).

Based on the results obtained from this study, test probiotic strains (HN001, HN017, and HN019) are devoid of any of the theoretical adverse effects in animals. Therefore further human clinical studies on these strains are likely to be safe and should be conducted before these strains are incorporated into food products. Following the introduction of these test probiotic strains into food products, their safety can be further monitored using epidemiological surveys on target population groups.

11.5 Conclusions

From the results obtained in this study, the following conclusions can be made:

- Probiotic LAB strains *Lb. rhamnosus* HN001, *Lb. acidophilus* HN017, and *B. lactis* HN019 are non-pathogenic in both conventional healthy and immunocompromised animals. They have no potential to induce systemic infections in rodents upon consumption at high dose.
- Strains HN001 and HN019 have no potential to exacerbate the development and progression of autoimmune disease (EAT).
- These strains (HN001, HN017, and HN019) do not have deleterious metabolic activity in respect to mucin degradation and/or platelet aggregation abilities.
- With respect to the antibiotic resistance attributes, these strains including *Lb. rhamnosus* HN067 are genetically stable.

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APPENDIX 1 Medium composition

A1.1 Brain heart infusion (agar)

Dehydrated Brain Heart Infusion (BHI) agar (Difco Laboratories, Detroit MI, USA)

	g/l
Calf brains infusion	200.0
Beef heart infusion	250.0
Bacto proteose peptone	10.0
Bacto dextrose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5
Bacto agar	15.0

The pH was adjusted to 7.4 ± 0.2 prior to autoclaving.

A1.2 BHI broth

Dehydrated BHI Broth (Difco Laboratories, Becton Dickinson, Sparks, MD, USA) contained the same components as BHI agar with the exception of Bacto agar which was not included in the BHI broth. The pH was adjusted to 7.4 ± 0.2 prior to autoclaving.

A1.3 Coliform agar

Coliform agar plates were purchased from Fort Richard Laboratory (cat: 1089, Auckland, New Zealand).

A1.4 Medium B

Minimal anaerobic culture medium (medium B) (Ruseler-van Embden *et al.*, 1995):

	g/l
Tryptone (Oxoid)	7.5
Casiton (Difco)	7.5
Yeast extract (Oxford)	3.0

Meat extract (Merck)	5.0
NaCl (BDH)	5.0
K ₂ HPO ₄ ·2H ₂ O	3.0
KH ₂ PO ₄	0.5
MgSO ₄ ·7H ₂ O	0.5
Cysteine HCl (Sigma)	0.5
Resazurin (BDH)	0.002

Glucose (BDH) 10 or 30, hog gastric mucin (HGM) (Sigma) 2 or 5, and agarose (Sigma) 15, were also included in specified cases. The pH was adjusted to 7.2 ± 0.2 .

A1.5 Mann-Rogosa-Sharpe (MRS) broth

The dehydrated MRS broth (BBL[®], Becton Dickinson, Cockeysville, USA):

	g/l
Pancreatic digest of gelatine	10.0
Beef extract	8.0
Yeast extract	4.0
Dextrose	20.0
Dipotassium phosphate	2.0
Polysorbate 80	1.0
Sodium acetate	5.0
Ammonium citrate	2.0
Magnesium sulphate	0.2
Manganese sulphate	0.05

The pH was adjusted to 6.2 ± 0.2 prior to autoclaving.

A1.6 MRS-C broth

5% (w/v) cysteine hydrochloride (cysteine-HCl) (sigma) in MQ-H₂O was prepared fresh before each use and was filter-sterilised (0.2µm). MRS-C broth was prepared by the addition of 1ml of 5% cysteine-HCl solution to 1L of autoclaved MRS broth prepared as above (A1.5).

A1.7 MRS agar

Dehydrated MRS agar (BBL[®], Becton Dickinson, Cockeysville, USA)

	g/l
Pancreatic digest of gelatine	10.0
Beef extract	8.0
Yeast extract	4.0
Dextrose	18.5
Dipotassium phosphate	2.0
Polysorbate 80	1.0
Sodium acetate	3.0
Ammonium citrate	2.0
Magnesium sulphate	0.2
Manganese sulphate	0.05
Agar	13.5

The pH was adjusted to 6.2 ± 0.2 prior to autoclaving.

A1.8 MRS-C agar

MRS-C Agar was prepared by addition of 1ml of 5% cysteine-HCl into 1L of MRS agar before pouring into petri dishes.

A1.9 Cell culture media

Complete RPMI medium (RPMI 1640 medium, GIBcoBRL Life Technologies, cat: 31800-022) contained:

L-glutamine	2 mM
2-ME (2-mercaptoethanol)	50 μ M
Penicillin	100U/ml
Streptomycin sulphate	100 μ g/ml

Foetal calf serum (FCS, heated 1 hr at 56°C) 5% (v/v)

Media was prepared according to the supplier's instructions; pH was adjusted to 7.3 ± 0.1 before filter (0.2 μ m) sterilization.

APPENDIX 2 Common buffers and solutions

The pH of buffers and solutions, unless otherwise stated, was adjusted with 1M NaOH or 1M HCl.

A2.1 Phosphate buffered saline (PBS)

	<i>g/l</i>
NaH ₂ PO ₄ (anhydrous)	0.23
Na ₂ HPO ₄ (anhydrous)	1.15
NaCl	9.0
pH	7.3 ± 0.1

A2.2 Carbonate coating buffer

	<i>g/l</i>
Na ₂ CO ₃	1.6
NaHCO ₃	2.9
NaN ₃	0.2
pH	9.5 ± 0.1

A2.3 PBS/Tween washing buffer (PBS-T) was prepared by the addition of 0.5 ml of Tween 20 (BDH) to 1 L of sterile PBS.

A2.4 ELISA sample buffer was prepared by the addition of 5 ml of heat-inactivated (1hr at 56°C) FCS (GIBcoBRL Life Technologies, Auckland, New Zealand, Cat: 10091-148) into 95 ml sterile PBS.

A2.5 5 X TBE buffer

	<i>g/l</i>
Tris (Sigma)	54.0
Boric acid	27.5
Na ₂ EDTA	4.6
pH	8.0 ± 0.1

This buffer was 10-fold diluted with MQ-H₂O to obtain a working solution of 0.5 x TBE.

A2.6 DNA sample buffer was prepared by the addition of 14 mg bromophenol blue (Sigma) and 13.4ml glycerin (Sigma) into 20ml MQ-H₂O.

A2.7 DNA agarose gel staining solution was prepared by the addition of 50µg ethidium bromide (Sigma) in 1L of MQ-H₂O.

A2.8 TE buffer contained 50mM Tris (Sigma); 250 mM Na₂EDTA , pH 8.0 ± 0.1.

A2.9 TES buffer contained 50mM Tris; 1mM Na₂EDTA; 6.7% sucrose (pH 8.0).

A2.10 SDS buffer contained 50mM Tris; 20 mM Na₂EDTA; 20% SDS. pH 8.0 ± 0.1.

A2.11 Agarose gel

Required amounts (0.75 to 2 gram as stated in specific experiments) of agarose (type 1-A, low EEO, Sigma Chemical co. USA) were suspended in 100ml of 0.5 x TBE buffer (A2.5) and melted by heating for 2 min in a microwave oven. The melted agarose was cooled to 45 - 50°C in a water bath before pouring into the gel cast. The gel was allowed to set at room temperature for 20 min before use.

A2.12 Bouins fluid contained (v/v) 24% formalin, 71% saturated picric acid, 5% glacial acetic acid.

A2.13 ACK lysis buffer

	g/l
NH ₄ Cl	8.29
KHCO ₃	1.00
Na ₂ EDTA	0.0372

pH 7.3 ± 0.1

A2.14 Alkaline phosphatase substrate kit (BIO-RAD Laboratories, Hercules, CA, USA, Cat: 172-1063)

Immediately before use, 1 ml of 5x diethanolamine buffer and 4 ml of MQ-H₂O were mixed and 1 substrate tablet (para-nitrophenol 1mg/ml, pH 9.8) was dissolved in the diluted buffer.

A2.15 DIFF-QUIK[®] stain set (Baxter Scientific Products, McGaw Park, Miami, USA) was used to stain the blood smears for differential leucocyte counts.

A2.16 Tyrode's buffer (Ginsberg *et al.*, 1990)

BSA	1%
MgCl ₂	2 mM/L
NaCl	137.5 mM/L
NaHCO ₂	12 mM/L
KCl	2.6 mM/L
pH	7.4

APPENDIX 3 Experimental equipment

A3.1 Centrifuges

One of the following centrifuges was used as stated in specific chapters.

- a) Megafuge 1.0 R (Heraeus Instruments, GmbH, Germany. Rotor 2708, 14.6 cm; Rotor 2252 16.2cm).
- b) Biofuge Pico (Heraeus Instruments, GmbH, Germany or Sorvall[®].MC 12V (Dupont, Newtown, Connecticut USA).
- c) Beckman Ultracentrifuge L8-M (Beckman, NVTi 90 rotor)

A3.2 Spectrophotometers

One of the following spectrophotometers (as stated in each specific chapter) was used for bacterial cell growth monitoring, carbohydrate and protein concentration determination, and cholesterol level analysis.

- a) Hitachi U-2000 Spectrophotometer (Hitachi, Ltd., Tokyo, Japan).
- b) Hitachi U-2001 Spectrophotometer (Hitachi, Ltd., Tokyo, Japan).

A3.3 Biohazard hood

BH2000 Series CLYDE-APAC (NSW, Australia). All sterile work was carried out within this hood.

A3.4 ELISA plate reader

Ceres 900 ELISA Plate Reader (BIO-TEK Instruments, Cortland, New York). All ELISA plate analysis was carried out using this equipment.

A3.5 Microplate washer

EL 404 Microplate Auto Washer (BIO-TEK Instruments, High Park, New York). All plates used in ELISA analysis were washed using this machine.

A3.6 PCR thermal cycler

One of the following thermal cyclers (as stated in each specific chapter) was used for amplification of DNA fragments.

- a) Teche PHC-3 Thermal Cycler (John Morris Scientific Ltd, Auckland, New Zealand.) (PHC).
- b) Perkin Elmer Gene Amp PCR System 2400 (USA) (Perkin).

A3.7 DNA agarose electrophoresis apparatus

A RAD-BIO sub TM mini DNA cell (BIO-RAD, Italy) was used to resolve PCR products and plasmid DNA.

A3.8 Flow cytometer

Becton Dickinson FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) was used for the analysis of lymphocyte phenotyping, leukocyte and macrophage phagocytosis, cell count, and platelet aggregation assays. Different excitation wavelength and emission wavelength for detection of MoAb labelled cells were used in different assays as indicated in the specific chapters. Light scatter and fluorescence data were obtained with gain settings indicated in specific chapters. The target cell population (e.g. lymphocytes, neutrophils, macrophages, platelets, etc.) were gated according to their light scatter profiles or by labelling with specific MoAbs (platelet).

A3.9 Microscope

An Olympus BH2-RFCA Microscope (Japan) was used for bacterial cell morphology and tissue histology examinations.

A3.10 Bacterial colony counter

All colony counting work was carried out on a IUL Colony Counter (IUL Instruments, Barcelona, Spain).

A3.11 Roche Cobas Minos Vet (ABX, Levallois, France) Analyser

This machine was used to determine the total leukocyte (WBC), red blood cell (RBC), and platelet counts (PLC), and mean corpuscular volume (MCV), haemoglobin concentration (HB), haematocrit (HT, or packed cell volume PCV), corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC).

A3.12 Sephacryl-300 gel column (Sephacryl S-300 HR Columns, cat: 17-0599-01, Pharmacia Biotech)

This column was used to separate MTg from thyroid extractions according to molecular size of proteins.

A3.13 Fast protein liquid chromatography (FPLC) system (Pharmacia Biotech)

This system was used to control and monitor the separation of mouse thyroglobulin.

A3.14 Liquid chromatography controller (LCC-500 plus) (Pharmacia Biotech) was used to automatically collect the fractions from the thyroid extracts eluted from the S-300 HR columns.

APPENDIX 4 Mouse diet composition

A4.1 Skim milk powder based diet (SMP)

	g/kg
Skimmed milk powder (NZDB, New Zealand) (380g protein/kg)	526
Mineral mix	50
Vitamin mix	50
Cellulose	10
Maize flour	284
Maize oil	80

These components were added together and mixed for 30 min to 1 hr using a diet maker. The diet was kept at -20°C before use.

A4.2 Probiotic LAB supplemented SMP

SMP diet was suspended in sterile MQ-H₂O (10%) and mixed well. Washed probiotic LAB cultures were suspended in PBS and the cell concentration was adjusted according to the absorbance of the culture. The cells were centrifuged and the cell pellets were resuspended in 10% SMP in a volume equal to that of the culture suspension prior to centrifugation. Probiotic-supplemented SMP diet was prepared freshly and kept on ice before use (in the same day).

APPENDIX 5 Peer-reviewed publications from this work

- 1 Acute oral toxicity and bacterial translocation studies on potentially probiotic strains of lactic acid bacteria.

Food and Chemical Toxicology 2000, 38, 153-161.

Zhou, J.S., Shu, Q., Rutherford, K.J., Prasad, J., Gopal, P.K. and Gill, H.S.

- 2 Safety assessment of potential probiotic lactic acid bacterial strains *Lactobacillus rhamnosus* HN001, *Lb. acidophilus* HN017, and *Bifidobacterium lactis* HN019 in BALB/c mice.

International Journal of Food Microbiology 2000, 56, 87-96.

Zhou, J.S., Shu, Q., Rutherford, K.J., Prasad, J., Birtles, M.J., Gopal, P.K. and Gill, H.S.

- 3 Probiotic lactic acid bacteria (*Lactobacillus acidophilus* HN017, *Lb. rhamnosus* HN001 and *Bifidobacterium lactis* HN019) have no adverse effects on the health of mice.

International Dairy Journal 1999, 9, 831-836.

Shu, Q., **Zhou, J.S.**, Rutherford, K.J., Birtles, M.J., Prasad, J., Gopal, P.K. and Gill, H.S.

- 4 Potential probiotic lactic acid bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) do not degrade gastric mucin *in vitro*.

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Comments on the items as requested by Examiners

- 1 *Why were same strains not used throughout the experimental program? Specifically, why was HN017 not studied in chapter 5 where the effect of probiotic strains on platelet aggregation is examined? This leaves the overall study incomplete.*

The study was funded by New Zealand Dairy Board (NZDB). Which particular strains were examined at any particular stage was dependent on the requirements of the NZDB. By the time I began to investigate the effects of probiotic LAB strains on platelet aggregation, the focus of research was restricted to HN001 and HN019 for commercial reasons. As a result, HN017 was not included in the list of probiotic strains being tested for their effect on platelet aggregation (chapter 5) or autoimmunity (chapter 10).

- 2 *On page 17 and 18 in the literature review, it is noted that improvement of diseases was demonstrated by the reduction in incidence, duration, and/or rotavirus shedding. The candidate advances no hypothesis explaining these observations. That bacterial cultures orally administered could have an influence on intestinal viruses, which are obligate intracellular parasites, is surely worth further comment. Please comment and clarify this point.*

The potential mechanisms by which probiotics influence the development and progression of intestinal viral infection, particularly rotavirus associated diarrhea, may include competitive adhesion to the intestinal mucosa (thereby reducing the chance of virus binding to the mucosa and preventing further invasion of the intestinal cells); stimulation of both innate (natural) and acquired (specific) immune responses, such as enhancing the T lymphocyte and natural killer cell activities, stimulation of cytokine and antibody production against the invading virus, or production of metabolites which may inhibit or prevent the proliferation of virus within the infected cells.

- 3 *Sherman's paper (1987) refers to an immunocompromised host- this should have been pointed out in the text, since it changes the significance of the "occasional clinical infections" described.*

As discussed in chapter 2 (Literature review, section 2.5.2.2), most reported clinical infections associated with LAB strains were identified in patients with preexisting conditions such as surgical procedures, cardiac valve abnormalities, impaired immune functions, or other severe diseases. This indicates that for the normal healthy population, consumption of probiotic LAB strains is unlikely to cause infections, but for subjects with abnormal immune functions, especially immunosuppressed subjects, consumption of probiotic preparations at a large dose need to be carefully monitored. For this reason, the safety of the probiotic strains HN001, HN017 and HN019 in immunocompromised and autoimmune animals was investigated in chapters 9 and 10 respectively.

- 4 *In the Material and Methods section (page 44) and in experimental section (page 126) inoculation of Salmonella onto coliform agar is mentioned. Since Salmonella organisms are in most cases lactose negative, an explanation for this unusual choice of technique should be provided. On page*

126 *“The majority of the tissue samples from these animals were culture positive on coliform agar plates” is taken as indicating that all Salmonella-challenged mice became infected with Salmonella typhimurium. The candidate has not indicated how he has distinguished Salmonella from coliform organisms on a medium that is designed to indicate coliforms. Were the isolates compared with the original inoculum to check the conclusion?*

Coliform agar plates (Ford Richard, Auckland) had previously been proven to be a good medium on which to cultivate the *Salmonella typhimurium* strain ATCC14028 in our Laboratory (Shu, Q., *et al.*, 2000 Microbiol Immunol 44, 213-222), and had been successfully used to cultivate this particular Salmonella strain within MHRC for several years. On this agar medium, 24 hours after aerobic incubation at 37°C, *Escherichia coli* appear as medium purple colonies, Salmonella as large colorless colonies, whereas other coliforms appear as pink colonies.

Initially the large colorless colonies with identical morphological characteristics to the pure culture of *Salmonella typhimurium* ATCC 14028 on coliform agar plates were identified as possible Salmonella colonies. As indicated in chapter 3, section 3.19, these colonies (at least 10% of identical colonies from each plate) then underwent further identification according to their cell morphology, oxidase and Gram stain reactions, indole test, and carbohydrate fermentation patterns (BBL CRYSTAL™, Becton Dickinson) to species level. Thus, all colonies recovered from the cultures of tissues from *Salmonella typhimurium* challenged animals were positively identified as *Salmonella typhimurium* using the above mentioned identification system.

5 *Page 67: What level of glucose might be expected in the gut of the normal healthy animal? Stepwise (diauxic) metabolism of substrate could explain the observed failure of mucinolytic activity. If one percent of the normal gut microflora degrade mucin, what is the significance or risk with probiotics? Please comment.*

Carbohydrate from food is the major source of glucose in the lumen. Carbohydrates are metabolised to glucose by digestive enzymes in the small intestine before they are absorbed into the blood stream. Glucose absorption from the gut lumen is a positive transfer process. So, the concentration of glucose within the gut lumen is highly dependent on the time phase after a meal. Within one hour or even longer after each meal, the glucose concentration within the lumen, particularly the small intestine, may be reasonably high, but once the digestive process is completed, little glucose is left within the lumen. The resident microflora of the large intestine has limited access to glucose because most of the glucose from the food is absorbed in the small intestine. During conditions of starvation, fasting or disease, there is even less glucose in the lumen. This may be the reason why some bacterial strains from the normal gut microflora degrade mucus, in order to generate an energy source to be used by themselves and/or other microorganisms. This process is hypothesized as a normal physiological adaptation. However, if there are more bacterial strains that have mucin degrading activities in the lumen, the mucin layer coating on the lumen surface of the intestines may be over-degraded; this may facilitate the entry of potential pathogens from the lumen into

enterocytes and tissues. The live probiotic LAB strains are intended for oral consumption by hosts at a relatively high daily dose (10^{6-7} cfu/day). These probiotics may transiently colonise and further proliferate within the gut lumen. If these strains possess mucin-degrading properties, there may be 'over mucin-degradation' and thus a weakening of the gut mucosal barrier. Thus, as a probiotic preparation, these strains should have no mucin degrading activity.

6 *Page 108: How is LD₅₀ calculated? Was there no significant difference between the control and HN017 at 5×10^{10} ? (There appear to be no statistics provided to resolve this, but the error bars on the graph suggest that there might be a difference). Comment briefly, please.*

All of the animals examined in this experiment were healthy and survived to the end of the experiment, even though the dosage of probiotics fed to mice was high (10^{11} cfu/mouse/day). The average mouse body weight was 20gm. 10^{11} cfu of LAB was assumed to be one gram of dry bacterial cells and therefore, the dosage used to feed mice in this experiment was 50g/kg/day. In-order to cause the death of 50% of the animals, the dose of probiotics should be higher than the dose used in this study, i.e. oral LD₅₀ of HN001, HN017 and HN019 is higher than 50g/kg/day.

All the data presented on page 113 were analyzed statistically. It appears that there may be differences in the water and feed intakes between the control group and HN017 fed mice in 5×10^7 group. However, statistical analysis indicated that there were no significant differences between the two groups. This may be due to the large standard error of the means.

7 *With respect to the localization of the LAB and bifidobacteria in the GI tract, the mouse and humans have different compartmentalization. As we know, the mouse is herbivorous, while the human is omnivorous. However, the object of the experiments in the thesis was to predict safety for humans and not for mice. Which of the conclusions might be affected if the experiments were to be carried out in humans? (We do not expect a prediction of the outcome of these experiments, but only to identify which of the conclusions from mice may not entirely valid for human study).*

In normal healthy mice, there is large number of LAB including bifidobacteria inhabiting their upper digestive tract (small intestine and even stomach). The defense system in these areas in mice may be more advanced than other animals. But, LAB and bifidobacteria mainly colonize the hindgut area (large intestine) in humans. Bacterial translocation was reported as occurring mainly in the small intestine. The dosage used to feed mice in this study did not cause LAB or bifidobacteria translocation, and did not cause any other adverse effects on the animals' health. However, it is difficult to predict, based on this data, that the same dose will also be safe for human consumption. Dose response studies in humans need to be undertaken to verify the safety and efficacy of potential probiotic strains prior to their use as food additives or dietary supplements.

Emendations

P= page; Para= paragraph; R-line = reverse line

Location	Errors	Corrections
P2, Para 3, R-line 3 P2, Para 4, line 4 P4, Para 3, line3	Gilland, 1989 Fonden, 1995 (Chapter 6)	Gilliand, 1989 Fond'en, 1995 (Chapter 6, HN001 and HN019)
P6, Para 1, R-line 7 P8, Para 1, line 4 P8, Para 2, R-line 1 P9, Para 2, R-line5 P11, Para 3, line 4 P11, Para 3, line 7 P11, Para 3, R-line 9 P13, Figure 2.1 P14, Para 4, R-line 1 P15, Para 1, line 6 P16, Para 3, R-line 1 P17, Para 2, R-line 5 P18, Para 3, line 2 P18, Para 3, line 4 P18, Para 3, line 6 P19, Para 3, line 5 P20, Para 2, line 10 P21, Para 4, R-line 2 P22, Para 2, line 5 P23, Para 2, title P25, Para 1, R-line 1 P26, Para 4, R-line 2 P27, Para 1, line 5 P27, Para 1, R-line 4 P27, Para 2, R-line 1 P29, Para 2, R-line 1 P33, Para 1, line 4 P35, Para 3, line 5 P35, Para 4, line 4 P47, Para 2, title P48, Para 2, Line 13 P49, Para 1, line 4 P51, Para 1, R-line 4	Hamilton-Miller, 1999 Tannock, 1990 under Majamma Tannock, 1994b Mitsuok et al., 1984 were Ps-Aeruginosa exist C. spp temporally Payne <i>et al.</i> , 1981; 1983 humoral, cellular, Fukashima <i>et al.</i> , 1998 Similarly Matsuzaki, 1998 carcinogenesis Hugh Cumming safety Salyer act as Aquirre E. spp hosts, Hoskins, 1981 <i>Lactobicillus</i> have Morelli & Salminen DNA extractions filmso proceded gram	Hamilton-Miller <i>et al.</i> , 1999 Tannock <i>et al.</i> , 19990 under discussion Majamaa Tannock, 1994 Mitsuok, 1984 are Ps-aeruginosa exists <i>Clostridium</i> spp temporarily Payne <i>et al.</i> , 1981 humoral and cellular omit Conversely omit carcinogenic Hughes Cummings Safety Salyers act as an Aguirre <i>Enterococcus</i> spp hosts; Hoskins & Boulding, 1981 <i>Lactobacillus</i> has Morelli & Wright DNA extractions for PCR film so proceeded Gram

P80, Para 2, line 12	Abram <i>et al</i>	Abrams <i>et al</i>
P90, Table 6.3	Title	Susceptibility of probiotics to various antibiotics
P93, Para 2, line 2	3 out of 4	three of the four
P106, Para 1, line 1	(1987)	(1997)
P115, Table 8.1	WBC ($\times 10^3/l$)	WBC ($\times 10^9/l$)
P128, Figure 9.5	WBC ($\times 10^{10}/l$)	WBC ($\times 10^9/l$)
P140, Para 4, line 1	microlitre	microlitres
P143, Figure 10.4	Y-axis title duplicated	omit one
P151, Para 2, R-line 3	Aquirre	Aguirre
P154, Para 3, line 5	Sator	Sartor
P154, Para 3, line 8	Wanger	Wagner
P164, R-line 4	Freud	Freund
P172, line 15	Macdonals	Macdonald