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

A thesis submitted for the degree of Doctor of Philosophy

at Massey University, Palmerston North,

New Zealand

Joseph Shengli Zhou

2001

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 (DR20^{TM}), *Lb. acidophilus* HN017 and *Bifidobacterium lactis* HN019 (DR10^{TM}) 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) finger-printing 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 x 10^7, 10^9 or 5 x 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 ~ 2.5 x 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 dysfuction, 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 x 10^8 cfu/mouse/day; HN019 2.16 x 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.

Acknowledgements

I wish to thank my chief supervisor Professor Harsharnjit Gill for providing much scientific, emotional and financial help throughout the course of these studies, for introducing me to scientific research and always believing that no problem was insurmountable. I also gratefully acknowledge the invaluable contribution of ideas and advice from my co-supervisors Dr Pramod Gopal and Dr Kay Rutherfurd.

I would like to thank a number of people for both their scientific contributions to this work, and their friendship over my time spent at Massey University: Dr Quan Shu, Dr Frank Cross, Ms Anne Broomfield, Miss Nicky Frearson, Ms Christine Booth, Miss Linley Fray, Mr Philip Moore, Miss Sarah Blackburn, Miss Kim Kennedy, Mr Daniel Johnson, Ms Fiona Doull, Miss Pauline Low, Ms Debbie Chesterfield, Ms Linda Ferry, Ms Jill Richards, Miss Fiona Hamilton, and Ms Nicky Rivers. I would also like to thank all other present and former members of Milk and Health Research Centre and the Small Animal Production Unit of Massey University, who have provided me with both technical assistance and friendly words of advice and encouragement over the years.

I gratefully acknowledge the considerable support given by research staff, particularly Dr Jaya Prasad, Dr Tim Coolbear and Dr Mark Lubbers, at the New Zealand Dairy Research Institute and for access to the laboratory facilities at the Microbiology, Nutrition, and Enzyme Section. Thanks also go to Garnett Davy for the preparation of some figures for this thesis. The histological work was conducted under the guidance of Mr Guy Hessell and Mr Mervyn Birtles in the Institute of Veterinary, Animal and Biomedical Science, Massey University.

I gratefully acknowledge financial support from the DNHP Mastorate Scholarship and MHRC Doctoral Scholarship from the Milk and Health Research Centre (Former Dairy Nutrition and Health Programme).

Finally, a special thanks to my wife Lily for her love, understanding, patience and support; special thanks also to my sons Punan and Daniel for the enjoyment they have brought to me.

Thank you all.

CONTENTS

List of tables

List of figures

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	*Lactobacillus rhamnosus* HN001 (DR20^{TM})
HN017	*Lactobacillus acidophilus* HN017
HN019	*Bifidobacterium lactis* HN019 (DR10^{TM})
HN033/Lb. GG	*Lactobacillus rhamnosus* GG (Lb. GG)
HN067	*Lactobacillus rhamnosus* 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_2O	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
SID	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.

- *In vitro* and *in vivo* safety assessment of probiotic lactic acid bacterial strains , Sixth Symposium of Lactic Acid Bacteria, Veldhoven, The Netherlands, September 19-23, 1999, ppJ43.

- Evaluation of safety profiles of probiotic lactic acid bacterial strains *Lactobacillus rhamnosus* HN001 (DR20™), *Lactobacillus acidophilus* HN017, and *Bifidobacterium lactis* HN019 (DR10™), In: A.C.J. Tuijtelaars, R.A. Samson, F.M. Rombouts & S. Notermans (editors), Food Microbiology and Food Safety into the Next Millennium. Proceedings of the Seventeenth International Symposium of the International Committee on Food Microbiology and Hygiene (ICFMH), Veldhoven, The Netherlands, October 1999, pp 877-881.

- *Bifidobacterium lactis* HN019 enhances host immunity and resistance to gastrointestinal pathogens. In: A.C.J. Tuijtelaars, R.A. Samson, F.M. Rombouts & S. Notermans (editors), Food Microbiology and Food Safety into the Next Millennium. Proceedings of the Seventeenth International Symposium of the International Committee on Food Microbiology and Hygiene (ICFMH), Veldhoven, The Netherlands, October 1999, pp 858-861.

- Establishment of an immunocompromised murine model in BALB/c mice, Australasian Society for Immunology Annual Meeting, University of Otago, New Zealand, December 5-8, 1999. pp7.9.

- *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, Mmicrobiology 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.