The biotherapeutic potential of *Lactobacillus reuteri* DPC16 and bovine lactoferrin in controlling some pathogens, genotoxicity and inflammation in the gut

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Abstract

This study investigated the effects of the probiotic bacterium, *L. reuteri* DPC16, alone and in combination with bovine lactoferrin, on intestinal pathogens, intestinal inflammation and carcinogenesis. Human and animal cellular model systems were designed and applied to this evaluation.

The identity of the *L. reuteri* DPC16 strain was confirmed using 16S rRNA analysis and its ability to produce the antibacterial compound, reuterin. It was able to tolerate pH 2 and physiological concentrations of bile salts in a simulated gastrointestinal tract environment in the presence of protective nutrients. It was able to adhere to a Caco-2 human epithelial monolayer (modelling the human GI tract) and it did not degrade mucin.

Both bovine lactoferrin and *L. reuteri* DPC16 inhibited the growth of the intestinal pathogens *Listeria monocytogenes, Staphylococcus aureus, Salmonella typhimurium and Escherichia coli* O157:H7, with much less effect on tested probiotic bacteria. Together, *L. reuteri* DPC16 and bovine lactoferrin showed synergistic inhibitory effects.

L. reuteri DPC16 was also able to remove indole from faecal water. Using human/animal cellular model systems, combined with the use of *E. coli* endotoxin and genotoxic factors present in faecal water, bovine lactoferrin was shown to down-regulate inflammation by affecting the signalling pathway on immune receptors that recognize the endotoxin, while both bovine lactoferrin and strain DPC16 were shown to have the potential to prevent epithelial cell DNA damage.

The study has demonstrated several significant properties of *L. reuteri* DPC16 and bovine lactoferrin, including antibacterial, antigenotoxic, and anti-inflammatory activities, and possible mechanisms for these activities have been proposed. Based on the information obtained from this work, a combination of the probiotic *L. reuteri* DPC16 and bovine lactoferrin could possibly be developed as a novel probiotic formula

for human consumption, to maintain beneficial bacteria while controlling harmful bacteria in the GI tract. However, advanced *in vitro* model systems and *in vivo* studies are suggested to confirm these findings in order to consider the feasibility of commercialisation.

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Abbreviations

AC	Aberrant crypt
ACF	Aberrant crypt foci
AAD	Antibiotic-associated diarrhea
AICR	The American Institute for Cancer Research
ANOVA	Analysis of variance
AOM	Azoxymethane
ATCC	American Type Culture Collection
AFB1	Aflatoxin B1
B(a)P	Benzo(a) pyrene
BCA	Bicinchoninic acid
BLAST	The Basic Local Alignment Search Tool
BLf	Bovine lactoferrin
BHI	Brain heart infusion broth
BM	Basal medium
BSA	Bovine serum albumin
CD	Crohn's disease
CFU/mL	Colony Forming Units per milliliter
CRC	Colorectal cancer
DC	Dendritic cells
DCA	Deoxycholic acid
Dg	Supernatant of DPC16 3h cell suspension in 250 mM glycerol PBS buffer
DMH	1, 2-dimethylhydrazine
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DS	DPC16 culture supernatant in MRS medium
DSg	DPC16 culture supernatant in 250 mM glycerol-containing MRS medium
CPS	Capsular polysaccharides
EaggEC	Enteroaggregative *E.coli*
EDTA	Ethylenediamine tetra-acetic acid

e.g.	*exempli gratia,* means "for example"
EHEC	Enterohaemorrhagic *E. coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *E. coli*
EPS	Extracellular polysaccharide
ETEC	Enterotoxigenic *E. coli*
FAO	Food and Agriculture Organization
FDA	The U.S. Food and Drug Administration
GI	Gastrointestinal
GIT	Gastrointestinal tract
HCAs	Heterocyclic amines
HMGB1	High-mobility group protein B1
HIV	Human immunodeficiency virus
HK cells	Heat-killed cells
3-HPA	3-hydroxypropionaldehyde
HFA	Human flora associated
IARC	International Agency for Research into Cancer
IBD	Inflammatory bowel diseases
IBS	Irritable bowel syndrome
i. e.	Abbreviation for id est. Latin meaning "that is,"
IFN-γ	Interferon-γ
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IQ	2- amino-3methylimidazo[4,5-*f*]-quinoline
LAB	Lactic acid bacteria
LCA	Lithocholic acid
LDH	Lactate dehydrogenase
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MAM	Methylazoxymethanol

MAP	Modified atmosphere packaging
MIC	Minimum inhibitory concentration
MICA/MICB	MHC class I chain-related genes
MLCK	Myosin light chain kinase
MW	Molecular weight
NAD+	Nicotinamide adenine dinucleotide
NADH	Reduced form of NAD+
NADP	Nicotine adenine dinucleotide phosphate
NK cells	Natural killer cells
NO	Nitric Oxide
NOD2	Nucleotide-binding oligomerization domain containing 2
NPD	4-nitro-o-phenylenediamine
NSP	Non-starch polysaccharides
NZMP	New Zealand milk products
OD	Optical density
PAMPs	pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PAD	Photodiode Array Detector
pH	.-Log10 (aH+)
PGM	Porcine gastric mucin
PMA	Phorbol myristate acetate
PRRs	Pattern-recognition receptors
r DNA	r RNA gene
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
r RNA	Ribosomal RNA
SCGE	Single cell gel electrophoresis
SCFAs	Short chain fatty acids
SD	Standard deviation
SDS	Sodium dodecyl sulphate

SE	Standard error
sIgA	Secretory Immunoglobulin A
TEER	Transepithelial electrical resistance
TNF-α	Tumour necrosis factor-alpha
TLRs	Toll-like receptors
UC	Ulcerative colitis
UV	Ultraviolet light
VLBW	Very low birth weight
WCRF	World Cancer Research Fund
WHO	World Health Organization

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Chapter 1
General Introduction

Overview

Gastrointestinal (GI) microflora (synonymous with microbiota or microbiome) play an important role in the host's health status. Environmental and physiological factors, such as diet, age and genotype may affect the composition of the GI microflora, and can be related to diseases in the body (Guarner & Malagelada 2003a). A new exciting discovery published in the journal "Nature" in 2010, and some later publications, revealed that humans can be classified to three distinct enterotypes by their gut microbiome (Arumugam *et al.* 2011), which supports the concept of tailoring functional food to human genetic signatures in the maintenance of health and well-being, and in the prevention of disease (Salminen *et al.* 1998; Stierum *et al.* 2001; Isolauri *et al.* 2002; Saarela *et al.* 2002; Siro *et al.* 2008; Cencic & Chingwaru 2010; Vella *et al.* 2013). For example, fermented milk and milk fractions have been demonstrated to confer a range of benefits on body function and health (Kano *et al.* 2002; Leblanc *et al.* 2004). The biological activities are wide and complex, including effects on functions related to digestion, nutrient absorption, functioning of the intestinal flora, immune defences and preventive effects against disease.

As Prakash *et al.* (2011) emphasized: gut microbiota is the next frontier in understanding human health and development of biotherapeutics. The work presented in this thesis focuses on investigating the efficacy and mechanisms of action of probiotics and health beneficial factors on the gut microbiota and gut health related events. It attempts to explain some of the functional claims of probiotic *L. reuteri* DPC16- and bovine lactoferrin-containing formulae. Outcomes are expected to contribute to characterisation of the probiotic strain *L. reuteri* DPC16 and its functions, and how it might interact when in combination with bovine lactoferrin as a probiotic formulation.

Literature review

1.1 Gastrointestinal microflora

The gastrointestinal tract, also called the alimentary canal, is the digestive tract wherein ingested food is acted upon by physical and chemical processes to provide the body with nutrients it can absorb, and where waste products are excreted. In the human the GI tract starts with the mouth and proceeds through the oesophagus, stomach, duodenum, jejunum, ileum, caecum, rectum and, finally, the anus. In an adult male human, the GI tract is approximately 6.5m long. The high moisture content, stable temperature and abundant nutrients provide an ideal environment for microorganisms. The microorganisms in the human digestive tract consist of 400 to 1000 different species (Guarner & Malagelada 2003a; Sears 2005; O'Hara & Shanahan 2006), with a number about 100 trillions that exceeds the number of body cells by a factor of ten (Blaut 2013).

1.1.1 The development of gastrointestinal microflora

The interior of a baby's intestine is essentially sterile before birth. However, as soon as the baby is born, the newborn is surrounded by microbes. Initially, the microflora of a new baby are strongly dependent on the mother's microflora, the mode of birth, hygiene, feeding practices and so on (Salminen & Gueimonde 2005). For example, the microbiota of vaginally delivered newborns represented the maternal vaginal and intestinal microbiota, while newborns delivered via cesarean delivery exhibited a microbiome representative of the maternal skin microbiota (Buccigrossi *et al.* 2013). The mother provides the first inoculum at birth, provides beneficial factors in breast milk, and introduces environmental bacteria through her skin and other contact with the infant. Therefore, the mother-infant contact has a crucial role in initial infant GI flora development. Studies have demonstrated that animals bred in a germ-free environment are highly susceptible to infections, thereby showing that the intestinal microflora are important constituents in the mucosal defensive barrier (Lievin *et al.* 2000; Meurens *et al.* 2007). In addition, the intestinal microflora provides an important stimulus for the development of the host immune system, and regulates innate and adaptive immunity.

Infant microflora and the first colonization steps play a crucial role in the development and maturation of the immune system and significantly impact on later health (Azad & Kozyrskyj 2012; Neu *et al.* 2013).

The development of individually optimized microflora is dependent on the unique environmental and host-specific factors possessed by each infant. All infants are initially colonized by large numbers of *E. coli* and streptococci. A major environmental influence is breast-feeding. During this time, the breast-fed babies' intestinal microbiota becomes dominated by bifidobacteria (Fanaro *et al.* 2003; Penders *et al.* 2006; Sherman *et al.* 2009), which make up 60-90% of the total faecal population. This starts to establish from the fifth to seventh day after birth, stabilises within a month, and continues for the next 12 months (Salminen & Gueimonde 2005; Coppa *et al.* 2006). This early establishment of the GI microbiota also influences the microbial development and diversity. Collectively, the bacterial inhabitants of the human gastrointestinal tract constitute a complex ecosystem and influence body health through an impact on the gut defence barrier, immune function and nutrient utilisation (Hooper & Gordon 2001; Guarner & Malagelada 2003b). Therefore optimal early establishment of a healthy microflora provides the first key step in long-term well-being for life.

1.1.1.1 The distribution of gastrointestinal microflora

In the human body, bacteria are distributed throughout the GI tract, with the most found in the lower part of the intestines. The microflora include beneficial, harmful and neutral microorganisms, including *Helicobacter, Bacteroides, Lactobacillus, Clostridium, Fusobacterium, Bifidobacterium, Eubacterium, Peptococcus, Peptostreptococcus, Escherichia and Veillonella* (Kierszenbaum 2002; Coico 2003; Carlson 2004; Silverthorn 2004). Species from the genus *Bacteroides* alone constitute about 30% of all bacteria in the gut, suggesting that this genus is especially important in the functioning of the host (Sears 2005). Because different microbes require different environmental conditions, the distribution of the intestinal microbes varies due to different GI environmental factors, such as pH, bile salt concentrations, oxygen content and the presence of bacteriolytic enzymes.

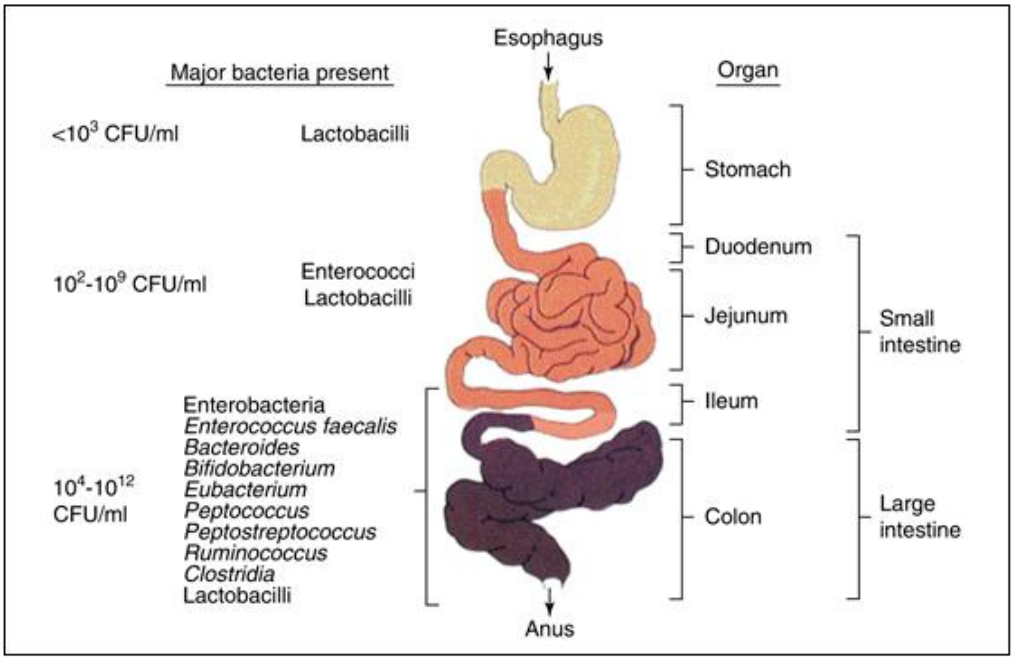


Figure 1.1 The distribution of microbes in the gastrointestinal tract of the human adult (adapted from Roccarina et al. 2010)

As **Fig. 1.1** shows, in the adult human the stomach and the upper segment of the small intestine contain a smaller number of microbes, as the gastric acid and the bile acids are both strongly antibacterial and suppress microbial proliferation. Bacterial numbers are approximately 10^1 –10^3 CFU/g in the region of the duodenum, and increase up to 10^4 – 10^9 CFU/g in the region of the ileum in the small intestine. However, the pH in the intestine gradually increases from the small intestine towards the large intestine. A bacterial conflict takes place in the intestine between putrefactive and fermentative bacteria. Fermentative bacteria produce organic acids which lower the pH and favour their growth and activity. Putrefactive bacteria produce ammonia which increases the pH, favouring their growth and activity. Some bacteria, such as *Lactobacillus*, can proliferate irrespective of the presence of oxygen (facultative anaerobe), and strongly colonize the small intestine. Bifidobacteria survive and grow in the absence of oxygen (obligatory anaerobes), and are predominant in the large intestine. There are 10^4– 10^{12} CFU/g in the colon (Cummings & Macfarlane 1991; Salminen *et al.* 1998; Roccarina *et al.* 2010), predominantly comprising facultative anaerobes (*e.g. Enterobacteria, Streptococcus, Staphylococcus, Lactobacillus, Propionibacterium and Bacillus*) in the upper part of the colon, but further down these change to strict anaerobes (e.g. *Bacteroides, Bifidobacterium, Eubacterium, Peptococcus, Fusobacterium and*

Clostridium) (Tannock 1999).

1.1.2 Factors influencing the composition of the intestinal microflora

Research has demonstrated that the maturation of microbiota into an adult-like configuration happens during the three first years of life. In infancy, among environmental factors, the diet exerts a major effect on the composition and activity of the immature intestinal flora. However, the populations of species in adults vary widely among different individuals but stay fairly constant within an individual over time, even though some alterations may occur with changes in lifestyle, diet and age. The faecal microbiota composition from some microarray data has shown that the intestinal microbiota contains a core community of permanent colonizers, and that environmentally introduced changes of the microbiota throughout adulthood primarily affect the abundance but not the presence of specific microbial species (Rajilic-Stojanovic *et al.* 2012).

1.1.2.1 Diet and intestinal microflora

Considering initiation of the intestinal microflora in infancy, the mother's milk is well-suited to promote the proliferation of bifidobacteria, and maintains their high levels during the first 12 months (Rinne *et al.* 2005; Rautava *et al.* 2006). This observation is consistent with data indicating that mother's milk contains substances including peptides and oligosaccharides that promote the growth of bifidobacteria (Rautava *et al.* 2006). Some studies have reported that the greatest differences between the microflora of breast-fed and formula-fed infants are in the numbers of lactic acid bacteria and the species of bifidobacteria present (Balmer & Wharton 1989; Harmsen *et al.* 2000; Salminen *et al.* 2005) (**Fig. 1.2**). Accordingly, the pH values present in these infant faeces are dramatically different from each other (Balmer & Wharton 1989) (**Fig. 1.3**).

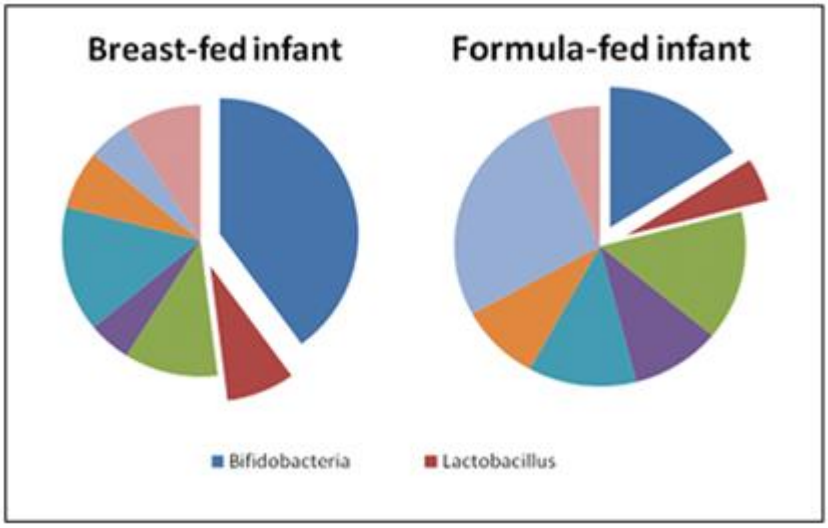


Figure 1.2 Faecal flora from 4-28 days of age in breast-fed babies and formula-fed babies (adapted from Balmer and Wharton, 1989)

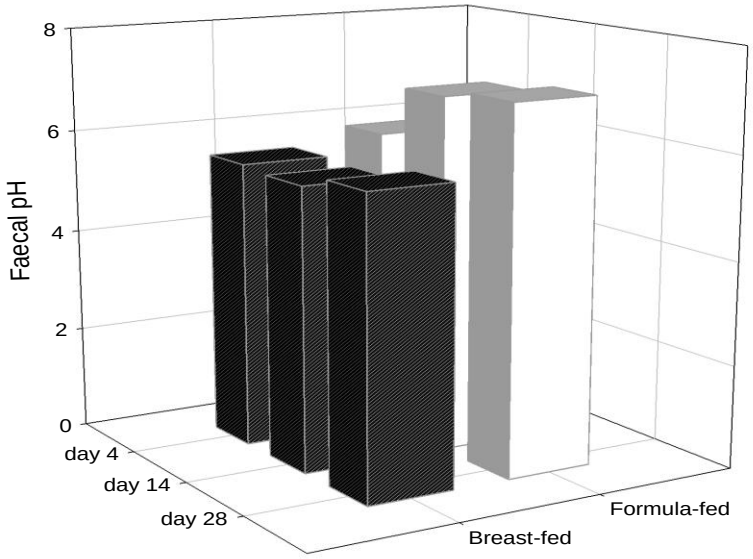


Figure 1.3 Faecal pH from 4-28 days of age in breast-fed babies and formula-fed babies (adapted from Balmer and Wharton, 1989)

However, as the babies' age increases, the solid foods they consume change, and a variety of bacteria begin to establish themselves in the intestine. Even if the bifidobacteria numbers remain unchanged, their proportion in the intestinal microflora decreases. As the adult-like microflora begin to establish, the levels of bacteroides increase gradually, whereas enterobacteria numbers decrease (Bruck *et al.* 2006).

In adulthood, the western diet with high red meat and high fat intake is associated with a

high risk of intestinal diseases (WCRF/AICR 2007) related to the harmful intestinal microflora. New studies and statistical analyses have identified the different bacterial genera in gut microbiota and their association with nutrient intake. "You are what you eat" is becoming an established concept. Gut microflora is mainly composed of three enterotypes: *Prevotella*, *Bacteroides* and *Ruminococcus*. There is an association between the concentration of each microbial community and dietary components. For example, *Prevotella* is related to carbohydrates and simple sugars, indicating an association with a carbohydrate-based diet more typical of agrarian societies, while *Bacteroides* enterotypes is associated with animal proteins, amino acids and saturated fats, components typical of a western diet. That means that one enterotype will dominate over the other depending on the diet - those who eat plenty of protein and animal fats have predominantly *Bacteroides* bacteria, while for those who consume more carbohydrates the *Prevotella* species dominate (Wu *et al.* 2011). For example, animal studies have demonstrated that bile acids, which aid in the digestion of fat, have an influence on the GI microflora composition (Sakai *et al.* 1980). Several harmful microbes in the intestine, such as *Veillonella, Clostridium* and *Proteus*, decompose food residues from undigested protein to cause intestinal putrefaction; this overgrown putrefactive microflora can release harmful gases and carcinogenic substances (Macfarlane & Macfarlane 1997; Salminen *et al.* 1998). In contrast, high vegetable and fruit diets, rich in dietary fibre, non-digestible starch, oligosaccharides and lactic acid bacteria, can help increase lactic acid bacterial populations such as *Lactobacillus* and *Bifidobacterium* (Wyatt *et al.* 1986; Fooks & Gibson 2002; Tlaskalova-Hogenova *et al.* 2004), and are regarded as beneficial to gut health. Reflecting this, short-chain fatty acids and ethanol concentrations produced by lactic acid bacteria appear highest in the caecum (of animals) and the ascending colon. In consequence, products of protein fermentation associated with putrefactive bacteria progressively increase from the right to the left colon, as does the pH of the gut contents (Macfarlane *et al.* 1992). Therefore, the toxins and carcinogenic substances usually accumulate in the lower part of intestine, the area with the highest risk of colorectal cancer.

Notably, the gut microbiome, however, can also be changed by following a long-term dietary change. Enterotype two people, whose diet is based on high levels of protein and fat, and their microbiome is predominantly Bacteroides, and who change their dietary patterns to a diet based on high levels of carbohydrates, will develop a Prevotella

enterotype in the long-term. This relationship may be interesting in the medical field as long term dietary interventions may allow modulation of an individual's enterotype to improve health (Wu *et al.* 2011).

1.1.2.2 Age and the intestinal microflora

Along with the maturation of the gut microflora, numbers of bifidobacteria remain at a constant level in adulthood, and then begin to decrease with old age. At the same time, *E. coli* and potential pathogens such as *Clostridium*, which are rarely found in the young, become dominant in the senile intestinal flora **(Fig. 1.4)** (Mitsuoka 1996; Isolauri *et al.* 2002; Woodmansey *et al.* 2004).

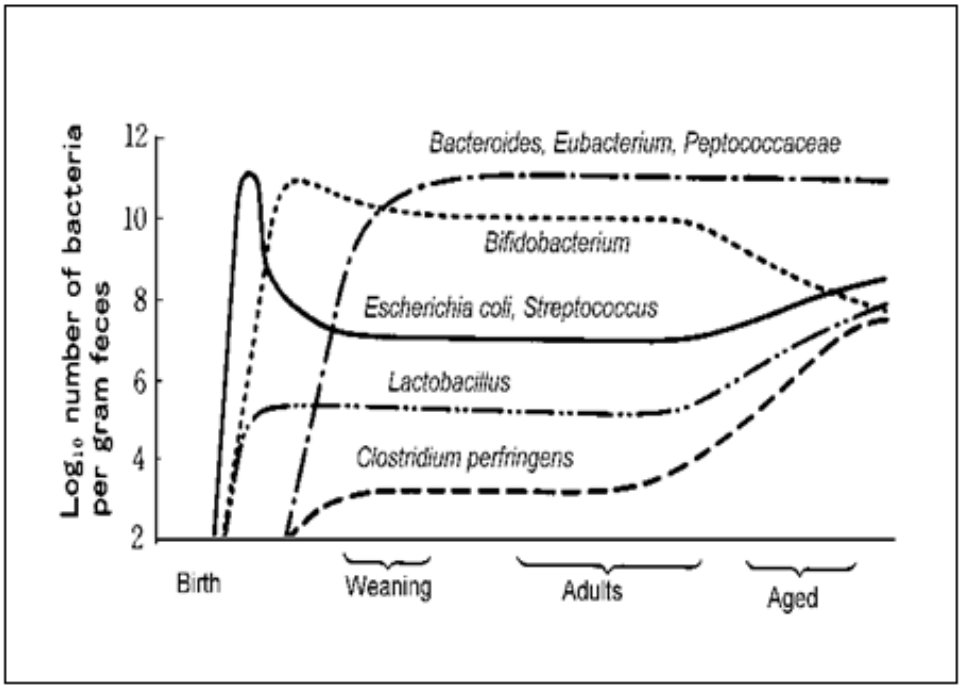


Figure 1.4 Changes in the faecal flora with increasing age in human (adapted from Mitsuoka 1996)

It is known that the modifications in gut physiology and function which occur with ageing can be linked to changes in the composition and metabolic activities of the microflora (Hebuterne 2003). A recent investigation of the faecal microflora in people aged 60 or more has shown that there are profound changes in the bacterial population at this time. Individuals with particular GI diseases (e.g. Crohn's disease, ulcerative colitis, irritable bowel syndrome) often exhibit specific bacterial profiles. Changes of mucosal innate immunity, or indirect consequences of age-related altered nutrition,

reflect direct changes in the gut microflora (Enck *et al.* 2009). Because the proportion of beneficial bacteria tends to decrease in the aged, allowing harmful microbes to proliferate and accelerate intestinal aging, it is essential to ensure the maintenance of the beneficial microbes in the intestinal environment and the suppression of unfavourable functions of harmful microbes in order to maintain health and wellbeing.

1.1.3 Imbalanced intestinal microflora and disease incidence

In the human body, the microflora in the gastrointestinal tract constitute a complex ecosystem that plays an important role in host health due to its involvement in nutritional, immunological and physiological functions. However, these microflora include both beneficial and harmful microbes (**Fig. 1.5**); therefore, they can conduct both favourable and unfavourable functions (Gibson & Roberfroid 1995).

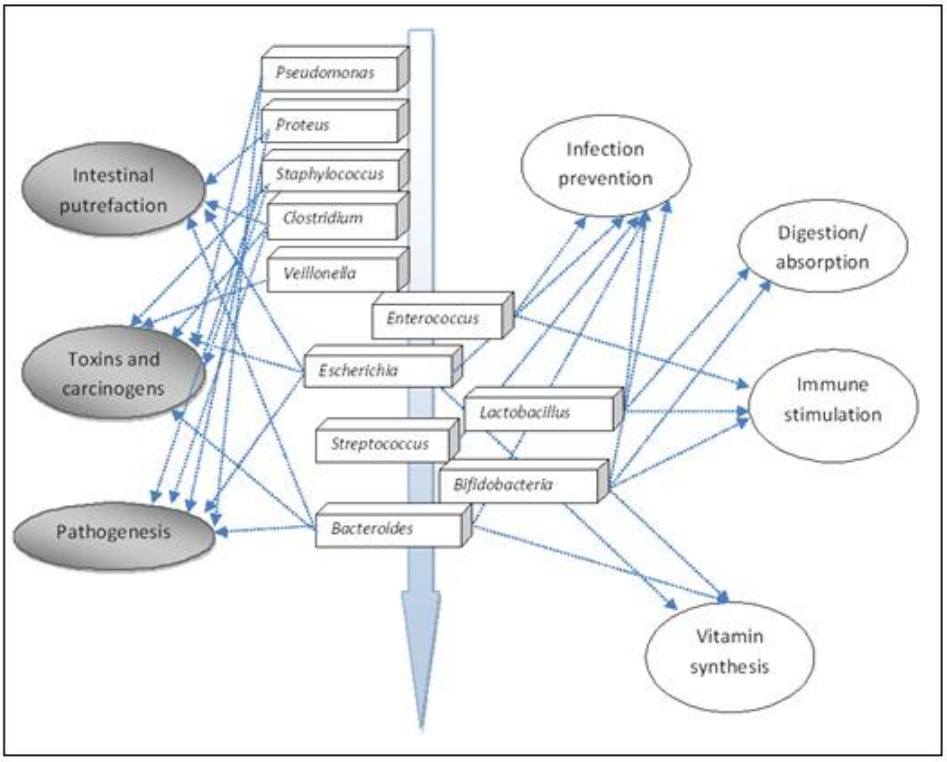


Figure 1.5 Functions of intestinal microbes in humans (modified from Gibson and Roberfroid 1995)

Under normal circumstances, the balanced microbiota cause neither pathogenesis nor inflammation in the host, but instead contribute to health maintenance by forming a barrier layer against colonization by pathogens and by aiding nutrient digestion and assimilation (Salminen *et al.* 1998). Additionally, the resident intestinal microflora play

other important physiological roles in health maintenance: deconjugation of potentially damaging oxidative metabolites and toxins in the gut; degradation of potentially allergenic food proteins; regulation of cholesterol and triglyceride uptake; increase in vitamin biosynthesis; and provision of immunosurveillance signals to limit GI inflammation (Collado *et al.* 2009). Thus stable, properly functioning and active intestinal microflora are essential to the maintenance of human health (Salminen *et al.* 1995). However, in some specific situations the balance of the intestinal microbial populations can be disturbed by various factors such as diet, surgical operations on the digestive tract, intake of antibiotics; genetic influences, aging and stress. In these cases, the harmful microbes can become dominant and the physical conditions may deteriorate. Perturbations of the resident microflora can lead to a deterioration of physiological function and a decline in health, including poor digestion and nutrient assimilation, immune dysfunction and susceptibility to infection by diarrhoea-causing pathogens (Guarner & Malagelada 2003a; Collado *et al.* 2009; Neish 2009).

1.1.3.1 Pathogens and infections

Predominant gut microflora such as *Bacteroides, Bifidobacterium, Lactobacillus,* and *Eubacterium* (Gibson & Roberfroid 1995) are generally recognized as beneficial towards GI health and wellness. These beneficial microflora, particularly the bifidobacteria and lactobacilli, may play critical roles in aspects of our immunological responses, by either helping to resist infection or by creating conditions which reduce the number of pathogenic bacteria. However, some pathogenic bacteria, such as *Salmonella, Shigella, Clostridium, Staphylococcus aureus, Campylobacter jejuni, Escherichia coli, Veillonella and Klebsiella,* and several strains of yeasts, most notably *Candida albicans*, can produce harmful local and systemic effects if they overgrow as a consequence of a gut microflora imbalance (Elmer *et al.* 1996).

Imbalances in the composition of the gut microflora have been reported in disease conditions such as infections, allergies, and irritable bowel disease (Juntunen *et al.* 2001; Turnbaugh *et al.* 2006; Collado *et al.* 2007; Nadal *et al.* 2007). Many pathogens are able to subvert the gut defences and immune system in order to enhance their survival, resulting in persistent infections (Kamada *et al.* 2013). Pathogenic effects associated with harmful GI microflora not only include colonic disorders, but also have

implications for possible vaginal infections (Reid & Bruce 2006) and systemic disorders. Thus, the harmful GI microflora may be designated as "internal environmental pollution" in the body.

Helicobacter pylori

Helicobacter pylori is a Gram-negative microaerophilic bacterium that can inhabit various areas of the stomach and duodenum. Over 80% of individuals infected with the bacterium are asymptomatic. *H. pylori*'s helix shape (from which the generic name is derived) is thought to have evolved to penetrate the mucoid lining of the stomach. It causes a chronic low-level inflammation of the stomach lining and is strongly linked to the development of duodenal and gastric ulcers and stomach cancer. The ability of *H. pylori* to chronically infect the gastric mucosa is due to its down-regulation of certain immune responses. Additionally, because human gastric epithelium lacks Toll-like receptor-4 (TLR-4), which is part of the protective innate immunity against organisms presenting lipopolysaccharide (LPS), such as *H. pylori*, the gastric epithelium is largely nonresponsive to this bacterium, and thus allows its chronic colonization in the stomach (Backhed *et al.* 2003).

Escherichia coli

Escherichia coli is a Gram-negative bacterium that is normally found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless, but some types have been linked to intestinal disease (Acheson & Luccioli 2004), including enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and enteroaggregative *E. coli* (EaggEC). For example, *E. coli* serotype O157:H7 can cause serious food poisoning in humans. Products of these harmful *E. coli* may alter immune homeostasis in the GI tract through inhibition of regulatory cytokine production, which could then contribute to bacterial pathogenesis.

Clostridium

Species of the genus *Clostridium* are all Gram-positive and have the ability to form spores. The genus contains some dangerous human pathogens, e.g. *C. difficile*. The toxins produced by certain members of the genus include botulin, produced by *C. botulinum*.

Salmonella

Salmonella is a genus of rod-shaped, Gram-negative, non-spore-forming, predominantly motile enterobacteria. They are facultative anaerobes, and most species produce hydrogen sulfide. In humans and many animals they cause illnesses such as typhoid fever, paratyphoid fever, and the foodborne illness, salmonellosis.

Shigella

Shigella is a genus of Gram-negative, non-spore-forming rod-shaped bacteria closely related to *Escherichia coli* and *Salmonella*. *Shigella* infection is typically via ingestion (faecal–oral contamination). *Shigella* causes dysentery that result in the destruction of the epithelial cells of the intestinal mucosa in the caecum and rectum. Some strains produce enterotoxin and Shiga toxin, similar to the verotoxin of *E. coli* O157:H7.

Staphylococcus

Staphylococcus aureus is a facultatively anaerobic, Gram-positive coccus, which appears as grape-like clusters. It is a common or commensal skin or foodborne organism of medical significance due to the consequences of wound or nosocomial infection (Safdar & Maki 2002).

Listeria monocytogenes

Listeria monocytogenes is a Gram-positive facultatively intracellular bacterium that is the causative agent of listeriosis. Listeriosis is a leading cause of death from foodborne bacterial pathogens, with fatality rates even exceeding those of *Salmonella* and *Clostridium botulinum*. *L. monocytogenes* can move within eukaryotic cells by explosive polymerization of actin filaments (known as comet tails or actin rockets). It is one of the most virulent foodborne pathogens with 20 to 30% of clinical infections resulting in death (Ramaswamy *et al.* 2007).

1.1.3.2 Mucosal barrier dysfunction and intestinal inflammation

The intestinal mucosal barrier is composed of a thick secreted layer of mucus, an epithelial cell layer and underlying leukocytes. The intestinal epithelial cell lining has a

complex array of agents on its lumenal surface, and organized lymphoid tissues designed to protect against harmful foreign antigens (Acheson & Luccioli 2004). Mucus is synthesized and secreted by the epithelia, and contains high molecular weight (~10^3 kDa) glycoproteins called mucin. Mucin is composed of a peptide core rich in serine and threonine residues which is linked to tandem oligosaccharide repeats *via O*- or *N*-glycosidic bonds (Macfarlane *et al.* 2005). By covering the epithelial enterocytes, mucus firstly provides protection against potential damage caused by acids, bile salts and digestive enzymes (Zhou *et al.* 2001; Macfarlane *et al.* 2005). Secondly, mucus provides protection against degradative bacterial enzymes. However, the mucus can be damaged by some colonised bacteria through degradation of the mucin oligosaccharide side chains (Rhodes *et al.* 1985). Studies have reported that a viable subpopulation of faecal flora collected from healthy human subjects is capable of degrading mucin (Ruas-Madiedo *et al.* 2008). Ruseler-Van Embden and co-workers (1995) have explained that any change in mucus content and structure may compromise the mucosal defence barrier and increase the numbers of bacteria, some of which are regarded as pathogenic and locally toxic, adhering to the enteric cell surface. Several early studies observed that none of *L. casei* strain GG, *L. acidophilus* or *Bifidobacterium bifidum* were able to degrade intestinal mucus glycoproteins (Ruseler-van Embden *et al.* 1995; Zhou *et al.* 2001). Therefore, mucin degradation has been considered as a useful indicator for distinguishing between commensal and pathogenic bacteria (Magalhaes *et al.* 2007). Although the multifunctional cellular and secreted barrier separates the microbial flora from host tissues, impaired mucin structure from enzymatic degradation may provide opportunities for invading microorganisms to penetrate to the underlying enterocytes. Interestingly, the intestinal response to pathogenic and commensal microbes is different. For the latter, sIgA (secretory Immunoglobulin A) is secreted into mucus and helps prevent penetration of the mucus barrier. In contrast, pathogenic bacteria break down the mucin polymer, cross the barrier and disrupt the epithelium, resulting in generation of an inflammatory response (McGuckin *et al.* 2009).

The normal intestinal epithelial enterocytes provide another barrier to block uptake of unwanted microbes and substances into the blood vessels. This epithelial barrier structure tightly controls the trans-cellular transport system by means of membrane pumps, ion channels and tight junctions. The tight junction proteins and cytoskeletal elements are believed to stabilise the tight junction and be critical to its regulation

(Turner 2000). It has been suggested that pathogens can somehow destabilise the tight junction, leading to increased permeability (Scott *et al.* 2002). It has also been suggested that the increased epithelial permeability is not only caused by exogenous factors such as infections, but that the immune system plays an important role in modulating intestinal permeability (Cunningham *et al.* 1993; Baumgart *et al.* 2005). Therefore, any abnormal interaction between the intestinal mucosal immune system and aberrant microflora in the intestinal tract may result in changed immunological function, and trigger the inflammatory response (Feillet & Bach 2004).

All immune systems make use of molecular recognition elements, disposal mechanisms, and a communication system. Microorganisms possess certain molecular configurations which are either not present in the host or are shielded in some way and are therefore known as pathogen-associated molecular patterns (PAMPs). PAMPs are highly conserved microbial structures that include lipopolysaccharide (LPS), peptidoglycans and bacterial DNA, which can initiate cellular signalling responses by interaction with innate host receptors. The host structures that recognize them are called pattern-recognition receptors (PRRs), such as the Toll-like receptor family (TLRs). These PRRs identify the pathogens that have crossed the mucosal barriers and trigger a set of responses that take action against the pathogen, which involve three main immunosensory cells: surface enterocytes, M cells and dendritic cells. The other class of PRRs are known as the nucleotide-binding oligomerization domain/caspase recruitment domain isoforms (NOD/CARD), which are cytoplasmic proteins that recognize endogenous or microbial molecules or stress responses and forms oligomers that activate inflammatory caspases. This would result in the cleavage and activation of important inflammatory cytokines and/or activation of the NK-κB signaling pathway to induce the production of inflammatory molecules (O'Hara & Shanahan 2006). Bacterial endotoxin (such as *E. coli* LPS) is recognized by TLR4 and flagellin by TLR5 (Hornef *et al.* 2002; Janeway & Medzhitov 2002). However, increased expression of host-derived molecules that signal stress (e.g. the major histocompatability complex homologues MICA/MICB that are detected by NK cells) or the release of molecules not normally exposed on living cells (e.g. chromatin-binding proteins such as HMGB1) can also trigger innate immune responses (Playfair & Bancroft 2008).

In normal situations, the mucosal barrier helps to maintain symbiosis between the host

animal and microbes residing in the gut, and the integrity of this barrier is regulated by a complex network of physical, physiological, and immune factors which include dietary influences, the host environment (which can be modified by age, external factors such as antibiotics, and immune competency), and the indigenous microbial flora of the gut. Modification and/or breakdown of these factors leads to ineffective clearance or degradation of harmful ingested antigens and/or disruption of regulatory cell function resulting in mucosal damage, increased gut permeability, and overgrowth of harmful pathogens (Acheson & Luccioli 2004). Reports have shown that intestinal disorders such as antibiotic-associated diarrhoea (AAD), inflammatory bowel diseases (IBD), colorectal cancer (CRC), necrotizing enterocolitis, and ileocolitis are often associated with bacterial translocation due to intestinal barrier failure (Gardiner *et al.* 1993; Solomons 1993). It is believed that abnormal intestinal immunological function, resulting from genetic susceptibility and certain environmental factors including chemical, physical and biological factors, contributes significantly to the inflammatory process of intestinal diseases (Oliva-Hemker & Fiocchi 2002).

Inflammatory bowel disease (IBD)

IBD is a chronic and recurrent inflammation generally affecting the colon or the small intestine, and includes ulcerative colitis (UC) and Crohn's disease (CD). Much evidence has shown that gut microflora (Bell *et al.* 2004; Darfeuille-Michaud *et al.* 2004) or intestinal contents (Kucharzik *et al.* 2006) play an important role in initiating and maintaining the mucosal inflammatory response in IBD. However, the mechanisms of inflammatory bowel diseases are still not entirely clear. Sartor (2004) suggested that IBD may be caused by a hyper-responsive cell–mediated immune response to intestinal commensal bacteria or by microflora aberrancies in genetically susceptible individuals. Later, several pathogens such as *Mycobacterium avium* subspecies *paratuberculosis* (Romero *et al.* 2005), *Listeria monocytogenes* (Thompson-Chagoyan *et al.* 2005) and *Escherichia coli* (Mylonaki *et al.* 2005) were suggested to cause intestinal infection and induce these diseases (Farrell & LaMont 2002). These bacteria influence mucosal immune development, structure, function, and integrity (Hooper *et al.* 2001). Further, some animal models of IBD have shown that colitis does not occur in a germ-free environment. This hypothesis of bacterial influence on the pathogenesis of IBD is strongly supported by the IL-10 (interleukin 10) knockout mouse model (Sellon *et al.*

1998). When kept in a germ free environment, IL-10-deficient mice had no evidence of colitis or immune system activation. However, when contaminated with specific pathogen-free flora, IL-10 knockout mice developed colitis at the time of contamination, and exhibited immune system activation. Hence it was clear that enteric bacteria were necessary for the development of spontaneous colitis and immune system activation in these mice. The evidence from human IBD patients has shown that the inflammation is present in those parts of the gut containing the highest bacterial concentrations (Schultsz *et al.* 1999), especially where pathogenic or potentially harmful bacteria concentrations are high and *Bifidobacterium* and *Lactobacillus* concentrations are low (Favier *et al.* 1997; Cummings *et al.* 2003). Moreover, the terminal ileum, caecum and rectum are areas of relative stasis, providing prolonged mucosal contact with lumenal contents. Enhanced mucosal permeability may play a critical role in maintaining a chronic inflammatory state, due to a genetic predisposition or as a result of direct contact with bacteria or their products (Linskens *et al.* 2001). In addition, several recent studies have proposed that possible mechanisms of IBD, such as intestinal mucosal dysfunction, with an increased translocation of LPS from Gram negative enterobacteria, play a role in the inflammatory pathophysiology of depression (Maes *et al.* 2008). Another study has suggested that IBD results from a breakdown of tolerance towards the indigenous flora in genetically susceptible hosts. When presented with LPS, the epithelial cells may produce abnormally high levels of cytokines (Baumgart *et al.* 2009). Two cytokines, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) have been found in high levels in intestinal mucosa in the context of inflammatory bowel diseases (MacDonald *et al.* 1990; Fais *et al.* 1994) and where there is also decreased barrier function *in vitro* (Madara & Stafford 1989; Mullin *et al.* 1992). Thus, a key step in the pathogenesis of inflammatory bowel disease may be myosin light chain kinase activation (MLCK) by IFN-γ and TNF-α *via* an immune response to bacterial antigens, leading to intestinal barrier dysfunction (**Fig. 1.6)** (Clayburgh *et al.* 2004).

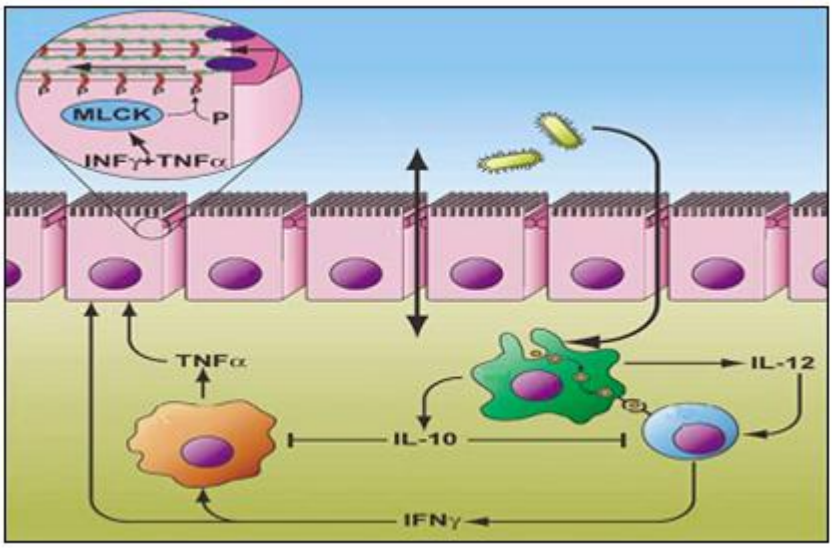


Figure 1.6 A model for the pathogenesis of inflammatory bowel disease

An initial barrier disruption leads bacteria to permeate and activates the antigen presenting cells (green) secreting cytokines, and stimulates T cells (blue) and macrophages (organge) activation. Through unknown mechanisms, two major cytokines, IFN-γ and TNF-α, then act on the epithelium to open the tight junction via MLCK activation. This leads to further loss of barrier function, continuing the cycle of disease progression (Clayburgh *et al.* 2004).

Pathogenic bacteria appear to trigger intestinal inflammation by secreting enterotoxins that increase epithelial cell permeability, dysregulate the mucosal immune system, and impair epithelial cell barrier functions (Wells *et al.* 1996; Robertson & Sandler 2001). In general, intestinal microflora may be involved in the pathogenesis of IBD in the following ways: specific pathogenic infection inducing abnormal intestinal mucosal inflammation; aberrant microflora components triggering the onset of IBD; abnormal host immune response losing normal immune tolerance to lumenal components; and lumenal antigens permeating through the defective mucosal barrier into the mucosal lamina propria and inducing an abnormal inflammatory response.

Irritable Bowel Syndrome (IBS) and other immune disorders

Irritable Bowel Syndrome (IBS) is another common debilitating, multifactorial GI disorder where a definitive aetiology has not been established and no uniformly successful treatment exists. Approximately 5-20% of the world population is estimated to suffer from IBS (Drossman *et al.* 2002; Hillila & Farkkila 2004). The main clinical symptoms of IBS include abdominal discomfort or pain, diarrhoea, constipation, bloating, and flatulence. The microflora in subjects with IBS have been shown to be less stable compared to those of healthy adults (Matto *et al.* 2005). The pathogenesis of IBS

remains unclear, but available evidence suggests that altered gut motility, visceral hypersensitivity, and dysregulation of the brain-gut axis are important mechanisms (Drossman *et al.* 2002). There is accumulating clinical evidence to suggest that an imbalanced intestinal microbial profile (Treem *et al.* 1996) and enteric bacteria-mediated mucosal inflammation (Chadwick *et al.* 2002) may be associated with IBS. There is a complex interaction between the bacteria within the developing gut and the immune system of the host, with long-term health consequences as diverse as eczema and allergic rhinitis (Conroy *et al.* 2009).

1.1.3.3 Harmful bacteria and the risk of cancer

In the body, under normal circumstances, cells exist within a fine balance between cell growth and cell death. However, DNA in cells is subjected to many events that could harm the information encoded by the genome. Some risk factors such as carcinogens, reactive oxygen species and UV exposure can cause mutations or activation of abnormal genes. If DNA repair systems are unable to repair the damage or the cell apoptosis systems fail to work **(Fig. 1.7)** (Friedberg 1995), the mutated cell will persist and replication will occur resulting in a population of altered daughter cells, which may develop into a tumour/carcinoma. Thus, cancer can be described as a disease of altered gene expression with three defined stages of cancer development: initiation, promotion and progression (Ruddon & Norton 1993).

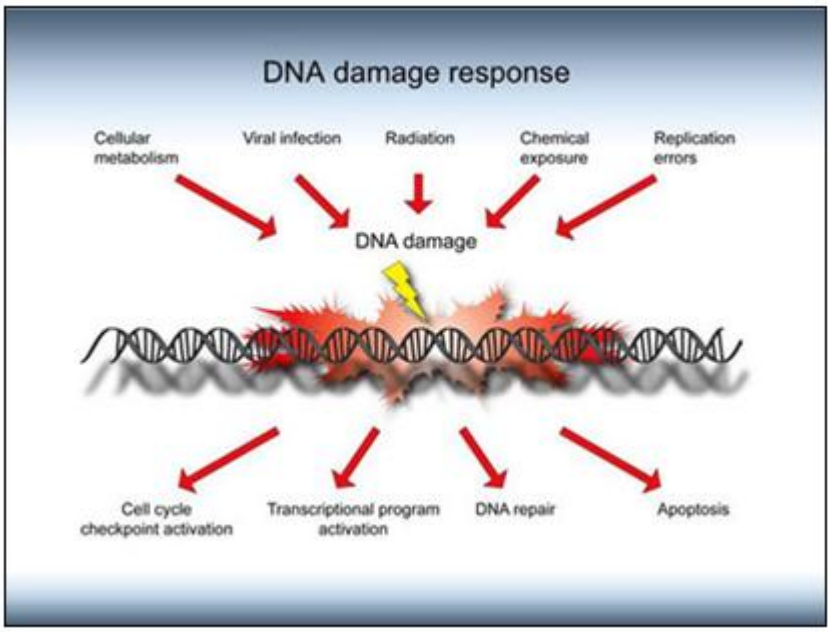


Figure 1.7 Summary of cancer risk factors on cellular DNA and consequences of DNA damage (Friedberg 1995)

Among cancers, colon cancer is the third most common cancer worldwide. The incidence of colon cancer is highest in New Zealand, Australia, the USA and parts of Europe (WCRF/AICR 2007). Carcinogenic agents are present in the diet or are formed during cooking and digestion. Many of these carcinogen formation mechanisms involve the metabolic activities of the bacterial flora normally resident in the colon. Toxins that accumulate in the colon can induce an alteration or mutation in the DNA of colon epithelial cells. This high environmental exposure means that the possibility of colorectal cancer occurring is relatively high. In one animal study, the addition of bacteria to germ-free rats increased both the number of aberrant crypt foci (ACF) and aberrant crypts (AC) (Onoue *et al.* 1997). Similarly, a T-cell receptor β chain and p53 double knockout mouse model showed that adenocarcinoma of the colon did not occur in the germ-free mice (Kado *et al.* 2001). In the colon, *Bacteroides* species have been suggested as factors which increase the risk of colon cancer, whereas some *Lactobacillus* species and *Eubacterium aerofaciens* have been associated with lower risk (Hill *et al.* 1971; Moore & Moore 1995). Similarly, mice mono-associated with *Mitsuokella multiacida*, *Clostridium butyricum* or *Bifidobacterium longum* showed a higher incidence of colonic adenoma (68%) than mice associated with *Lactobacillus acidophilus* (30%), when treated with 1,2-dimethylhydrazine (DMH). It was suggested that the *L. acidophilus* effect may be mediated by faecal pH (Horie *et al.* 1999). Some years ago, it was suggested that colonic tumour formation is dependent upon the presence of intestinal flora and certain intestinal bacterial enzymes (Reddy *et al.* 1974). Several streptococci *(Biarc et al. 2004), Clostridium paraputrificum, Clostridium clostridiiform, Escherichia coli* and *bacteroides* (Rowland *et al.* 1985) have been reported to produce xenobiotic metabolising enzymes such as β-glucuronidase, nitroreductase, azoreductase and steroid 7-α-dehydroxylase (Nakamura *et al.* 2002), all of which can convert procarcinogens to carcinogens (Ling *et al.* 1994; de Roos & Katan 2000). Colon carcinogens such as DMH, azoxymethane (AOM) and methylazoxymethanol (MAM) require deconjugation in order to induce carcinogenesis. In humans, faecal β-glucuronidase activity was shown to be 1.7 times higher in colon cancer patients when compared to healthy controls (Parvez *et al.* 2006). Therefore, there are two mechanisms by which bacteria may be associated with cancer. One is the induction of chronic inflammation following bacterial infection, and the other is the production of toxic bacterial metabolites. Although these mechanisms remain largely unclear, the view that IBD patients have an increased risk of colorectal cancer has been

suggested by several studies (Aspinall 2004; Martin *et al.* 2004). In support of bacteria and inflammation causing cancer, *Helicobacter pylori* infection is known to increase the risk of adenocarcinoma of the distal stomach; this bacterium is the first to be termed a definitive cause of cancer by the International agency for Research into Cancer (IARC) (Santacroce *et al.* 2008).

Chronic inflammatory molecules

Possible links between intestinal inflammation and cancer development have been suggested to be through abnormal interaction between the intestinal microflora and the mucosal immune response (Hussain & Harris 2007). Furthermore, enhanced mucosal permeability may enhance the chronic inflammatory state due to a genetic predisposition or as a result of direct contact with bacteria or their products (Linskens *et al.* 2001), or the accumulation of specific products as a result of DNA damage. Nitrative DNA damage, as well as oxidative DNA damage, has been associated with inflammation-related carcinogenesis (Hussain & Harris 2007). During bacterial infection the active immune system induces phagocytes such as monocytes, macrophages, and neutrophils to generate inflammatory molecules, e.g. pro-inflammatory cytokines and nitric oxide, to kill invading bacteria. Normally inflammation is self-balanced; the innate immune cells, e.g., macrophages, mast cells, dendritic cells (DC) and natural killer (NK) cells can initiate the inflammatory response by releasing pro-inflammatory cytokines (Th1 cytokines IL-1, IL-6, TNF-α and IFN-γ) and anti-inflammatory cytokines (Th2 cytokines IL-1, IL-10 or IL-13), and reactive oxygen (ROS) and nitrogen (RNS) species, which lead to the elimination of pathogens and repair of tissue damage. There is evidence that inducible nitric oxide synthase (iNOS), induced by two signaling pro-inflammatory cytokines, IFN-γ and TNF-α (Gorczyniski & Stanley 1999), is present at high levels in intestinal mucosa involved in IBD (MacDonald *et al.* 1990; Fais *et al.* 1994). These intestinal mucosal cells showed decreased barrier function *in vitro* (Madara & Stafford 1989; Mullin *et al.* 1992). The dysregulated mucosal barrier may result in increased uptake of antigens and pro-inflammatory molecules, including bacterial products and endotoxins, into the *lamina propria,* followed by activation of *lamina propria* immune cells, further secretion of pro-inflammatory cytokines, products of reactive oxygen metabolites and proteases, and further mucosal damage (Kennedy *et al.* 2002). In this manner, a vicious cycle is created

in which barrier dysfunction allows further leakage of lumenal contents, thereby triggering an immune response that can in turn feedback on the intestinal barrier to promote further leakiness (Clayburgh *et al.* 2004). Therefore, sustained generation and accumulation of ROS and RNS, e.g., OH^-, NO^-, O_2^-, $OONO^-$, can inflict oxidative and nitrative damage on critical genes and proteins leading to a pro-tumorigenic microenvironment (Hussain & Harris 2007). The chronic inflammation contributed by IBD leads to an increased risk of colorectal cancer (Hussain *et al.* 2000; Aspinall 2004; Nair *et al.* 2006; Sawa & Ohshima 2006; Meira *et al.* 2008). Thus, cancer may be at least partly due to microbial-triggered inflammation in the GI tract (Kipanyula *et al.* 2013).

Dietary mutagens

Aromatic and heterocyclic nitrogenous compounds are extensively used in industrial processes, and nitrate is a common contaminant of food and drinking water. These substances are reduced by bacterial nitroreductases to toxic N-nitroso- and N-hydroxy-compounds and nitrite in the human colon (Allison & Macfarlane 1989; Macfarlane & Macfarlane 1997). In addition, some fungal toxins, such as aflatoxin B1 (AFB1) which is a secondary metabolite of *Aspergillus* fungi that grow on a variety of food and feed commodities (Chu 1991), are activated within the gut (Allison & Macfarlane 1989) to form potent DNA alkylating agents (Mirvish 1995; Tricker 1997). A range of heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs)(**Fig. 1.8**) such as 2-amino-3-methylimidazo[4,5-*f*]-quinoline (IQ), 2-amino-3,4-dimethylimidazol[4,5-*f*] quinoline (MeIQ), 2-amino-3,8-dimethylimidazol[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) are formed from amino acids when meat and fish are cooked at high temperatures (Turesky 2007; Nowak & Libudzisz 2009). 3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) have been isolated and identified as mutagens in pyrolysates of tryptophan (Sugimura 1997); and benzo(a) pyrene (B(a)P) (Lijinsky 1991), from combustion products of organic material, and found in charbroiled food (Sugimura 1997), has been linked to some cancers (Ferguson 1994).

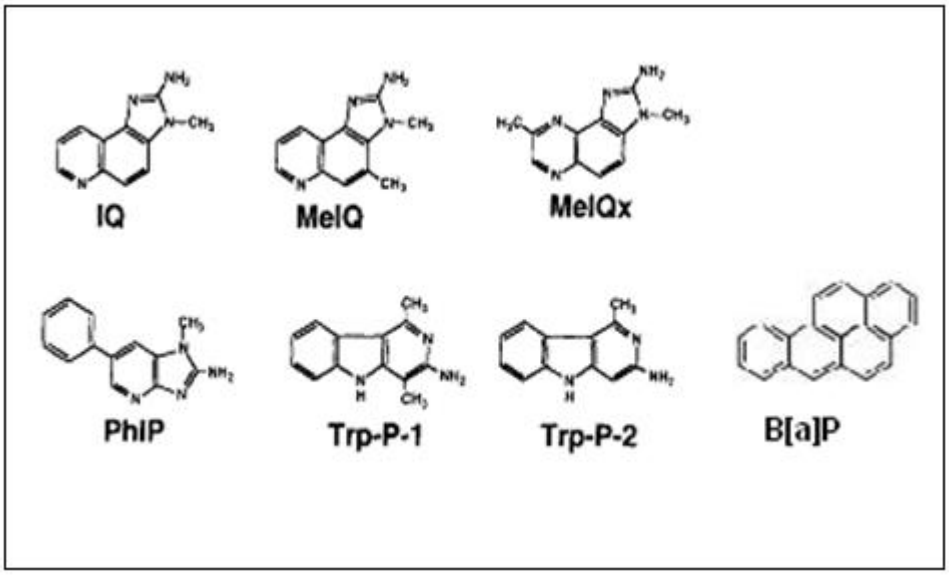


Figure 1.8 Structure of heterocyclic amines and polycyclic aromatic hydrocarbons (modified from Sugimura, 1997)

In these HCAs, IQ is a carcinogen commonly used to induce cancer in animal models of colon cancer (Reddy & Rivenson 1993), while its mutagenic 7-keto derivative (7-OHIQ), a toxic bacterial metabolite, has been found in faeces of individuals consuming a fried meat-rich diet (Carman *et al.* 1988). This metabolite has been shown to be a direct-acting and potent mutagen in *Salmonella typhimurium* and an inducer of DNA damage in colon cells *in vitro* (Ohgaki *et al.* 1991; Rumney *et al.* 1993b). The DNA damage was observed to be 65% greater in colonocytes and hepatocytes from IQ-treated human flora-associated rats when compared to IQ-treated germ-free rats, and it was concluded that intestinal bacteria play a role in the health risks caused by dietary carcinogens (Kassie *et al.* 2001). The data suggest that the intestinal microflora often convert heterocyclic aromatic amines to genotoxic metabolites.

Harmful metabolites

Some human and animal studies have suggested that there is a higher risk of colon cancer with increased meat intake (Rumney *et al.* 1993a; Welfare *et al.* 1997). Further, high consumption of cooked red meat and saturated fat has been related to colon, prostate and breast cancers (Lijinsky 1999; Norat *et al.* 2002). Epidemiological studies have suggested that the incidence of colon cancer is influenced by a high red meat and fat diet, and by a diet low in starch, non-starch polysaccharides (NSP) and vegetables (Potter 1999a, b). More recently, studies have shown that diet can substantially

modulate bacterial activities. Different food components that remain undigested can provide growth substrates for different groups of bacteria in the colon. It has been estimated that in the human body around 60-80 g/day of food reaches the colon (Holm 2001). The large intestine provides the ideal environment for microbial fermentation of undigested carbohydrates (fibre and resistant starch). This in turn produces short-chain fatty acids (SCFAs) which are the main energy source for the epithelial cells of the colon and which can inhibit pathogens by lowering the pH value. In addition, some of these microbial carbohydrate-fermenters produce vitamins K and B (Gibson & Roberfroid 1995). In contrast to the beneficial effects of fibre, protein breakdown can favour putrefactive bacteria which can result in high populations of *Clostridium* and *Bacteroides*, groups of bacteria which express enzymes known to be involved in the metabolism of procarcinogens and tumour promoters (Rowland 1988). Overgrown putrefactive organisms such as *E. coli*, *Enterococcus faecalis*, *Proteus* and *Clostridium* are known to produce toxins e.g. ammonia, amines, nitrosamines, phenols, cresols, indole and skatole, and estrogens (Cummings & Bingham 1987; Mitsuoka 1996; O'Sullivan 2008). Moreover, sulphate, sulphites and sulphur-containing amino acids from protein-rich foods are metabolised by sulphate-reducing bacteria and amino acid fermenting bacteria, producing hydrogen sulphide (H_2S) (Macfarlane *et al.* 1992). Human studies have shown that faecal concentrations of H_2S are higher in patients with untreated ulcerative colitis compared to healthy controls (Pitcher *et al.* 2000).

The bile acids that are synthesised from cholesterol in the liver and secreted in the bile are metabolized by colonic bacteria. The secondary bile acids, deoxycholic acid and lithocholic acid, formed by 7-α-dehydroxylase, are postulated to play an important role in the aetiology of colon cancer by acting as promoters of the tumorigenic process. Concentrations of these secondary bile acids have been shown to be significantly higher in faecal water from patients with colonic polyps or cancer when compared to controls with normal colons (Stadler *et al.* 1988). The accumulated *in vitro* and *in vivo* data have shown that secondary bile acids can cause DNA damage (Pool-Zobel & Leucht 1997), increased colonic crypt foci formation (Sutherland & Bird 1994) and disruption of the colonic mucosal cell membrane integrity leading to a compensatory increase in mucosal proliferation (Nagengast *et al.* 1995). The biological activities of the major toxins produced by bacteria in the colon can be summarized as shown in **Fig. 1.9**.

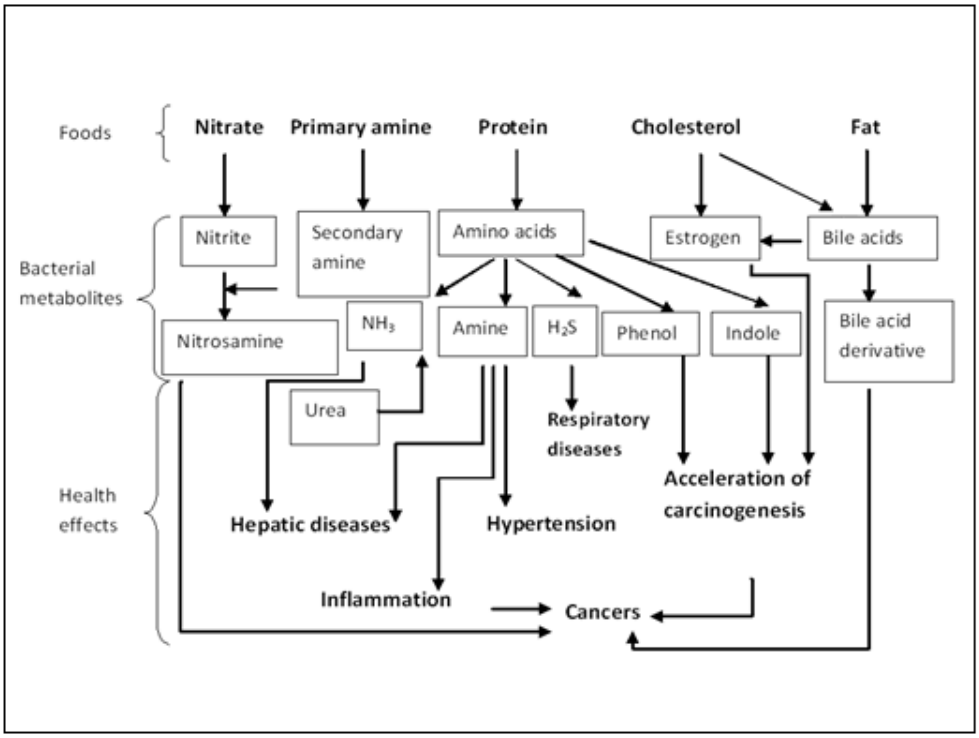


Figure 1.9 The major toxic metabolites produced by intestinal bacteria and their proposed negative health effects

Overall, intestinal bacteria can be described as a "fountain of disease"; in fact, the colon is associated with the greatest number of different diseases of any human organ (Benno 2004). The intestinal tract is also on the "front line of immunity," as it is connected to the outside world. If these harmful substances are introduced and absorbed into the systemic circulation throughout the body by the blood without being decomposed or detoxified in the liver, they may cause damage to various organs and increase the risk of cancers elsewhere (Pool-Zobel & Leucht 1997; Gill & Rowland 2002; Collins & Ferguson 2004; Kapiszewska 2006). In addition, recent molecular biological studies suggest that toxic substances produced by intestinal bacteria may promote cholesterol deposition in blood vessels, inducing arteriosclerosis, which in turn causes heart and cerebrovascular disease. As **Fig. 1.10** shows, intestinal bacteria can be the causes of carcinogenesis, ageing, and various pathological conditions, including arteriosclerosis, liver damage, dementia, autoimmune diseases, and weakened immunity (Benno 2004).

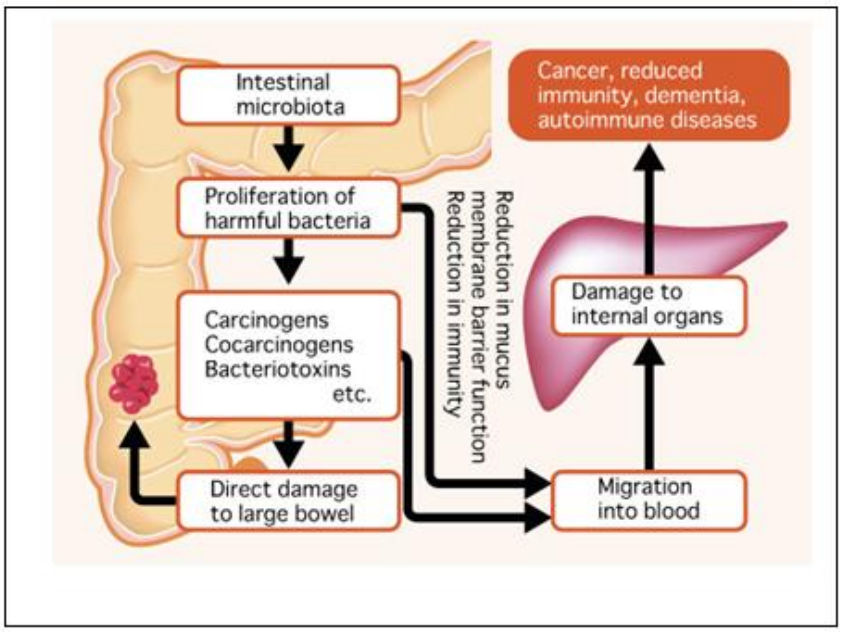


Figure 1.10 Intestinal microflora and disease incidence (from Benno, 2004)

1.1.4 Summary

According to the literature cited above, the balance of intestinal microbiota can be disturbed by physical and environmental factors. A shift in the balance of microbiota composition such that it may become deleterious to host health is termed "dysbiosis". Dysbiosis of the gut microbiota has been implicated in numerous disorders, ranging from intestinal maladies such as inflammatory bowel diseases and colorectal cancer to disorders with more systemic effects such as diabetes, metabolic syndrome and atopy. The potentially important therapeutic options include narrow spectrum antibiotics, novel probiotics, dietary interventions and more radical techniques such as faecal transplantation, which aim to suppress clinical dysbiosis, restore intestinal microbiota diversity and improve host health (Walker & Lawley 2013).

1.2 Probiotics

The word probiotics is derived from the Greek words “pro bios”, literally meaning “for life”. In 1907, Eli Metchnikoff suggested that "the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (Metchnikoff 1907). In 1930, Minora Shirota first isolated *Lactobacillus casei* from the human intestine. Until the 1950s, the probiotic product was licensed by the United States Department of Agriculture (Orrhage *et al.* 1994). Later, Lilly and Stillwell (1965) defined probiotics as

"growth promoting factors produced by microorganisms". Later, Parker (1974) indicated that probiotics were organisms and substances which contribute to intestinal microbial balance. Fuller (1989) defined probiotic as a "live microbial feed supplement". In 1994, the World Health Organization (WHO) deemed probiotics to be the most important immune defence system after prescribed antibiotics (Kailasapathy & Chin 2000). More recently, an official definition of probiotics has been made by the Food and Agriculture Organization (FAO) and WHO as "live microbial food ingredients that, when administered in adequate amounts, confer a health benefit on a host" (Reid *et al.* 2003). Nowadays, probiotics, as members of the healthy gut microflora, assist in mimicking the healthy microflora of both infants and adults. The aim of the probiotic approach is to repair the deficiencies in the gut flora and restore the protective effect. However, the possible ways in which the gut microbiota is being influenced by probiotics is yet unknown (Bezirtzoglou & Stavropoulou 2011). Therefore, the contribution of modulation of an unbalanced indigenous microflora by means of consumption of these beneficial bacteria to the prevention of disease is yet to be confirmed. There is, however, increasing information in the literature to support the hypothesis that administration of probiotic cultures in high doses to human subjects will impact on the intestinal microflora (O'Toole & Cooney 2008). It should be stressed, however, that the available clinical evidence does not provide unambiguous proof of efficacy, and it is apparent that some manufacturers of probiotics may be making some unwarranted claims.

1.2.1 Probiotics characteristics and selection criteria

Most probiotics are intestinal commensal bacteria which normally fall into the lactic acid bacteria group. The most commonly used are *Lactobacillus* and *Bifidobacterium* species, which have a long standing "health image". Specific strains of *Lactobacillus*, *Bifidobacterium* (Cabana *et al.* 2006) and, more recently, *Propionibacterium*, have been shown to have probiotic properties (Ouwehand *et al.* 2000), and have been introduced into food products (Laniewska-Moroz *et al.* 2001) due to their purported health benefits (Cabana *et al.* 2006; Parvez *et al.* 2006; Meile *et al.* 2008).

Lactobacillus

Lactobacillus is a genus of Gram-positive facultatively anaerobic or microaerophilic bacteria. They are a major part of the lactic acid bacteria group, named as such because

most of its members convert lactose and other sugars to lactic acid. In humans they are present in the vagina and in the GI tract, where they are symbiotic and make up a small portion of the gut flora. Many species are prominent in decaying plant material. The production of lactic acid makes its environment acidic, which inhibits the growth of some harmful bacteria. Many lactobacilli are unusual in that they operate using homofermentative metabolism (that is, they produce only lactic acid from sugars, in contrast to heterofermentative lactobacilli which can produce both ethanol and lactic acid from sugars) and are aerotolerant despite the complete absence of a respiratory chain. This aerotolerance is manganese-dependent; they do not require iron for growth and have an extremely high tolerance to hydrogen peroxide (Lee *et al.* 2005).

Bifidobacterium

Bifidobacterium is a genus of Gram-positive, non-motile, often branched anaerobic bacteria inhabiting the GI tract and vagina. It is one of the major genera that make up the gut flora. *Bifidobacterium* produces vitamins, largely of the B-group (Gibson & Roberfroid 1995), and short chain fatty acids which can reduce intestinal pH. An effect of lowering the gastrointestinal pH might be the protonation of toxic ammonia (NH_3) to produce the ammonium ion (NH_4^+), which is non-diffusable and could result in lower blood ammonia levels and a reduced hepatic load. The other important effect is inhibition of the growth of many potential pathogens and putrefactive bacteria.

The selection of a strain to be used as an effective probiotic is critical. Probiotics must fulfil the following criteria: be resistant to acid and bile, be able to attach to human intestinal mucosal cells, be able to colonize the human intestinal tract, produce antimicrobial substances, have demonstrable probiotic activity and survive modern manufacturing processes (O'Sullivan 2001). In addition to these criteria, the potential strain must be non-pathogenic, non-mucin-degrading, and not be associated with intestinal diseases (Saarela *et al.* 2000; Zhou *et al.* 2001; Saarela *et al.* 2002; Fernandez *et al.* 2005). The strain needs to be identified using reliable techniques, for example, 16S rRNA identification. Furthermore, the strain also must not carry horizontally transmissible antibiotic resistance genes (Tuomola & Salminen 1998; Saarela *et al.* 2000).

Table 1.1 Commonly used probiotics

Genera	Species
Lactobacillus	*acidophilus*
	casei
	delbrueckii
	fermentum
	helveticus
	johnsonii
	plantarum
	salivarius
	rhamnosus
Bifidobacterium	*breve*
	infantis
	lactis
	longum
	thermophilum
Enterococcus	*E. faecium*
Streptococcus	*S.salivarius*
Saccharomyces	*S.boulardii*

Adapted from Cabana *et al.* (2006)

The effects of probiotics on human health have been studied both within food matrices and as single or mixed culture preparations (Timmerman *et al.* 2004; Salminen *et al.* 2005). Several well-characterized strains of *Lactobacillus* and *Bifidobacterium* are available for human use to (nominally) reduce the risk of gastrointestinal infections or to treat such infections (Tuomola & Salminen 1998). **Table 1.1** shows some of the most commonly used probiotics.

1.2.2 Probiotic research and the existing gaps

Probiotics and their potential benefits to humans have become attractive research topics over last fifty years. The international Life Sciences Institute (ILSI) has evaluated the probiotic benefits to humans in four areas: metabolism, chronic intestinal inflammatory and functional disorders, infections and allergy (Rijkers *et al.* 2010). These have been summarised by Parvez (2006) as shown in **Fig. 1.11.**

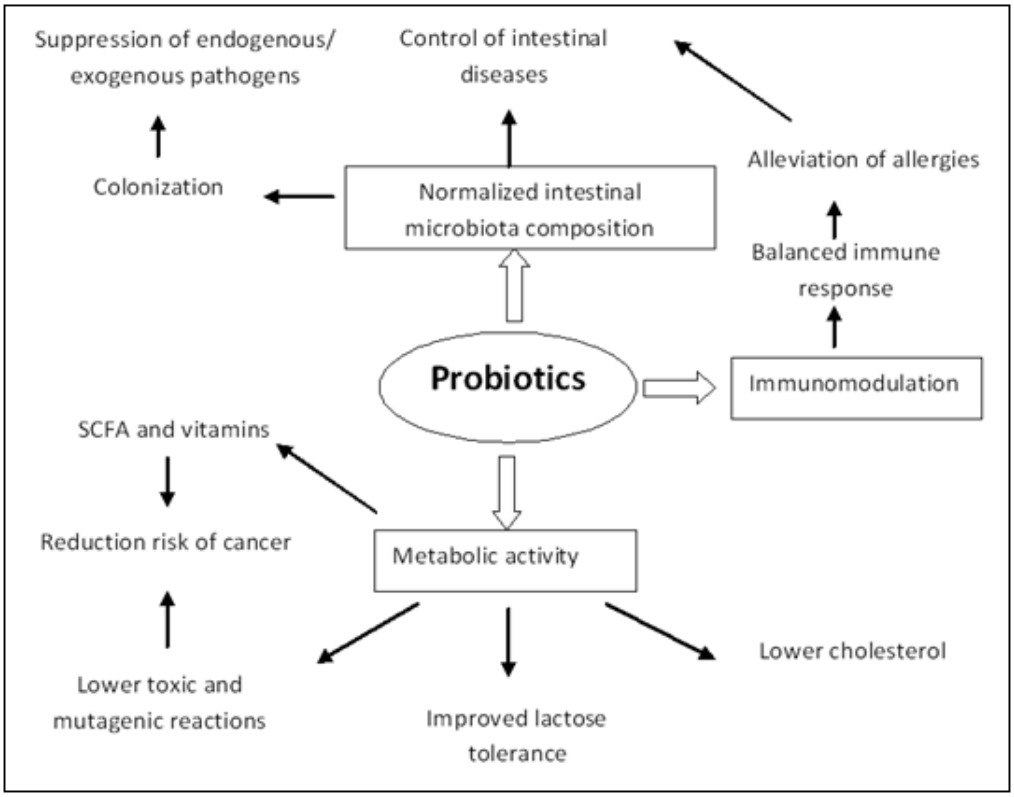


Figure 1.11 Beneficial effects of probiotics on human health (adapted from Parvez *et al.*, 2006)

It is known that probiotics have been increasingly incorporated into functional foods and health supplements for clinical applications in recent years. Some publications have reviewed the effects of probiotics on humans in the treatment of allergies, *H. pylori* infection, intestinal disorders such as irritable bowel syndrome, travellers' diarrhoea, inflammatory bowel disease, liver functions and some cancers (Perdigon *et al.* 2001; Marteau *et al.* 2002; Cesaro *et al.* 2011). Probiotics in fermented and unfermented milk products appear to be promising for the treatment of certain GI tract disturbances such as lactose maldigestion, antibiotic-associated diarrhoea, ulcerative colitis, pouchitis, *Clostridium difficile*-associated diarrhoea and the duration of diarrhoea in infants with rotavirus enteritis (Rosenfeldt *et al.* 2002b). Based on clinic evidence, most of the other potentially beneficial effects are still in the "infancy" of research. Some publications report the use of *in vitro* assays or animal models (including gene knock-out models) but these do not provide proof of efficacy *in vivo* in humans. The use of such models is because of the difficulty of establishing long-term human trials and/or of controlling the human physiological baseline. For these reasons, it remains difficult to draw clear conclusion of clinical benefits of probiotics in humans (Gurusamy *et al.* 2008; Rayes *et al.* 2009; Rayes *et al.* 2012). Moreover, studies have suggested that although probiotics

can be useful in the treatment and prevention of allergies when administered in infancy, it is less useful in adults. Regarding probiotic treatment for chronic inflammatory bowel diseases, results obtained so far have been disappointing (Rijkers *et al.* 2010; Sondergaard *et al.* 2011; Mercer *et al.* 2012). Hence, probiotic research has a long way to go in terms of suitable models and biomarkers selection, and appropriately designed clinic trials.

Although the majority of probiotics tested in previous research systems have reasonable efficacy and safety records, several papers have noted that the properties of different probiotic species vary and can be strain-specific; therefore, care must be taken using probiotic strains for human or clinical practice without validation in separate systems. Among these probiotics, there are some cases of bacterial sepsis related to their use in human, such as liver abscess, bacteremia and endocarditis. The strains mentioned are *Lactobacillus rhamnos*us GG and *Baccillus subtilis* (Boyle *et al.* 2006).

The description of *Lactobacillus reuteri* as a probiotic strain is relatively recent. However, it has increasingly been reported as a functional food component and health supplement because some *L. reuteri* strains present unique beneficial properties based on their genetic characteristics.

1.2.3 *Lactobacillus reuteri*

Lactobacillus reuteri was first isolated from human faecal and intestinal samples by a German microbiologist, Gerhard Reuter, in the 1960s. He subsequently separated it from *L. fermentum* and re-classified it as *L. fermentum* biotype II (Reuter 1965). Kandler *et al.* (1980) eventually identified *L. reuteri* as a distinct species based on phenotypical and genetic characteristics, and proposed it as a new species of heterofermentative *Lactobacillus* found in the large intestine (Reuter 2001). It can ferment glucose alone, with lactate, ethanol, and CO_2 as end products, but a characteristic of most *L. reuteri* is the ability to convert glycerol to 3-hydroxypropionaldehyde (3-HPA) (Talarico *et al.* 1988; Luthi-Peng *et al.* 2002) using the enzyme glycerol dehydratase (Morita *et al.* 2008). 3-HPA may be further reduced to 1, 3-propanediol, as illustrated in **Fig. 1.12**.

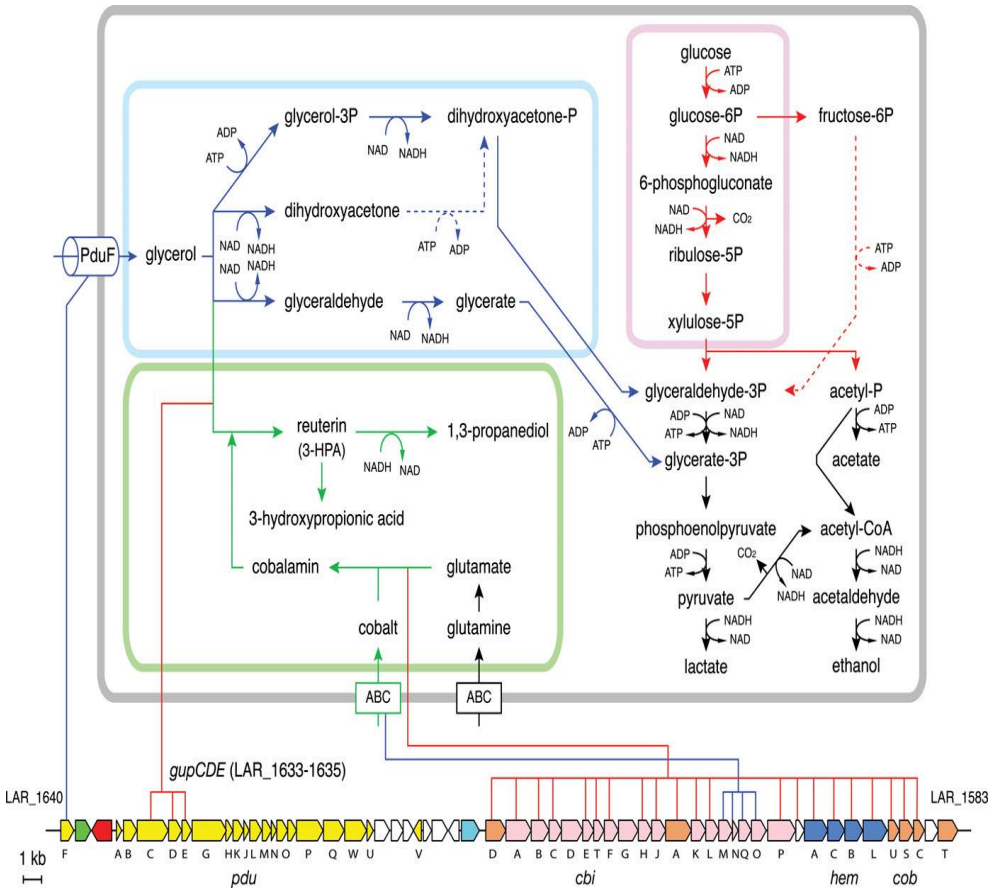


Figure 1.12 A proposed glucose (pink outline) and glycerol (blue outline) metabolic pathway of *L. reuteri* JCM112T (from Morita *et al.* 2008)

Reuterin biosynthesis is outlined in green. The bottom part of the figure shows the gene cluster containing the gupCDE genes responsible for reuterin biosynthesis.

3-HPA is often called "reuterin" (Talarico *et al.* 1988), and is an inhibitory compound that affects both Gram-positive and Gram-negative bacteria, as well as yeasts, fungi, and protozoa (Talarico & Dobrogosz 1989; Liang *et al.* 2003; Spinler *et al.* 2008). The inhibitory mode of reuterin has been suggested as interference with DNA synthesis by inhibition of the conversion of ribonucleotides to deoxyribonucleotides (Talarico & Dobrogosz 1989; Dobrogosz & Lindgren 1995; Lindgren & Dobrogosz 1998). It has been found that concentrations of reuterin in the range of 15-30 µg/mL effectively inhibit growth of many bacteria, but much higher concentrations are required to kill lactic acid bacteria, including *L. reuteri* itself (Axelsson *et al.* 1989; Chung *et al.* 1989; Casas & Dobrogosz 2000).

1.2.3.1 Safety and efficacy of *L. reuteri*

Animal and human experiments have shown that *L. reuteri* is transmitted from mothers

to newborn animals or infants during birth and the nursing process, mainly *via* the mammary duct (Casas & Dobrogosz 2000), and is thus considered to be one of the few true autochthonous (indigenous) *Lactobacillus* species in the human GI tract (Reuter 2001). The viability and activity of some *L. reuteri* strains have been investigated in *in vitro* studies, demonstrating their ability to resist pH 3 and 0.3% bile (Guo *et al.* 2010), and to strongly adhere to an intestinal epithelial model cell-line (Caco-2), indicating potential ability to re-colonise the host GI tract (Nissen *et al.* 2009). Further, orally administered *L. reuteri* has been shown to survive gastric acid and bile salts in the stomach and upper intestine, bind to the gut mucus and epithelial cells, and to colonise the host intestine (Casas & Dobrogosz 2000). A human trial conducted in Mexico with child subjects also confirmed that oral intake of *L. reuteri* was well-tolerated and it colonised in high numbers (Ruiz-Palacios *et al.* 1996). Valeur *et al*. (2004) performed clinical trials on *L. reuteri* strain ATCC 55730 and claimed that this strain can colonise the human stomach, duodenum and ileum and exert its probiotic effects in humans.

Speck *et al.* (1993) first proposed that *L. reuteri* was safe to be used in the food industry and can be consumed as a supplement without adverse effect. The feeding of *L. reuteri* strains to animals has confirmed not only safety, but has also demonstrated growth-promoting and therapeutic value to all tested animal hosts. In human studies, the first clinical safety trial of *L. reuteri* was conducted in 1995 in Finland. The strain was isolated from breast-milk of a healthy human female, and the double-blind trials were conducted on infants aged from 6-36 months hospitalized with viral or bacterial infections. It was concluded that administration of *L. reuteri* was accompanied by good GI colonisation and had no adverse effect (Casas & Dobrogosz 2000). Another group found that the use of *L. reuteri* as a formula supplement in early infancy was safe, well-tolerated and did not adversely affect growth, stooling habits or infant behaviour (Weizman & Alsheikh 2006), as was found in a clinical trial in healthy adults (Mangalat *et al.* 2012). A recent study has confirmed that *L. reuteri* strains are safe with regard to their low amine and high lactic acid production, antibiotic resistance pattern and lack of haemolytic activity (Ruiz-Moyano *et al.* 2009). Overall, supplementation with *L. reuteri* has been proven to be safe and effective with no reported side effects (Klein & Stevens 2008). In addition to safety, it has been claimed that *L. reuteri* confers favourable physiological properties and therapeutic values on the host by reducing respiratory or gastrointestinal diseases (Tubelius *et al.* 2005).

In 2000, Saavedra suggested that an immunological mechanism is responsible for the beneficial effects of *L. reuteri* against rotaviral diarrhoea (Saavedra 2000). From 2002 to 2004, Rosenfeldt and coworkers conducted a series of randomized placebo-controlled trials with lyophilized *L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246. These probiotic strains were effective in reducing the duration of diarrhoea of children in day-care centres (Rosenfeldt *et al.* 2002b), ameliorating acute diarrhoea in hospitalized children and reducing the period of rotavirus excretion, and reducing the length of hospital stay. The beneficial effects were most prominent in children treated early in the diarrhoeal phase (Rosenfeldt *et al.* 2002a). *L. reuteri* also stabilized intestinal barrier function and decreased gastrointestinal symptoms in children with atopic dermatitis (Rosenfeldt *et al.* 2004). Later, some research data provided a novel explanation for beneficial probiotic effects on visceral pain (Kamiya *et al.* 2006), in that oral administration of either live or killed probiotic bacteria, or "conditioned medium", inhibited the constitutive cardio-autonomic response to colorectal distension in rats through effects on enteric nerves. This concept was supported by Savino *et al.* (2007), who demonstrated that *L. reuteri* improved colicky symptoms in breastfed infants within one week of treatment, suggesting that probiotics may have a role in the treatment of infantile colic. Weizman *et al.* (2005) showed that infants fed a formula supplemented with *L. reuteri* had fewer and shorter episodes of diarrhoea, but there was no effect on respiratory illnesses. However, a recent study has supported a role for *L. reuteri* in attenuating the allergic airway response, the major characteristic of the asthmatic response, in a mouse model of allergic airway inflammation, following oral treatment with live cells (Karimi *et al.* 2009).

As a beneficial food supplement, *L. reuteri* was first introduced into the human consumer market in Sweden, in 1991, in a mixture of cultures (*Bifidobacterium animalis*, *L. reuteri* and *L. acidophilus*) in milk. From then on, *L. reuteri* has been incorporated into various food and drinks as a potent health-beneficial ingredient worldwide. *L. reuteri* strains such as B-54 and RC-14 are commercially available in Austria (Reid & Bruce 2006). Clinical data have demonstrated that the consumption of yoghurt supplemented with *L. rhamnosus* GR-1 and *L. reuteri* RC-14 was associated with significant anti-inflammatory effects (Lorea Baroja *et al.* 2007). *L. reuteri* food supplementation also improved feeding tolerance and gut function in formula-fed

preterm newborns (Indrio *et al.* 2008). The Swedish company BioGaia, a world-leader in probiotics, provides *L. reuteri*-containing products globally. To date, 67 clinical studies using BioGaia's human strains of *L. reuteri* have been performed in almost 4,500 individuals of all ages. The results (up to February 2010) are published in 37 articles in scientific journals covering the human health areas of acute diarrhoea, antibiotic-associated diarrhoea, general health, *Helicobacter pylori* infection, infant colic, and oral health. In general, *L. reuteri* has significant efficacy in inhibiting pathogenic invasion of not only the intestinal area, but also in stomach infections caused by *H. pylori* (Mukai *et al.* 2002; Lionetti *et al.* 2006; Imase *et al.* 2007), as well as in dental health (Twetman *et al.* 2009). Recently, a group has demonstrated the concept of artificially boosting the lactobacilli numbers through probiotic administration, including using *L. reuteri* (Reid & Bruce 2006), to reduce vaginal infection (Saunders *et al.* 2007), and improve treatment of bacterial vaginosis (Martinez *et al.* 2009). A novel therapeutic approach using *L. reuteri* RC-14, genetically modified to produce anti-HIV proteins, was capable of blocking the three main steps of HIV entry into human peripheral blood mononuclear cells (Liu *et al.* 2007) which may potentially lower the sexual transmission of HIV. Additionally, *L. reuteri* has been reported to attenuate the production of IL-6, IFN-γ and nitric oxide by peritoneal cells (Tejada-Simon *et al.* 1999), and to suppress TNF-α production induced by LPS in THP-1 cells (Jones & Versalovic 2009). A clinical trial has shown that *L. reuteri* (strain ATCC 55730) positively modulated immune responses in the human GI tract (Valeur *et al.* 2004), while another strain has been reported to be effective in preventing hypercholesterolemia in mice, producing a 17% increase in the ratio of high-density lipoprotein to low-density lipoprotein (Taranto *et al.* 2000). There is evidence for efficacy of some strains of lactobacilli, including *L. reuteri*, in acute gastroenteritis (Vandenplas *et al.* 2007). Finally, *L. reuteri* has been claimed to prevent side effects produced by a nutritional vitamin B deficiency (Molina *et al.* 2009). This species may also represent a treatment option for lactose intolerance (Ojetti *et al.* 2010).

In conclusion, the accumulating evidence suggests that *L. reuteri* can colonise the host, secrete potent substances against harmful pathogens, reduce some infection and disease symptoms, and positively interact with the host cells and the immune system. However, the underlying mechanisms are complicated, with little available information as to how *L. reuteri* colonises the mammalian GI tract, or how its metabolites may modulate the GI microbiota *in vivo*. In addition, other postulated health benefits of *L. reuteri* as

proposed in recent years, such as protection against cancers and treatment of inflammatory diseases still lack evidence in both mechanistic and clinical aspects.

1.2.3.2 *Lactobacillus reuteri* DPC16

Lactobacillus reuteri DPC16 was originally isolated from faecal samples of a healthy Caucasian male by Bioactive Research New Zealand (Auckland, New Zealand). This strain was genetically characterised using 16S rRNA PCR and subsequent sequencing. This identification revealed that this strain was affiliated to the *Lactobacillus* genus with 99.3-99.6% similarity to known species of *L. reuteri*, and at least 0.5% sequence differences in the DNA genome from other *L. reuteri* strains. Also, a 279 bp fragment was detected with a sequence corresponding to a gene encoding glycerol dehydratase in *L. reuteri* species, associated with 3-HPA (reuterin) production (Lu 2007). Hence, this DPC16 strain was identified as a novel *L. reuteri* strain, and registered for a patent by Bioactive Research New Zealand (Shu & Liu 2008). In recent years, some *in vitro* and *in vivo* approaches have been undertaken to understand the characteristics and health-promoting properties of the *L. reuteri* DPC16 strain, while the fermentation process and storage conditions have also been investigated (Liu *et al.* 2004a; Liu *et al.* 2004b; Liu *et al.* 2004c; Chen 2007; Bian 2008). Strong antimicrobial activity of DPC16 against common foodborne pathogens has been noted, and this strain has been proposed as a potential food preservation agent in the New Zealand seafood industry (Lu 2007). However, to date, knowledge on its probiotic properties and the ways by which this strain links to health benefits are very limited. Applications of this strain as a biotherapeutic or nutraceutical supplement, possibly in combination with other dietary agents, still lack underlying theoretical knowledge. Therefore, accumulating solid research experimental data on *L. reuteri* DPC16 strain appears to be urgent.

1.3 Milk beneficial factors

Human and animal trials have indicated the possible strategy of boosting the beneficial bacteria numbers in the host GI tract by using probiotics. However, the provision of other dietary agents or prebiotics to enhance beneficial microflora is a relatively new concept, but one that has stimulated research in many areas of both nutrition and medical science (Roberfroid 2007).

According to the mother-infant model, the mother provides the first bacterial inoculum at birth, and then promotes a bifidogenic environment through the beneficial factors in her milk. The microflora and pH values present in breast-fed and formula-fed infants' faeces are dramatically different (Balmer & Wharton 1989). The promotion of the growth of beneficial bacteria including lactobacilli and bifidobacteria may relate to the components in the breast milk (Coppa *et al.* 2006). In recent years, Dr. David Mills' group in USA has focused on the milk oligosaccharides in the infant intestine in their Functional Glycobiology Program. Their research evidence has revealed that some human milk oligosaccharides are selectively metabolized by some bifidobacteria strains (LoCascio *et al.* 2007). Coppa and co-workers suggested that, in addition to the oligosaccharides in the colostrum which may promote the growth and activity of beneficial bacteria, several antimicrobial proteins such as immunoglobulins (IgG), ê-casein, lysozyme, lactoferrin, haptocorrin, á-lactalbumin, and lactoperoxidase, may contribute to the defence of breastfed infants against pathogenic bacteria and viruses due to their relative resistance to proteolysis in the GI tract. A recent report described that human milk proteins including lactoferrin may be largely resistant to digestion in the gastrointestinal tract, and be only partially digested into bioactive peptides which may have some relationship with the health of human infants (Lonnerdal 2013). These bioactive factors may control unwanted bacteria and stimulate the growth of beneficial bacteria (Coppa *et al.* 2004; Coppa *et al.* 2006). In combination, breast-milk proteins and derived amino acids may assist in providing adequate nutrition to breastfed infants while simultaneously aiding in the defence against infection and facilitating optimal development of important physiological functions in newborns (Lonnerdal 2003). Among these beneficial factors, the glycoprotein lactoferrin has been regarded as a particularly attractive commercial option due to the possibility of isolation from cow's milk with the available technology, high values and the demands of nutraceutical application.

1.3.1 Lactoferrin

Lactoferrin (Lf) is found in colostrum, milk, and other secretions and cells of most mammalian species and is a major component of the mammal's innate immune system (Lonnerdal 2003). It is an iron-binding glycoprotein, and acts as a first-line defence

agent against infections by inhibiting the entry and growth of pathogenic bacteria/viruses in the body (Levay & Viljoen 1995; Iigo *et al.* 2009).

Lactoferrin was first identified in bovine milk by Sorensen and Sorensen in 1939 and subsequently isolated and characterised from human milk in 1960 (Montreuil *et al.* 1960). It is synthesized mainly by granular epithelial cells and secreted in the apo-form in milk, where its concentration varies widely among animal species. In mature bovine milk, its concentration is about 0.03 g/L, whereas in human milk it varies from 1 g/L (mature milk) to 7 g/L (colostrum) (Montreuil & Mullet 1960; Montreuil *et al.* 1960).

Bovine lactoferrin (BLf), isolated from cow's milk, was produced commercially by Oleofina Company in Belgium in 1985. Nowadays, a cation-exchange chromatography system is used for large-scale production by manufacturing companies (Tomita *et al.* 2009). The New Zealand company Fonterra built a processing plant at Hautapu in 2004, in order to meet growing demand for lactoferrin in Asian countries. The market for bovine lactoferrin is thought to be worth around NZ$200 million per year, with annual growth of about 20 per cent (PIS 2010). In 2013, another bovine lactoferrin producer, Synlait Milk in New Zealand, reported that the company is investing $15 million to upgrade its special milks drier to further meet the $15 billion a year demand for infant formula in China. The investment will enable Synlait Milk to become one of only two manufacturers in the world to produce lactoferrin as a spray dried powder, and will also allow the company to manufacture dairy ingredients to a pharmaceutical standard. The beneficial effects of BLf-containing products on health have been shown in clinical and animal studies (Okuda *et al.* 2005; King *et al.* 2007). Lactoferrin has been used as an ingredient in a range of consumer products such as infant formula, yoghurt, dietary supplements, speciality nutritional formulations, and fresh milk, to boost the body's immunity. However, in 2012 the New Zealand Ministry for Primary Industry (MPI) published a guidance on " the Addition of Nutritive Substances Derived from Milk in Infant Formula Products" according the Food Standard Australia and New Zealand (FSANZ), which restricts the source and quantity of lactoferrin to be used in infant formula. Therefore, the use of bovine lactoferrin in the food applications needs to follow the country's regulations.

1.3.2 Structure

Lactoferrin is a single-chain protein with a molecular mass of 80 kDa (Rey *et al.* 1990; Pierce *et al.* 1991). The cDNA and amino acid sequences of bovine Lf were reported in 1990 (Mead & Tweedie 1990). The primary sequences of human and bovine Lf consist of 692 and 689 amino acid residues, respectively (Metz-Boutigue *et al.* 1984; Pierce *et al.* 1991). The three-dimensional structures of human and bovine Lf have been characterised using crystallographic studies. Human Lf contains intramolecular disulphide (SS) bonds but no free sulfhydryl (SH) groups, has an isoelectric point of pH 8.7, and tends to associate with other molecules due to charge interactions. The two globular lobes (N and C) are linked by an extended α-helix (H), and the two domains have a similar amino acid sequence. Each lobe contains one iron-binding site with the capacity to reversibly bind one ferric iron (Fe^{3+}), and one sugar chain **(Fig. 1.13**). A conformational change in the iron-binding cleft, from open to closed forms, accompanies iron binding (Anderson *et al.* 1989; Anderson *et al.* 1990; Moore *et al.* 1997; Baker & Baker 2009).

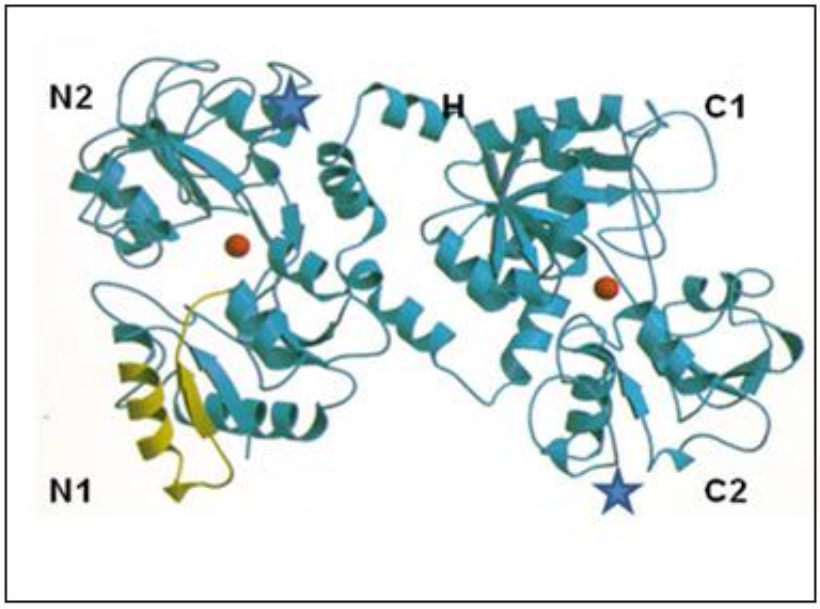


Figure 1.13 Three-dimensional structure of bovine lactoferrin (adapted from Baker and Baker, 2009)

Two iron-binding sites (red dots), two glycosylation sites (blue stars) and the antibacterial lactoferricin domain (highlighted with yellow).

1.3.3 Functions

Lactoferrin is considered to be an important host defence molecule and is known to

confer many biological activities, such as antimicrobial, antiviral, antioxidative, antiinflammatory, anticancer, and immune regulatory properties (Lonnerdal 2003; Wakabayashi *et al.* 2006; Yamauchi *et al.* 2006; Pan *et al.* 2007; Zimecki *et al.* 2007; Lonnerdal 2009; Zimecki *et al.* 2009). This plethora of activities is made possible by mechanisms of action implementing not only its capacity to bind iron but also interactions with molecular and cellular components of both host and pathogens (Legrand *et al.* 2008).

Protection against infections

The biological properties of lactoferrin have been the subject of scientific research since its discovery. Initially, investigations were confined largely to its antimicrobial activity, but now its multifunctionality has been recognized. The antimicrobial activity has been attributed mainly to three mechanisms (Farnaud & Evans 2003): a) its iron-chelating ability deprives microorganisms of a source of iron and leads to inhibition of bacterial proliferation (Arnold *et al.* 1977; Arnold *et al.* 1980); b) direct binding of lactoferrin to the microbial membrane, especially to lipopolysaccharide in Gram-negative bacteria, causes lethal structural damage to outer membranes and inhibition of bacteria replication (Appelmelk *et al.* 1994; Nibbering *et al.* 2001); and c) prevention of microbial attachment to epithelial cells or enterocytes (Hanson *et al.* 2002).

During the past two decades, *in vitro* and *in vivo* studies of lactoferrin and its derivatives have been performed against a wide range of pathogens, including enteropathogenic *E. coli, Clostridium perfringens, Candida albicans, Haemophilus influenzae, Helicobacter pylori, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella typhimurium, S. enteritidis, Staphylococcus aureus, Streptococcus mutans, Vibrio cholerae,* hepatitis C, G and B viruses, HIV-1, cytomegalovirus, poliovirus, rotavirus, and herpes simplex virus (Bhimani *et al.* 1999; Farnaud & Evans 2003; Pan *et al.* 2007). These studies have demonstrated that orally administered lactoferrin and related compounds suppress the overgrowth of certain intestinal bacteria, such as *E. coli, Streptococcus,* and *Clostridium* strains (Teraguchi *et al.* 1995a; Teraguchi *et al.* 2004; Wakabayashi *et al.* 2006; Zimecki *et al.* 2007), reduce infections of *H. pylori* (Okuda *et al.* 2005) and *Candida* strains (Takakura *et al.* 2004), and prevent clinical symptoms of influenza virus infection (Yamauchi *et al.* 2006). A clinical study in infants using bovine lactoferrin preparations

has demonstrated that oral administration increased the number of bifidobacteria in the faecal flora, while the numbers of *Enterobacteriaceae*, *Streptococcus*, and *Clostridium* decreased (Wharton *et al.* 1994b). Another human study showed that lactoferrin supplementation to healthy infants for 12 months was associated with fewer lower respiratory tract illnesses when compared to the control group, which received regular infant formula (King *et al.* 2007). Furthermore, several antimicrobial peptides, such as lactoferricin B and lactoferrampin, which can be cleaved from lactoferrin by the action of pepsin, have strong potential antibacterial activities (Saito *et al.* 1991; Ogata *et al.* 1998) against a broad range of Gram-positive and Gram-negative bacteria *in vitro* (Bullen 1972; Saito *et al.* 1991). These reports indicate that lactoferrin or its hydrolysed products may be able to influence the composition of the intestinal microflora.

Immune system, anticancer and other health claims

Lactoferrin also displays immunological properties influencing both innate and acquired immunity. One *in vitro* study suggested that it plays a regulatory role during cytokine responses, suppressing the release of IL-1, IL-2, and TNF-α from mixed lymphocyte cultures (Crouch *et al.* 1992). Chodaczek *et al.* (2006) demonstrated that a complex of lactoferrin with monophosphoryl lipid A is an efficient adjuvant of the humoral and cellular immune responses. Its stimulating effect on the immune system concerns mainly the maturation and differentiation of T lymphocytes, the Th1/Th2 cytokine balance and the activation of phagocytes. The endotoxin-neutralizing capability of lactoferrin has been observed by several investigators in *in vitro* and *in vivo* studies (Zhang *et al.* 1999). Its up- or down-regulatory effects are related to its ability to interact with pro-inflammatory bacterial components, mainly LPS, or specific cellular receptors on a wide range of epithelial and immune cells. This results in the modulation of the production of various cytokines and of the recruitment of immune cells at the infected sites (Legrand *et al.* 2008).

Recently, Yamauchi *et al.* (2006) reported that intake of bovine lactoferrin may promote systemic host immunity. Kruzel *et al.* (2006) reported that lactoferrin decreases the recruitment of eosinophils, and reduces pollen antigen-induced allergic airway inflammation in a murine model of asthma. Cornish *et al.* (2004) claimed that oral lactoferrin administration to mice regulated bone cell activity and increased bone

formation. Most importantly, the antitumour activity of lactoferrin has been studied intensively over the last decade (Sekine *et al.* 1997a; Sekine *et al.* 1997b; Tsuda *et al.* 1998; Ushida *et al.* 1998; Iigo *et al.* 1999; Masuda *et al.* 2000; Tsuda *et al.* 2000; Gopal *et al.* 2001; Tsuda *et al.* 2002; Parodi 2007). Many mechanisms for the activity have been suggested, e.g. iron-chelation-related antioxidative properties and immunoregulatory and anti-inflammatory functions, but there appears to be no clear consensus regarding this.

Overall, lactoferrin treatment appears to result in beneficial preventive and therapeutic effects on infection, inflammation, and cancer, as well as enhancing iron status in vulnerable groups (Lonnerdal 2009). However, the mechanisms of action of lactoferrin as a modulator of inflammatory and immune responses, or as a host-protective agent against microbial infections, or its role in many physical disorders such as allergies, arthritis, neurodegenerative disorders (Zimecki & Kruzel 2007) and carcinogen-induced tumours in the colon, oesophagus, lung, tongue, bladder, and liver (Sekine *et al.* 1997a; Tsuda *et al.* 2002) are complicated and the details of these mechanisms remain largely unknown. Therefore, selection of lactoferrin as a research subject in the area of health function appears to be very challenging.

1.4 Probiotics and lactoferrin

Probiotic supplements are becoming increasingly popular, as a definite association exists between probiotics and gut health. In addition, there appears to be a natural association between some of the milk proteins/peptides, such as lactoferrin, and probiotics. The milk proteins are believed to positively influence immunomodulation and beneficial microflora, and to aid in disease prevention (Gopal *et al.* 2001), but the mechanisms behind the effects are unclear.

The link between lactoferrin and beneficial bacteria and the initiation of healthy gut flora has stimulated research to explore their function in combination. However, relevant scientific reports are very limited. Before 1993 there was only one report, where the effect of diet on intestinal ecology was studied in germ-free mice that were inoculated with predominant intestinal microflora components isolated from the faeces of breast-fed human infants (Hentges *et al.* 1992). In this study, the addition of bovine

lactoferrin to the whey-dominant formula resulted in significantly greater counts of *Bifidobacterium*, *Bacteroides*, *Enterococcus* and total aerobes in the small intestine when compared with mice fed unsupplemented formula. *Bifidobacterium* was present in large numbers in both the caecum and small intestine of mice fed the lactoferrin-supplemented formula. However, in 1994, Wharton *et al.* claimed that bovine lactoferrin does not influence gut microflora (Wharton *et al.* 1994a). This observation indicates that the actions of lactoferrin *in vivo* may be complex. Later, Sherman *et al.* (2004) demonstrated that human lactoferrin and a probiotic bacterium can be therapeutic agents that may reduce necrotizing enterocolitis and gut-related sepsis in preterm human infants. Further, some results of a human study suggested that the addition of bovine lactoferrin and probiotics could improve the standard eradication therapy for *H. pylori* infection (de Bortoli *et al.* 2007). Moreover, another human study demonstrated that a regular, long-term intake of various synbiotics (probiotics plus prebiotics), including one lactoferrin-containing formula, may improve health by reducing the incidence and severity of respiratory diseases during the cold season (Pregliasco *et al.* 2008). A very recent clinical trial was established to observe if bovine lactoferrin, alone or in combination with a probiotic bacterium, reduces the incidence of late-onset sepsis in "very low birth weight" neonates. The results so far have been positive (Manzoni *et al.* 2009).

1.5 Aim of this study

Normally, only breast-fed infants are exposed to, and are able to benefit from, some milk proteins, such as lactoferrin contained in colostrum. However, if it is added to infant formulae and baby foods, lactoferrin may be able to exert its biological activities in a wider population. The mother-to-infant transfer of both microbes and beneficial milk factors can be considered as a model for the development of future functional foods. Carefully designed bovine lactoferrin adjuvant probiotic formulae could offer a means for creating and maintaining a healthy microflora. The addition of a combination of lactoferrin and probiotics to infant formulae and adult dietary supplements may benefit both infants and vulnerable adults. Based on the literature information, for infant immune development both ingredients (probiotics and lactoferrin) are proposed to relate to a healthy GI microbiota that confers benefits upon host well-being and health. However, not enough is yet known to design specific clinical care practices that support

a healthy microbiota (Murguia-Peniche *et al.* 2013; Neu *et al.* 2013). Therefore, to investigate this concept, the novel commercial strain *L. reuteri* DPC16, alone and in combination with bovine lactoferrin, was selected for study to investigate its health benefits using an *in vitro* cellular model approach, with immunological and microbiological methodologies. The following points were studied:

- Characterisation and validation of the probiotic properties of *Lactobacillus reuteri* DPC16.
- Evaluation of the effects of bovine lactoferrin on the growth of bacteria.
- Investigation of the protective effect of *L. reuteri* DPC16 against harmful bacterial metabolites.
- Evaluation of the effects of bovine lactoferrin and *L. reuteri* DPC16 on faecal genotoxin-induced epithelial DNA damage.
- Evaluation of the effects of bovine lactoferrin and *L. reuteri* DPC16 against endotoxin-induced inflammation.

The expected outcomes from this study are to determine the properties of the combination of *L. reuteri* DPC16 and bovine lactoferrin, and the feasibility as a novel probiotic formulation on the gut immune system and on disease prevention. Although *in vitro* cellular models have limitation to make a clear conclusion, the experiments could be a good starting point to provide justification for the applications.

Chapter 2
Characterisation of the probiotic properties of *Lactobacillus reuteri* DPC16

2.1 Introduction

As documented in the literature, the selection of a probiotic strain to be used in a human dietary application needs to be based on a large quantity of research work. To evaluate a candidate strain to meet the criteria of probiotics, the first important issue is strain identification using reliable techniques, for example, 16S rRNA identification (Higuchi *et al.* 2008; Guo *et al.* 2010), and the strain for human use should preferably be isolated from the healthy human GI tract (Saarela *et al.* 2000). The second criterion is based on probiotic safety requirements, which include that the strain must be non-pathogenic, not degrade mucin, must not carry horizontally transmissible antibiotic resistance genes (Tuomola & Salminen 1998; Saarela *et al.* 2000) and must not be associated with intestinal diseases (Saarela *et al.* 2000; Zhou *et al.* 2001; Fernandez *et al.* 2005; Magalhaes *et al.* 2007; Abe *et al.* 2010). The third commonly employed criterion is the viability assessment for the candidate strain in the stressful human gastrointestinal tract (GIT) conditions of low pH and high bile salts concentrations (Noriega *et al.* 2004; Ritter *et al.* 2009), followed by the ability to colonise the GIT, which is related to adhesion to mucus and/or the intestinal epithelium (Gopal *et al.* 2001; Riedel *et al.* 2006). In addition to all the foregoing, the last important criterion for a probiotic is its antibacterial activity through the production of antimicrobial molecules or the ability to inhibit/displace the adhesion of pathogens (Talarico *et al.* 1988; Ganzle *et al.* 2000).

2.1.1 The background of the *Lactobacillus reuteri* DPC16 strain

The patented *Lactobacillus reuteri* DPC16 strain was originally isolated from the faeces of a healthy Caucasian male (Shu & Liu 2008). Biochemical API 50CH identification, 16S rRNA gene isolation, PCR and subsequent sequencing were performed to identify the strain genus (Bian 2008). The 16S rRNA genetic approach revealed that the strain

was affiliated to the *Lactobacillus* genus with 99.3-99.6% similarity to the known species *L. reuteri*, and the sequence differences in the DNA genome from other *L. reuteri* strains confirmed that it is a novel *L. reuteri* (Lu 2007). Preliminary research has demonstrated that *L. reuteri* DPC16 possesses a wide range of health-enhancing effects, e.g. resistance to pathogen and rotavirus infections and enhanced immune response in animal models (Liu *et al.* 2004a; Liu *et al.* 2004b; Liu *et al.* 2004c). One *in vitro* study also investigated the industrial application of incorporating *L. reuteri* DPC16 into modified atmosphere packaging (MAP) for the control of seafood pathogens and extension of shelf-life of seafood products (Lu 2007). However, to recruit *L. reuteri* DPC16 into a lactoferrin-adjuvant human probiotic formula, the safety and efficacy in human use still lacks information. To fill these gaps, this thesis was initiated to understand the characteristics of this organism as a probiotic strain with gut-beneficial functions.

2.1.2 Aims of this chapter

Previous studies have shown that *L. reuteri* DPC16 is a novel strain isolated from humans (Shu & Liu 2008), which exerts strong antimicrobial activity against selected Gram-positive and Gram-negative foodborne pathogens (Lu 2007), without any adverse effects on selected probiotics (Bian 2008) in *in vitro* conditions. Lu (2007) also detected a 279 bp DNA fragment in *L. reuteri* DPC16 with a sequence corresponding to a gene encoding glycerol dehydratase, an enzyme associated with production of an antimicrobial molecule, "reuterin" (3-hydroxypropionaldehyde). This information indicated that *L. reuteri* DPC16 may be able to convert glycerol to reuterin, which may be responsible, at least in part, for the antibacterial activity against pathogens. However, to validate the probiotic concept and the feasibility of application of *L. reuteri* DPC16 in an ingestible probiotic formula for health benefits, it is important to characterize its viability during transit through the GIT, assess its adhesion to the gut epithelium and determine its affect on mucin degradation, as well as inhibition of intestinal pathogens. These characteristics require examination through a variety of *in vitro* tests, *in vivo* model systems and human clinical trials, Hence, in this chapter, after initial reconfirmation of its identity, *in vitro* experiments were performed to assess *L. reuteri* DPC16 in some aspects of its antibacterial mechanisms, its tolerance to GIT conditions, and its ability to adhere to the intestinal epithelium.

2.2 Materials and Methods

2.2.1 Chemicals

Glycerol was purchased from Biolab Ltd (Auckland, New Zealand). Acrolein was purchased from Sigma (St. Louis, MO, USA).

2.2.2 Bacterial strains and culture media

The probiotic and pathogenic bacteria used in these experiments were provided by Bioactive Research New Zealand (Auckland, NZ) and were stored at -80 °C in 15% glycerol stock solution (made in individual growth medium). *L. reuteri* DPC16, *L. acidophilus* DPC201, *L. plantarum* DPC206, *Pediococcus acidilactici* DPC209, *Bifidobacterium lactis* HN019 and *L. rhamnosus* HN001 were grown in MRS broth (see ***Appendix I***) at 37 °C for 18 h for culture activation before use in assays. The four pathogens, *Listeria monocytogenes* Scott-A ATCC49594, *Staphylococcus aureus* ATCC 25932, *Salmonella enterica* serovar Typhimurium ATCC 1772 and *Escherichia coli* O157:H7 strain 2988 were grown in Brain Heart Infusion (BHI) broth (see ***Appendix I***) at 37 °C for 24 h for culture activation before use in assays.

2.2.3 16S rRNA analysis

2.2.3.1 Genomic DNA extraction from *L. reuteri* DPC16

The stock *L. reuteri* DPC16 strain was thawed and grown at 37 °C overnight in MRS broth. The overnight culture was streaked onto a MRS agar plate (see ***Appendix I***), and incubated at 37 °C in an anaerobic condition generated by GasPak^{TM} EZ Gas Generating Pouch Systems (BD, Sparks, MD, USA) for 24 h. A typical colony was picked off and inoculated into MRS broth and incubated for 18 h at 37 °C. The cell growth was monitored by measuring the optical density (OD) at 550 nm, to ensure that the bacterial cell concentration was in the OD range of 1.2-1.8 using a spectrophotometer (Geneva, Jenway Ltd, Essex, UK). The culture was centrifuged at 4,000 × *g* for 10 min, the supernatant was removed, and the cell pellet was resuspended in 1x PBS (see ***Appendix I***), to an OD value between 1.2 and 1.8. Genomic DNA extraction followed the procedure described in the product manual (QIAamp DNeasy

Blood & Tissue Mini Kit, Qiagen, Biolab New Zealand). The DPC16 cells were harvested in a 1.5 mL micro-centrifuge tube by centrifugation at 5,000 × *g* (7,500 rpm) for 10 min, and resuspended in 180 µL enzymatic lysis buffer (20 mM Tris·Cl, pH 8.0; 2 mM sodium EDTA; 1.2% Triton X-100; 20 mg/mL lysozyme). The suspension was incubated at 37 °C for 30 min before addition of 25 µL proteinase K and 200 µL Buffer AL, vortex mixing, and incubation at 70 °C for 30 min. RNase A (4 µL) was added and the mixture was incubated at room temperature for 2 min. Next, 200 µL of ethanol (96-100%) were added to the sample and mixed thoroughly by vortexing. The mixture was pipetted into a DNeasy Mini spin column placed in a 2 mL collection tube, centrifuged at 6,000 × *g* (8,000 rpm) for 1 min and the flow-through and collection tube were discarded. The DNeasy Mini spin column was placed in a new 2 mL collection tube followed by addition of 500 µL Buffer AW1. The column was centrifuged for 1 min at 6,000 × *g* (8,000 rpm) and the flow-through and collection tube were discarded. The column was re-inserted in a new 2 mL collection tube followed by addition of 500 µL Buffer AW2. The column was centrifuged for 3 min at 20,000 × *g* (14,000 rpm) to dry the DNeasy membrane. Finally, the DNeasy Mini spin column was carefully removed and placed in a clean 1.5 mL or 2 mL microcentrifuge tube, and 200 µL of buffer AE were directly pipetted onto the DNeasy membrane, incubated at room temperature for 1 min, and then centrifuged for 1 min at 6,000 × *g* (8,000 rpm) to elute. The DNA sample was stored at -20 °C before further processing.

2.2.3.2 Amplification of the 16S rRNA gene using the polymerase chain reaction (PCR)

PCR amplification of the 16S rRNA gene was carried out using an Eppendorf thermal cycler (Mastercycler gradient, Hamburg, Germany) with universal primers M27F (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1522R (5′ AAG GAG GTG ATC CAG CCG CA 3′)(Lu 2007). The PCR Master Mix contained a cocktail solution of 10 µL of mixed PCR buffer (contains 60 mM Tris-SO_4 (pH 9.1), 18 mM $(NH_4)_2SO_4$, 1-2 mM $MgSO_4$), 1 µL Elongase Enzyme Mix [a mixture of *Taq* DNA polymerase (Recombinant) and Pyrococcus species GB-D thermostable DNA polymerase] (Invitrogen, New Zealand), 0.2 µM each of forward and reverse primer, 200 µM of each deoxynucleoside triphosphate (Invitrogen, New Zealand), and 1 µL of genomic DNA as a template in a total volume of 50 µL.

The amplification was programmed, on the basis of the primers used, for 1 cycle at 94 °C for 3 min; 35 cycles at 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 45 sec; and 1 cycle of 72 °C for 7 min. The PCR product was checked using agarose gel electrophoresis.

2.2.3.3 Gel electrophoresis of PCR products

Firstly, 1% (w/v) agarose gel was prepared by adding 1 g of agarose (Invitrogen, Carlsbad, USA) into 100 mL of 0.5x TAE buffer (see ***Appendix I*)** using a microwave oven to heat until the agarose was dissolved entirely. After the solution cooled down to about 50 °C, ethidium bromide (Bio-Rad, Hercules, CA, USA) was added to a concentration of 0.5 μg/mL before pouring into the gel tray with the combs placed appropriately. The 5 mm depth of agarose gel was allowed to solidify at room temperature before use. Secondly, the solidified agarose gel was gently placed in the electrophoresis chamber after the combs were removed, and 0.5x TAE electrophoresis buffer was added to cover the gel. Lastly, after mixing 1 μL of DNA sample with 3 μL of 6x gel loading dye (see ***Appendix I***), the mixture and 5 μL DNA mass ladder (Invitrogen, Auckland, New Zealand) were loaded into the individual wells on the agarose gel. The running conditions of the gel electrophoresis device (Bio-Rad, Hercules, CA, USA) were set at 100 V for running between 30-60 min until the dye markers had migrated an appropriate distance. The gel was visualised under UV light and photographed using a Kodak gel documentation system (Kodak, New Haven, CT, USA).

2.2.3.4 Purification of PCR products

The amplified DNA fragments were purified using a DNA clean & Concentrator^{TM}-5 Kit (Zymo Research, USA) according to the procedure described in the product manual. Briefly, 24 mL of 100% ethanol were added to the 6 mL concentrated DNA wash buffer to obtain the final DNA wash buffer solution. Five volumes of DNA binding buffer were mixed with one volume of DNA sample in a 1.5 mL microcentrifuge tube, and mixed briefly by vortexing. The mixture was then transferred into a Zymo-Spin^{TM} column in a collection tube, centrifuged at ≥10,000 × *g* for 30 s, and the flow-through was discarded.

Wash buffer (200 µL) was added to the column and centrifuged at 10,000 × *g* for 30 s. This washing step was repeated. Milli Q water (6 µL) was added directly to the column matrix, and the column was transferred to a new 1.5 mL microcentrifuge tube and centrifuged at ≥10,000 × *g* for 30 s to elute the DNA.

The resulting purified PCR product was quantified on an agarose gel with a low DNA mass ladder (Invitrogen, Auckland, New Zealand), and stored at -20 °C before DNA sequencing.

2.2.3.5 DNA sequencing

The concentration and purity of the purified PCR product were measured using a spectrophotometer (NanoDrop ND-1000 Spectrophotometer, USA) and the sample was sent to the Allan Wilson Centre Genome Sequencing Service (AWCGS, Massey University, Palmerston North, New Zealand). The sequencing was carried out using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kits in a Gene Amp PCR system 9700 machine, and analyzed on an ABI PRISM 377 DNA sequencer. The 16S rRNA gene sequences obtained based on primers M27F and 1522R were assembled, overlapping sequences were pieced together and the contigs were generated using Geneious Pro 4.8.4 software. The contigs were compared to the *L. reuteri* DPC16 sequence presented in the patent documents (New Zealand Patent 526544) using the Basic Local Alignment Search Tool (BLAST) programme (Altschul *et al.* 1997), available on the internet (http://blast.ncbi.nlm.nih.govt/).

2.2.4 Preparation of *L. reuteri* DPC16 culture supernatants

To obtain culture supernatants from different fermentative conditions, a pre-activated *L. reuteri* DPC16 culture was inoculated into MRS medium or MRS medium containing 250 mM glycerol, and incubated anaerobically at 37 °C for 18 h. The culture supernatants, designated DS and DSg, respectively, were obtained by centrifugation (4,000 × *g*, 10 min, at 4 °C) (Centrifuge, Model 5810R, Eppendorf, Hamburg, Germany) followed by filter-sterilization through a 0.2 µm syringe-filter system. Additionally, stationary phase cells from 10 mL of a MRS culture were harvested and washed with PBS buffer by centrifugation (conditions as above) and transferred into 10 mL of 250

mM glycerol (in PBS buffer, pH 7.0) and incubated at 37 °C for 3 h. The culture supernatant, designated Dg, was collected and filter-sterilized as above. All of the supernatants were stored at 4 °C until the time of use.

2.2.5 Agar diffusion assay

An agar diffusion assay, modified from previous work (Tagg *et al.* 1976; Ghrairi *et al.* 2004; Martin *et al.* 2005), was used to determine the antibacterial activities of *L. reuteri* DPC16 culture supernatants. In the assay, a minimal anaerobic culture basal medium (BM) agar (***Appendix 1***) containing 2% (w/v) glucose was selected as a co-cultivation medium to allow both *L. reuteri* DPC16 and *E. coli* O157:H7 to grow. In some experiments, the basal medium agar was further supplemented with 250 mM glycerol. After the agar had cooled to 48 °C in a water bath, *E. coli* O157:H7 was added to a final concentration of $1x10^5$ CFU/mL, and mixed as quickly as possible. Immediately, each mixture was poured into a petri dish with a volume of 20 mL, and allowed to solidify at room temperature in a Class II cabinet. Pre-activated *L. reuteri* DPC16 culture (20 µL) was spotted onto the agar (20 µL of heat-killed culture was a negative control) and allowed to dry at room temperature for 10-15 min in the cabinet. The triplicate petri dishes were then inverted and incubated anaerobically at 37 °C for 24 h. The diameter of inhibition zone was measured from different angles and recorded.

2.2.6 Spectrophotometric turbidity bioassay

Double strength bacterial culture medium was used in experiments to test the inhibitory effects of *L. reuteri* DPC16 cell-free culture supernatants on the growth of selected pathogens and probiotics. A high-throughput bioassay procedure (Rosendale *et al.* 2008) was used. Briefly, a series of two-fold dilutions with sterilised distilled water (effectively 1.56%, 3.13%, 6.25%, 12.5%, 25% and 50% in v/v dilution) of the culture supernatants were transferred into a flat-bottom 96-well culture plate (BD FALCON, USA) at a volume of 50 µL per well. Each of the bacteria under test (at a final bacterial cell number of 10^5 CFU/mL) was diluted in double strength BHI or MRS medium, and 50 µL of each were added to each well of the 96-well plate in triplicate. Each plate was monitored by measuring the optical density at 620 nm during 24 h incubation at 37 °C using an ELISA plate reader (Model Multiskan EX, Thermo Electron Corporation, Vantaa, Finland). The culture medium without *L. reuteri* DPC16 supernatant was used

as a negative control (NC) while the medium containing antibiotics [including streptomycin (100 µg/mL) and penicillin (100 U/mL)] was a positive control (PC).

2.2.7 Analysis of reuterin and short chain fatty acids in *L. reuteri* DPC16 supernatants

As shown in **Fig. 2.1,** when reuterin (3-hydroxypropionaldehyde, 3-HPA) is produced from glycerol by *L. reuteri*, it can be further converted into 3-HPA hydrate, 3-HPA dimer and acrolein (Bauer *et al.* 2010a) Therefore, the determination of reuterin in *L. reuteri* DPC16 culture supernatants followed the "acrolein method" used in previous studies (Circle *et al.* 1945; Lu 2007). Reuterin was converted by dehydration into acrolein, and then acrolein, in an acidic alcoholic solution with tryptophan, forms a violet-coloured tryptophan-acrolein complex, which can be detected using spectrophotometry.

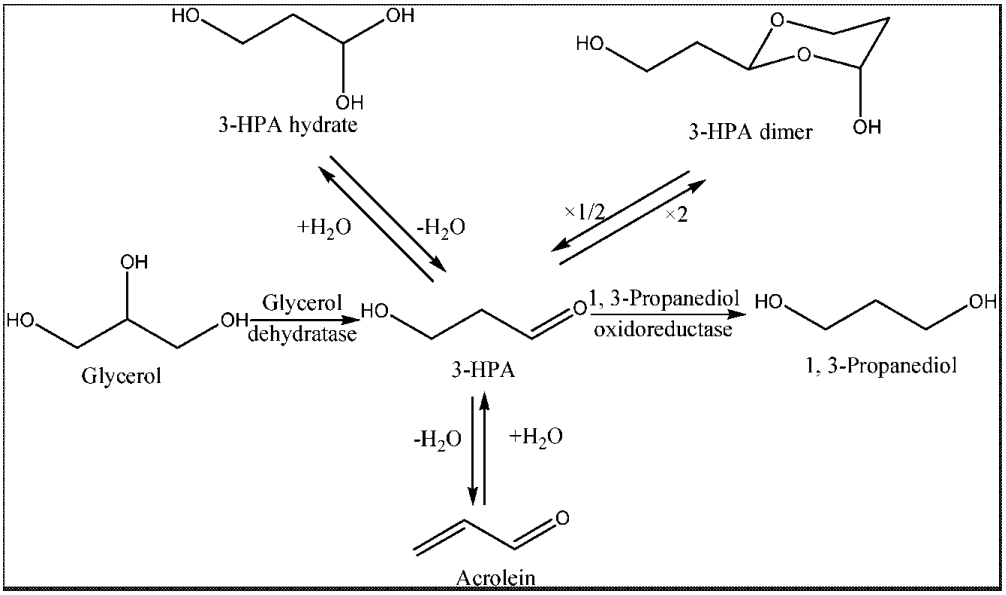


Figure 2.1 Other derivatives of Reuterin (3-hydroxypropionaldehyde, 3-HPA) produced from glycerol (modified from Bauer *et al.* 2010a)

A 100 µg/mL stock acrolein solution (Sigma, USA) was made using 95% ethanol, and a series of acrolein standards (2 mL each) was made from this stock solution. A 0.01 M tryptophan solution was made by dissolving the requisite quantity of crystalline *DL*-tryptophan in 0.05 M HCl, and 0.5 mL of the tryptophan solution was added to each acrolein standard. Subsequently, 6.3 mL of 12 N HCl (37%, J. T. Baker Chemical Co.;

USA) were added to each acrolein standard. Finally, an additional 1.2 mL of 95% ethanol were added to each standard mixture to make the total volume to 10 mL. All standards were prepared in an ice bath to prevent premature heating. Once prepared, the standards were transferred immediately to a 40 °C water bath, and incubated for 50 min to reach the maximum colour development. After incubation, all standards were placed immediately back to the ice bath to stabilize the colours, and were subsequently measured for absorbance at 490 nm using a spectrophotometer (Geneva, Jenway Ltd, Essex, UK). A reagent control containing 2 mL of ethanol to replace the acrolein standard was used as a blank. As several authors have explained, during reuterin determination in the supernatants of *L. reuteri*, 3-HPA as a precursor can be converted to acrolein spontaneously through the secession of H_2O under the conditions of low acidity and/or heat. This method allows exact molar quantification of 3-HPA using acrolein as a standard (Luthi-Peng *et al.* 2002; Bauer *et al.* 2010b) Therefore, the supernatants DS, DSg and Dg prepared from *L. reuteri* DPC16 were diluted appropriately with 95% ethanol to ensure that they fitted within the standard curve. An aliquot of each diluted supernatant (1 mL) was then mixed with 95% ethanol (1 mL) and subjected to the same procedure as above. Acrolein concentrations were measured in triplicate, and the concentration of reuterin present in *L. reuteri* DCP16 fermentation culture supernatants was determined.

The short chain fatty acids (SCFA) were analysed using high performance liquid chromatography (Dionex ICS 2000 Ion Chromatography System with an AS11-HC column), in the School of Engineering & Advanced Technology, Massey University, Palmerston North, New Zealand. The acids were eluted using a concentration gradient of KOH from 0.7 mM to 95 mM. Lactate was measured using a YSI 2700 Select Biochemistry Analyser with a lactate membrane.

2.2.8 *L. reuteri* DPC16 environmental tolerance assays

The physiological food transit time and the conditions in the GIT during digestion, have been described by several authors (Huang & Adams 2004; Noriega *et al.* 2004; Ritter *et al.* 2009), and are summarised in **Table 2.1**.

Table 2.1 pH values, bile salts concentrations and food retention times in the human intestinal tract

	Stomach	**Small intestine**
pH	1.5-2.0	6.2-7.4
Bile salts		0.3-2% (w/v)
Retention time	1.5 h	1-4 h

The tolerance of *L. reuteri* DPC16 to low pH and high bile salts concentrations was tested in both nutrient-sufficient and nutrient-deficient conditions.

2.2.8.1 Tolerance to low pH and high bile salts concentrations in nutrient-sufficient conditions

L. reuteri DPC16 was grown in 10 mL of MRS broth for 18 h. The culture was centrifuged at 4,000 × *g* for 10 min, and the cell pellet was resuspended into pH=2 MRS broth (10 mL, pH was adjusted using 1 M HCl) followed by incubation at 37 °C for 90 min. The pH=2 MRS broth was removed by centrifugation and the cell pellets were transferred into 10 mL MRS broth containing different concentrations of bile salts (containing 50% sodium cholate and 50% sodium deoxycholate, Sigma, USA), with the final pH between 6.9-7.2, for a further 60 min incubation at 37 °C. The culture was centrifuged at 4,000 × *g* for 10 min; cell pellets were washed with PBS and diluted with MRS broth in 10-fold serial dilutions using a 96-well plate (**Fig. 2.2**). Each individual suspension (20 µL) was plated on MRS agar plates in triplicate using the 4 x 4 drop plate method as displayed in **Fig. 2.3**. After the spotted drops were dried at room temperature for 10-15 min in the cabinet, the petri dishes were then inverted and incubated in anaerobic conditions. The bacterial colonies were counted after 24 h incubation at 37 °C to examine the bacterial cell survival after low pH and high bile salt challenges. The other probiotic bacteria *L. acidophilus* DPC201, *L. plantarum* DPC206, *P. acidilactici* DPC209, *B. lactis* HN019 and *L. rhamnosus* HN001 were subjected to the same experiment.

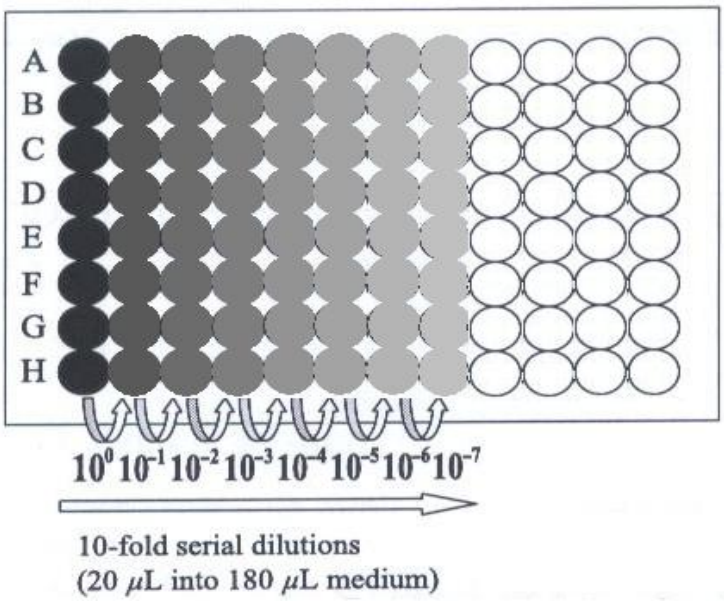


Figure 2.2 A 10-fold serial dilution of a bacterial suspension conducted in a 96-well plate

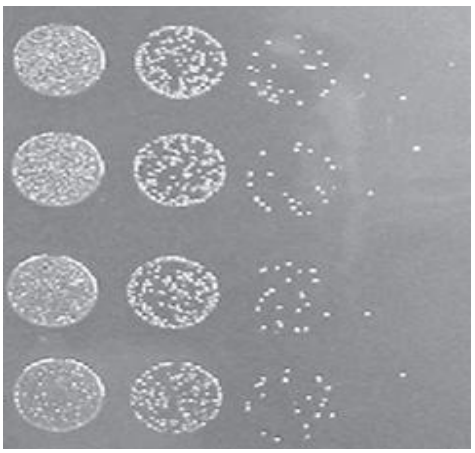


Figure 2.3 4x4 drop plate count method: 4 samples displayed vertically with 4 dilutions displayed horizontally on an agar plate

2.2.9 Tolerance to low pH values and high bile salts concentrations in nutrient-deficient conditions

To further investigate the resistance of *L. reuteri* DPC16 to low pH and high bile salts concentrations, cell pellets of *L. reuteri* DPC16, prepared as described in **Section 2.2.8.1**, were resuspended in PBS buffer (adjusted to pH 2 using 1 M HCl), instead of MRS broth, and incubated at 37 ^{o}C for 90 min. Also, cells were separately incubated in PBS buffer, pH 6.5, in the presence of various concentrations of bile acids for 60 min at 37 ^{o}C. The 4 x 4 drop plate method was then applied to count the number of surviving cells.

2.2.10 Human intestinal epithelium (Caco-2) adhesion assays

The human colon adenocarcinoma Caco-2 cell line was obtained from Plant and Food Research Ltd., Auckland, New Zealand. The cells were precultured in Minimum Essential Medium (MEM) (see ***Appendix I***) supplemented with 10% (v/v) fetal bovine serum (FBS) (heat inactivated at 60 °C for 45 min), 100 μg/mL streptomycin, 100 U/mL penicillin, 1% non-essential amino acids (NEAA) and 1 mM *s*odium pyruvate as a complete MEM medium (all cell culture media reagents obtained from Invitrogen, Auckland, New Zealand), with passage number less than 40. Incubation was at 37 °C in a 5% CO_2 humidified atmosphere (Cell culture CO_2 incubator: *SANYO* Japan). Caco-2 cell differentiation was assessed microscopically on a cell culture Transwell. A monolayer of polarized cell coupled by tight junctions can be observed.

The assays to assess the adhesion of probiotic cells to human intestinal epithelium (Caco-2 cells) were designed according to previous reports (Tuomola & Salminen 1998; Gopal *et al.* 2001; Riedel *et al.* 2006; Tallon *et al.* 2007) with some modifications. Briefly, Caco-2 cells were plated in a 24-well plate at a density of 2.3 x 10^5 cells/mL and 1 mL/well in complete MEM medium. The Caco-2 cells were maintained with medium changes every other day for 15 days after the cells reached confluence. *L. reuteri* DPC16 and the other probiotic bacteria were grown in MRS broth at 37 ^oC for 18 h before harvest. Each bacterial culture was centrifuged at 4,000 × *g* for 10 min, and cell pellets were washed twice with PBS and resuspended in complete MEM medium without antibiotics. Immediately, 1 mL of each probiotic suspension, after adjustment of the bacterial density to the OD range 1.2-1.8 at 550 nm, was exposed to the Caco-2 monolayer by replacing the growth medium and incubated at 37 °C in a 5% CO_2 incubator for 1.5 h. The Caco-2 monolayer was then washed twice with PBS to remove the unattached probiotic bacteria. The monolayer was trypsinised with 0.25% trypsin-EDTA solution for 10 min and the mixture of Caco-2 cells and probiotic bacteria was serially diluted, and then plated on MRS plates using the 4 x 4 drop plate technique. The bacterial colonies were counted after 24 h anaerobic incubation at 37 °C to examine the number of the adhering bacteria on the epithelial monolayer.

2.2.11 Mucin degradation studies

2.2.11.1 Mucin preparation

Partially purified porcine gastric mucin with 0.5-1.5% (w/v) bound sialic acid (Type III, Sigma; USA) was suspended at 1% (w/v) in PBS buffer and stirred for 1 h. The pH was adjusted to 7.2 using 0.1 M NaOH. With the addition of a few drops of toluene for preservation, this mixture was continuously stirred for another 24 h at room temperature, after which it was centrifuged at 10,000 x *g* at 4 °C for 10 min. The supernatant was removed and immediately put in an ice-bath. The pellet was washed with PBS buffer and re-centrifuged to collect the resulting supernatant. This supernatant was added to that previously collected to obtain the final supernatant, which contained dissolved mucin. Pre-chilled absolute ethanol was added into the cooled final supernatant to a final concentration of 60% (v/v). The precipitated mucin was collected and washed twice with ethanol. The final precipitate was then rotary-evaporated to remove ethanol, and dissolved in distilled water. This mucin solution was gradually frozen and then freeze-dried, and the lyophilized mucin was stored at -20 °C (for indefinite time) and later used as a source of purified mucin for the degradation studies.

2.2.11.2 Faecal flora preparation

Faecal samples were obtained from a healthy, non-vegetarian, non-smoking female with no history of gastrointestinal disease. The faecal sample was homogenized in a stomacher (Masticator, IUL, Spain) with PBS (ratio 1:5) for 5 min and centrifuged at 3,200 × *g* for 30 min at 4 ^{o}C (Centrifuge, Model 5810R, Eppendorf, Hamburg, Germany). After the faecal particles were discarded, the supernatant was distributed to 1.5 mL sterilized Eppendorf tubes, followed by a secondary centrifugation at 13,000 × *g* for 10 min (Mini centrifuge, Model 5415D, Eppendorf, Hamburg, Germany) to separate the faecal flora from the aqueous phase. The cell pellets were washed twice and resuspended in PBS to an optical density of 1.2-1.8 at 550 nm.

2.2.11.3 Mucin degradation assay

The mucin degradation assay on agarose plates was based on methods previously described by Colina *et al.* (1996) and modified by Zhou *et al.* (2001). Purified porcine

gastric mucin was incorporated into an agarose (1.5% (w/v) in distilled water, DNA-grade, BDH, England) medium, to a final concentration of 0.3% (w/v). After the agarose had solidified in a petri dish, 20 µL aliquots of bacterial culture (optical density was adjusted to 1.2-1.8 at 550 nm) of *L. reuteri* DPC16, the other probiotic bacteria, pathogenic bacteria and the faecal flora culture, were inoculated onto the surface of the agarose plates. After drying for 10 min at room temperature, the plates were incubated anaerobically at 37 °C for 72 h. After incubation, the plates were stained with standard Coomassie Blue R-250 staining solution [containing per L, 2.5 g of Coomassie Blue R-250; 450 mL of analytical grade ethanol; 450 mL of distilled water; and 100 mL of glacial acetic acid (100%, BDH; UK)] for 45 min, and subsequently washed with 1.2 M acetic acid until the discoloured zone within and around the colony of bacteria appeared. According to the authors (Zhou *et al.* 2001; Abe *et al.* 2010), mucin degradation activity is indicated as the appearance of a mucin lysis zone. The present mucin degradation assay was extended to score the degree of the colour loss (grade-1: very weak colourless, grade-2: mild colourless, grade-3: partial colourless, grade-4: completely colourless) and the size of the lysis zone (grade-1: diameter of zone <10mm, grade-2: diameter of zone between 10mm-12mm, grade-3: diameter of zone between 12mm-14mm, grade-4: diameter of zone >14mm). The sum of grades from these parameters indicates the mucinolytic activity of each bacterial strain tested.

2.2.12 Statistical analysis

Data were processed on Excel spreadsheets. The counts of viable cells in GI tolerance assays and adhesion assays were transformed to log values. The concentration responses obtained from the turbidity and adhesion experiments, were analysed based on a "Sigmoidal concentration-response (variable slope)" model (identical to the four-parameter logistic equation or Hill equation), shown in **Fig. 2.4**.

Model: Y=Bottom + (Top-Bottom)/(1+10^(($LogEC_{50}$-X)*HillSlope))

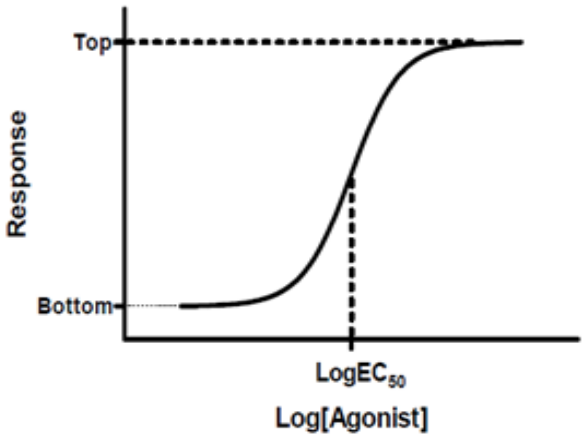


Figure 2.4 A sigmoidal concentration-response (variable slope, four-parameter logistic equation) model

X is the logarithm of agonist concentration and Y is the response. The variable Bottom is the Y value at the bottom plateau; Top is the Y value at the top plateau, and $LogEC_{50}$ is the X value when the response is halfway between Bottom and Top. The steepness of the curve is quantified by the Hill slope, also called a slope factor. When the curve goes downhill, usually called inhibition response, $LogIC_{50}$ is used instead of $LogEC_{50}$.

The standard sigmoidal concentration-response curves were plotted using the *logarithm* of the concentration, rather than the concentration itself, in order to avoid the hyperbolic shape when there was no surety that the absolute value of HillSlope was greater than 1.0 or not. Also, the advantage of this expression is that the response values are equally spaced on a log scale, so that the resulting Log EC_{50} distribution is much closer to a Gaussian distribution.

For the bacterial growth time course study, the data were collected and fitted to a growth kinetics sigmoidal model (Gompertz equation): Y=OD+C*exp(-exp((2.718*μ/C)*(Lag-X)+1)) [OD, initial optical density; C, difference between initial and final OD values; Lag, delay before growth; μ, maximum specific growth rate; X is time, Y is OD value](Zwietering *et al.* 1990).

Results were expressed as means ± SD. A D'Agostino & Pearson omnibus normality test, or graph plotting for normality of residuals, was performed, and the significance of differences was determined using global curve fitting analysis or ANOVA and post-hoc analysis using Prism 4.03 software (GraphPad Software, Inc). Differences were considered statistically significant at *P value* <0.05. Graphs were made using Prism 4.03 and Microsoft Excel software.

2.3 Results

2.3.1 *L. reuteri* DPC16 genotype confirmation

To confirm the identity of the DPC16 strain, the partial 16S rRNA gene sequence (approximately 1500 bp) was obtained and analysed. Following the steps of DNA extraction, PCR and purification, the purified PCR product from DPC16 strain was visualised on an agarose gel as a single band of about 1500 bp (**Fig. 2.5**). The concentration of the purified PCR product was 204 ng/mL and the purity was 1.89 (A260/A280) as determined using a spectrophotometer (NanoDrop ND-1000 Spectrophotometer, USA).

The 16S rRNA gene sequence data were compared to *L. reuteri* DPC16 sequence presented in the patent documents (New Zealand Patent 526544) using the Basic Local Alignment Search Tool (BLAST) programme. The identification of the DPC16 strain was confirmed by 100% match with the original culture (see sequences and alignment in ***Appendix III***).

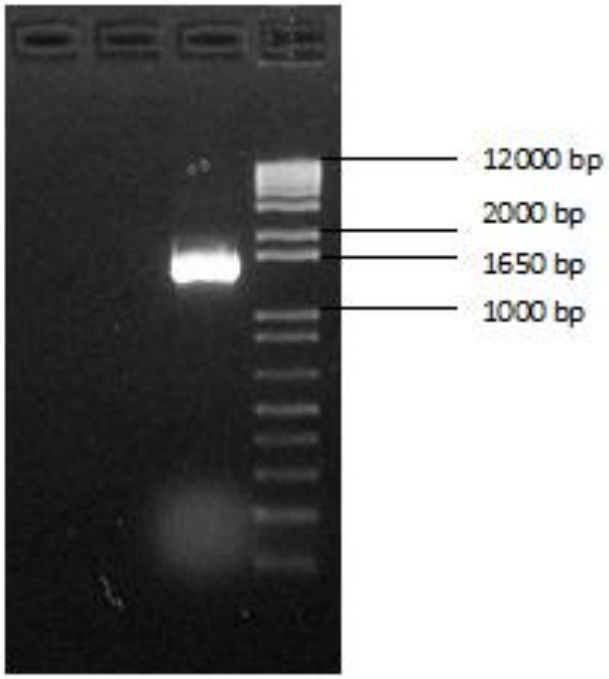


Figure 2.5 Agarose gel electrophoresis of 16S rRNA gene PCR product from *L. reuteri*

The PCR product size is compared with selected bands from the low DNA mass ladder, and is approximately 1500 bp.

2.3.2 Antibacterial activities of *L. reuteri* DPC16 against pathogens

The most important characteristic of the *L. reuteri* species is its ability to utilise glycerol, in the presence of glucose, and convert it to 3-hydroxypropionaldehyde (3-HPA, termed "reuterin") (Luthi-Peng *et al.* 2002), though not all *L. reuteri* can do this (Cadieux *et al.* 2008). The broad spectrum antimicrobial activity of *L. reuteri* against Gram-positive and Gram-negative bacteria, as well as yeasts, fungi, and protozoa, has been observed by a number of investigators (Talarico & Dobrogosz 1989; Liang *et al.* 2003).

A modified agar diffusion assay was carried out in this study to test the antibacterial activities of *L. reuteri* DPC16 against the indicator pathogen *E. coli* O157:H7. Usually, an agar diffusion assay is designed to test the antibacterial activity of substances e.g. antibiotics or bacterial supernatants, against one indicator pathogen. In the present work, however, this assay was used to examine if one substrate in the growth medium is able to allow live *L. reuteri* DPC16 cells to produce different end metabolic products with different antibacterial effects on the indicator pathogen. This was done to investigate the activities of *L. reuteri* DPC16 metabolic end products and reveal the possible metabolic pathways of this strain. A basal medium agar, containing glucose [2% (w/v)] was used to allow the growth of both *E. coli* O157:H7 and *L. reuteri* DPC16. This medium could be further supplemented with 250 mM glycerol.

The data for the inhibitory zone measurements were analyzed by performing a One-way ANOVA and Tukey's Multiple Comparison Test after their residuals appeared to be close to normal distribution. As shown in (**Fig. 2.6**), live *L. reuteri* DPC16 in the glycerol-negative medium produced small inhibitory zones, but the statistical analysis showed that the inhibitory effect was not significant compared to that of the negative control (*P value* >0.05). In contrast, the inhibitory zones and the statistical analysis both demonstrated that the inhibition was significantly more pronounced in the presence than in the absence of glycerol (*P value* <0.01). This preliminary result indicated that *L. reuteri* DPC16 may be able to utilise glycerol to form some antibacterial molecules such as SCFA and "reuterin" (Talarico *et al.* 1988; Lu 2007). Unfortunately, an appropriate positive control (another *L. reuteri* strain known to conduct similar pathways) could not be found to include in the experiment (the normal antibiotic paper disc was not considered as a suitable positive control in this case). Thus, some further experiments are needed to confirm this finding and to compare the different activities of these

metabolic substances.

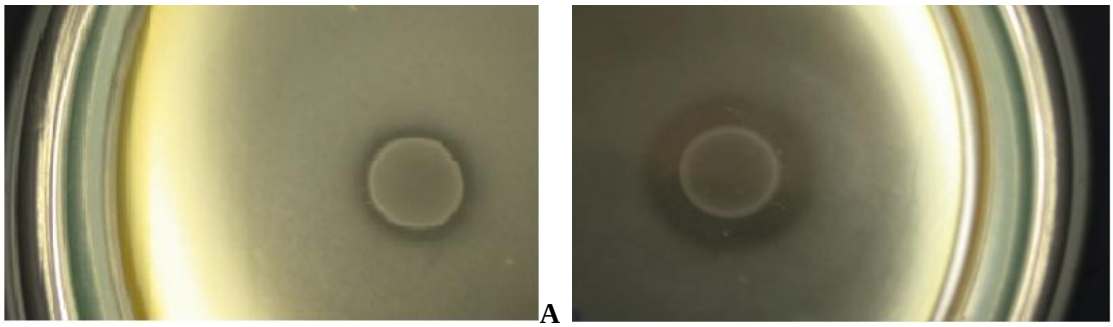


Figure 2.6 Antibacterial activities of *L. reuteri* DPC16 against growth of the indicator pathogen *E.coli* O157:H7 in an agar diffusion assay

The images show the diameters of inhibition zones on individual basal agar plates in the absence [A. 9.4±1.5 (mm)] and presence of 250 mM glycerol [B. 17.2±1.9 (mm)] after *L. reuteri* DPC16 was spotted and plates were incubated at 37 ^{o}C for 24 h. The heat-killed *L. reuteri* DPC16 used as a negative control showed no inhibition zone around the spot (image is not shown). The statistical analysis is shown in *Appendix II*.

Subsequently, a high-throughput spectrophotometric turbidity bioassay was performed to further determine the antibacterial activities of different *L. reuteri* DPC16 culture supernatants produced during growth in MRS medium alone (DS), MRS medium containing 250 mM glycerol (DSg) or during 3 h incubation in 250 mM glycerol in PBS buffer (Dg), against Gram-positive and Gram-negative pathogens, i.e. *Listeria monocytogenes, Staphylococcus aureus, Salmonella typhimurium and Escherichia coli* O157:H7, in liquid medium for 24 h incubation. Based on the optimised conditions obtained from preliminary turbidity experiments, the final experimental data were fitted to a variable slope sigmoidal model: Y=Bottom + (Top-Bottom)/(1+10^((LogEC_{50}-X)*HillSlope)). This experiment aimed to obtain the MIC_{50} (the minimum dilution value that gave 50% inhibition of growth of each pathogen) for each supernatant. However, the data points at the lower concentrations of some *L. reuteri* DPC16 supernatants appeared greater than that of the negative control (100%) indicating the presence of stimulatory effects in these cell-free supernatants that appeared after the inhibitory effects were diluted out. In addition, the growth was not completely inhibited by the highest concentration of some supernatants. Therefore, in this model, the values of top and bottom plateaux should be considered sharing the same top and bottom values for all data sets but not be constrained to "100" and "0". A relative IC_{50} (the value that gave **"**half way" inhibition of growth of each strain between the top and bottom plateaux of the curve, also called EC_{50} in above model) rather than the absolute IC_{50} (the

value that gave 50% inhibition of growth of each pathogen between 0% and 100%) was defined and was considered suitable to assess the inhibitory effects of a mixture of substances (containing not only inhibitory but also stimulatory factors). The curves as shown in **Fig. 2.7,** indicated that the supernatants (DS: red, DSg: green and Dg: purple) inhibited the growth of all tested pathogens in a concentration-dependent manner. A global fitting (the residuals of all data sets were fully normally distributed, including the single data set of Dg against *Staphylococcus aureus* by excluding one value from the data set) was performed to analyse whether the family concentration-response curves shared the same parameters (LogIC50 and Hillslope) between all the data sets (with a null hypothesis of one curve for all data sets, or an alternative hypothesis of a different curve for each data set). In all cases, the conclusion of the analysis was a different curve for each data set by rejecting the null hypothesis (*P value* <0.0001).

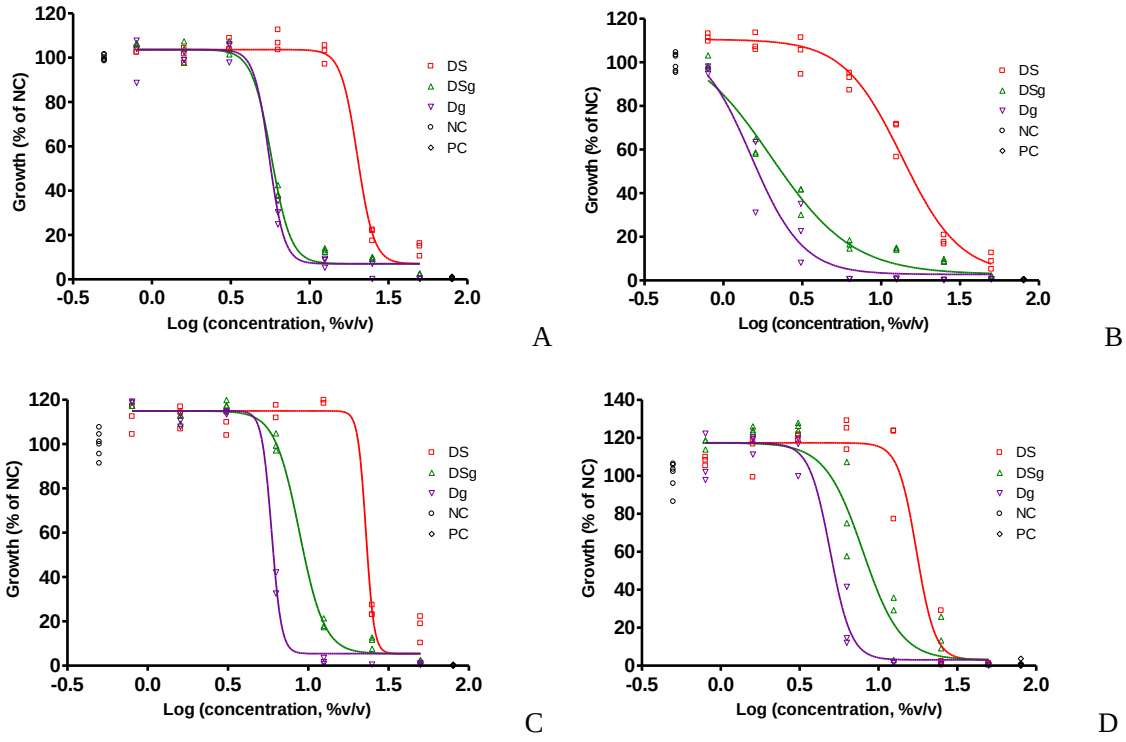


Figure 2.7 Antibacterial concentration responses of three *L. reuteri* DPC16 culture supernatants

The effects of DS, DSg and Dg against pathogens *Listeria monocytogenes* (A), *Staphylococcus aureus* (B), *Salmonella typhimurium* (C) and *Escherichia coli* O157:H7 (D) were determined in triplicate in the experiment by using the spectrophotometric turbidity assay in 96-well plates after incubation at 37 ^{o}C for 24 h. [X: Log (concentration) Y: response (% of negative control). Both negative control (medium only) and positive control (antibiotics: 100 µg/mL of streptomycin and 100 U/ml of penicillin) were included in the experiment. The statistical analysis is shown in *Appendix II*.]

The resulting individual relative MIC_{50} values for each supernatant are listed in

Table 2.2. The pH values of the culture supernatants were 4.3, 4.3 and 4.8 for DS, DSg and Dg, respectively.

Table 2.2 Relative MIC50 of different *L. reuteri* DPC16 supernatants (DS, DSg and Dg) against pathogens

Pathogens	DS (% v/v)	DSg (% v/v)	Dg (% v/v)
Listeria monocytogenes	20.1	5.7	5.5
Staphylococcus aureus	13.5	2.0	1.5
Salmonella typhimurium	22.9	8.8	5.9
Escherichia coli	17.4	8.0	4.9

Using *E. coli* O157:H7 as an example, some further experiments were performed in which the pH values of the supernatants DS and DSg were adjusted to 7.0 prior to the assay. The experimental data were fitted to the "sigmoidal concentration-response model (variable slope)" by sharing the same top plateau values only or by sharing both the same top and bottom plateau values for the data sets. The results shown in **Fig. 2.8**, indicate that the antibacterial activities of DS and DSg were reduced upon pH-neutralization, particularly those of DS (none of the pH-adjusted concentrations inhibited the growth of *E. coli)*, but the activity was retained in DSg. The relative MIC_{50} of pH-neutralized DSg was greater than that of initial DSg, the value rising from 8.0 (% v/v) to 13.0 (% v/v). However, these results indicate that the acidic components in the supernatants were unlikely to be the only cause of the antibacterial activity.

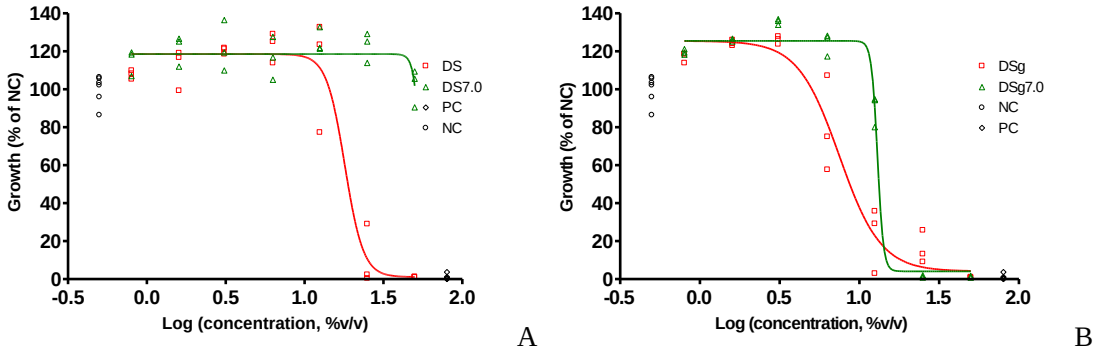


Figure 2.8 Comparison of the antibacterial effects of original and pH-neutralized *L. reuteri* DPC16 supernatants

The effects of DS and pH-adjusted DS (A) and DSg and pH-adjusted DSg (B) against *E. coli* O157:H7 were determined in triplicate using the spectrophotometric turbidity assay in 96-well plates after incubation at 37 ^{o}C for 24 h. [X: Log (concentration) Y: response (% of negative control). Both negative control (medium only) and positive control (antibiotics: 100 µg/mL of streptomycin and 100 U/ml of penicillin) were included in the experiment. The statistical analysis is shown in

Appendix II.]

To ensure that glycerol itself was not responsible for the inhibitory activity, the 250 mM glycerol solution in PBS was tested without inoculation with strain DPC16. The results, analysed using the same principle as above, confirmed that it is a metabolic product (Hillslope =-4.8) rather than glycerol itself (Hillslope =15.6) that is responsible for the antibacterial activity (**Fig. 2.9**).

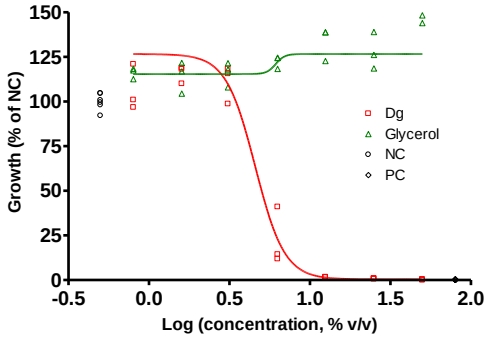


Figure 2.9 Comparison of the inhibitory effects of glycerol and Dg

The effects of 250 mM glycerol solution and Dg against *E. coli* O157:H7 were determined in triplicate using the spectrophotometric turbidity assay in 96-well plates after incubation at 37 ^{o}C for 24 h. [X: Log (concentration) Y: response (% of negative control). Both negative control (medium only) and positive control (antibiotics: 100 µg/mL of streptomycin and 100 U/ml of penicillin) were included in the experiment. The statistical analysis is shown in *Appendix II.*]

2.3.3 Identification of the antibacterial substances in *L. reuteri* DPC16 fermentation products

In the above experiments, all of the *L. reuteri* DPC16 supernatants, DS, DSg and Dg, demonstrated inhibitory effects against both Gram-positive and Gram-negative pathogens. Of these supernatants, DSg and Dg showed much stronger activities against these pathogens than did DS. Therefore, the different supernatants were analysed to attempt to identify the metabolites responsible for the antibacterial activities. Based on the fermentation pathways of *L. reuteri* species illustrated in **Fig. 1.10**, the possible major fermentation products are short chain fatty acids and reuterin (3-HPA). Therefore, *L. reuteri* DPC16 supernatants, DSg and DS, were selected for analysis in order to compare the values of the proposed metabolites produced from MRS medium. Supernatant Dg was not analysed for the short chain fatty acids as it was produced during incubation in PBS buffer rather than in a growth medium, but the concentration of reuterin (3-HPA) in Dg was determined.

As shown in **Table 2.3,** the pH values of the MRS cultures dropped to 4.3 after 24 h incubation, in both the presence and absence of glycerol. The major short chain fatty acids produced were lactic and acetic, with considerably more of the latter being produced in the presence of glycerol; however, no further analysis was conducted to support this conclusion.

Table 2.3 pH and SCFA analysis of MRS growth medium and *L. reuteri* DPC16 culture supernatants (DS and DSg)

	MRS broth	DS	DSg
pH	6.5-6.7	4.3	4.3
Acetate (mg/L)	1077	1589	5265
Propionate (mg/L)	0	0	66
Isobutyrate (mg/L)	547	0	0
Butyrate (mg/L)	0	0	0
Isovalerate (mg/L)	0	0	0
Lactate (mg/L)	980	7520	7160

The concentrations of reuterin in the culture supernatants DSg, Dg and DS were determined and calculated according to the standard curve of acrolein (2-propenal) (**Fig. 2.10)**.

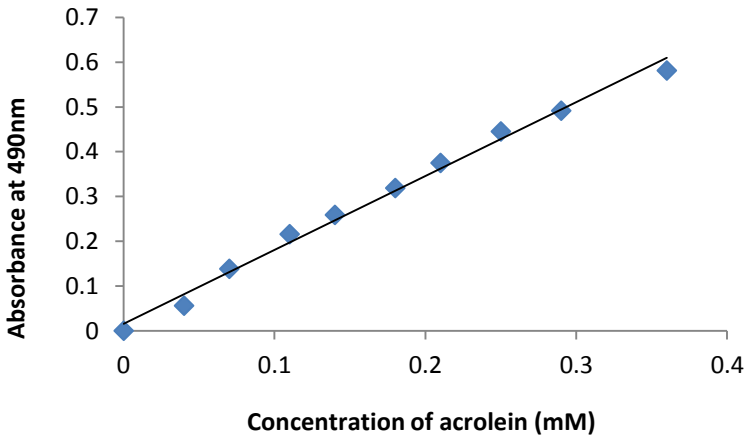


Figure 2.10 An acrolein standard curve for determination of reuterin using the colorimetric method (Circle *et al.* 1945)

Reuterin concentrations were 0.02 mM and 0.14 mM when *L. reuteri* DPC16 was grown for 24 h in MRS broth and in MRS broth containing 250 mM glycerol, respectively. In contrast, the concentration of reuterin in Dg (stationary phase cells incubated for 3 h in 250 mM glycerol in PBS, pH 7.0) was 15.91 mM.

Accordingly, these experiments confirmed that the strain DPC16 belongs to the reuterin-producing *L. reuteri* species with strong antibacterial activity against pathogens. The major antibacterial molecules appear to be SCFA and reuterin. The former were produced by *L. reuteri* DPC16 in both the presence and absence of glycerol in MRS medium; however, the highest concentration of reuterin was produced by harvested stationary phase cells when glycerol was provided in a buffer solution.

2.3.4 The effect of DSg on the growth kinetics of some pathogens and probiotics

In the large intestine, both glucose and glycerol can be derived from carbohydrate and lipid sources, thus DSg can be considered as an artificial *L. reuteri* DPC16 fermentation complex produced under colonic conditions. Based on this premise, 7.5 (% v/v) of DSg (above the average relative MIC_{50} of DSg for all tested pathogens) was used in a time course study to observe the effects of DSg on the growth of some pathogens and probiotics. A bacterial growth kinetics model was fitted and the family curves fitting analysis was performed (the initial OD values were constrained to the same values), with the null hypothesis that there was no difference between the two curves, i.e. they share the same parameters (differences of initial and final OD values, delay before growth and maximum growth rate). As shown in **Fig. 2.11**, DSg, at a concentration of 7.5% in BHI medium, inhibited the growth of pathogens *Listeria monocytogenes*, *Salmonella typhimurium* and *Escherichia coli* O157:H7 during 24 h incubation at 37 °C. In each case, the null hypothesis was rejected (*P value* <0.0001), there was extension of the lag phase, and a reduction in the final concentration of cells, but the exponential growth rate was less affected. It was noted that the data sets of DSg against *Staphylococcus aureus* showed less confidence on normality residuals compared to the others; however, the inhibitory trends were similar to those for the other pathogens. Additionally there was a huge extension of the lag phase of *Staphylococcus aureus*.

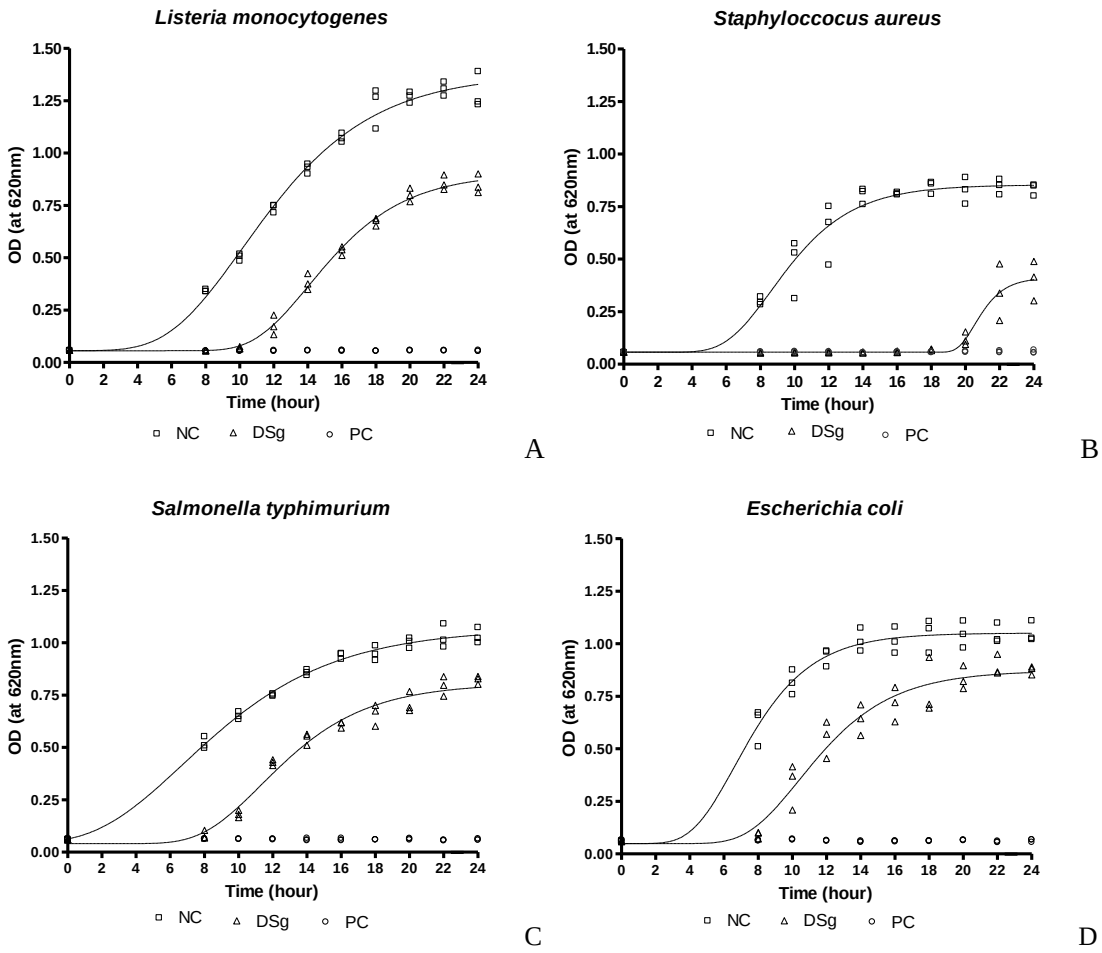


Figure 2.11 A time course study of *L. reuteri* DPC16 supernatant (DSg) effects on the growth of pathogens

The effects of *L. reuteri* DPC16 supernatant DSg, at a concentration of 7.5% in BHI medium, on the growth of *Listeria monocytogenes* (A), *Staphylococcus aureus* (B), *Salmonella typhimurium* (C) and *Escherichia coli* O157:H7 (D) were determined in triplicate by using the spectrophotometric turbidity assay in 96-well plates during 24 h incubation at 37 ^{o}C. [X: Incubation time Y: Optical Density measured at 620nm. Both negative control (medium only) and positive control (antibiotics: 100 µg/mL of streptomycin and 100 U/ml of penicillin) were included in the experiment. The statistical analysis is shown in *Appendix II.*]

In contrast, there was much less inhibitory effect of DSg on the growth of the probiotics based on these parameters (lag phase, final growth and the exponential growth rate) under similar experimental conditions (**Fig. 2.12**), although in all cases the null hypothesis that there was no difference between the curves was rejected (*P value* <0.005). Nevertheless, the lengths of the lag phase and the final growth levels were similar for the DSg treatments and the control on these probiotics. Hence the inhibitory effects of *L. reuteri* DPC16 supernatant DSg on different groups of bacteria can vary considerably.

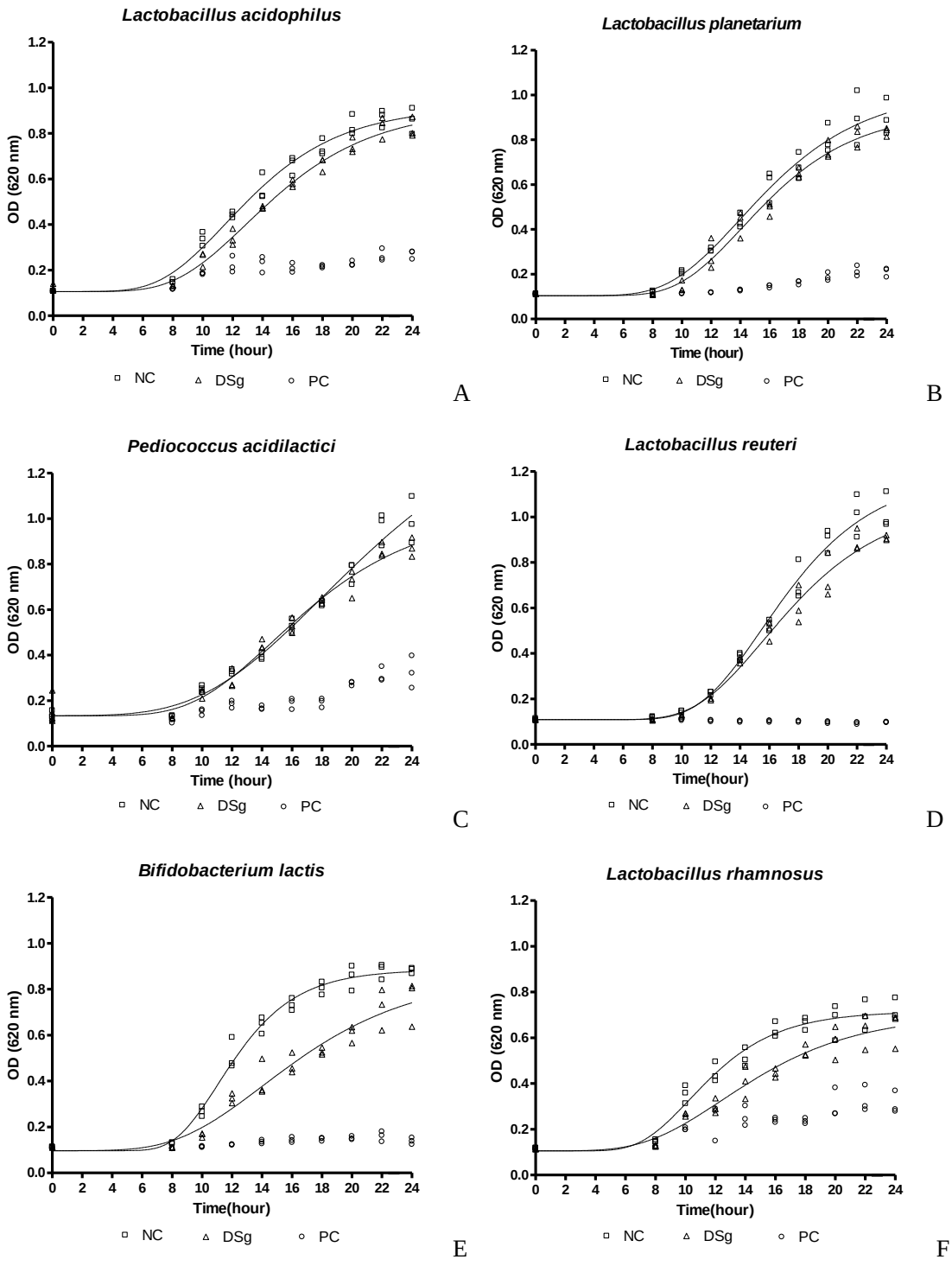


Figure 2.12 A time course study of *L. reuteri* DPC16 supernatant (DSg) effects on the growth of probiotics

The effects of *L. reuteri* DPC16 supernatant DSg, at a concentration of 7.5% in MRS medium, on the growth of *L. acidophilus* DPC201 (A), *L. plantarum* DPC206 (B), *P. acidilactici* DPC209 (C), *L. reuteri* DPC16 (D), *B. lactis* DR10 (E), and *L. rhamnosus* DR20 (F) were determined in triplicate by using the spectrophotometric turbidity assay in 96-well plates during 24 h incubation at 37^{o}C under anaerobic conditions. [X: Incubation time Y: Optical Density measured at 620 nm. Both negative control (medium only) and positive control (antibiotics: 100 µg/mL of streptomycin and 100 U/ml of penicillin) controls were included in the experiment. The statistical analysis is shown in *Appendix II.*]

2.3.5 Tolerance of *L. reuteri* DPC16 to pH and bile salts under nutrient-sufficient and nutrient-deficient conditions

The second part of the investigation in this chapter was focused on evaluating the viability of *L. reuteri* DPC16 in a simulated GI environment. Firstly the survival of *L. reuteri* DPC16 in a low pH and high bile salts environment was assessed in the presence of sufficient nutrients. The tolerance to such GI conditions was compared among *L. reuteri* DPC16 and other probiotic strains *L. acidophilus* DPC201, *L. plantarum* DPC206, *P. acidilactici* DPC209, *B. lactis* HN019 and *L. rhamnosus* HN001. The residuals of data collected from each treatment among these strains were plotted and visualized to be reasonably normally distributed. A Two-way ANOVA and Bonferroni post tests were performed. As shown in **Fig. 2.13,** *L. reuteri* DPC16 demonstrated the same responses as the other probiotics, i.e. it survived incubation at pH 2 for a period of 90 min. The tolerance to intestinal bile salts during subsequent incubation at pH 7 was reduced as the bile salt concentration increased, with a significant viability reduction (*P value* <0.001) in the 1% bile salts treatment under the nutrient-sufficient conditions.

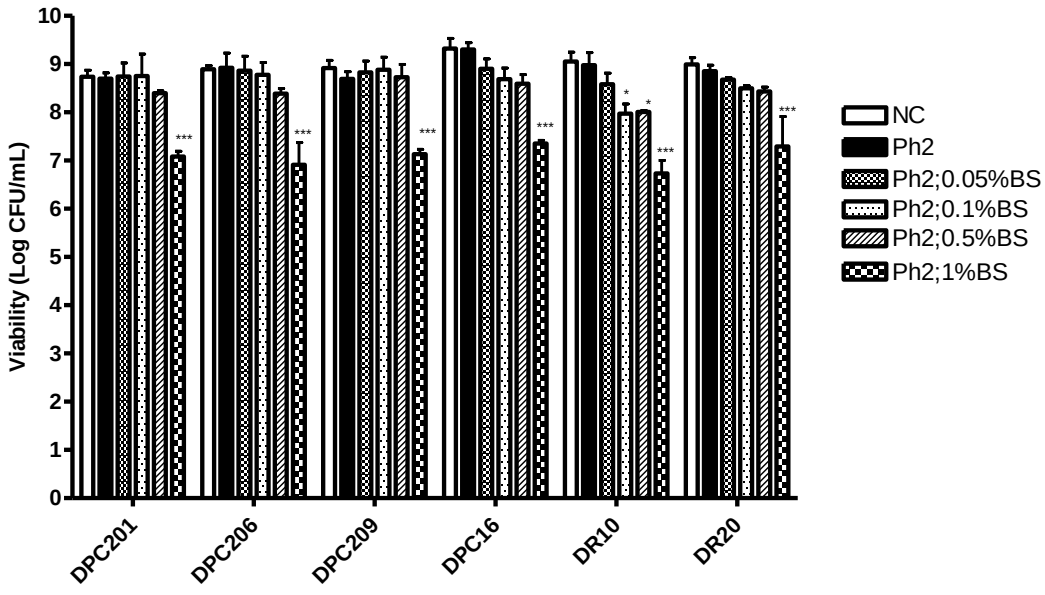


Figure 2.13 Survival of *L. reuteri* DPC16 and other probiotics challenged with low pH and high bile salts under conditions of nutrient sufficiency

The viabilities of probiotic strains *L. reuteri* DPC16, *L. acidophilus* DPC201, *L. plantarum* DPC206, *P. acidilactici* DPC209, *B. lactis* HN019 (DR10) and *L. rhamnosus* HN001 (DR20) were examined in triplicate using the 4x4 drop plate count method after these strains were exposed to pH 2 for 90 min, followed by 60 min in the presence of bile salts at pH 7. [X: strain Y: viability (Log CFU/mL) of tested probiotics. The negative controls were these probiotics without low pH and high bile salts challenges. The error bars are SD, while "***" and "*" indicate that the viability of the strain under the treatment condition was significantly different (*P value* <0.001and <0.05, respectively) to that of the control. The statistical analysis is shown in *Appendix II*.]

In general, there was no statistical difference between the strains in their response to the various treatments except that strain *B. lactis* HN019 (DR10) showed less resistance than the other strains to the subsequent 0.1% and 0.5% bile salts treatments (*P value* <0.05).

Following these results, some preliminary experiments performed under nutrient-deficient conditions indicated that the probiotic bacteria tested could not survive at pH 2 coupled with a subsequent bile salts challenge (results not shown). Therefore, experiments were conducted to test the resistance of these probiotic bacteria to either acidic or bile salts challenges in nutrient-deficient conditions. **Fig. 2.14** shows the results for pH 2 and 0.05% w/v bile salt conditions. Although the data set size for each strain and treatment condition was too small to do a formal normality test, a plot of residuals appeared relatively normal. Two-way ANOVA and Bonferroni post tests were performed and the results showed that under nutrient-deficient conditions, there were significant effects of the strain, the treatment and interactions between them (*P value* <0.0001).

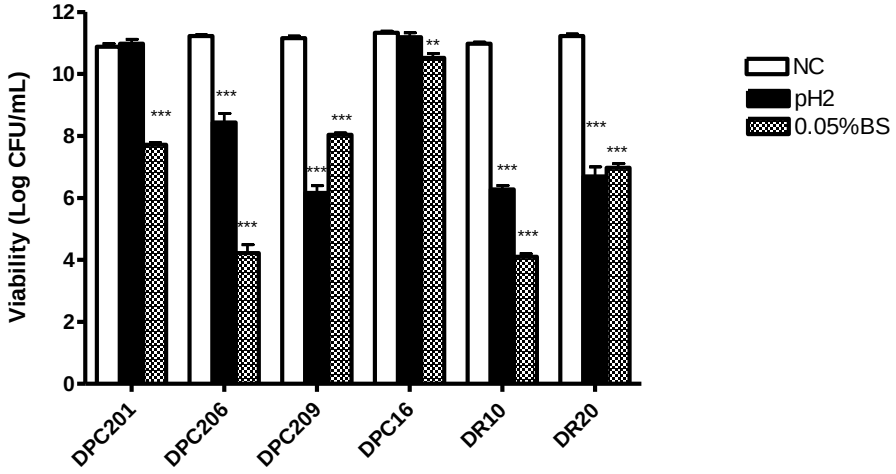


Figure 2.14 Survival of probiotics challenged with low pH or high bile salts under conditions of nutrient deficiency

The effect of low pH or bile salts on the survival of probiotic strains *L. acidophilus* DPC201, *L. plantarum* DPC206, *P. acidilactici* DPC209, *L. reuteri* DPC16, *B. lactis* HN019 (DR10) and *L. rhamnosus* HN001 (DR20) was tested in triplicate using the 4x4 drop plate count method after these strains were incubated either at 37°C for 90 min at pH 2 or at 37^{o}C for 60 min at pH 7 in the presence of 0.05% bile salts under nutrient-deficient conditions [X: probiotic strains Y: viable cells (Log CFU/mL) of probiotics. The error bars are SD, while "***" and "**" indicate that the viability of the strain under the treatment condition was significantly different (*P value* <0.001 and <0.01, respectively) to that of the control. The statistical analysis is shown in *Appendix II.*]

Among the strains, two, *L. acidophilus* DPC201 and *L. reuteri* DPC16, were resistant to the pH 2 condition (*P value* >0.05); the remainder were much less resistant (*P value* <0.001). The viability of all strains was significantly reduced (*P value* <0.01) under bile salts treatment at a concentration of 0.05% (v/v) in these nutrient-deficient conditions.

2.3.6 Adhesion of *L. reuteri* DPC16 to an intestinal epithelial Caco-2 monolayer

A Caco-2 monolayer was prepared as a model of the intestinal epithelium, and the adhesion of *L. reuteri* DPC16 to this monolayer was assessed as described in **Section 2.2.9.** The data were analysed by fitting a "sigmoidal dose-response (variable slope)" model due to the possible saturation of adhesion sites when the bacteria were over loaded and the non- regression curve fitting analysis showed that the curve plotted as uphill and the Hillslope was great than 1.0. As the results demonstrated, in **Fig. 2.15,** the number of *L. reuteri* DPC16 bacteria adhering to the Caco-2 monolayer depended on the number of bacteria added to the monolayer in the experimental conditions, with a positive correlation.

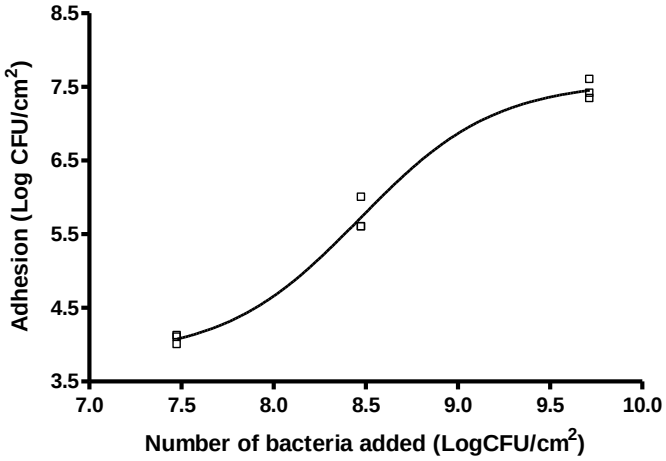


Figure 2.15 Correlation of added *L. reuteri* DPC16 numbers and those adhered to the Caco-2 monolayer

The numbers of adhered *L. reuteri* DPC16 were determined in triplicate using the 4x4 drop plate count method after incubation, washing and trypsinising together with Caco-2 epithelial cells. [X: bacteria numbers added (Log CFU/cm^2) Y: bacterial numbers adhered (Log CFU/cm^2). The statistical analysis is shown in *Appendix II.*]

Further experiments and statistical analysis were performed to compare the adhesion ability of different probiotic strains. The data were processed with a normality of residuals estimation and One-way ANOVA. The results (**Fig. 2.16**) revealed that the

other probiotic bacteria did not demonstrate a significantly different adhesion level to *L. reuteri* DPC16 (*P value* >0.05).

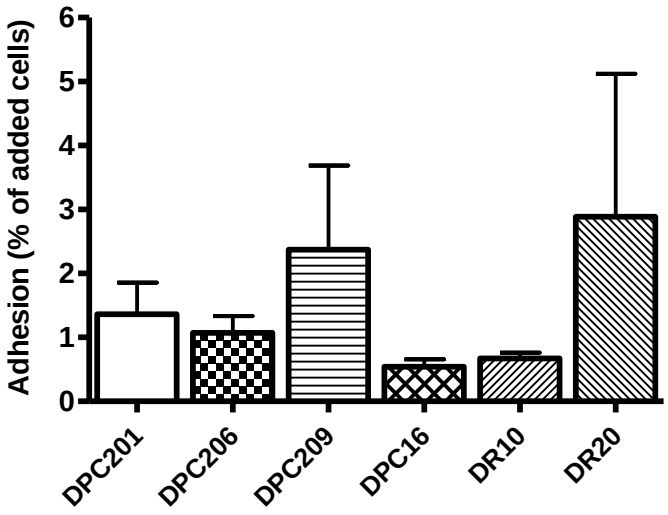


Figure 2.16 The epithelial adhesion of *L. reuteri* DPC16 and other probiotic strains

The adhered *L. reuteri* DPC16, *L. acidophilus* DPC201, *L. plantarum* DPC206, *P. acidilactici* DPC209, *B. lactis* HN019 (DR10) and *L. rhamnosus HN001* (DR20) were plate-counted in triplicate after incubation, washing and trypsinising together with Caco-2 epithelial cells. [X: strains Y: adhesion (% of added cells). The statistical analysis is shown in *Appendix II*.]

2.3.7 Mucin degradation assessment of *L. reuteri* DPC16 and other bacteria

Probiotic bacteria are generally recognised by their low mucin degradation ability. This assessment is critical to *L. reuteri* DPC16 "safety of use" in human consumption preparations. In a mucinolytic assay, as shown in **Fig. 2.17**, according to the scores (as described in **Section 2.2.10.3)** obtained from the degree of colour loss and the size of the lysis zone present on the agarose plates (the data are shown in ***Appendix II***), *L. reuteri* DPC16 and most of the probiotic strains [including two control strains, *Bifidobacterium lactis* HN019 (DR10) and *L. rhamnosus* HN001(DR20), which showed no degradation of gastric mucin in a previous study (Zhou *et al.* 2001)] showed extremely low degradation of mucin (activity grades were 2-3). In contrast, the four pathogens tested, *L. monocytogenes* Scott-A ATCC 49594, *S. aureus* ATCC 25932, *S. enterica* serovar Typhimurium ATCC 1772 and *E. coli* O157:H7 strain 2988, all showed mucin degradation capabilities (activity grades were 6-8). A faecal flora sample was included as a positive control, according to other studies (Zhou *et al.* 2001; Abe *et al.* 2010); however, a *pooled* faecal flora sample should have been used in the assay due to human inter-individual variation. Additionally, the inability to control the concentration

of mucin-degrading bacterial cells in the faecal flora sample made analysis of the size of the lysis zone too difficult to assign a grade.

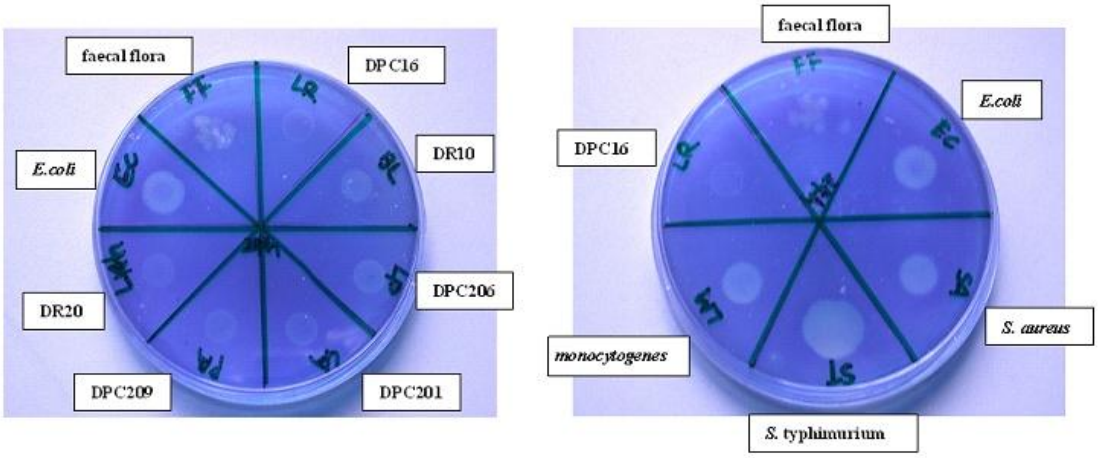


Figure 2.17 Agarose plate mucinolytic assay

The assay was conducted in triplicate in mucin-containing agarose petri dishes. Two non-mucin degrading probiotic strains *Bifidobacterium lactis* HN019 (DR10) and *L. rhamnosus* HN001 (DR20) and one faecal flora (FF) sample were included in the experiments as negative controls and a positive control, respectively. Four pathogenic strains produced extended lysis zones on the Coomassie blue-stained agarose gel; in contrast, *L. reuteri* DPC16 and the most of probiotic strains showed no extended decoloured zones on the Coomassie blue-stained agarose gel after 37 ℃ for 72 h anaerobic incubation.

2.4 Discussion

In this chapter, the bacterial strain identification technique of 16S rRNA extraction, PCR and subsequent sequencing was performed to confirm the DPC16 strain identity. The DPC16 strain was confirmed as belonging to the *L. reuteri* species that had been used in a previous study (Lu 2007). The investigation of antibacterial activities of *L. reuteri* DPC16 was designed to test three different types of *L. reuteri* DPC16 culture supernatants using *in vitro* assessment techniques. Using curve fitting to determine the minimum inhibitory concentration required to achieve a half reduction in growth (relative MIC_{50}) between the top and bottom plateaux of selected Gram-positive and Gram-negative pathogens, the antibacterial activities of these supernatants in this experiment were shown to differ from each other, with greater activity observed in those supernatants that had been obtained from cultures containing glycerol.

The strain identification and the preliminary antibacterial results provided evidence that

the DPC16 strain belongs to the *L. reuteri* species, with antibacterial activities, derived from glycerol fermentation, against the pathogenic bacteria *Listeria monocytogenes* Scott-A ATCC49594, *Staphylococcus aureus* ATCC 25932, *Salmonella enterica serovar* Typhimurium ATCC 1772 and *Escherichia coli* O157:H7 strain 2988. According to the metabolic pathways of *L. reuteri* species shown in **Fig. 1.10**, and the reported presence of the glycerol dehydratase gene in strain DPC16 (Lu 2007), this strain appears able to produce reuterin from glycerol. The chemical analyses in the present study elucidated that SCFA and reuterin in DPC16 spent culture media are probably responsible for the antibacterial activities of this strain. The components of the short-chain fatty acids in two supernatants (DS and DSg) were analysed by HPLC, and the results in **Table 2.3** showed that in the presence of glycerol, there was an increase in acetate production, with little effect on lactate. This result agrees with a previous study, which showed that when glycerol was included in the culture medium of *L. reuteri*, lactate production remained constant but a significant increase in acetate production and a decrease in ethanol production were observed (Talarico *et al.* 1990). However, replicated experimental data are needed to analyse for significant differences among the supernatants in their relative MIC_{50} values against pathogens, and the components within the supernatants.

In a recent study on *L. reuteri* DPC16, freeze-dried cells incubated in a glycerol-water system showed no reuterin production, and the author concluded that this strain may not be capable of producing reuterin without the presence of fermentable carbohydrate (Bian 2008). However, in the present work it was observed that strain DPC16, in a secondary fermentation buffer (PBS) system, was able to produce reuterin (15.91 mM) when incubated for 3 h in the presence of 250 mM glycerol. Similar findings of glycerol conversion to reuterin by strains of *L. reuteri* during a secondary fermentation have been reported by other investigators (Cadieux *et al.* 2008; Spinler *et al.* 2008). Talarico *et al* (1988) noted that reuterin production is not affected by the presence of glucose when glycerol concentrations are above 100 mM.

The primary antimicrobial data showed that the culture supernatant (DS) harvested from a non-glycerol-containing fermentation had significant concentration-dependent inhibitory effects against the target pathogens, but that these effects were reduced in their pH-neutralized counterparts. This indicates that the major antimicrobial activities

in DS were most likely due to its acidic components. The short chain fatty acids (SCFAs), such as lactic acid, acetic acid, formic acid and succinic acid, produced by probiotic lactobacilli, have been reported to be responsible for inhibiting pathogen invasion by not only affecting their growth activities (De Keersmaecker *et al.* 2006) but also their adhesion (Lehto & Salminen 1997). De Keersmaecker *et al.* (2006) concluded that lactic acid produced by probiotic *Lactobacillus rhamnosus* GG exerts the key antibacterial activity against *Salmonella typhimurium*. The affect of SCFA on the growth of pathogens, and its mechanism, was suggested in early studies (Booth 1985). The undissociated SCFAs, in a low pH environment, are able to penetrate the bacterial membrane and subsequently dissociate at the higher intracellular pH values. The released free hydrogen ions generate a large transmembrane proton gradient which interferes with essential metabolic functions of the affected bacteria.

The supernatants DSg and Dg, obtained from a glycerol-containing fermentation and from a secondary fermentation in a glycerol-buffer solution, respectively, had much stronger inhibitory effects than did DS, and although the antibacterial effects of DSg were also reduced by pH-neutralization, considerable activity remained at this higher pH value. These results support the concept that the antibacterial activities of DSg and Dg were due to not only SCFA but also to a metabolite of glycerol, i.e. reuterin, but not to glycerol itself.

A time course investigation has provided further information on the inhibitory effectiveness of DSg on pathogen growth kinetics. An extension of the lag phase was observed, in addition to a reduction in the final concentration of cells produced, but there was less effect on the exponential growth rate. These observations reveal that DSg may exert both bacteriostatic and bactericidal actions, but the activities diminish with increased time of exposure. These experiments were performed in BHI culture medium, at a pH value close to neutrality, so it is likely that reuterin was the major effective antimicrobial metabolite. In contrast, there was much less inhibitory activity against the probiotic strains, even though the decrease in pH value that accompanies growth of these organisms would have been expected to increase such activity due to the presence of the SCFA. Spinler *et al.* (2008) also observed that the growth of specific *Lactobacillus* species, including *L. acidophilus* and *L. gasseri,* was not inhibited by *L. reuteri.* However, the reason for the resistance of the probiotic strains is unclear at this

stage. Little information is available on the mechanism by which reuterin inhibits bacteria, but there is a report that it interferes with DNA synthesis (Dobrogosz & Lindgren 1995).

In the second part of this chapter, experiments were performed to investigate the activity and viability of strain DPC16 in a simulated GI environment. The evaluation of the survival of probiotics in stressful conditions is normally studied using the standard microbiology plate count method (Kim *et al.* 2001; Saarela *et al.* 2004). Recently some other methodologies have been explored. For example, the cell integrity of *L. reuteri* DPC16 in stressful process/storage conditions has been studied using flow cytometry (Chen 2007). Comparing the cost and accuracy of these assays, a high efficiency 4x4 drop plate count method was developed and used in the present study. The results showed that under conditions of nutrient sufficiency *L. reuteri* DPC16 (and other tested probiotics), is able to tolerate pH 2 and physiological concentrations of bile salts (1%) in some degree. In nutrient-deficient conditions all of the probiotic bacteria tested were much less resistant to high bile salts concentrations than in nutrient-sufficient conditions. Clearly, the nutrients confer some protective effect on the cells. This information indicates that *L. reuteri* DPC16 is a candidate strain for a probiotic formula in that a relatively high number of viable cells should survive passage through the stomach and small intestine provided that it is ingested with food. After probiotic bacteria reach the intestine, the next important step is colonization. Some *in vitro* model systems have been suggested to study the ability of putative probiotic strains to adhere to the intestinal epithelium, using cellular lines obtained from human colon adenocarcinomas such as Caco-2 and HT29 (Tuomola & Salminen 1998; Gopal *et al.* 2001; Riedel *et al.* 2006; Tallon *et al.* 2007). Although the HT29-MTX cell line (mucus-secreting subpopulation of HT29 cells) is the ideal cell line (Hao & Lee 2004) for bacterial adhesion study in GI tract, by using an available human intestinal epithelial model cell-line (Caco-2) in this study, the results indicated that *L. reuteri* DPC16 possesses the ability to adhere to the human GI epithelium, as do other probiotic bacteria. This result is supported by the work of Nissen *et al.* (2009). Last but not least, mucin degradation has been considered as a useful indicator for distinguishing commensal from pathogenic bacteria (Ruseler-van Embden *et al.* 1995; Magalhaes *et al.* 2007). As the results showed, *L. reuteri* DPC16 produced very little mucinolytic activity and was similar to the commercial strain *L. rhamnosus* HN001 (DR20), and so it meets the safety criterion

of a probiotic strain on mucin degradation. However, experiments using human mucins should be performed in a future study to support this conclusion. Some *in vitro* studies (Servin & Coconnier 2003) have revealed that probiotic bacteria (Coconnier *et al.* 1993a), in addition to the ability to adhere to epithelial cells, may inhibit pathogen adhesion in a strain and concentration- and/or pH-dependent manner (Coconnier *et al.* 1993b; Lehto & Salminen 1997; Forestier *et al.* 2001; Lee & Puong 2002), and thus prevent pathogen invasion (Lievin *et al.* 2000; Gopal *et al.* 2001). The postulated mechanisms include competition for host-cell binding sites (Reid *et al.* 2001) and effects of pH on adhesion (Lehto & Salminen 1997). This ability was not studied in the present work, but it may also influence the intestinal microflora and the protective functions of the intestinal mucosa (Vesterlund *et al.* 2006).

The genomic information of some probiotic strains has revealed the presence of several molecules able to adhere to different components of the intestinal mucosa and to exchange signals with the intestinal immune system (Salminen *et al.* 2005) indicating an adaptation of probiotics to the gut environment. As the literature has revealed, through many animal and human studies, administration of *L. reuteri* has been reported to be safe and to have no adverse effect on the body. Indeed, these studies have shown beneficial properties on health such as reducing respiratory or gastrointestinal infections (Wolf *et al.* 1998; Tubelius *et al.* 2005). The *in vitro* data reported in the present work provide initial indications of positive efficacy for *L. reuteri* DPC16. However, although the results for both antibacterial activity and the ability to adhere to epithelial cells are positive *in vitro*, it would be dangerous to assume that similar results would be observed *in vivo*. It is clear that data from *in vivo* trials are required before final conclusions can be drawn.

In summary, several critical characteristics of *L. reuteri* strain DPC16 have been identified, such as physical tolerance to unfavourable conditions, the ability to adhere to intestinal cells and inability to degrade mucin. Thus, this strain can be recognized as a good candidate for probiotic application. Most importantly, the phenomenon of significant antibacterial activity reflects the glycerol dehydratase gene expression in strain *L. reuteri* DPC16. This specific characteristic of the DPC16 strain in conducting heterofermentation equips it with stronger anti-pathogen properties with the potential to modulate the intestinal flora by suppressing the pathogens when introduced into a host

GI tract. However, it is worth repeating that these findings were obtained under simple *in vitro* conditions. Animal and human studies would provide multiple physiological interactive factors so that any *in vivo* results may not replicate those obtained *in vitro*.

Taking the whole into account, the experimental outcomes of this chapter have provided useful information to support the possibility of using *L. reuteri* DPC16 as an effective commercial probiotic strain for human consumption.

Chapter 3
Effects of bovine lactoferrin on the growth of bacteria and the possible mechanisms

Introduction

As indicated in the literature, the balance of the intestinal microbial population can be disturbed by environmental factors such as diet, surgical operations on the digestive tract and intake of antibiotics; or by physiological factors such as genetic composition, aging and stress (Mitsuoka 1996; Isolauri *et al.* 2002; Woodmansey *et al.* 2004; Enck *et al.* 2009). The classical example is a baby's intestinal microflora, which is dominated by bifidobacteria during the breast-feeding period (Fanaro *et al.* 2003; Penders *et al.* 2006; Sherman *et al.* 2009). The greatest difference between the microbiota of breast-fed and formula-fed infants is the number of lactic acid bacteria present, particularly the species of bifidobacteria (Balmer & Wharton 1989; Harmsen *et al.* 2000; Salminen *et al.* 2005). The mother's milk appears well-suited to promote the proliferation of bifidobacteria in the baby's intestine during the breast-feeding period which may affect baby's life-long health (Harmsen *et al.* 2000; Coppa *et al.* 2006; Penders *et al.* 2006). Based on the mother-baby model, it is now proposed that ingestion of one of the major components of colostrum, lactoferrin, may help to initiate and maintain a healthy microflora in the gut of infants and adults.

3.1.1 Lactoferrin

Lactoferrin, a cationic protein, is a member of the transferrin family of iron-binding glycoproteins. It has been passed as safe by the US Food and Drug Administration (FDA) (Taylor *et al.* 2004), and bovine lactoferrin preparations are prepared commercially using chromatography technology. Recent studies have indicated that oral supplementation with bovine lactoferrin (BLf) in humans may assist in preventing some diseases (Talukder & Harada 2007; Tang *et al.* 2009). The biological properties of lactoferrin were initially confined largely to its antimicrobial activity. But, increasingly,

more findings regarding its multifunctionality have been recognized. Lactoferrin has been demonstrated to have antibacterial properties against a number of Gram-negative and Gram-positive bacteria, including *Escherichia coli, Salmonella typhimurium, Shigella dysenteriae, Listeria monocytogenes, Bacillus stearothermophilus, Bacillus subtilis* and *Micrococcus luteus* (Bullen 1976; Haug *et al.* 2007; Murdock *et al.* 2007). Since lactoferrin is able to pass through the stomach and enter the intestine unaltered (Percival 1999; Fischer *et al.* 2007), it could play a role in the colon by inhibiting the growth of pathogenic bacteria (Coppa *et al.* 2006). Some studies have shown that even if lactoferrin were partially hydrolyzed by acid or acidic proteases (such as pepsin), the hydrolysates have strong potential antibacterial activities (Saito *et al.* 1991; Teraguchi *et al.* 1995b; Ogata *et al.* 1998). A peptide isolated from a pepsin hydrolysate of bovine lactoferrin, known as lactoferricin, has been shown to be highly effective against a broad range of Gram-positive and Gram-negative bacteria *in vitro* (Bullen 1972; Saito *et al.* 1991). Several peptides that have been identified as originating from lactoferrin have been shown to have capacities to promote bifidobacteria at very low concentrations *in vitro* (Coppa *et al.* 2006), and bovine lactoferrin and recombinant bovine lactoferrampin-lactoferricin have shown effects on the intestinal microflora in adult dog (Hellweg *et al.* 2008) and weaned piglet models (Tang *et al.* 2012). These reports indicate that lactoferrin, or its hydrolysed products, may allow modulation of the gastrointestinal microflora that confer benefits upon host well-being and health.

3.1.2 Aim of this chapter

In the previous chapter, *L. reuteri* DPC16 was shown to be a good candidate probiotic strain. Based on this information, and on the current knowledge regarding lactoferrin, it is proposed that bovine lactoferrin could be used in combination with *L. reuteri* DPC16 to influence the intestinal microflora. Since there are insufficient data available on the effects of BLf on different groups of bacteria in the gut, the present work was designed to provide this information, and to attempt to find the reason why lactoferrin in breast milk contributes to high numbers of lactic acid bacteria in the colon during breast-feeding of infants. Finally, the effects of a combination of BLf and *L. reuteri* DPC16 on the growth of some pathogens were studied to determine if there are any synergistic or additive effects

3.2 Materials and Methods

3.2.1 Bovine Lactoferrin

Bovine lactoferrin (NZMP 7100) was kindly supplied by Fonterra Co-operative Group, New Zealand. In this product the protein content is >90% and iron saturation is 10-20%. The pH value (5% solution at 20 °C) is 5.5-6.5.

3.2.2 Bacterial strains and culture media

Bacterial strains and culture media used in the experiments were as described in **Section 2.2.2.**

3.2.3 Skim milk medium

Commercial liquid skim milk (Anchor) was obtained from a local supermarket (Countdown, Auckland, New Zealand). The milk was filter-sterilized through a 0.45 µm filter (the particle sizes of fat-free protein, lactose and minerals are less than 0.10 µm, which allow passing through 0.45 µm filters to remove most of bacteria). Several filters were used in medium preparation to achieve this purpose; the filtered skim milk was stored at 4 °C before use as a growth medium.

3.2.4 Spectrophotometric turbidity bioassay and bacterial drop plate count technique

Bacterial growth responses were monitored using both the spectrophotometric turbidity bioassay and the bacterial drop plate count technique as described in **Section 2.2.6** and **Section 2.2.8.1**.

3.2.5 Statistical analysis

Data were processed on Microsoft Excel spreadsheets. Some of the bacterial growth turbidity assays were analysed based on linear and non-linear regression curve fitting analysis. Results were expressed as means ± SD. D'Agostino & Pearson omnibus normality test or graph plotting for normality of residuals was performed, and the significance of differences was determined using global curve fitting analysis or ANOVA and post-hoc analysis using Prism 4.03 software (GraphPad Software, Inc). Differences were considered statistically significant at P *value* <0.05. Graphs were

made using Prism 4.03 software.

3.3 Results

3.3.1 The effects of bovine lactoferrin on the growth of some pathogenic and probiotic bacteria

The effects of bovine lactoferrin on the growth of four pathogens, including Gram positive and Gram negative strains, and some probiotic bacteria, were determined by measuring the bacterial optical density after 24 h incubation at 37^{o}C. To confirm that the effects were due to lactoferrin *per se* rather than proteins in general, bovine serum albumin (BSA) was used as a negative control using the same experimental conditions. The residuals of data were visualized to be normally distributed, and the data were analysed to determine differences between the effects of bovine lactoferrin (BLf) and bovine serum albumin (BSA) on different bacteria, by performing a Two-way ANOVA analysis and Bonferroni post test on each concentration of BLf and each bacterial strain used in the experiment. As **Fig. 3.1** and **Fig. 3.2** showed, the positive control (a treatment of antibiotics: 100 μg/mL of streptomycin and 100 U/ml of penicillin) significantly inhibited the growth of all bacteria tested in the experiment (*P value* < 0.001). **Fig. 3.1** shows that BLf at a high concentration (20 mg/mL) inhibited the growth of all pathogens (*P value* < 0.001) but of only two of the five probiotics (*P value* < 0.05), and these to a lesser extent than of the pathogens. BLf at some lower concentrations showed some enhancement of probiotics growth (*P value* < 0.05) but not of pathogens. These results indicate that BLf at some higher concentrations may inhibit the growth of pathogens and some probiotics, whereas at some lower concentrations it may promote the growth of some probiotics but not of pathogens.

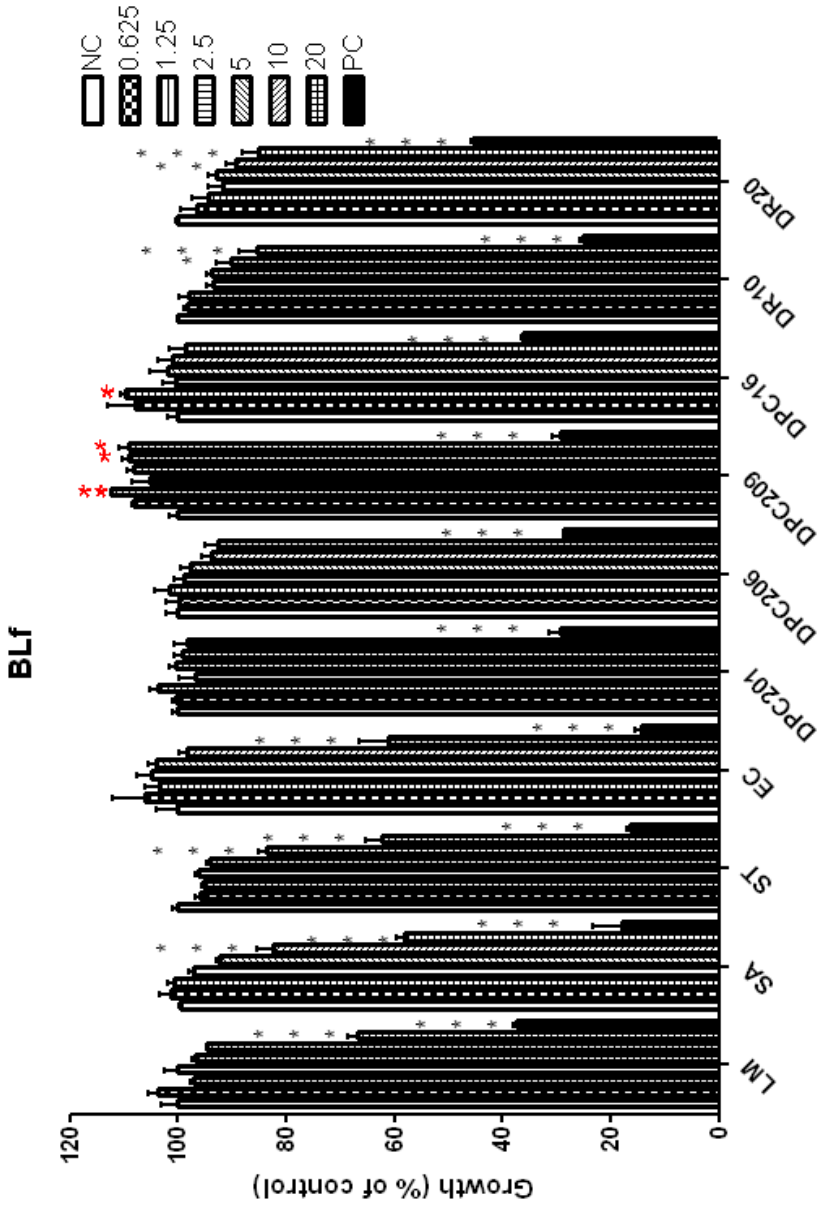


Figure 3.1 The effects of bovine lactoferrin on the growth of pathogens and probiotics

The growth effects of BLf on four pathogens [*L. monocytogenes* (LM), *S. aureus* (SA), *S. typhimurium* (ST) and *E. coli* (EC)] and six probiotics [*L. acidophilus* (DPC201), *L. plantarum* (DPC206), *P. acidilactici* (DPC209), *L. reuteri* (DPC16), *B. lactis* (DR10) and *L. rhamnosus* (DR20)] were tested in triplicate during incubation at 37 ^{o}C for 24 h in BHI and MRS medium, respectively. [X: strains Y: growth (% of negative control). Both negative control (medium only) and positive control (antibiotics: 100 μg /mL of streptomycin and 100U/ml of penicillin) were included in the experiments. The error bars are SD. The black stars- "*", "**" and "***" indicate that the treatments significantly (*P value* < 0.05, *P value* < 0.01 and *P value* < 0.001) inhibited the growth of some bacteria; the red stars-"*" and "**"indicate that the treatments significantly (*P value* < 0.05 and *P value* < 0.01) promoted the growth of some probiotics. The statistical analysis results are shown in *Appendix II*.]

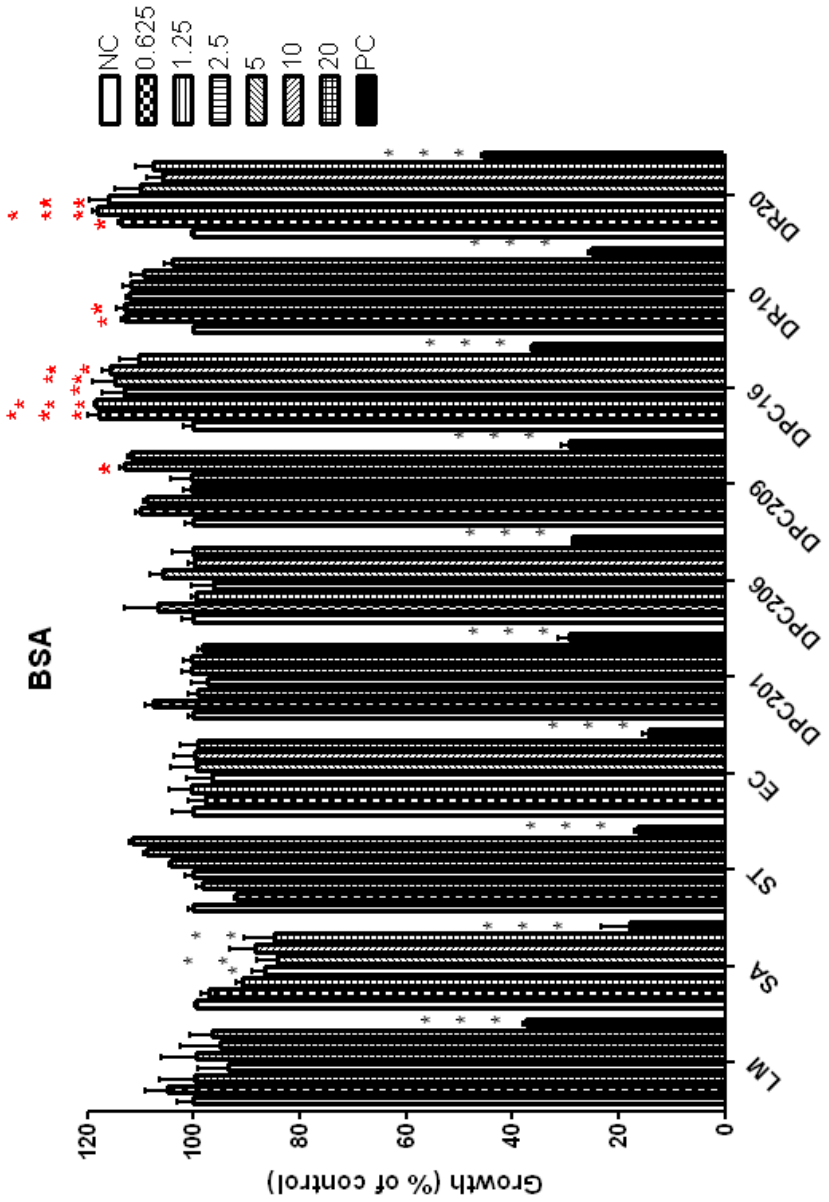


Figure 3.2 The effects of bovine serum albumin on the growth of pathogens and probiotics

The growth effects of BSA on four pathogens [*L. monocytogenes* (LM), *S. aureus* (SA), *S. typhimurium* (ST) and *E. coli* (EC)] and six probiotics [*L. acidophilus* (DPC201), *L. plantarum* (DPC206), *P. acidilactici* (DPC209), *L. reuteri* (DPC16), *B. lactis* (DR10) and *L. rhamnosus* (DR20)] were tested in triplicate during incubation at 37 ^{o}C for 24 h in BHI and MRS medium, respectively. [X: strains Y: growth (% of negative control). Both negative control (medium only) and positive control (antibiotics: 100 μg /mL of streptomycin and 100 U/ml of penicillin) were included in the experiments. The error bars are SD. The black stars- "*", "**" and "***" indicated that the treatments significantly (*P value* < 0.05, *P value* < 0.01 and *P value* < 0.001) inhibit the growth of some bacteria; the red stars-"*", "**" and "***" indicate that the treatments significantly (*P value* < 0.05, *P value* < 0.01 and *P value* < 0.001) promoted the growth of some probiotics. The statistical analysis results are shown in *Appendix II*.]

Fig. 3.2 shows that BSA at some concentrations significantly (*P value* < 0.05) inhibited only one pathogen, *S. aureus* (SA), but not the other pathogens or any of the probiotics. Indeed, BSA showed enhancement of the growth of some probiotics (*P value* < 0.05) but of none of the pathogens. These results indicate that BSA at some concentrations may inhibit some pathogens but not the probiotics. The lower concentrations may also promote the growth of some probiotics but not pathogens.

The drop plate count method was used to confirm that BLf, but not BSA, significantly inhibited growth of pathogenic bacteria. **Fig. 3.3** shows the colonies of *E. coli* O157:H7 growing on BHI agar plates after growth in BLf- and BSA-supplemented BHI medium.

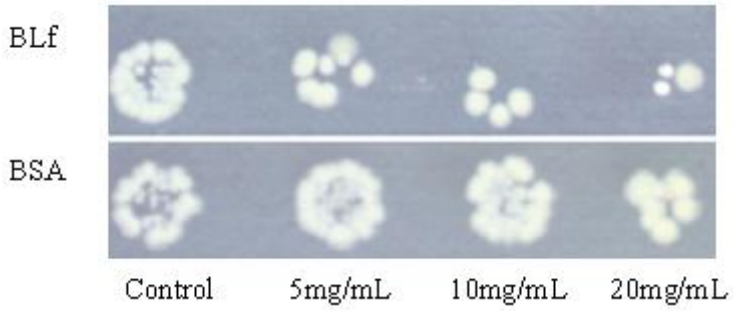


Figure 3.3 The effects of bovine lactoferrin and bovine serum albumin on the growth of *E. coli* (EC) in BHI medium tested using the standard drop plate count method

Bovine lactoferrin (BLf), at a concentration of 5 mg/mL to 20 mg/mL, showed growth inhibition of *E. coli*, but the same concentrations of bovine serum albumin (BSA) showed less effect.

The above figures demonstrate that bovine lactoferrin inhibited the growth of tested pathogens but with less inhibitory effect on probiotics, in the liquid growth media. However, the pathogens were grown in BHI medium and the probiotics in MRS medium. Further experiments were performed where the selected bacteria were grown in a skim milk medium, to ensure that the medium was not the cause of the different effects. The drop plate count technique was used to enumerate the bacteria due to the difficulty of optical density measurement for the skim milk medium. As shown in **Fig. 3.4**, growth of two pathogens, i.e. one Gram-negative (*E. coli*) and one Gram-positive (*S. aureus*) were selected and compared with six probiotic bacteria in the skim milk medium on addition of BLf at 20 mg/mL. The residuals of data were plotted and visualized to be reasonably normally distributed, and the result of One-Way ANOVA and Bonferroni's Multiple Comparison Test indicated that the inhibitory effects of lactoferrin (20 mg/mL) on the tested bacteria showed some significant differences (*P*

value < 0.05) in the skim milk medium. There were no significant differences between the two pathogens or between any of the probiotics (*P value* > 0.05), but both *E. coli* and *S. aureus* showed significant growth differences to some of the probiotics (*P value* < 0.05) under this experimental condition.

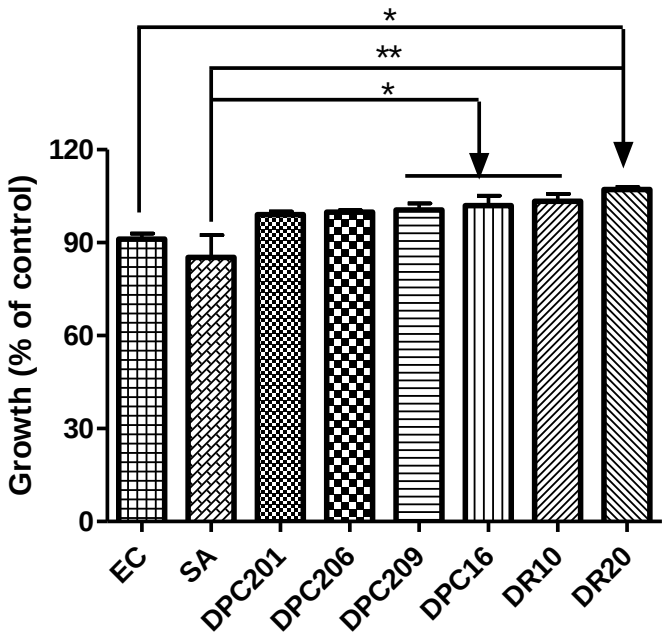


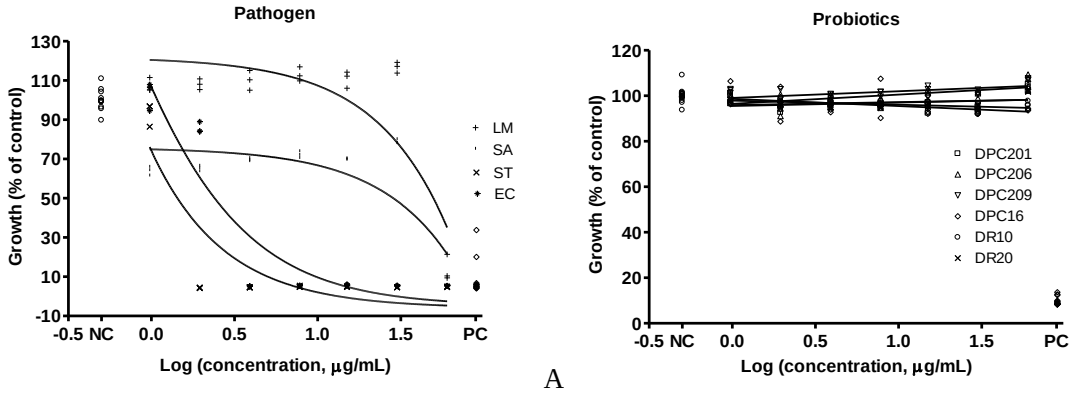
Figure 3.4 The effects of bovine lactoferrin, at 20 mg/mL, on the growth of selected bacteria in skim milk medium

E. coli (EC) and *S. aureus* (SA), and *L. acidophilus* (DPC201), *L. plantarum* (DPC206), *P. acidilactici* (DPC209), *L. reuteri* (DPC16), *B. lactis* (DR10) and *L. rhamnosus* (DR20) were incubated in skim milk medium supplemented with 20 mg/mL of bovine lactoferrin at 37 ^{o}C aerobically (pathogens) or anaerobically (probiotics) for 24 h.. Bacterial colonies were counted in triplicate using the drop plate count method. [The negative controls were medium only. The error bars are SD. "*" and "**" indicate that lactoferrin (BLf, 20 mg/mL) inhibited the growth of the two pathogens and that these effects are significantly different from those on some of the probiotics (*P value* < 0.05 and *P value* < 0.01). The statistical analysis is shown in *Appendix II*.]

3.3.2 The effect of polymyxin B on the growth of selected pathogens and probiotics

The results described above indicate that lactoferrin inhibits the growth of pathogenic bacteria but with less effect on probiotic bacteria. An attempt was now made to investigate the possible antibacterial mode of action of lactoferrin. Polymyxin B is a bactericidal agent that is used for controlling Gram-negative bacteria, but which has little or no effect on Gram-positive bacteria. Polymyxins are cationic, basic proteins that bind to the cell membrane and alter its structure to increase its permeability. Since lactoferrin is a cationic protein, it was hypothesized that its mode of action may be

similar to that of polymyxin B. Hence, experiments were performed to assess the antibacterial activity of polymyxin B against the pathogens and probiotics used in the present study. The group of data for the inhibition by polymyxin B of pathogens fitted a sigmoidal concentration-response curve (same inhibition manner), with a global fitting analysis that showed the curves were sharing different parameters (different concentration-dependent manner between Gram-negative and Gram-positive bacteria). As **Fig. 3.5 (A)** demonstrates, polymyxin B strongly inhibited the growth of the Gram-negative pathogens *S. typhimurium* and *E. coli* O157:H7 above a concentration of 4 µg/mL, but that a concentration approaching 60µg/mL was required before significant inhibition of the Gram-positive pathogens *L. monocytogenes* and *S. aureus* was observed. However, the data for the inhibition by polymyxin B of most probiotics fitted better to a linear-regression model than to the concentration-response model (the residuals of data were found to be relatively normally distributed). **Fig. 3.5 (B)** shows that polymyxin B, even at 60 µg/mL, did not inhibit most of the probiotic bacteria.



A

B

Figure 3.5 The effects of polymyxin B on the growth of selected bacteria

The growth of pathogens (A) *L. monocytogenes* (LM), *S. aureus* (SA), *S. typhimurium* (ST), *E. coli* (EC), and probiotics (B) *L. acidophilus* (DPC201), *L. plantarum* (DPC206), *P. acidilactici* (DPC209), *L. reuteri* (DPC16), *B. lactis* (DR10) and *L. rhamnosus* (DR20) were tested in triplicate during incubation aerobically (pathogens) or anaerobically (probiotics) at 37 ^{o}C for 24 h when polymyxin B was supplemented in BHI or MRS media. [X: Log concentration of polymyxin B (µg/mL) Y: growth (% of negative control). Both negative control (medium only) and positive control (antibiotics: 100 µg /mL of streptomycin and 100U/ml of penicillin) were included in the experiments. The linear and non-linear regression analysis results are shown in *Appendix II*.]

As a comparison, the data groups for the inhibitory combination of two antibiotics (penicillin and streptomycin) on the pathogens and probiotics mostly fitted a sigmoidal model (same inhibition manner), with a global fitting analysis showing that the curves were sharing different parameters (different concentration-dependent manner) (the data sets of LM and DPC209 should be ignored due to the lower P values of normality

residuals). However, these probiotic strains displayed very similar responses and were more susceptible than were the pathogens to the action of a combination of penicillin and streptomycin (PS) in the growth medium at concentrations of up to 100 U/mL penicillin and 100 μg/mL streptomycin (**Fig. 3.6**).

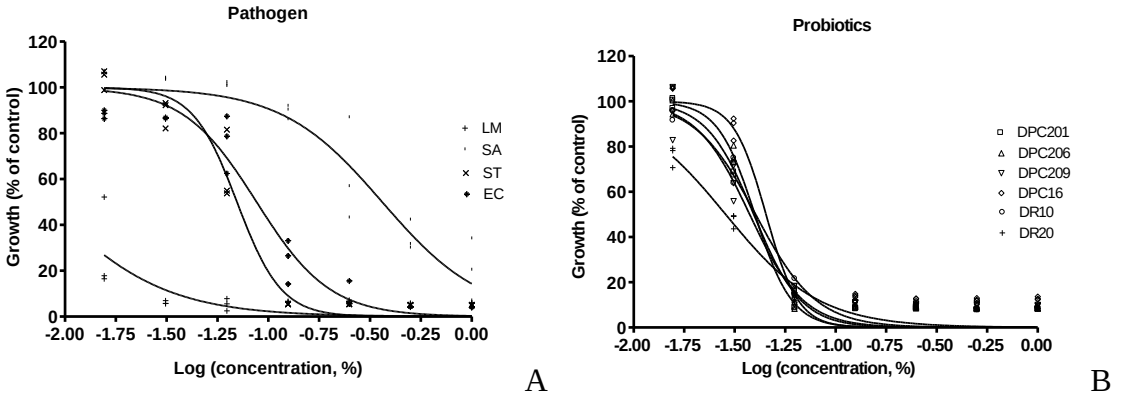


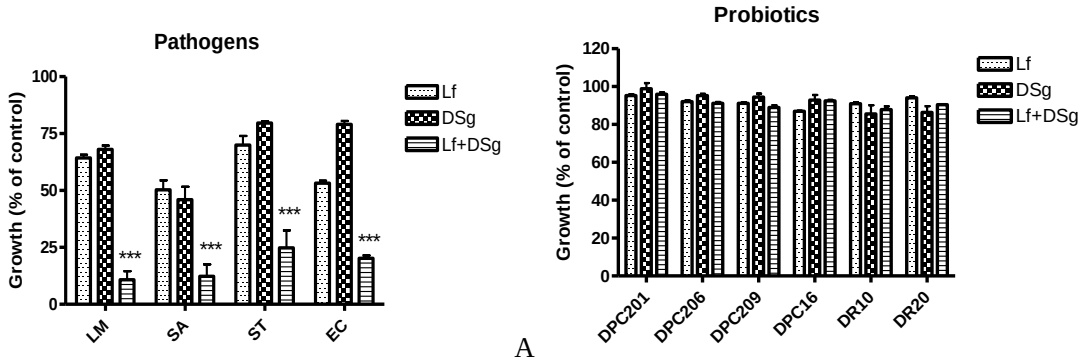
Figure 3.6 The effects of penicillin and streptomycin on growth of selected bacteria

The growth of pathogens (A) *L. monocytogenes* (LM), *S. aureus* (SA), *S. typhimurium* (ST), *E. coli* (EC), and probiotics (B) *L. acidophilus* (DPC201), *L. plantarum* (DPC206), *P. acidilactici* (DPC209), *L. reuteri* (DPC16), *B. lactis* (DR10) and *L. rhamnosus* (DR20) were tested in triplicate during incubation aerobically (pathogens) or anaerobically (probiotics) at 37 ^{o}C for 24 h when a combination of antibiotics (penicillin and streptomycin) in the concentration range from 0 to 1% [1% refers to the dilution of a commercial concentrated stock (PS, Gibco Invitrogen), and contains penicillin (100 U/mL) and streptomycin (100 μg/mL)] was added to BHI or MRS media. [X: Log concentration of PS (%) Y: growth (% of negative control). The negative controls were medium only. The non-linear regression analysis results are shown in *Appendix II*.]

3.3.3 The effect of bovine lactoferrin in combination with a probiotic supernatant on the growth of selected pathogens and probiotics

According to previous results (Chapter 2), the cell-free supernatant of *L. reuteri* DPC16 inhibits growth of pathogens with minimal effects on other probiotics, and these probiotic bacteria are also relatively resistant to bovine lactoferrin from the results in the present Chapter. Hence, a combination of lactoferrin/probiotic may help in manipulating the relative numbers of pathogens and probiotics in the GI tract. Therefore, the effects of bovine lactoferrin (10 mg/mL) in combination with DPC16 cell-free supernatant [DSg, 12.5 % (v/v)] were tested on the growth of pathogens and probiotics, using the same methodology as described in **Section 3.2.4**. The residuals of data generally appeared as normally distributed and the analysis of results of two-way ANOVA and Bonferroni post test on each treatment and each bacteria, indicated significant differences (*P value* < 0.001) among the combination treatment compared to each

individual treatment on the pathogens, with an augmentation of pathogen inhibition. In contrast, the combination of BLf and DSg on probiotics showed no significant difference (*P value* > 0.05) to each individual treatment with neither inhibitory nor enhancing effects (**Fig. 3.7**).



A B

Figure 3.7 Effects of a combination of BLf and DSg on the growth of pathogens and probiotics

The growth of four pathogens LM, SA, ST and EC (A.) and six probiotics DPC201, DPC206, DPC209, DPC16, DR10 and DR20 (B.) was tested in triplicate after each fraction or the combination of bovine lactoferrin (10 mg/mL) and DPC16 supernatant (12.5 % (v/v) were supplemented in BHI or MRS media and incubated aerobically (pathogens) or anaerobically (probiotics) at 37 ^{o}C for 24 h. [X: pathogens or probiotics Y: growth (% of negative control). The negative controls were medium only. The error bars are SD. "***" indicates significant inhibitory effects (*P value* < 0.001) of the combination treatment on all pathogens compared to each individual treatment. The statistical analysis results are shown in *Appendix II*.]

In terms of the significant differences between the combination treatment using 10 mg/mL of BLf and 12.5 % (v/v) of DSg and the individual fraction treatment on these pathogens, a synergism analysis was performed by generating the Combination Index (CI) according to the multiple drug-effect equation used by several authors (Chou & Talalay 1984; Murdock *et al.* 2007; Lopez-Exposito *et al.* 2008), where the Combination Index is related to the sum of fractional inhibitory concentration-index (FIC index). A CI <0.9 indicates synergism; a CI >0.9 but <1.10 means additive; a CI >1.10 means antagonism.

$CI_{(a, b)} = FIC_{(a)} + FIC_{(b)}$

= [Activity of (a+b) /Activity of fraction a] + [Activity of (a+b)/Activity of fraction b]

The results show that the combination of BLf (10 mg/mL) and DSg [12.5 % (v/v)] had synergistic effects on all pathogens tested (**Table. 3.1**).

Table 3.1 Combination Index of the antibacterial activities of BLf (10 mg/mL) and DSg [12.5 % (v/v)] against pathogens

Strain	Lf	DSg	Lf +DSg	CI	Effect
LM	64.2857	68.0372	10.775	0.32598	Synergistic
SA	50.3242	45.9862	12.3024	0.511988	Synergistic
ST	69.9656	79.5813	24.8231	0.66671	Synergistic
EC	53.2101	79.0362	20.203	0.6353	Synergistic

3.4 Discussion

The effects of bovine lactoferrin on the growth of four pathogens and six probiotic bacteria were studied. The results demonstrated that BLf inhibits the growth of all tested Gram-negative and Gram-positive pathogenic bacteria but had less inhibitory effect on the probiotics. Since bovine serum albumin, as a negative control, did not show clear inhibitory effects on most of the pathogens or the probiotics the conclusion from the experiments is that BLf is a special protein that possesses antibacterial capabilities to some, but not all, bacteria (at the concentrations tested).

Several possible antibacterial mechanisms of action of BLf have been proposed. The first is that its iron-binding action leads to lack of iron resources for bacterial growth (Bullen *et al.* 1972). However, other studies have indicated that its action is not restricted to bacteria with iron requirements (e.g. coliforms), because apo-BLf has also been shown to possess bactericidal activity against a wide range of microorganisms (Dionysius *et al.* 1993). Secondly, a direct interaction between the BLf positive charge and charged elements in the bacterial outer layers, such as lipopolysaccharide in Gram-negative bacteria and lipotechoic acid in Gram-positive bacteria, has been suggested (Appelmelk *et al.* 1994; Elass-Rochard *et al.* 1995). Later, the antibacterial and endotoxin-neutralizing capabilities of BLf were suggested to be related to its ability to bind lipopolysaccharides with high affinity (Zhang *et al.* 1999). Finally, the mechanism of the antibacterial properties has been shown by electron microscopy to involve the loss of membrane integrity and alteration of cytoplasmic structure in the target bacteria (Tomita *et al.* 1994).

There are two basic mechanisms of action of antibacterial peptides, including polymyxin B, as illustrated in **Fig. 3.8** (Jenssen *et al.* 2006). One is membrane permeabilization, shown as A to D, and the other is non-membrane permeabilizing actions (E to I).

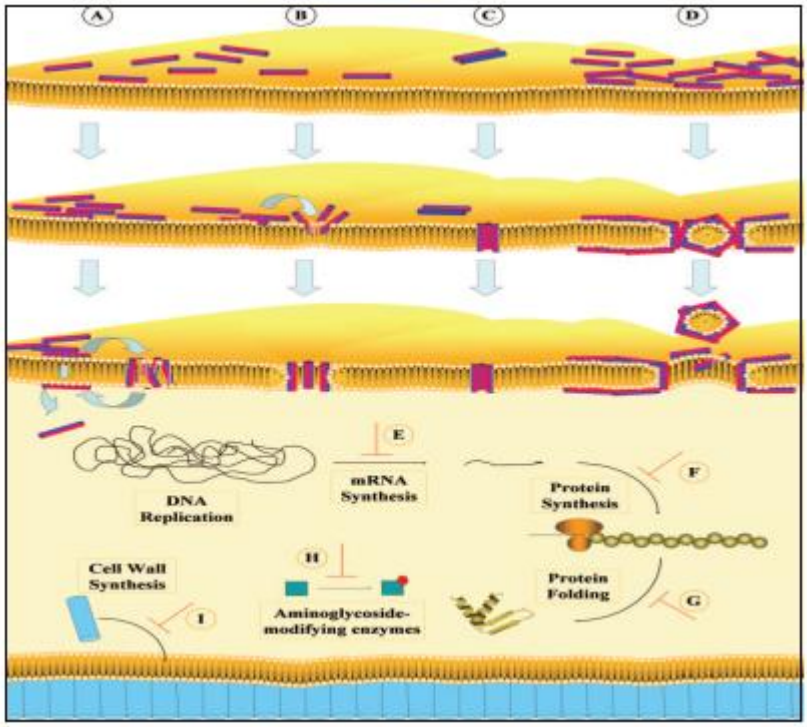


Figure 3.8 Mechanisms of action of antibacterial peptides (from Jenssen *et al.* 2006)

A to D show mechanisms of membrane permeabilization, E to I show mechanisms of non-membrane permeabilization.

In the "aggregate" model (A), peptides re-orient to span the membrane as an aggregate with micelle-like complexes of peptides and lipids, but without adopting any particular orientation. The "toroidal pore" model (B) proposes that peptides insert perpendicular to the plane of the bilayer, with the hydrophilic regions of the peptides associating with the phospholipid head groups while the hydrophobic regions associate with the lipid core. In this process, the membrane also curves inward such that the bilayer also lines the pore. In the "barrel-stave" model (C), the peptides insert in a perpendicular orientation to the plane of the bilayer, forming the "staves" in a "barrel"-shaped cluster, with the hydrophilic regions of the peptides facing the lumen of the pore and the hydrophobic regions interacting with the lipid bilayer. The "carpet" model (D) proposes that peptides aggregate parallel to the lipid bilayer, coating local areas in a "carpet-like" fashion. At a

given threshold concentration, this is thought to result in a detergent-like activity, causing formation of micelles and membrane pores (Jenssen *et al.* 2006).

It is not clear which of these four permeabilization mechanisms is used by lactoferrin, though it is known that polymyxin B, as a cationic protein, alters cytoplasmic membrane permeability by binding to a negatively charged site in the lipopolysaccharide layer (Koch *et al.* 1999).

The present results have provided evidence that lactoferrin inhibits not only Gram-negative bacteria, but also some Gram-positive bacteria. However, the Gram-positive lactic acid probiotic bacteria were much less sensitive to both this protein and to polymyxin B (**Fig. 3.1** and **Fig. 3.5**). Thus, it appears that the probiotics tested in the present study have some special cell structures that confer some resistance to both bovine lactoferrin and polymyxin B. In contrast, these bacteria are as susceptible to penicillin and streptomycin as are other bacteria.

The *in vitro* results in the present study support the concept that lactoferrin can inhibit the growth of pathogens (Bullen 1976; Haug *et al.* 2007; Murdock *et al.* 2007), with only either mild inhibiting or mild promoting effects on the growth of some probiotic bacteria. Further, the effects of lactoferrin on the different bacteria place emphasis on its inhibitory effects on pathogenic bacteria rather than on a stimulus to the growth of probiotic bacteria. This study investigated the antibacterial effects of only a native form of lactoferrin (10-20% iron-saturated) which is commercially isolated from bovine milk. However a recent study (Chen *et al.* 2013) has provided information concerning apo-BLf and BLf hydrolysate, which support the previous conclusion that hydrolyzed lactoferrin (Saito *et al.* 1991; Ogata *et al.* 1998) and bovine lactoferrin peptide (lactoferricin) are inhibitory against a broad range of Gram-positive and Gram-negative bacteria *in vitro* (Bullen 1972; Saito *et al.* 1991). The study determined the MIC of apo-BLf and BLf hydrolysate on thirteen probiotics and six foodborne pathogens, and the results confirmed that the inhibitory effects of apo-BLf and BLf hydrolysate on foodborne pathogens and probiotics are strain- dependant and concentration-dependant (Chen *et al.* 2013). The inhibitory effects were more apparent on pathogens than on probiotics, but any possible growth promoting effects on the probiotics were not reported. Therefore, whether or not lactoferrin enhances the growth of some probiotic

bacteria (Kim *et al.* 2004) still needs to be clarified.

As shown in **Fig. 3.7**, the combination of lactoferrin with *L. reuteri* DPC16 has significantly greater inhibitory effect compared to that of each individual treatment, at the same concentration, on the growth of the pathogens but on none of the probiotics**.** Further, the synergism assessment demonstrated that the combination has synergistic effects on all the pathogens tested in the experiment. Chen *et al.* (2013) have subsequently confirmed a partial synergism or synergism of probiotics and apo-BLf/ BLf hydrolysate against foodborne pathogens using strains of *L. fermentum*, *B.lactis* and *B. longum*. Conceptually, a two-pronged attack on the pathogens, with lactoferrin affecting cell permeability, and SCFA (produced by probiotics) and/or reuterin (in *L. reuteri* DPC16 supernatant, DSg) interfering with enyzmes or DNA synthesis, is potentially a very useful means of manipulating the composition of the microflora in the GI tract. However, it should be cautioned that the concentration of BLf used in the present work (10 mg/mL) is rather high and that maintenance of this concentration *in vivo* may be a challenges (Troost *et al.* 2002). The technique of microencapsulation may provide a means of delivering it to its target site at an effective concentration.

Fig. 3.9 proposes a scheme by which lactoferrin and reuterin (in DSg), may work in concert with each other to inhibit bacterial growth. It is proposed that lactoferrin acts by releasing LPS from bacterial outer membranes, and assists penetration of the reuterin (as the data revealed), and the latter acts by interfering with DNA synthesis (Dobrogosz & Lindgren 1995). Nevertheless, separate models and better scientific tools are needed to give additional evidence to the proposed mechanism.

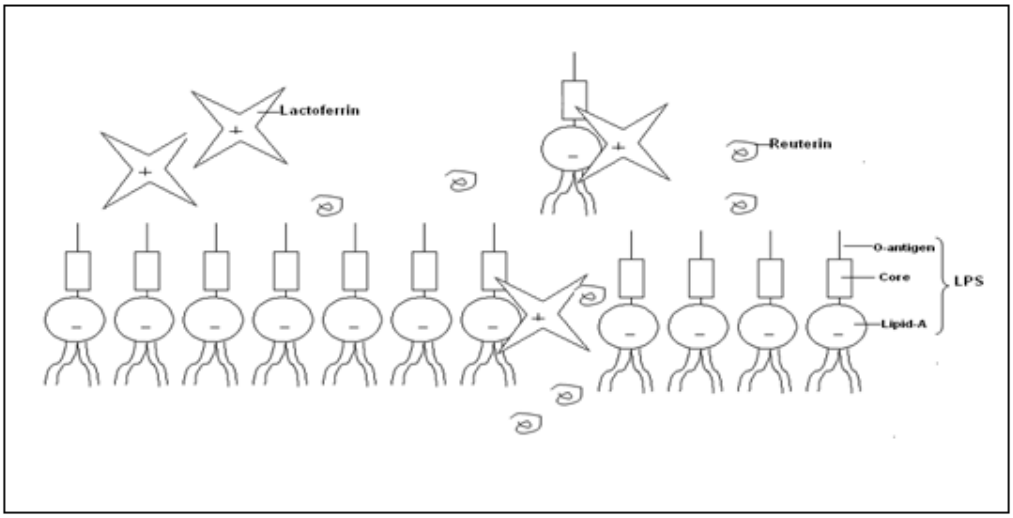


Figure 3.9 A proposed model for the interaction of lactoferrin with LPS in Gram-negative bacterial outer membranes

Electrostatic interactions occur between the positively charged lactoferrin and the negatively charged LPS in lipid-A. Lactoferrin becomes integrated within the membrane, neutralizing the charge on the membrane and releasing LPS, rendering the membrane unable to inhibit the penetration of water and other compounds, e.g. reuterin.

Finally, this study does not explain how the probiotic bacteria resist lactoferrin or reuterin. Their special cell wall structures or the protective materials related to their thick cell walls (e.g. exopolysaccharide) may contribute to this resistance, by affecting the accessibility or activity of cationic proteins, including lactoferrin and polymyxin B, to the negatively charged elements in the bacterial membranes. This would prevent change to the permeability of these bacterial cell membranes when exposed to these cationic proteins.

Chapter 4

Protective effects of *L. reuteri* DPC16 and bovine lactoferrin on faecal genotoxin-induced epithelial cell DNA damage

4.1 Introduction

Among intestinal diseases, colorectal cancer (CRC) is a major cause of death in humans. Epidemiological studies suggest that the incidence of colon cancer is influenced by lifestyle factors, especially dietary intake (WCRF/AICR 2007). The rates of CRC are relatively high with increased meat intake (Rumney *et al.* 1993a; Welfare *et al.* 1997), and this may be associated with protein-derived genotoxins accumulating in the colon area (in faeces) (Potter 1999a, b; WCRF/AICR 2007; Windey *et al.* 2012b). Carcinogenic agents may be present in the diet or formed during digestion, and many of the mechanisms may involve the metabolic activities of the bacterial flora normally resident in the colon. Five major groups of colonic carcinogens have been identified in diet and faeces, including polycyclic aromatic hydrocarbons, heterocyclic amines, N-nitroso compounds (NOC), bile acids, and fecapentaenes (de Kok & van Maanen 2000). Bacteria such as *E. coli* and clostridia produce toxins (Rafter *et al.* 2007) and some may be associated with carcinogens such as nitrosamines, heterocyclic amines, phenolic/indolic compounds, nitrated polycyclic aromatic hydrocarbons, azo-compounds, ammonia (Macfarlane & Macfarlane 1997) and H_2S (Pitcher *et al.* 2000). For example, indole and some indolic derivatives are products of metabolism of aromatic amino acids derived from dietary proteins. Indole-positive bacteria such as *E. coli* produce tryptophanase, an enzyme that cleaves tryptophan, producing indole and other products (Rowland *et al.* 1985; Goldin 1986; Smith & Macfarlane 1996; Saikali *et al.* 2004) (**Fig. 4.1**).

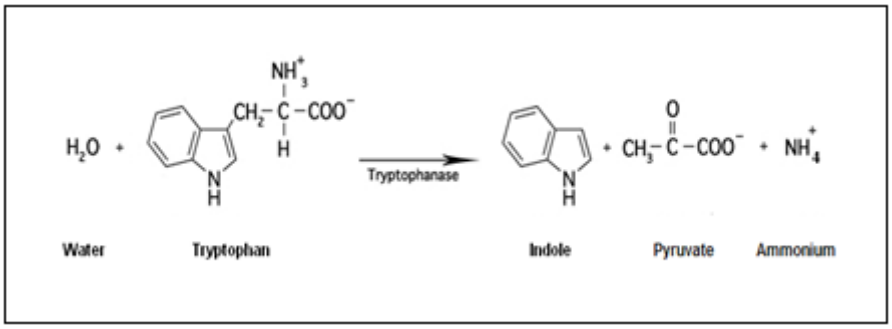


Figure 4.1 The metabolism of tryptophan to indole by trytophanase

It has been shown that indole is a cocarcinogen, because it enhances nitrosation of secondary amines by nitrite and thus acts as a cancer promoter (Dunning *et al.* 1950a, b; Dunning & Curtis 1958b, a; Bryan 1971). Additionally, secondary bile acids (Pool-Zobel & Leucht 1997) or reactive oxygen species (ROS) (Hussain *et al.* 2003) in the colon can induce mutations in the DNA of epithelial cells and increase the risk of cancer (Pool-Zobel & Leucht 1997; Gill & Rowland 2002; Collins & Ferguson 2004; Kapiszewska 2006).

4.1.1 DNA damage and carcinoma development in the colon

Colon carcinogens such as 1, 2-dimethylhydrazine (DMH), azoxymethane (AOM) and methylazoxymethanol (MAM) induce carcinogenesis after deconjugation (Okazaki *et al.* 2002; Dihal *et al.* 2006). These carcinogens/genotoxins induce an alteration or mutation in the DNA of colon epithelial cells (Hirayama & Rafter 2000). When DNA repair systems are unable to repair the damage or the cell apoptosis systems fail to work, the turn-on oncogenes and turn-off tumor suppressor genes in the mutated cells allow the cells to persist. Replication will occur resulting in a population of damaged daughter cells which may develop into a tumour/carcinoma. For example the APC gene is a tumor-suppressor gene; when inherited DNA changes turn off this "brake" on the cell's growth, hundreds of polyps can be formed (**Fig. 4.2**) in the colon. Further changes on other genes such as KRAS, TP53, and SMAD4 can lead the cells to grow and spread uncontrollably (Gill & Rowland 2002). Thus, CRC can be described as a intestinal disease of altered gene expression (Ruddon & Norton 1993). Initiation, promotion and progression are three defined stages of cancer development.

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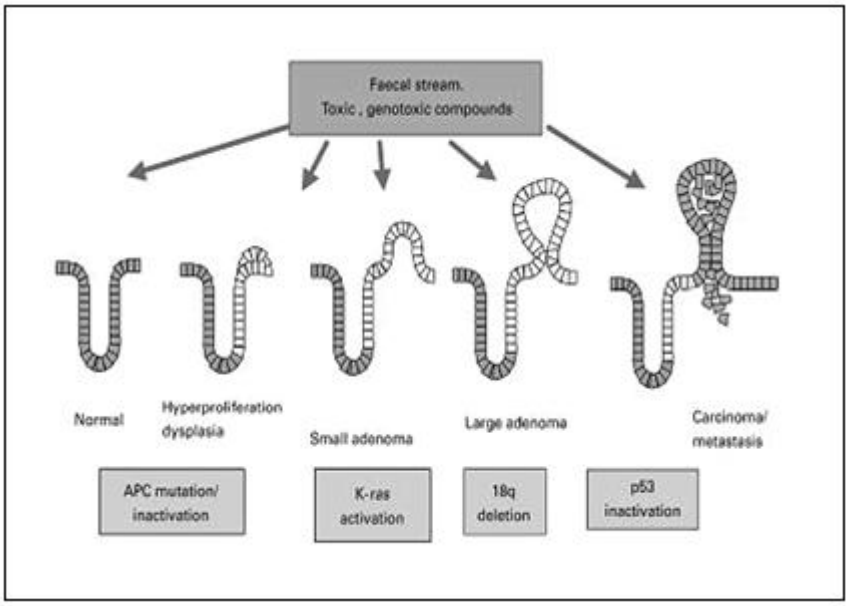


Figure 4.2 Carcinoma development in the colon (adapted from Gill and Rowland 2002)

4.1.2 Genotoxicity of human faecal water and probiotic interventions

As described in **Section 4.1.1**, DNA damage is an essential component of the genesis of colonic cancer. Gut microbial products and food components are thought to be principally responsible for the damage that initiates disease progression (Gratz *et al.* 2011). In recent years, the aqueous phase of human faeces (faecal water) has been selected as a source of biomarkers for colon cancer research. The multiple risk factors involved in the aetiology of colon cancer are associated with the aqueous phase of the faecal stream in the gut although the composition of faecal water is not fully understood. Nevertheless, faecal water has been shown to contain different mutagenic and genotoxic compounds and a large number of bacteria in the colon are involved in their metabolism. These biologically active substances are cytotoxic to mammalian cells (Rafter *et al.* 1987), and have been considered to be an important source of inducers and modulators of carcinogenesis in the colon. Methods exist for assessing biological activities related to CRC development (Karllson 2005; Gill *et al.* 2007). It has been shown, using the Comet (single cell gel electrophoresis) assay, that approximately 43% of faecal water samples from human volunteers are genotoxic, to some extent, towards a human colon cell line (Venturi *et al.* 1997). Human studies have shown that faecal concentrations of H_2S are higher in patients with untreated ulcerative colitis compared to healthy controls (Pitcher *et al.* 2000). Furthermore, the bile acids deoxycholic acid and lithocholic acid are postulated to play an important role in the aetiology of colon cancer by acting as

promoters of the tumorigenic process. The concentrations of secondary bile acids have been shown to be significantly higher in faecal water from patients with colonic polyps or cancer as compared to controls with normal colons (Stadler *et al.* 1988). A variety of *in vitro* and *in vivo* studies have proven that secondary bile acids with toxic effects on colonic epithelial cell DNA (Pool-Zobel & Leucht 1997; Ou *et al.* 2013) can cause increased colonic crypt foci formation (Sutherland & Bird 1994) and disruption of colonic mucosal cell membrane integrity, leading to a compensatory increase in mucosal proliferation (Nagengast *et al.* 1995).

Table 4.1 Summary of dietary studies assessing genotoxicity in human faecal samples

Diet or compounds	Methodology	Genotoxicity	References
Bifidobacterium longum 913 & *Lactobacillus acidophilus* 145	Comet	Decreased	Oberreuther-Moschner *et al.* (2004)
Lactobacillus casei (in meat)	Comet	No effect	Osswald *et al.* (2000)
Lactobacillus rhamnosus	Comet (biopsy)	Decreased	Rafter *et al.* (2007)
Lactobacillus acidophilus LA-2	Ames	Decreased	Hosoda *et al.* (1996)
Bifidobacterium lactis LKM512	SOS (umu)	Decreased	Matsumoto *et al.* (2001); Matsumoto and Benno (2004)
Lactobacillus paracasei Lpc-37, *Lactobacillus acidophilus* 74-2, and *Bifidobacterium animalis subsp. lactis* DGCC 420	Comet	Decreased	Roessler *et al.* (2012)
Red meat (0-420g/day)	Comet	No effect	Hughes *et al.* (2002); Cross *et al.* (2006); Joosen *et al.* (2009); Joosen *et al.* (2010)
	Transcriptomic analyses	Increased	Hebels *et al.* (2012)
Fat	Ames	No effect	Venitt *et al.* (1986)
	Ames	No effect	Suzuki *et al.* (1992)
Dairy rich diet	Comet	No effect	Glinghammar *et al.* (1997)
Vegetarian	Ames	Decreased	Johansson *et al.* (1992)
"High risk" diets	Comet	Increased	Rieger *et al.* (1999)
	Ames	Increased	Kuhnlein *et al.* (1983)
Heterocyclic amines and vitamin D	Comet	No effect	Mai *et al.* (2009)
Protein	Comet	No effect	Windey *et al.* (2012a)
Polydextrose	Comet	Decreased	Costabile *et al.* (2012)
Galacto-oligosaccharides	Comet	No effect	Walton *et al.* (2012)
Weight-loss diets	Cytokinesis-block micronucleus cytome assay	No effect	Benassi-Evans *et al.* (2010)
Blackcurrant seed press residue	Comet	Increased	Helbig *et al.* (2009)
Case-control studies	Ames	No effect	Schiffman *et al.* (1989)
	Comet	No effect	Nordling *et al.* (2003)

Since Bruce and Dion (1980) discovered the mutagenic properties of human faeces, the Comet assay has become commonly used to study the effect of diet on DNA damage and to identify harmful and protective dietary components. Many studies have examined the relationship between diet and faecal mutagenicity/genotoxicity using the Ames, Comet assay and other approaches (**Table 4.1**). Among these studies, most of the dietary interventions failed to change faecal mutagenicity or genotoxicity. So far, the only dietary modifications that convincingly alter faecal potential to damage DNA are probiotics, although different strains have been used in each study (Gratz *et al.* 2011).

4.1.3 Aim of this chapter

As mentioned previously, several bacterial species in the colon have been implicated in increasing the risk of colon cancer, while depressed populations of lactobacilli and bifidobacteria were associated with increased faecal mutagenicity in a study of 52 subjects (Savitskaia & Bondarenko 2008). Evidence for the protective effects of probiotics against cancer is accumulating from *in vitro* studies, animal models, epidemiology and human intervention studies. However, the precise mechanisms are presently unknown (Gursoy & Kinik 2006; Orlando & Russo 2013). The aim of the present study was to explore the potential protective effects of probiotic bacteria and lactoferrin against faecal genotoxins-induced DNA damages using a colonic cellular model system. The results in previous chapters demonstrated that *L. reuteri* DPC16 and bovine lactoferrin have the potential to influence the gut microflora, and it was appropriate to investigate their effects in systems related to carcinogenesis. Thus, in the present chapter, genotoxic human faecal water, which includes a variety of carcinogenic/genotoxic factors, was selected as a source of biomarkers and was used in conjunction with the human colonic epithelial cell line HT29 and the Comet assay as a carcinogenesis cellular model. This human colonic cellular model approach was considered ideal to study the potential antigenotoxicy activities of proposed probiotic formulae and hopefully answer some fundamental questions related to the mechanisms of action of specific dietary interventions that lead to reduced colonic DNA damage.

4.2 Materials and methods

4.2.1 Bacterial strains and growth conditions

The bacterial strains used in this study included probiotic bacteria *L. reuteri* DPC16, *L. acidophilus* DPC201, *B. lactis* HN019 (DR10), and pathogenic bacteria *E. coli* O157:H7 strain 2988 and *S. aureus* ATCC 25932. The probiotic bacteria were grown in MRS broth at 37 °C for 18 h anaerobically before use in the assays. The pathogens were grown in BHI broth at 37 °C for 24 h aerobically before use. The bacterial culture growth was monitored using the optical density value at 550 nm (Genova MK3 uv/visible spectrophotometer, Jenway Ltd, England), to ensure that the appropriate stage of growth was reached.

4.2.2 Bacterial supernatant and cells preparation

The overnight bacterial culture was harvested by centrifugation (4,000 × *g*, 10 min, at 4 °C) (Centrifuge, Model 5810R, Eppendorf, Hamburg, Germany). The supernatants were collected, filter-sterilized by passage through a 0.20 µm syringe-filter system, and the pH was adjusted to 7.0. The supernatants were stored at 4 °C until the time of use. The cell pellets were washed twice with PBS then resuspended in this buffer (optical density at 550 nm was adjusted to 1.2-1.8) and divided into two parts. One part of the cell suspension was heated to 95 °C for 30 min using a digital dry bath (AccuBlock™ Digital Dry Bath, Labnet, USA) to provide non-viable cells (this heat-killed cell suspension was plated on agar and incubated at 37 °C for 24 h to validate complete cell death). The viable cell suspension and the heat-killed cell suspension were both stored at 4 °C for a short period of time before use.

4.2.3 Human faecal water and faecal flora preparation

Faecal samples were obtained from a healthy non-vegetarian, non-smoking female with no history of gastrointestinal disease, and faecal water was prepared as described elsewhere (Klinder *et al.* 2004). Briefly, the complete faecal sample was homogenized in a stomacher (Masticator, IUL, Spain), at a ratio of 1:5 faecal sample to PBS, for 5 min and then centrifuged at 3,200 × *g* for 30 min at 4 ^{o}C. After the faecal particles were discarded, the supernatant was distributed to 1.5 mL sterilised Eppendorf tubes, followed by centrifugation at 13,000 × *g* for 10 min (Eppendorf Mini centrifuge, 5415D,

Germany) to separate the faecal flora from the aqueous phase. The top layer of faecal water was sterilised by filtration through a 0.20 μm pore size filter; the pH was measured (pH 7.0-7.2), and aliquots were stored at −20 °C prior to use. The separated faecal flora cell pellets were washed twice and resuspended in PBS to an optical density (550 nm) of 1.2-1.8. The viable and heat-killed faecal flora were prepared (as in **Section 4.2.2**) and stored at 4 °C for a short period of time before use.

4.2.4 Cell lines and growth conditions

The human colon carcinoma cell line HT29 was obtained from the School of Medical Sciences, The University of Auckland (Auckland, New Zealand). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, New Zealand) (see ***Appendix I***) supplemented with 10% (v/v) fetal bovine serum (heat inactivated at 60 °C, 45 min), 1% antibiotics [including streptomycin (100 μg/mL) and penicillin (100 U/mL)] at 37 °C in a 5% CO_2 humidified atmosphere (Cell culture CO_2 incubator: *SANYO*, Japan). For the experiments, HT29 cells were cultured until a density of 10^6 cells/mL was achieved (counted using a haemocytometer).

4.2.5 Cytotoxicity determination (MTT assay)

The human colon carcinoma HT29 cell line was used to determine the cytotoxicity of the faecal water as described by van Munster *et al.* (1993). Pre-cultured cells in the complete DMEM (as described above) were harvested from a cell culture flask by centrifugation at 500 x *g* for 5 min after trypsinisation with 0.25% trypsin-EDTA solution for 10 min. The resulting cell pellet was resuspended to 2 x10^5 cells/mL in complete DMEM, and distributed in amounts of 100 μL to each well of a 96-multiwell plate. The cells were cultured for 24 h at 37 °C in a 5% CO_2 humidified atmosphere. The cultured cells were then incubated at 37 °C for 5 to 120 min in the presence of sterilised faecal water, at final concentrations of 5%, 10% and 50% (v/v) in cell culture medium. PBS buffer was used as a negative control, while 300 μM bile salts (Sigma, USA) was the positive control. Wells were washed with PBS, and the surviving cells were cultured for another 24 hours at 37 °C in a 5% CO_2 humidified atmosphere under the same conditions as above. Thereafter, the MTT assay was performed (MTT cytotoxicity kit, Roche, USA), following the manufacturer's instructions. In this assay, the yellow tetrazolium MTT salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide) is reduced in cell mitochondria in metabolically active cells to form insoluble purple formazan crystals, which are later solubilised by the addition of a detergent, dimethyl sulfoxide. The colour can be quantified by spectrophotometry at a wavelength of 550 nm. The reduction of tetrazolium takes place only when mitochondrial reductase enzymes are active, and therefore conversion (and absorbance) is directly related to the number of viable (living) cells (**Fig. 4.3**).

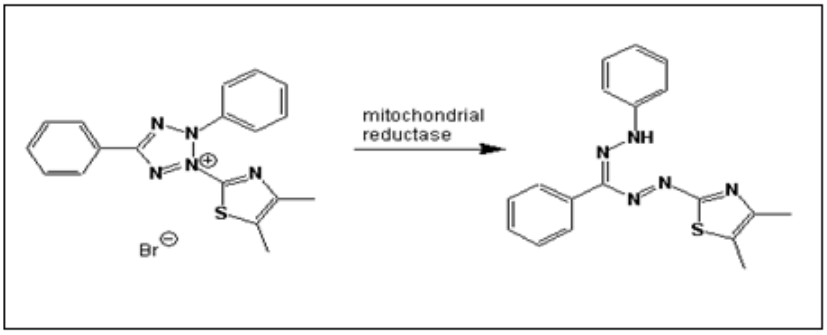


Figure 4.3 Molecular structure of MTT and its corresponding colorimetric reaction product in the presence of mitochondrial reductase enzyme activity

An enzyme-linked immunosorbent assay plate reader (Microplate Reader Model ∑960, Metertech Inc., Taiwan) was used to measure the absorbance at 550 nm and at a reference wavelength of 650 nm. The value of Absorbance 550 nm – Absorbance 650 nm was directly related to the viable cell numbers as shown in equation (1).

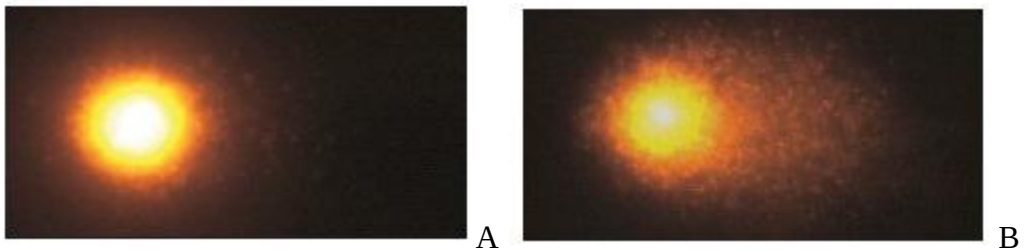
$$\text{Cell viability (\% control)} = \left\{ \frac{\triangle_{(A550\text{-}A650)} \text{ of experimental group}}{\triangle_{(A550\text{-}A650)} \text{ of control group}} \right\} \times 100 \quad \text{——————(1)}$$

4.2.6 Colon carcinogenesis model and genotoxicity measurement

The human colon carcinoma cell line HT29 was used in the model for cell carcinogenesis. The cells were harvested and were exposed to faecal water samples with a concentration of 50% (v/v) to assess the genotoxocity of the latter. The antigenotoxicity of potential protectants (bacterial cells and culture supernatants, and

lactoferrin) was assessed by incubating them with faecal water prior to exposing the HT29 cells to this mixture. The Comet assay was performed to measure the degree of DNA damage (genotoxicity) in the HT29 cells after exposure to the faecal water samples.

The Comet assay is a technique for detecting DNA damage and is also known as single cell gel electrophoresis (SCGE). In this technique, the cell is lysed in an agarose gel; the damage to the DNA is then represented by an increase of DNA fragments that have migrated out of the cell nucleus, using electrophoresis after the DNA has unwound. Under fluorescence this forms a distinctive comet tail, the length and fragment content of which are directly proportional to the amount of DNA damage (**Fig. 4.4**).



A B

Figure 4.4 Comet image showing original cell DNA (A) and damaged DNA (B) spread out by electrophoresis and highlighted using a fluorescent stain

The amount of DNA fragments outside the nucleus form a comet-like tail, the intensity of which is an estimate of the total DNA strand breaks in the cell.

The protocol used was based on that of Venturi *et al.* (1997) and Burns & Rowland (2004). Pre-cultured HT29 cells were harvested from cell culture flasks by centrifugation at 500 x *g* for 5 min after trypsinisation with 0.25% trypsin-EDTA solution for 10 min. The cell pellet was resuspended in complete DMEM medium and separated into a number of vials with a cell density of 2 x 10^5 cells/mL. Mixtures for testing were prepared as shown in **Table 4.2.**

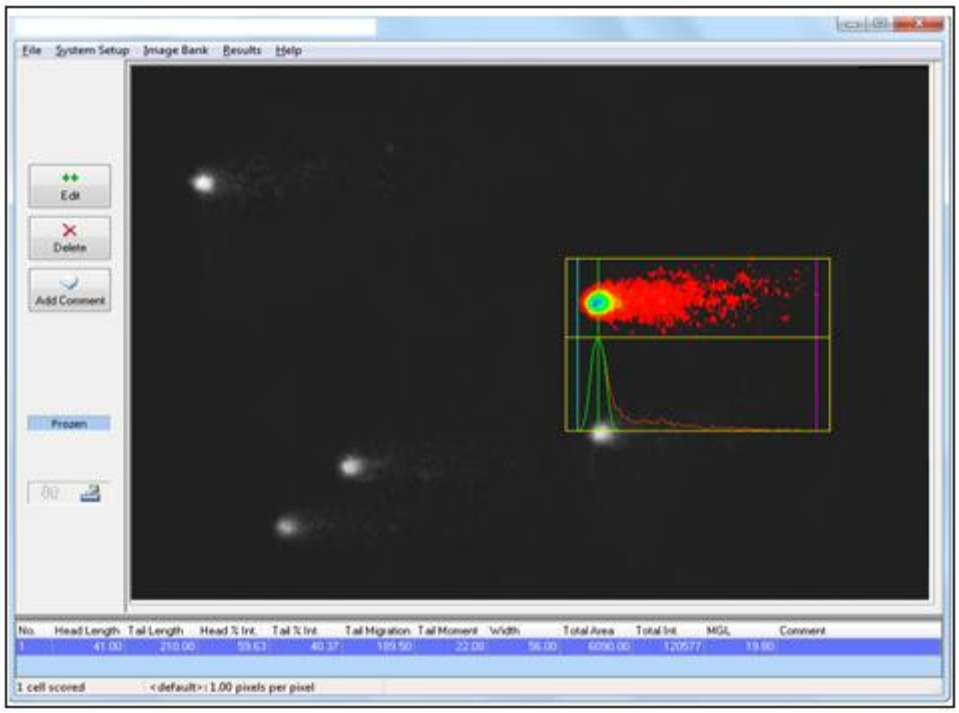
Table 4.2 Treatment preparation for antigenotoxicity assays

Sample	**Mixture rate =1:1**
Negative control (PBS)	PBS: H_2O
Positive control (Hydrogen peroxide)	H_2O_2: H_2O*
Faecal water	Faecal water: H_2O
Bacterial supernatants	Bacterial supernatant: H_2O
Faecal water + bacteria supernatant	Faecal water: bacterial supernatant
Faecal water + viable bacteria or heat-killed cells	Faecal water: bacterial cells**
Faecal water + bovine lactoferrin or bovine serum albumin in certain concentrations	Faecal water: BLf/BSA

* The final concentration of H_2O_2 was 300 µM
** The OD value of bacterial suspension was adjusted to 1.2-1.8 in PBS at the wavelength of 550 nm

The individual mixtures were incubated at 37 °C for 30 min followed by centrifugation at 3,500 x *g* for 5 min to collect the supernatants. The HT29 cells (100 µL, 2 x 10^5 cells/mL) were then exposed to each supernatant (100 µL) for 5 min on ice, and then centrifuged at 500 x *g* for 5 min. The supernatants were discarded and the cell pellet was resuspended in 75 µL of 0.85% (w/v, in PBS) low melting point agarose (LMPA) and maintained in a digital dry bath (Labnet, model D1100, USA) at 40 °C. The suspensions were added to the pre-coated agarose gels (normal agarose; 1% w/v in PBS) on frosted slides and cover slips were added. The gels were chilled at 4 °C for 20 min and a further protective layer of LMPA was added. After the cover slips were removed, the gels on the slides were immersed in lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, and 10 mM Tris) for 1 h at 4 °C and then placed in the buffer (0.001 M Na_2EDTA, 0.3 M NaOH) and the DNA allowed to unwind for 20 min before running at 25 V, 300 mA for 20 min using the gel electrophoresis device (Bio-Rad, Hercules, CA, USA). After electrophoresis, gels were washed in neutralization buffer (0.4 M Tris, pH adjusted to 7.5 using concentrated HCl) three times (4 °C). Gels were stained with 20 µL of ethidium bromide (10 µg/mL) for 20 min in the dark prior to scoring. Images were

analysed at 400× magnification using a fluorescence microscope (Olympus BX51, Japan). The percentage of tail DNA and length of DNA tail migration were recorded for evaluation as the degree of DNA damage using the image analysis software (Olympus Soft Imaging system analySIS LS FIVE). For each slide 100 cells were scored. The degree of DNA damage in HT29 cells was represented by the DNA tail moment with equation (2) and (3) (**Fig. 4.5**).



$$\text{DNA tail moment} = (\text{the length of DNA tail} \times \%\ \text{tail DNA}) \div 100 \quad \text{——(2)}$$

$$\text{DNA tail moment (\% control)} = \left[\frac{\text{DNA tail moment of experimental group}}{\text{DNA tail moment of control group}}\right] \times 100 \quad \text{——(3)}$$

Figure 4.5 Diagram of typical comet and DNA tail moment analysis

4.2.7 Indole determination

Both qualitative and quantitative methods were used to determine indole production by *E. coli* O157:H7 and *L. reuteri* DPC16 cultures, and the indole level in human faecal water samples. The minimal anaerobic culture basal medium (BM) (see ***Appendix I***), containing 2% (w/v) glucose, was used to grow *E. coli* O157:H7 and *L. reuteri* DPC16

individually. Firstly, a biochemical qualitative indole test (Mac Faddin 1980) was performed to determine the ability of the organism to produce indole from tryptophan. Ehrlich's reagent (0.1 g of *para*-dimethylaminobenzaldehyde was dissolved in 9.5 mL of 95% ethanol, then 2.0 mL of concentrated HCl was added and mixed) was prepared and kept at 4 °C before use. The bacteria were incubated anaerobically at 37 °C for 24 h; 50 µL of Ehrlich's reagent was then added to 950 µL of culture. A positive result was shown by the presence of a red color in the surface alcohol layer of the broth, while a negative result appeared yellow. Further, High Performance Liquid Chromatography (HPLC) was employed to determine the indole concentration in tested samples, using a method modified from a recent publication (Nowak *et al.* 2008). Before the experiments, indole was prepared in methanol to a final concentration of 0.5% (w/v). A range of indole concentrations (1.25, 2.5, 5, 10 µg/mL), chosen according to literature data related to the concentration of indole in the colon (Smith & Macfarlane 1996; Nowak & Libudzisz 2006), were prepared in BM. The HPLC apparatus (Ultimate 3000, Dionex, Sunnyvale, CA) was equipped with an UltiMate 3000 Photodiode Array Detector for indole determination. Chromatographic separations were performed by injection of 20 µL of sample onto a Phenomenex C18 (2) Luna column (250 × 4.6 mm i.d.; 5 µm particle size) fitted with a C18 guard column (Phenomenex, Torrance, CA) at a column temperature of 30 °C. The mobile phase contained water and acetonitrile (50:50) and the flow rate was 1 mL/min. Indole was identified by its retention time and spectral data as compared to an authentic standard. Quantification was accomplished at the maximum absorbance detected in the spectrum of indole (at wavelength of 270 nm) by comparing integrated chromatographic peak areas from the samples to peak areas of known amounts of indole. To investigate the ability of *L. reuteri* DPC16 to reduce the indole concentration in human faecal water, stationary bacterial cells from *L. reuteri* DPC16 and *E.coli* cultures were centrifuged and suspended into a heat-inactivated faecal water [these liquids were heated to 85 °C for 20 min using a digital dry bath (AccuBlock™ Digital Dry Bath, Labnet, USA) before use], and incubated anaerobically at 37 °C for 24 h. After 24, incubated faecal water samples were centrifuged, filtered through a 0.2 µm filter and stored at 4 °C prior to analysis.

4.2.8 Statistical analysis

Data were processed on Microsoft Excel spreadsheets. The faecal water cytotoxicity

time series assay and genotoxicity/antigenotoxicity assays were analysed based on curve & regression analysis.

The data for faecal water cytotoxicity to the human colonic cell line-HT29 were fitted to a model of plateau followed by One-phase exponential decay [equation: Y= IF (X<X0, Plateau, Plateau*exp (-k*(X-X0))); plateau until X=X0, then exponential decay to zero].

A "Sigmoidal concentration-response (variable slope)" model was used to plot the best fit curves for the data for genotoxicity/antigenotoxicity assays.

Results were expressed as means ± SD. D'Agostino & Pearson omnibus normality test or graph plotting for normality of residuals was performed, and the significance of differences was determined using global curve fitting analysis or ANOVA and post-hoc analysis using Prism 4.03 software (GraphPad Software, Inc). Differences were considered statistically significant at *P value* <0.05. Graphs were made using Prism 4.03 and Microsoft Excel software.

4.3 Results

4.3.1 Cytotoxicity of human faecal water

The cytotoxicity of sterile human faecal water to HT29 cells was assessed at different concentrations (the range from 5% to 50% v/v) during 5 to 120 min exposure time. A model of plateau followed by One-phase exponential decay was employed to fit all data sets. The plateau principle has widely applicability in pharmacology, physiology, nutrition, biochemistry and system dynamics (Jeong *et al.* 1997; Ayoub & Pin 2013). The cytotoxicity of faecal water to HT29 cells through the accumulation of cell DNA damages (a steady state) to an exponential cell viability decrease. Faecal water genotoxic examination assays need establish the treatment within the period of time X0. The results, shown in **Fig. 4.6**, demonstrate that the data for cell viability of HT29 in response to the faecal water at concentrations of 5% (red), 10% (green) and 50% (purple) (v/v), and the exposure time, fitted to the model [Y= IF (X<X0, Plateau, Plateau*exp (-k*(X-X0)))]. A global fitting was performed to analyse whether the three curves shared the same parameter (plateau) between all the data sets (with a null hypothesis of the

same plateau for all data sets or an alternative hypothesis of a different plateau for each data set). The conclusion of the analysis was the same plateau for all data sets by not rejecting the null hypothesis. The three curves showed that the cytotoxicity of the faecal water correlated with faecal water concentration and exposure time. The cell viability reduced as the time increased beyond an exposure time of X0 [5% of faecal water, X0=50.60 min; 10% of faecal water, X0=35.15 min; and 50% of faecal water, X0=23.94 min). Hence, the time points of 5 min or 15min in the experiment (shorter than each minimum X0 for all concentrations of faecal water tested) could be used as a test condition for the following faecal water genotoxicity study as such a condition for any of the concentrations of 5%, 10% and 50% (v/v) faecal water did not cause major cell death.

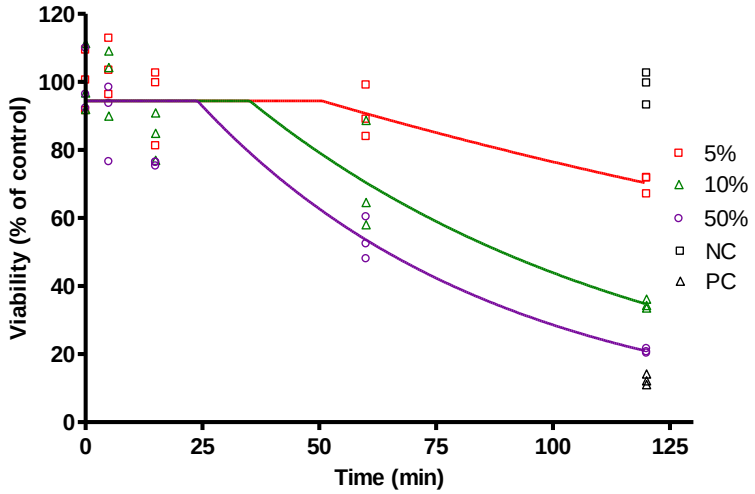


Figure 4.6 Cytotoxicity of faecal water measured using the MTT assay

HT29 cells were incubated for 5 min to 120 min with the sterile faecal water at final concentrations of 5% (red), 10% (green) and 50% (purple) (v/v in complete DMEM) in cell culture medium at 37 °C in a 5% CO_2 humidified atmosphere. The cytotoxicity of faecal water at each concentration and exposure time was determined in triplicate using the MTT assay. [X: exposure time Y: viability (% of negative control). PBS buffer and 300 μM bile salts were negative and positive controls, respectively. The statistical analysis is shown in *Appendix II.*]

4.3.2 Genotoxicity of human faecal water and the effects of potential protectants

An assessment of faecal water genotoxicity on epithelial cell DNA, including its possible variability, was made using the procedure described in **Section 4.2.6.** Three faecal samples were obtained on three different days from a healthy, non-smoking female with no history of gastrointestinal disease, and stored at -20 °C before use. The sterilised faecal water samples, prepared as described in **Section 4.2.3**, at final concentrations of 2.5%, 5%, 10% and 50% (v/v in complete DMEM) were used to

challenge HT29 cells for 5 min on ice. The Comet assay was performed to evaluate the genotoxicity of each faecal water sample in triplicate. Based on the DNA tail moment responses obtained from the experiment, a "Sigmoidal concentration-response (variable slope)" model (Kitchin & Brown 1996) was used to plot the best fit curves for these three faecal samples (FW1: red, FW2: green and FW3: purple) (**Fig. 4.7)**. A global fitting analysis disproved the null hypothesis of one curve for all data sets (*P value* =0.0025) by accepting a different curve for each data set with the different parameters (Top, Bottom, LogEC50 and HillSlope). The conclusion made from this experiment was that the genotoxic effects of faecal water were in a concentration-dependent manner and there was significant variability between the samples.

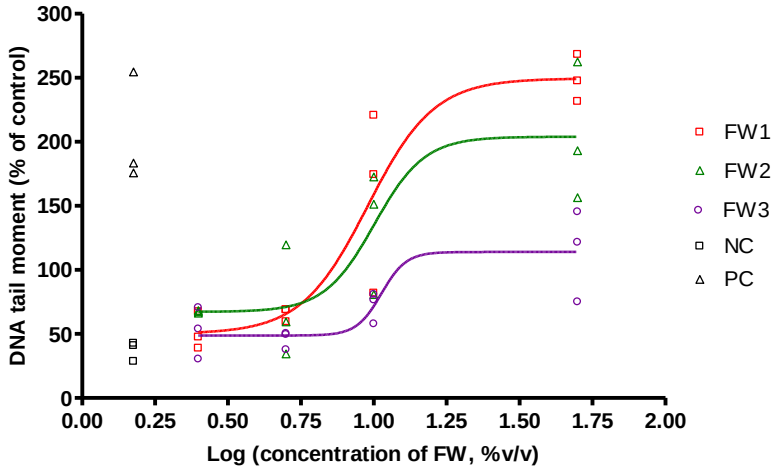


Figure 4.7 Genotoxicity of three faecal water samples

The genotoxicity of three faecal water samples (FW1-red, FW2-green and FW3-purple) at final concentrations of 2.5%, 5%, 10% and 50% was measured on HT29 cells in triplicate using the Comet assay. [X: Log (concentrationof FW) Y: DNA tail moment (% of negative control). PBS buffer and 300 µM hydrogen peroxide were included in the experiment as negative and positive controls, respectively. The statistical analysis is shown in *Appendix II.*]

Next, the hypothesis of antigenotoxic effects of potential protectants was examined using Comet assays. The *L. reuteri* DPC16 strain and two other probiotic bacteria, *L. acidophilus* DPC201 and *B. lactis* HN019 (DR10), and two pathogenic bacteria, *S. aureus* (SA) and *E. coli* O157:H7(EC), were tested for their antigenotoxic properties. After confirmation that the culture supernatants of these bacteria, and fresh MRS and BHI media, have no genotoxic effect themselves (results not shown), the preliminary experimental results indicated that all three probiotic strains reduced 50% (v/v) faecal water (FW)-induced colon cell DNA damage more effectively than did *S. aureus* or *E. coli*. In addition, the culture media BHI and MRS showed no protective effects (**Fig.**

4.8).

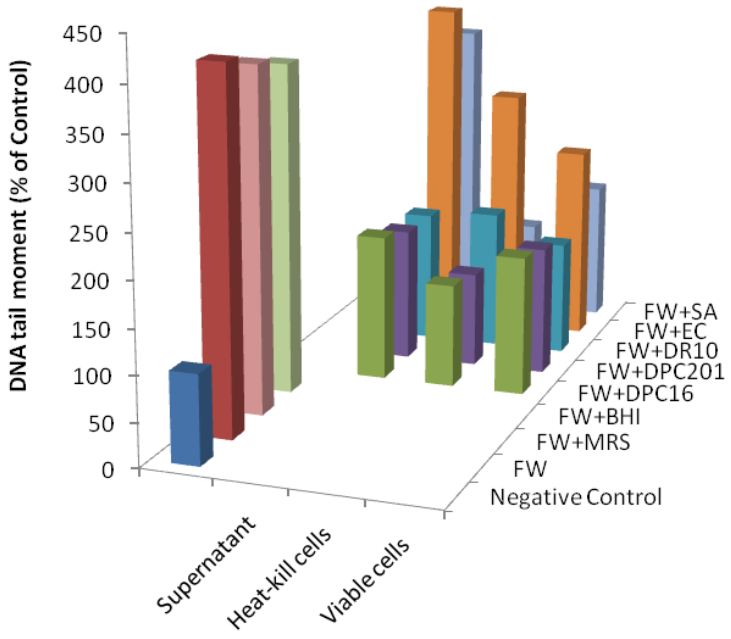
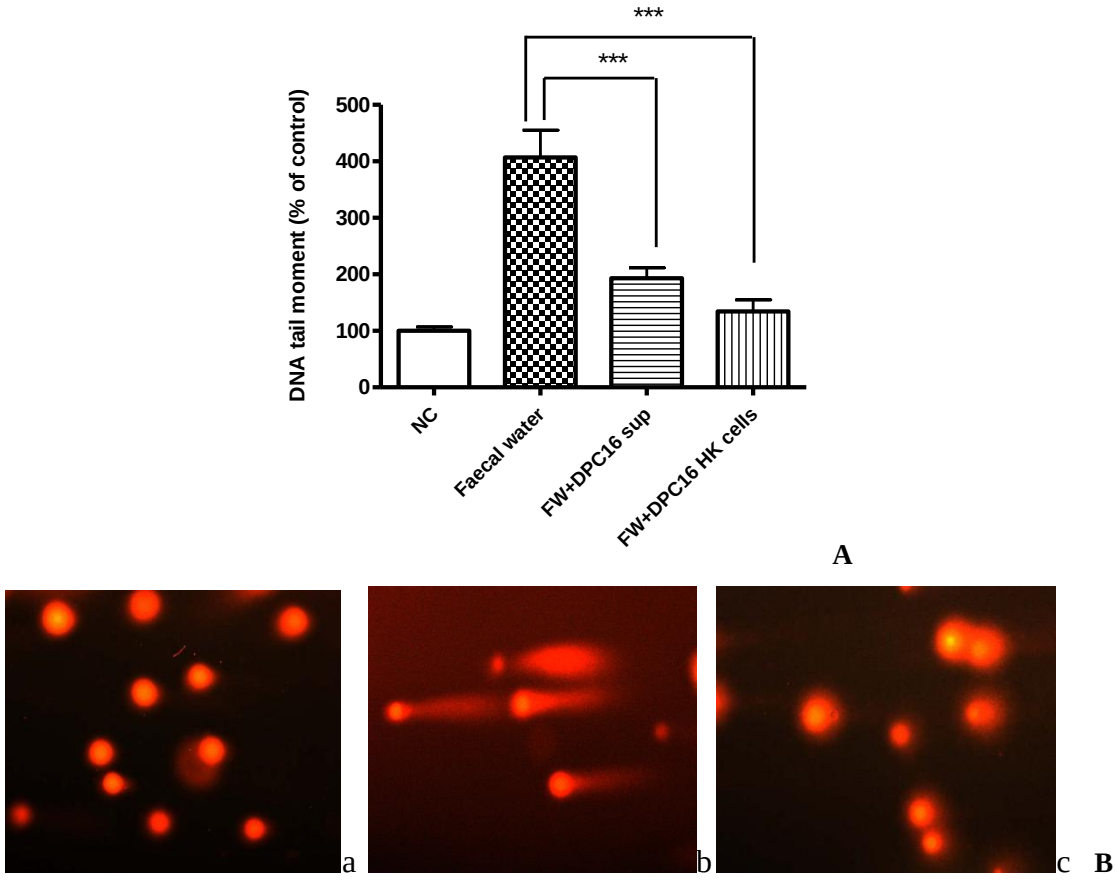


Figure 4.8 The antigenotoxic effects of tested probiotics and pathogens

Three probiotic strains (DPC16, DPC201 and DR10) and two pathogenic strains (ST and EC) were selected to evaluate the protective effects on HT29 DNA damage induced by faecal water. Faecal water (FW) was co-cultured with culture media, viable cells, heat-killed cells or culture supernatants at 37 °C for 30 min. After removal of the cells by centrifugation, the mixtures were used to treat HT29 cells for 5 min on ice. The degree of DNA damage of each treatment was tested in triplicate using Comet assay.

After the pre-assessment, further assays of antigenotoxicity of *L. reuteri* DPC16 were made. The results were analysed by performing a D'Agostino & Pearson omnibus normality test and then a One-Way ANOVA followed with a Tukey's Multiple Comparison Test, and, as shown in **Fig. 4.9,** both culture supernatant and heat-killed cells (comet image shown) of *L. reuteri* DPC16 significantly inhibited 50% (v/v) faecal water-induced DNA damage on HT29 cells (*P value* <0.001).

Together, the data in **Fig. 4.8** indicate that in addition to lactic acid bacteria, other groups of bacteria may mitigate the effects of faecal genotoxins, with the Gram-positive bacteria exhibiting a greater level of protection than the Gram-negative *E. coli.* However, the faecal flora collected from the same donor as provided the faecal water showed no such protective effect (results not shown). The numbers of antigenotoxic bacteria contained in the tested sample might be too few; more experiments are needed to clarify these hypotheses.



a. Negative control (PBS treatment of HT29 cells)
b. Faecal water treatment of HT29 cells
c. Faecal water + DPC16 HK cells, followed by treatment of HT29 cells

Figure 4.9 The antigenotoxic effects of *L. reuteri* DPC16 heat-killed cells and cell-free culture supernatant

The heat-killed cells or supernatant of *L. reuteri* DPC16 were incubated individually with faecal water at 37 °C for 30 min, followed by centrifugation (3,200 x g 10 min) before treatment of HT29 cells for 5 min on ice. The antigenotoxic effects were detected using the Comet assay. [X: treatment and controls Y: DNA tail moment (% of negative control). PBS buffer and faecal water were included in the experiments as negative and positive controls, respectively. The data were collected in triplicate from three experiments , the error bars are SD. Compared to faecal water (FW), both *L. reuteri* DPC16 supernatant and HK cells (B) significantly protected against faecal water-induced colonic epithelial HT29 cell DNA damage (*P value* <0.001, marked as *** in A). The statistical analysis is shown in *Appendix II.*]

4.3.3 Antigenotoxic effects of bovine lactoferrin on faecal water-induced colon epithelial cell DNA damage

Subsequently, the antigenotoxic properties of lactoferrin on 50% (v/v) faecal water-induced DNA damage in HT29 cells were determined, and bovine serum albumin was included as a comparison. A graph plotting confirmed the normality of data residuals. The results were analysed by performing One-Way ANOVA followed with a Tukey's

Multiple Comparison Test. **Fig. 4.10** shows that bovine lactoferrin (BLf) conferred protection from DNA damage in HT29 cells induced by faecal water, in a concentration-dependent manner. At the concentrations of 10mg/mL and 20mg/mL lactoferrin significantly protected faecal water-induced DNA damage in colonic cells (*P value* <0.01). In contrast, the bovine serum albumin (BSA) at the same concentration range, did not show a protective effect. **Fig. 4.11** displays the comet images of BLf treatment compared to BSA treatment, at concentrations of 10 mg/mL.

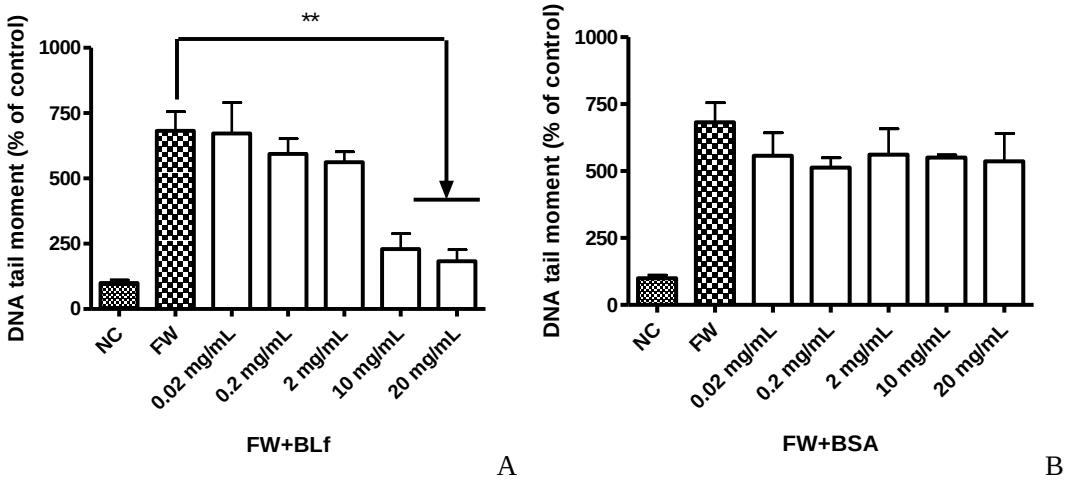


Figure 4.10 Antigenotoxic effects of bovine lactoferrin and bovine serum albumin

Bovine lactoferrin (BLf) and bovine serum albumin (BSA) were incubated with faecal water individually at 37 °C for 30 min prior to exposure to HT29 cells. The protective effects of these proteins at different concentrations against faecal water-induced HT29 cell DNA damage were tested in triplicate using the Comet assay. [X: Log (concentration) Y: DNA tail moment (% of negative control). PBS buffer and faecal water were included in the experiment as negative and positive controls respectively. The statistical analysis is shown in *Appendix II.*]

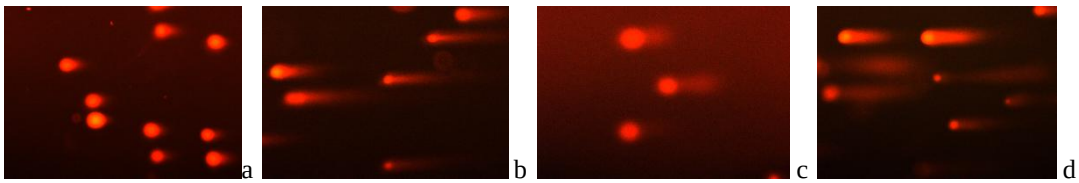


Figure 4.11 Comet images showing the effects of bovine lactoferrin and bovine serum albumin on faecal water-induced HT29 cell DNA damage

Image a, is the negative control (PBS). Image b, is the positive control (faecal water). Image c and d show the colonic epithelial HT29 cell DNA damage caused by faecal water in the presence of BLf and BSA (at a concentration of 10 mg/mL), respectively.

4.3.4 Indole determination

The factors involved in the colon epithelium DNA damage caused by faecal water are complicated. Previous researchers claimed indole as one of the bacterial metabolites responsible for inducing DNA damage in HL-60 cells (Nowak *et al.* (2008). Indole was detected in six faecal water samples, collected from the same individual on different days. The indole levels detected were variable, ranging between 1.1 µg/mL to 11.2 µg/mL in these six samples. However, indole alone in these physiological ranges, did not show a genotoxic effect on HT29 cells in our experimental conditions (results not shown) but some of the faecal water samples containing indole showed genotoxicity in the same experimental conditions. These preliminary results indicated that the possible factors in faecal water that induce DNA damage in HT29 cells may not be only indole, but other undefined components may act synergistically with indole. However, in subsequent experiments, indole was used as a "toxin reduction marker" to study the effects of *L. reuteri* DPC16 on the removal of genotoxins. Firstly, *E. coli* O157:H7 and *L. reuteri* DPC16 were cultured for 24 h in a basal medium in anaerobic conditions. The culture of *E. coli* 0157:H7 showed positive (red ring) while *L. reuteri* DPC16 showed negative for indole production (**Fig. 4.12A**) as described in **Section 4.2.7**. Based on the linear standard curve (**Fig. 4.8**) obtained using the peak areas on HPLC, the chromatograms (wavelength at 270 nm, retention time from 7.5 to 8.0 min) in **Fig. 4.12B** gave further evidence that indole was produced by *E .coli* O157:H7 but not by *L. reuteri* DPC16.

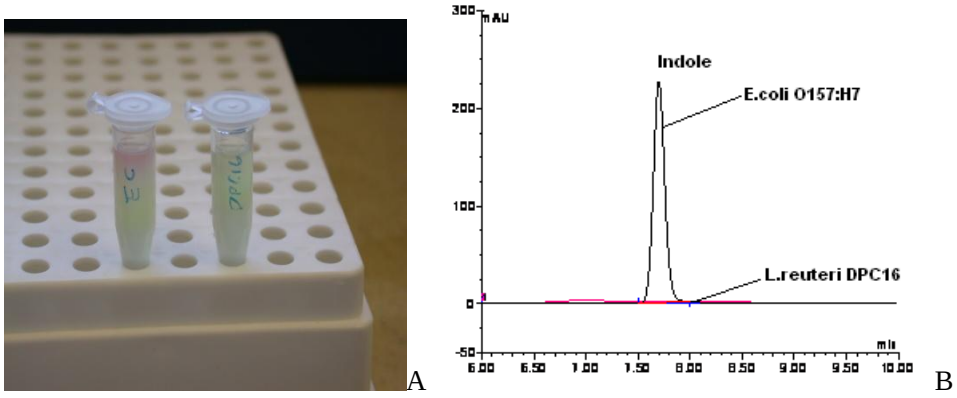


Figure 4.12 Biochemical test and HPLC analysis for indole production

Both detection methods (A) and (B) showing indole production by *E.coli* 0157:H7 but not by *L. reuteri* DPC16

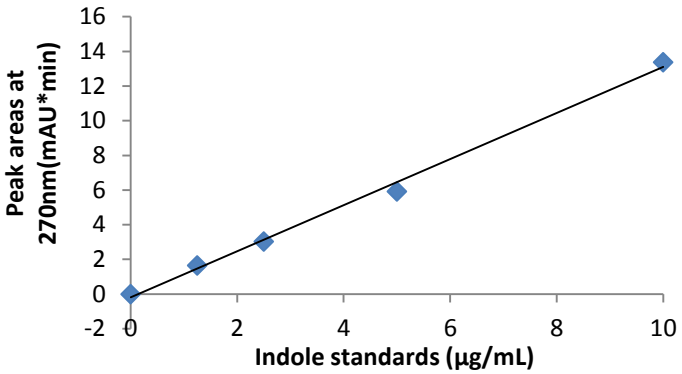


Figure 4.13 Indole standard curve obtained using HPLC analysis

The curve was constructed using a set of known amounts of indole standard at wavelength of 270 nm.

Next, the cells of *L. reuteri* DPC16 and *E.coli* were collected from 1mL of 24h-culture media, and resuspended into 1mL of heat-inactivated faecal water. These faecal water samples were processed in triplicate together with a control (faecal water alone) incubated anaerobically at 37 °C for further 24 h. After 24h incubation, the indole level in each case was determined as described in **Section 4.2.7**.

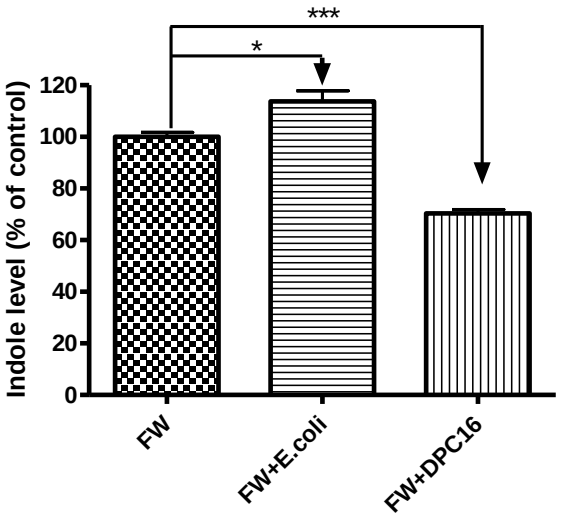


Figure 4.14 The effect of *L. reuteri* DPC16 and *E.coli* on indole concentration in faecal water

The residues of indole were determined in triplicate when *L. reuteri* DPC16 and *E.coli* 0157:H7 were incubated in inactivated human faecal water at 37 °C for 24 h under anaerobic conditions. [X: FW and treated samples Y: Indole level (% of control) after 24h incubation. The error bars are SD. "*" and "***" marked above indicate that the indole were significantly increased by *E.coli* (*P value* <0.05), but significantly reduced by DPC16 (*P value* <0.001). The statistical analysis is shown in *Appendix II.*]

The results (**Fig. 4.14**) demonstrated that the indole in faecal water was significantly reduced by *L. reuteri* DPC16 strain (*P value* <0.001), but not by *E.coli* 0157:H7. In fact, *E.coli* 0157:H7 significantly increased the indole level in the faecal water (*P value* <0.05). Although further experiments need to be performed to validate this finding, the capability of *L. reuteri* DPC16 remove indole from faecal water supports its property of reducing faecal genotoxicity in the colonic epithelium HT29 model described in **Fig 4.9**.

4.4 Discussion

As mentioned in the literature review and in **Section 4.1.2**, in comparison with multiple components in dietary intervention studies to modify colon genotoxicity, the use of probiotics seems to be simple and the accumulated research outcomes appear to be exciting. For example, a number of studies have demonstrated that pre/probiotics can detoxify carcinogens, suppress urinary and faecal mutagenicity (Lidbeck *et al.* 1992; Hayatsu & Hayatsu 1993), suppress tumour formation (Reddy 1998; Burns & Rowland 2004), and reduce the development of aberrant crypts (precancerous) (Onoue *et al.* 1997; Rowland *et al.* 1998; Reddy 1999; Femia *et al.* 2002). Directly related to reduction of faecal genotoxicity, Pool-Zobel's team has demonstrated the ability of a probiotic application of *L. casei* shirota to inhibit DNA damage in the colon of rats exposed to the mutagen *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (Pool-Zobel *et al.* 1993). Further, Goldin *et al*. (1996) observed a lower incidence of colonic tumours in rats fed *L. rhamnosus GG* before, during and after exposure to 1,2-dimethylhydrazine. They concluded that the probiotics inhibit the initiation or early promotion phase of carcinogenesis. Zsivkovits *et al*. (2003) demonstrated that oral administration to rats of *Lactobacillus* and *Bifidobacterium* inhibited DNA damage induced by heterocyclic aromatic amines in the colon mucosa. An important human study has clearly demonstrated that consumption by healthy volunteers of yoghurt (300 g/day) containing probiotic bacteria can modify the genotoxicity of faecal samples, thus providing direct evidence that probiotic consumption may have a beneficial influence on events related to colon cancer in human subjects (Oberreuther-Moschner *et al.* 2004).

In the present study, an *in vitro* colon epithelium carcinogenesis model was established using the colon epithelial HT29 cell line in conjunction with human faecal water. This

model system was used to confirm the genotoxicity of faecal water and, subsequently, to assess the antigenotoxicity of potential protectants such as probiotics and lactoferrin. The extent of genotoxicity was measured using the Comet assay. Initially, the faecal water was assessed for its cytotoxicity to the HT29 cell line, as any such property could result in ambiguous results for genotoxocity. The assessment revealed that under the experimental conditions used to evaluate genotoxicity, cytotoxicity was not a complicating factor. As expected, the results as shown in **Fig. 4.7,** demonstrated that there was considerable variability observed in the intensity of the genotoxicity of faecal water samples prepared on different days from the same individual, which agreed with other studies (Venturi *et al.* 1997; Osswald *et al.* 2000; Woods *et al.* 2002), who claimed both interindividual and intraindividul variability were high in human faecal water samples. However, the interexperimental variability was relatively low. That suggests that selection of some genotoxic faecal water samples and incorporation with either HT29 or Caco-2 cells can create some reasonable reliable bioassay systems (Osswald *et al.* 2000). Using this model approach, the study found that *L. reuteri* DPC16 (viable cells, heat-killed cells and cell-free culture supernatants) and other probiotic bacteria can protect against faecal water-induced cell DNA damage. In contrast, two pathogenic bacteria that were tested in a similar manner showed a weak antigenotoxicity, although some of the cells of these pathogens did show a small protective effect. The results also showed that heat-killed *L. reuteri* DPC16 cells are possibly more efficient antigenotoxic agents than are the corresponding viable cells. Similar findings have been reported by Nowak *et al.* (2008), who showed that dead *Lactobacillus* cell walls adsorb indole better than do viable cells. This suggests that removal of the genotoxins by adsorption to cells is more likely than their inactivation by metabolism. This indicates that the cell wall-related components of these bacteria may be involved in the antigenotoxic effect.

The polysaccharide components of bacterial cell walls and bacterial metabolites of probiotics have been claimed to have antimutagenic properties (Zhang & Ohta 1991; Sreekumar & Hosono 1998; Dinesh & Zhang H. 2008). In the present work, preliminary results using exopolysaccharide (EPS) secreted from *L. reuteri* DPC16 showed some protective effect from colon cell DNA damage induced by genotoxins in human faecal water (results not shown). However, no conclusions can be drawn until further work is conducted. Nevertheless, it is proposed that the cell wall material of *L. reuteri* DPC16 may be the active antigenotoxic agent present in the viable and non-viable cells, and

that EPS, sloughed off from the cell wall, may be the active factor in the cell-free culture supernatants. Such cell wall materials may bind and inactivate faecal genotoxins.

The protective effects of lactoferrin on colon carcinogenesis have been observed in animal models and clinical trials (Sekine *et al.* 1997a; Tsuda *et al.* 2002). However, the detailed molecular mechanisms of action remain unclear. In the present work, bovine lactoferrin has been demonstrated to possess antigenotoxic properties. In contrast, bovine serum albumin, when tested at the same concentration, showed no such properties (**Fig.4.10**). Based on the known binding properties of lactoferrin (Baker & Baker 2009), it is now proposed that lactoferrin may act by somehow binding to, and thus inactivating, the genotoxic chemicals present in faecal water. The experimental data in this chapter support the view that some glycoproteins, including lactoferrin, can detoxify genotoxins, thus providing a possible route to prevent colon carcinogenesis. However, the *in vitro* antigenotoxic activity of lactoferrin was observed at the concentration of 10 mg/mL-20mg/mL, the way to maintain such concentration in the human gut might need a suitable delivery system.

In the last part of this chapter, indole, as a known protein metabolite of *E. coli*, was selected as an example of faecal toxins. It is certain that the colonic carcinogenesis is initiated by cell DNA damage which correlates to faecal gentotoxins (Hirayama & Rafter 2000). Protein fermentation is widely recognized to be detrimental to gut health because protein fermentation results in the production of branched chain fatty acids (BCFA: isobutyric and isovaleric acid) and short chain fatty acids (SCFA: acetic acid, propionic acid and butyric acid) but also of metabolites such as ammonia (NH3), amines, indolic, phenolic and sulphur-containing compounds (Blachier *et al.* 2007). Indole was found inducing DNA damage in HL-60 cells by Nowak *et al.* (2008). However, indole alone, in physiological concentrations, did not induce DNA damage in HT29 cells in the present study. This indicates that indole is not the only toxin in the faecal water. It may or may not act synergistically as a co-genotoxin that causes DNA damage in HT29 cells.

A recent study identified some protein metabolites in faecal water, but their role in gut toxicity is unclear (Windey *et al.* 2012a). There are some known risk factors in processed food, and it is known that some harmful bacteria involved in the activation of

carcinogens. There include metabolites of protein, fat, heme and heterocyclic amines (Windey *et al.* 2012b). However, their role in faecal toxins is unclear. Thus, the exact nature and source of faecal toxins remains unknown, as does the mechanisms of dietary intervention. A preliminary result in the present study showed that *L. reuteri* DPC16 has a capability to remove indole from faecal water. Although it is not clear at this stage that indole removal is due to physical means or metabolism by *L. reuteri* DPC16, it indicates that this probiotic strain can not only inhibit the growth of *E. coli* by producing antibacterial substances (as shown in Chapter 2), but it can also remove its harmful metabolites such as indole. However, this needs further experiments to confirm. Nevertheless, it can be postulated that when *L. reuteri* DPC16, as a probiotic bacterium, is introduced into a host GI tract, it may be able to utilise both glucose and glycerol from food digestion to produce antibacterial substances (SCFA and reuterin) to inhibit the growth of some harmful bacteria such as *E. coli* O157:H7, and it may also react with harmful bacterial metabolites, as shown in **Fig. 4.15**.

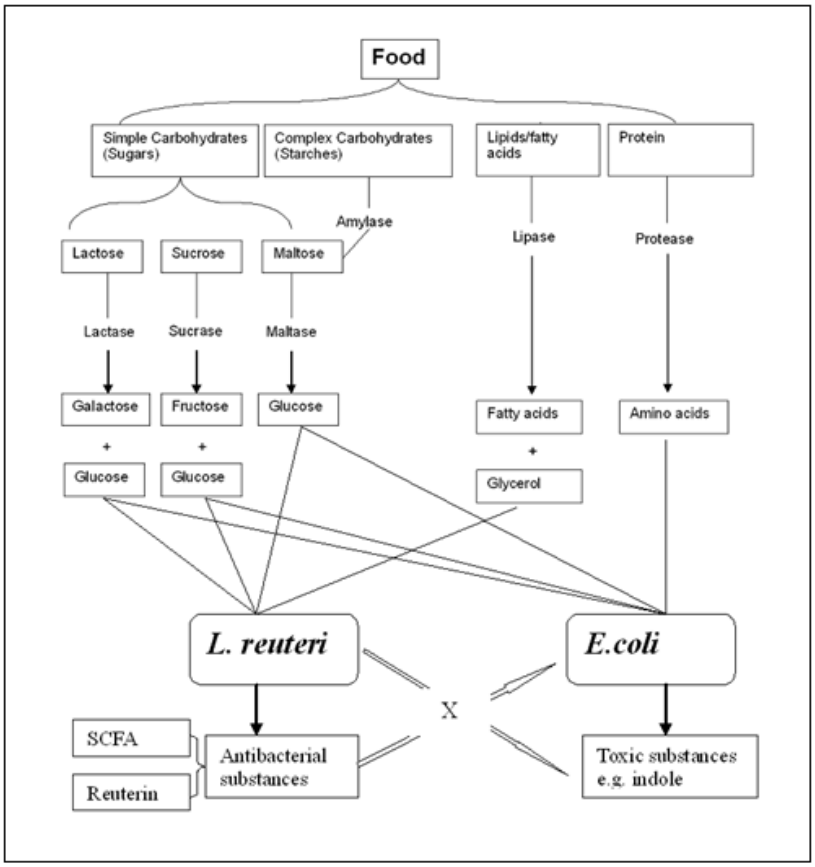


Figure 4.15 A proposal for mechanisms by which *L. reuteri* may counteract *E.coli* in the large intestine

In summary, this *in vitro* study has demonstrated some promising results of antigenotoxicity of *L. reuteri* DPC16 and lactoferrin against faecal water-induced DNA damage in colonic HT29 cells, and further supported the indole removal from faecal water by *L. reuteri* DPC16. However, due to the largely unknown genotoxins contained in the faecal water and the uncontrollable variability in faecal water concentrations and genotoxicity, the *in vitro* conclusions need to be validated by repeatable results using pooled genotoxic faecal water resources from different individuals (may have different toxic components at different concentrations) to make up for the limitation of the *in vitro* settings. Further, although Caco2 and HT29 cells are colon carcinoma derived cell lines, and both are widely used in colonic DNA damage assays, there are some questions regarding the events that occur in the normal gut epithelium are different to these cells, such as are the capacity to activate pro-carcinogens and repair DNA damage. Thus, it must be cautioned that *in vitro* results do not always translate to the *in vivo* situation. To test the potential protectants in real *in vivo* systems would be the best way to support the *in vitro* findings.

Chapter 5

The effects of *L. reuteri* DPC16 and bovine lactoferrin on models of endotoxin- induced intestinal inflammation

5.1 Introduction

It has been shown that abnormal intestinal immunological function, resulting from genetic susceptibility and certain environmental factors, contributes significantly to the inflammatory process of intestinal diseases (Oliva-Hemker & Fiocchi 2002). Abnormal interactions between the intestinal mucosal immune system and microbiota in the intestinal tract results in changed immunological function and triggers the inflammatory response (Feillet & Bach 2004). Inflammatory bowel disease (IBD) is a chronic and recurrent inflammation generally affecting the colon or the small intestine. Evidence has been provided showing that the gut microflora play an important role in initiating and maintaining the mucosal inflammatory response in IBD (Bell *et al.* 2004; Darfeuille-Michaud *et al.* 2004). There is accumulating clinical evidence to suggest that intestinal dysbiosis and enteric bacteria-mediated mucosal inflammation may be associated with another common, debilitating gastrointestinal disorder, Irritable Bowel Syndrome (IBS) (Treem *et al.* 1996; Chadwick *et al.* 2002) .

5.1.1 Endotoxin –lipopolysaccharide (LPS)

Endotoxins are toxins associated with certain bacteria. The prototypical examples of endotoxin, found on the outer layer of the outer membrane of various Gram-negative bacteria, are lipopolysaccharide (LPS) and lipo-oligo-saccharide (LOS), which contribute to the ability of the bacteria to cause disease (Tzeng *et al.* 2002). Endotoxins are approximately 10 kDa in size but can form large aggregates up to 1000 kDa. LPS consists of a lipid region, termed lipid A, covalently linked to a polysaccharide region. The polysaccharide region is composed of three separate domains; an inner core, an outer core, and an O-specific chain or O-polysaccharide. Each domain has distinct structural and functional properties. This type of architecture is found in a number of

bacterial genera, such as *Salmonella*, *Escherichia*, *Aeromonas* and *Rhizobium*. However, some Gram-negative bacteria, such as *Bordetella pertussis*, *Neisseria meningitidi*s and *Campylobacter jejuni* have LPS without an O-polysaccharide but with an extended core region, termed lipooligosaccharide.

Lipid A in LPS is responsible for the toxic effects, while the polysaccharide chain is highly variable amongst different bacteria. LPS is a prototypical endotoxin because it binds to the CD14/TLR4/MD2 receptor complex (Ouburg *et al.* 2005) of target cells (Hao *et al.* 2009) (**Fig. 5.1**), which promotes the secretion of pro-inflammatory cytokines in many cell types, but especially in macrophages. In immunology, the term "LPS challenge" refers to the process of exposing a subject to an LPS which may act as a toxin. Thus, LPS, as an endotoxin, induces a strong response from normal animal immune systems. Injection of a small amount of endotoxin into human volunteers produces fever, a lowering of blood pressure, and activation of inflammation and intravascular coagulation (Slofstra *et al.* 2006).

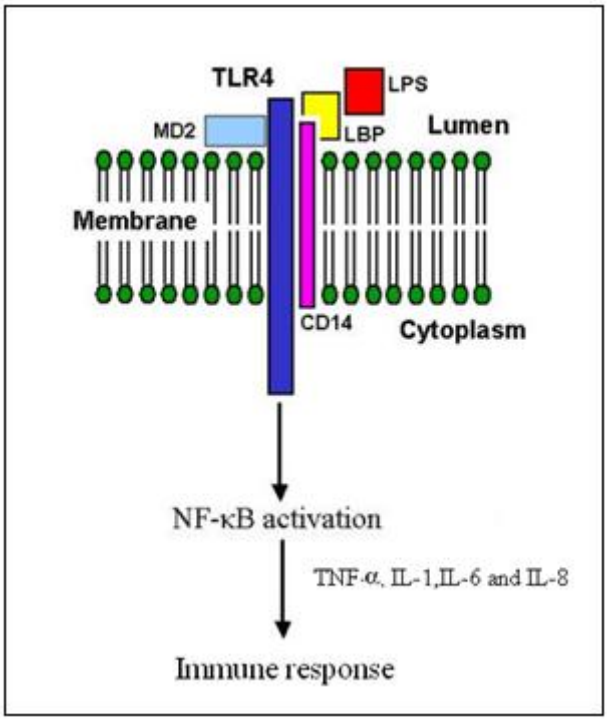


Figure 5.1 CD14/TLR4/MD2 receptor complex

This receptor complex of the innate immune system recognizes LPS and induces NF-**κ**B associated immune responses including the release of cytokines (TNF-**α**, IL-1, IL-6, and IL-8) to trigger an immune response. (TLR4=Toll-Like Receptor 4; LBP=LPS Binding Protein; NF-**κ**B=Nuclear Factor **κ**B) (modified from Ouburg *et al.* 2005).

5.1.2 Lipopolysaccharide-induced inflammatory response

During a bacterial infection, chemical signals attract phagocytes (monocytes, macrophages, neutrophils, dendritic cells and mast cells) to places where the pathogen has invaded and the receptors on the phagocytes' surface will bind the bacteria and engulf them. Some phagocytes generate oxidants or nitric oxide (Fang 2004) to kill invading bacteria, as part of the human immune response. After phagocytosis, macrophages and dendritic cells can also participate in antigen presentation, this process is important in building immunity. Nitric oxide, when secreted as part of an immune response, is a free radical and is toxic to bacteria, the mechanism of which includes DNA damage (Wink *et al.* 1991; Nguyen *et al.* 1992; Coppa *et al.* 2006) and degradation of iron sulfur centers into iron ions and iron-nitrosyl compounds (Hibbs *et al.* 1988). Phagocytes are armed with inducible nitric oxide synthase (iNOS), which is activated by interferon gamma (IFN-γ) or by tumor necrosis factor (TNF) (Gorczyniski & Stanley 1999). Transforming growth factor-beta (TGF-β) provides a strong inhibitory signal to iNOS, while interleukin-4 (IL-4) and interleukin-10 (IL-10) provide weak inhibitory signals. In this way the immune system may regulate the arsenal of phagocytes that play a role in inflammation and immune responses. However, under certain conditions, the inflammation response can backfire: severe infection (sepsis) causes excess production of nitric oxide by phagocytes, leading to vasodilation, probably one of the main causes of hypotension in sepsis. The cytokines IFN-γ and TNF-α are found at high concentrations in intestinal mucosa that are involved in inflammatory bowel diseases (MacDonald *et al.* 1990; Fais *et al.* 1994) and they also effect decreasing epithelial barrier function *in vitro* (Madara & Stafford 1989; Mullin *et al.* 1992). Thus, a key step in the pathogenesis of inflammatory bowel disease may be the activation of IFN-γ and TNF-α *via* the immune response to bacterial antigens, leading to intestinal barrier dysfunction (Clayburgh *et al.* 2004). Then, in the inflammatory bowel disease state, the dysregulated mucosal barrier allows an increased uptake of antigens and pro-inflammatory molecules, including bacterial products and endotoxins, into the *lamina propria*, followed by activation of immune cells in the *lamina propria*, secretion of pro-inflammatory cytokines and products of reactive oxygen metabolites, and further mucosal damage (Kennedy *et al.* 2002). In this way, a vicious cycle is created in which barrier dysfunction allows further leakage of lumenal contents, thereby triggering an immune response that can in turn feedback on the intestinal barrier to promote further leakiness (Clayburgh *et al.* 2004). Although

inflammatory mediators produced by activated inflammatory cells play infection-fighting roles for various defence mechanisms, they can also raise the cancer risk in chronic inflammatory diseases (Hussain *et al.* 2000; Nair *et al.* 2006). It has been suggested that, when the repair system or pro-apoptosis fails, the toxic immune mediators that are over-produced in a chronic inflammation state may be produced in increasingly large amounts, possibly leading to the development of cancer (Ueno *et al.* 2006).

Animal studies and clinical trials have indicated the beneficial effects of probiotics in several intestinal diseases including inflammatory bowel disease and chronic inflammation-associated colon carcinogenesis (Perdigon *et al.* 2001; Marteau *et al.* 2002). Recently, there have been indications that the ingestion of some bioactives, such as lactoferrin, may exert antibacterial and antiviral activities in the intestine, in part through a direct effect on pathogens as shown in Chapter 3 of this thesis, but possibly also *via* an effect on mucosal immune function (Legrand *et al.* 2008) *via* an endotoxin-neutralizing capability (Zhang *et al.* 1999).

5.1.3 Aim of this chapter

The aim of this chapter was to identify the mechanisms by which probiotics and lactoferrin may interact with the immune response and bacterial endotoxins. The cellular model approach was considered ideal for this study. A number of studies have used the murine macrophage RAW 264.7 cell line to investigate inflammation and anti-inflammation mechanisms (Taechowisan *et al.* 2007; Chon *et al.* 2009). Macrophages are tissue-based phagocytes derived from monocytes; they can be activated by microbial components, such as endotoxin, lipopolysaccharides (LPS) and lipoteichoic acids (LTA). Activated macrophages phagocytose micro-organisms, secrete pro-inflammatory cytokines and nitric oxide (NO), present antigens to T helper cells, and contribute to immune defence mechanisms of the host in response to external stimuli. Therefore, an immune model was used employing LPS-activated murine macrophage RAW 264.7 cells to create an *in vitro* inflammation status. This allowed assessment of the effects of probiotic bacteria and lactoferrin by measuring the changes in inflammatory-associated parameters (cytokines and NO). However, since the results from one murine cell model may not be sufficient, a second immune model was employed using the human acute

monocytic leukemia THP-1 cell line. This cell line can be differentiated into macrophage-like cells (Cheng *et al.* 2005) and further manipulated into an inflammatory model after stimulation using LPS. This human immune cellular model system allowed further exploration of the target sites of the probiotics and lactoferrin, and their involvement in the anti-inflammatory process. Since literature reports have indicated that chronic inflammation can result in vascular damage and intestinal mucosal barrier dysfunction, especially in Inflammatory Bowel Disease and Irritable Bowel Syndrome, a co-culture cell model was used to imitate inflammation events in the gastrointestinal tract, by co-cultivation of human intestinal enterocyte-like Caco-2 cells and human monocyte THP-1 cells in separate compartments of a transwell culture system. This allowed investigation of the potential of probiotics and lactoferrin to prevent the intestinal barrier dysfunction caused by inflammation-associated events.

5.2 Materials and Methods

5.2.1 Chemicals

Lipopolysaccharide (LPS, from *Escherichia coli* O127:B8), phorbol myristrate acetate (PMA), camptothecin [$(CH_3)_2SO$], sulphanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride were purchased from Sigma (Life Sciences, USA).

LPS was diluted in sterile distilled water to give 1 mg/mL. The solution was aliquoted into 50 µL volumes and stored at -20 °C.

A stock solution of PMA at 1 mg/mL (162 µM) was made up in DMSO, and stored as 20 µL aliquots at -20 °C.

Camptothecin was dissolved in DMSO to obtain a stock solution of 10 mM for cytotoxicity analysis. The stock solution was stored at -20 °C.

5.2.2 Bacterial supernatants and cell preparations

The probiotic cell-free supernatants and cells were prepared as described in **Section 4.2.2**.

5.2.3 Human and murine immune cell lines and culture conditions

Murine macrophage RAW 264.7 cell line was obtained from Bioactive Research New Zealand Ltd. (Auckland, New Zealand). Human acute monocytic leukemia THP-1 cell line was provided by the New Zealand Institute for Plant & Food Research Ltd. These two cell lines were cultured in RPMI Medium1640 (see ***Appendix I***), supplemented with 10% (v/v) fetal bovine serum (heat inactivated 60 °C, 45 min), 100 μg/mL streptomycin and 100 U/mL penicillin, at 37 °C in a 5% CO_2 humidified atmosphere (Cell culture CO_2 incubator, *SANYO*, Japan).

5.2.4 Human colon adenocarcinoma Caco-2 cell line and culture conditions

The human colon adenocarcinoma Caco-2 cell line was obtained from the New Zealand Institute for Plant & Food Research Ltd. The cells (used at passages between 35 and 65) were subcultured in complete Minimum Essential Medium (1x) (MEM)(see ***Appendix I***) as described in **Section 2.2.9**.

5.2.5 Inflammatory cellular models

Both murine and human cellular models were developed to study the inflammatory responses when the cells were exposed to LPS or other pro-inflammatory agents (after non-cytotoxic concentrations had been identified). The murine macrophage RAW 264.7 cell model was used to study production of nitric oxide using the Griess reaction assay, while production of the pro-/anti-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin 6 (IL-6) and interleukin 10 (IL-10) was measured using flow cytometry. The relationships between nitric oxide production and cell growth (MTT assay), in the presence of LPS and in combination with other agents were evaluated. The human monocyte THP-1 cell model was used to confirm the inflammation responses to LPS in the presence or absence of other agents. Release of the key pro-inflammatory cytokine, TNF-α, was measured by enzyme-linked immunosorbent assay **(**ELISA).

5.2.6 Intracellular cytokine detection (Flow Cytometry)

Pre-cultured RAW 264.7 cells were seeded in 12-well plates at a concentration of $1x10^5$ cells/mL in 2 mL of complete RPMI medium, and incubated for 24 h to allow adhesion

of the cells to the surface of the wells. The LPS (1.0 µg/mL) and PBS buffer additions acted as positive and negative controls, respectively. After addition of the materials under test, the plate was incubated at 37 °C for 16-18 h in a 5% CO_2 humidified incubator. Twenty µL (500 µg/mL) of protein transport inhibitor, GolgiPlug™ (BD, Biosciences, San Diego, CA), containing Brefeldin A (as a perturbant of intracellular traffic, which reversibly disassembles the Golgi complex), was added to each well 4 h before the measurement of the cytokines. After the cells were harvested by scraping and centrifugation (130 x *g* for 5 min) at room temperature, the cells were fixed in 100 µL Cytofix/ Cytoperm buffer (BD, Biosciences, San Diego, CA) at 4 °C for 15 min, and then labelled with different fluorescent cytokine antibodies prepared as individual master-mix, including 0.2 mg/mL of PE-conjugated rat anti-mouse IL-6 (Biosciences, San Diego, CA), 0.5 mg/mL of PE Rat anti-mouse IL-10 (Biosciences, San Diego, CA), 0.2 mg/mL of FITC labelled anti-mouse IFN-γ (Biosciences, San Diego, CA) and 0.2 mg/mL of FITC rat anti-mouse TNF (Biosciences, San Diego, CA) in the dark at 4 °C for 30 min. The labelled cells were then washed once with 1x Perm/Wash buffer (BD, Biosciences, San Diego, CA), and resuspended in 1x Perm/Wash buffer. Finally, the intracellular cytokines were detected using Flow Cytometry (BD, LSDII, the University of Auckland).

5.2.7 Nitric oxide determination (Griess reaction assay)

As described above, RAW 264.7 cells, at a concentration of $1x10^5$ cells/mL, were seeded in a 96-well cell culture plate at 100 µL/well. After 24 h incubation, the cells were challenged with LPS at different concentrations (0.125 µg/mL, 0.25 µg/mL, 0.5 µg/mL and 1.0 µg/mL) for a further 24 h. The culture supernatants from the optimised level of LPS treatment, in the presence and absence of other agents, were collected and analysed for nitric oxide using the Griess reaction. Briefly, 100 µL of supernatant was mixed with 50 µL of 1.0% sulphanilamide in HCl and 50 µL of 0.12% N-(1-naphthyl)-ethylenediamine dihydrochloride for 15 min at room temperature. The absorbance was measured at 550 nm using a plate reader (Microplate Reader Model ∑960, Metertech Inc., Taiwan). Nitrite concentrations were calculated on the basis of a standard curve prepared using sodium nitrite (BDH, Biolab, New Zealand).

5.2.8 Cell proliferation assessment (MTT assay)

The proliferation of murine macrophage RAW 264.7 cells was monitored after challenge with LPS and other agents using an MTT Assay Kit (MTT cytotoxicity kit, Roche, USA). Pre-cultured RAW 264.7 cells (10^6 cells/mL in RPMI Medium 1640) were harvested from cell culture flasks by scraping and centrifugation (130 x *g* for 5 min). The cells, at a concentration of $1x10^5$ cells/mL, were seeded in a 96-well cell culture plate at 100 µL/well and incubated for 24 h. After addition of the test reagents, the plate was incubated for another 24 h. Thereafter, the MTT assay was performed according to the manufacturer's instructions, by measuring the absorbance at 550 nm and 650 nm using a microplate reader (Microplate Reader Model ∑960, Metertech Inc., Taiwan).

5.2.9 TNF-α analysis (ELISA)

Human acute monocytic leukemia THP-1 cells were incubated with 10 nM PMA in complete RPMI medium diluted from the PMA stock as described in **Section 5.2.1** to allow cell differentiation to occur (approximately after 48 h and entirely completed in 72 h). The differentiated THP-1 cells were stimulated with 100 ng/mL of LPS, in the presence and absence of test materials, for 4 h. The supernatants were then collected for TNF-α analysis using the ELISA method. The standard curve was made by preparing TNF-α concentrations up to 500 pg/mL. The absorbance was measured at 450 nm and 620 nm using an ELISA plate reader (Model Multiskan EX, Thermo Electron Corporation, Vantaa, Finland).

5.2.10 Intestinal epithelium/immune co-culture model (Caco-2/THP-1)

An intestinal epithelium/immune co-culture model (Caco-2/THP-1) was established to imitate inflammation events in the gastrointestinal tract. This model system allowed co-cultivation of human enterocyte-like Caco-2 cells (in the apical compartment) and human acute monocytic leukemia THP-1 cells (in the basolateral compartment) in separate compartments of a transwell culture (the transwell system including an insert with a 0.4 µM pore transparent membrane and a companion 24-well plate, BD, Falcon, supplied by Becton Dickinson Ltd, Auckland, New Zealand) to simulate intestinal inflammation-associated events and to investigate the protective potential of probiotics and bovine lactoferrin. The system is illustrated in **Fig. 5.2**.

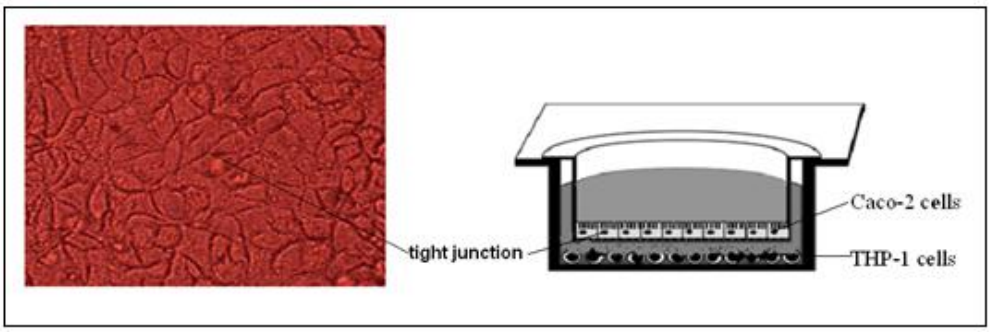


Figure 5.2 Caco-2/THP-1 co-culture system in separate compartments of a transwell culture

The development of the system is outlined in the flow chart **(Fig. 5.3)**:

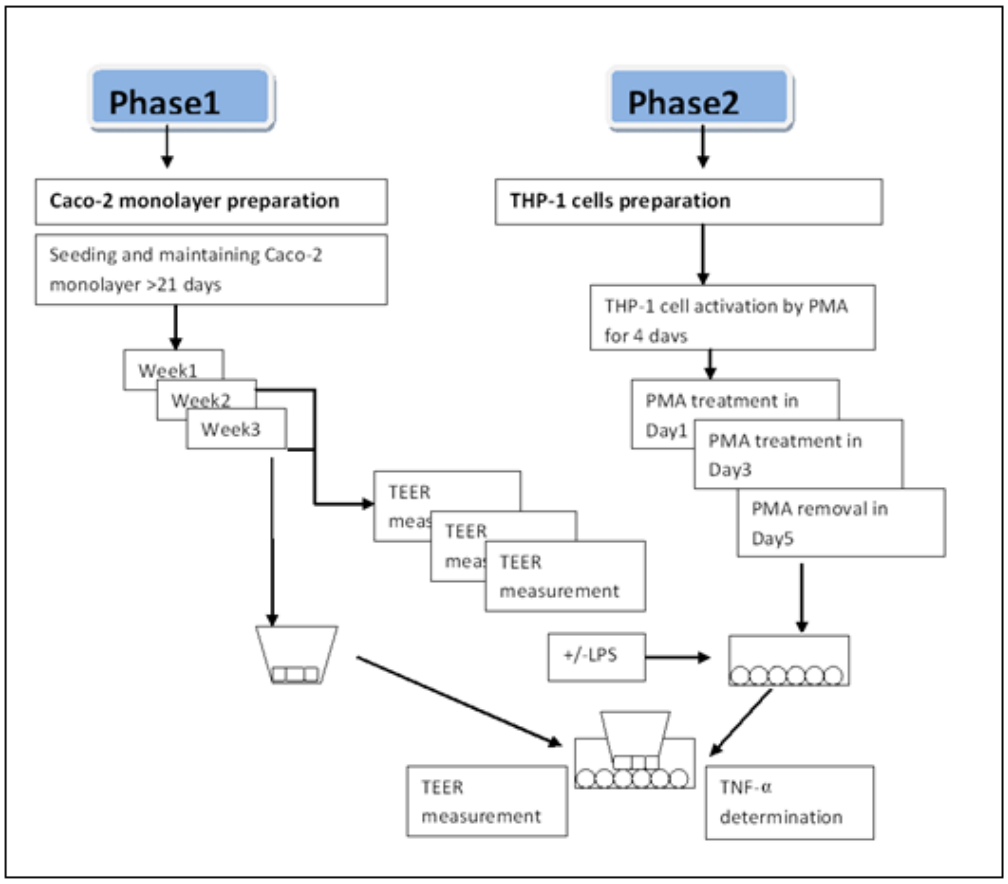


Figure 5.3 Caco-2/THP1 co-culture system work flow chart

(TEER: transepithelial electrical resistance)

At initiation, $2.3x10^5$ cells/mL of human tumor adenocarcinoma Caco-2 cells (used at passages between 35 and 65) were seeded into the transwell inserts with a medium volume of 0.35 mL/insert. The growth medium was switched from complete MEM to

complete DMEM [supplemented with 10% (v/v) fetal bovine serum (heat inactivated 60 °C, 45 min), 100 μg/mL streptomycin and 100 U/mL penicillin] (see ***Appendix I***), during the first few cultivation days and continually maintained using complete DMEM. A medium change was conducted every other day for 21 days to form an epithelial monolayer. The monolayer tight junction was monitored by measuring the transepithelial electrical resistance (TEER) values from day 14 to day 21 using an EndOhm-6 electrode chamber connected to a Millicell-ERS electrical resistance meter (World Precision Instrument Inc., USA) **(Fig. 5.4)**. A TEER value > 150 $\Omega \cdot cm^2$ was considered adequate for this study. Concurrently, the THP-1 cells, at a concentration of $1.5x10^5$ cells/mL, were seeded in a companion 24-well plate with a medium volume of 1 mL/well. Different concentrations of PMA were added as cell stimulators, to differentiate the cells for 96 h, to achieve high TNF-α production. The growth medium for THP-1 cells was switched from RPMI to DMEM prior to the experiment.

At the start of experiments, the Caco-2 monolayer inserts with 0.35 mL/insert of DMEM were transferred to the wells containing the activated THP-1 cells with 0.9 mL/well of DMEM, and appropriate treatments were applied to the apical and basolateral media. The cells were then cultured together, and TEER values were measured after 48 h cultivation to assess any interruption to the Caco-2 monolayer tight junction.

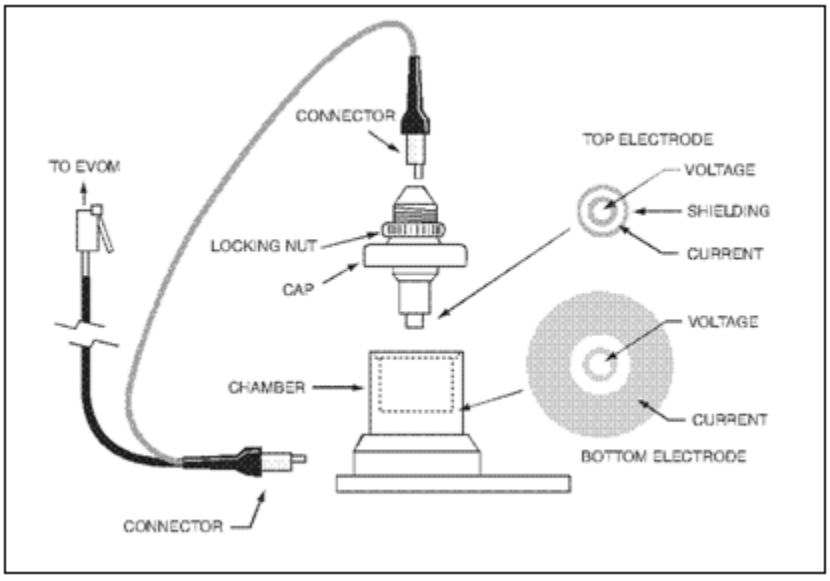


Figure 5.4 EndOhm-6 chamber for measurement of transepithelial electrical resistance

5.2.11 Cell death determination (LDH assay)

Lactate dehydrogenase (LDH) is a stable enzyme, present in all cell types, that is rapidly released into the cell culture medium upon damage to the plasma membrane. The LDH assay was used to detect cell death and cell lysis, based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the medium. The enzyme activity was measured using an LDH assay kit (Roche Ltd, Auckland, New Zealand), according to the manufacturer's instructions.

5.2.12 Statistical analysis

Data were processed on Microsoft Excel spreadsheets. The concentration responses obtained from nitric oxide determination, cell proliferation and TNF-α ELISA assays were analysed based on a "Sigmoidal concentration-response (variable slope)" model. Results are expressed as means ± SD. A D'Agostino & Pearson omnibus normality test, or graph plotting for normality of residuals, was performed, and the significance of differences was determined by global curve fitting analysis or ANOVA and post-hoc analysis using Prism 4.03 software (GraphPad Software, Inc). Differences were considered statistically significant at *P value* <0.05. Graphs were made using Prism 4.03.

5.3 Results

5.3.1 Establishment of the RAW 264.7 cell model system

To establish a murine inflammation system, the murine macrophage RAW 264.7 cell line was grown in the complete RPMI Medium1640 as described in **Section 5.2.3**. The cell number $1x10^5$ cells/mL was selected as the RAW 264.7 cell seeding concentration, while a suitable concentration of LPS of 1.0 µg/mL was selected to challenge the RAW cells. Hence, the samples were tested in this murine *in vitro* system by determination of intracellular cytokine profile, nitric oxide production, and cell proliferation as described in **Section 5.2.6, Section 5.2.7** and **Section 5.2.8,** to assess the degree of inflammation or anti-inflammatory effects using 1.0 µg/mL of LPS as a positive control or an inflammation inducer.

5.3.1.1 Effects of *L. reuteri* DPC16 and lactoferrin on the cytokine profile of RAW 264.7 cells

As described in **Section 5.1.1 and Section 5.1.2**, LPS from Gram-negative bacteria is recognised as being involved in inflammatory events, and two pro-inflammatory cytokines, TNF-α and IFN-γ, are produced during inflammation (Gorczyniski & Stanley 1999). Conversely, TGF-β, IL-4 and IL-10 provide inhibitory signals to stop the continuing inflammation process but the roles of probiotics and bioactives, e.g. lactoferrin, in inflammation-linked disease are unclear. An experiment was now performed to investigate the effects of probiotic strain DPC16 cell-free supernatant and bovine lactoferrin on the production of different cytokines by RAW 264.7 cells, and to compare the effects with that of LPS. The cytokines that were measured in the experiment were three pro-inflammatory cytokines, TNF-α, INF-γ, and IL6, and one anti-inflammatory cytokine, IL-10. The Flow cytometry dot plot graphs (**Fig. 5.5)** and the cytokine profile histogram (**Fig. 5.6)** show that LPS at the concentration of 1 µg/mL and *L. reuteri* DPC16 supernatant (12.5% v/v, pH 7.0) demonstrated a similar inflammatory response; these treatments induced much more pro-inflammatory cytokine TNF-α production (range 7- 20 fold) than did the negative control. In contrast, bovine lactoferrin (10 mg/mL) created a different cytokine profile, showing no induction of pro-inflammatory cytokine TNF-α production. However, no definitive conclusions can be made until further experimental data are collected to allow a full analysis.

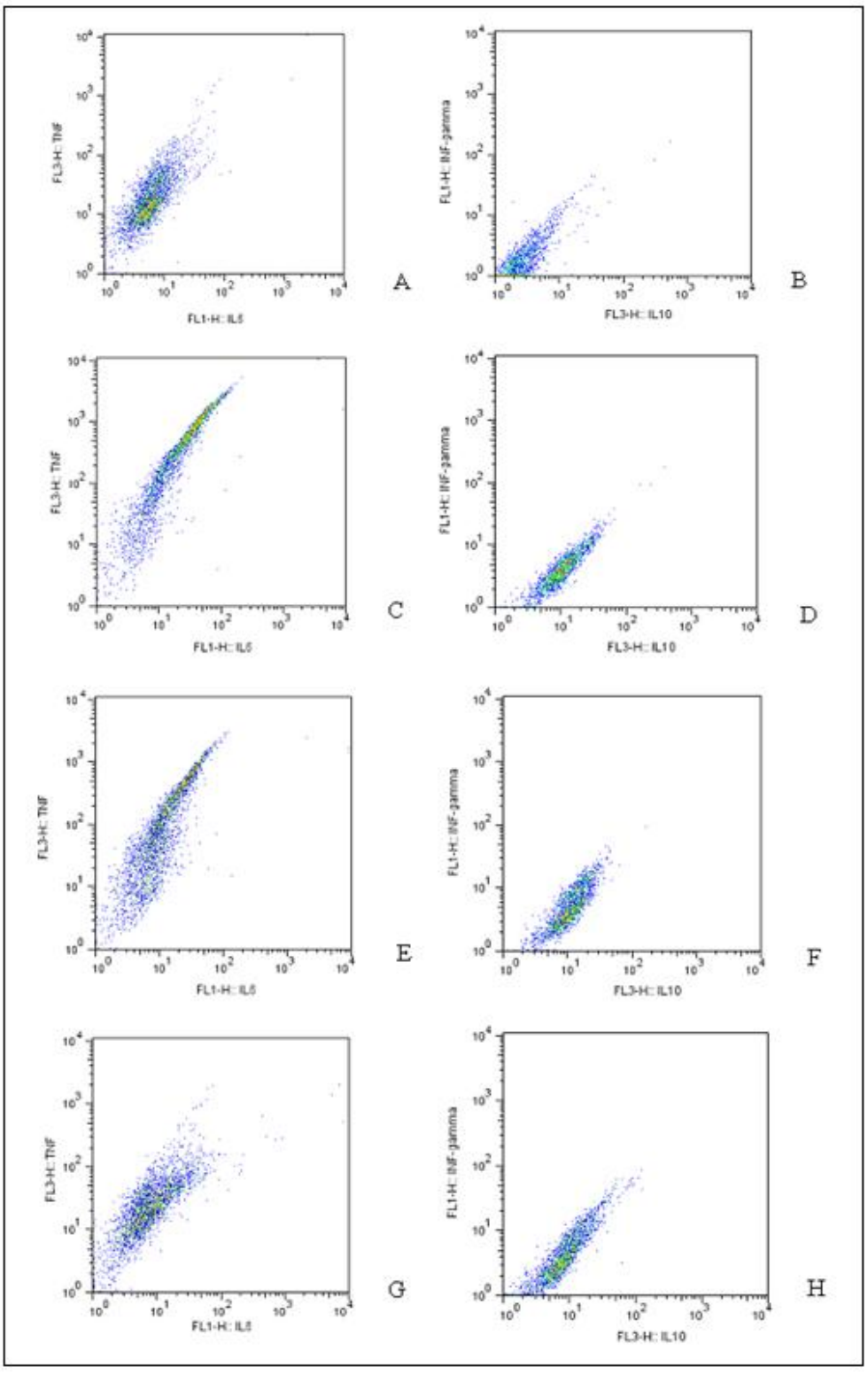


Figure 5.5 Flow cytometry dot plot graphs

The two-parameter flow cytometric dot plot graphs showing the macrophage cytokine profiles (TNF-α, IL-6, IFN-γ and IL-10) from the negative control (A and B), positive control (1 µg/mL of LPS, C and D), in the presence of 12.5 % (v/v) *L. reuteri* DPC16 supernatant (E and F) and 10 mg/mL of bovine lactoferrin (G and H) after 18 h incubation at 37 °C.

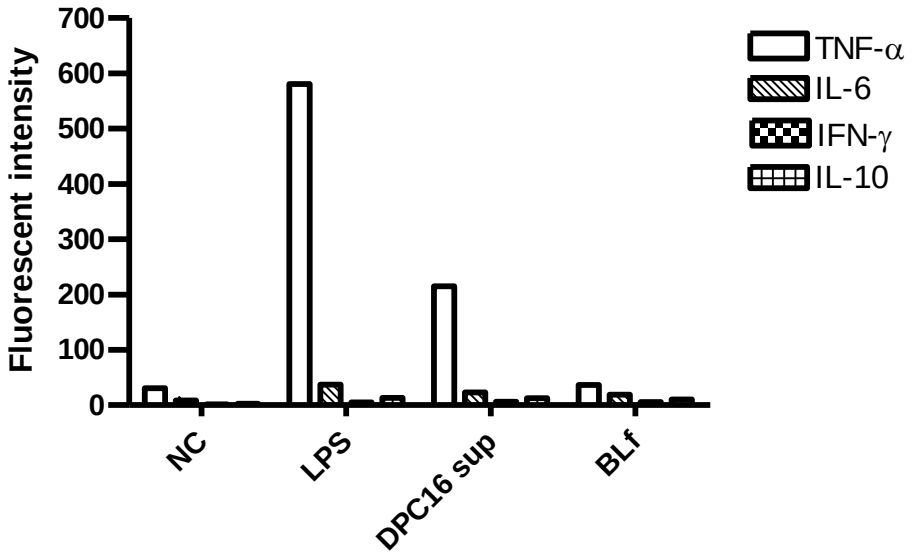


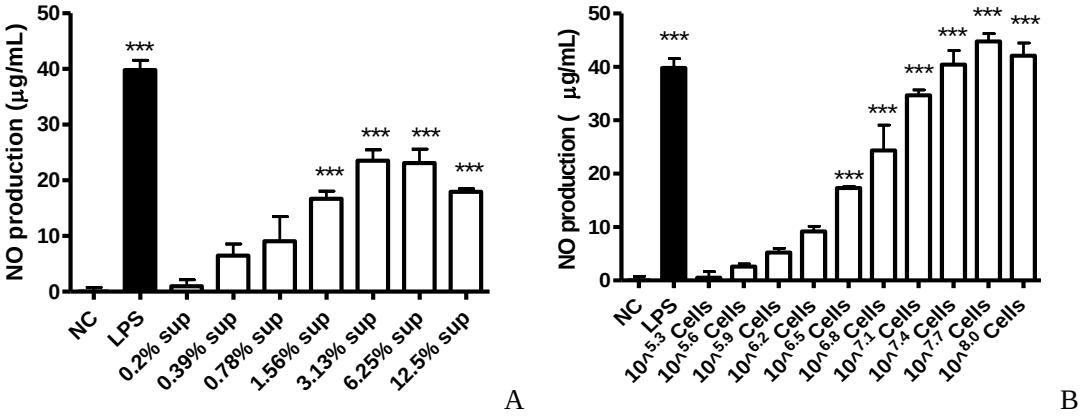
Figure 5.6 Flow cytometry cytokine histogram

The cytokine profile histogram showing the macrophage cytokine profiles (TNF-α, IL-6, IFN-γ and IL-10) from the negative control (NC), positive control (LPS), in the presence of 12.5 % (v/v) *L. reuteri* DPC16 supernatant (DPC16 sup) and 10 mg/mL of bovine lactoferrin (BLf) after 18 h incubation at 37 °C. [X: control and treatment Y: fluorescent intensity RPMI medium alone was the negative control (NC) and 1 µg/mL of LPS treatment was the positive control.]

5.3.1.2 Effect of *L. reuteri* DPC16 and bovine lactoferrin on nitric oxide production by RAW 264.7 cells

In addition to the above investigation of the cytokine profile, the production of another major inflammatory parameter, nitric oxide, from active murine macrophages was determined. The bacterial supernatant, heat-killed cells of *L. reuteri* DPC16 and bovine lactoferrin were tested for their effects on nitric oxide production by RAW 264.7 cells. LPS (1 µg/mL) was used as a positive control. The results (**Fig. 5.7),** performed by graph plotting for normality of residuals and then analysed by a One-way ANOVA and Tukey's Multiple Comparison Test, showed that LPS (1 µg/mL) significantly induced nitric oxide production (*P value* <0.001) compared to the negative control. Both of *L. reuteri* DPC16 supernatant and heat-killed cells stimulated nitric oxide production from RAW 264.7 cells in a concentration-dependent manner in the certain ranges (the top level of concentrations for both supernatant and heat-killed cells might affect the growth of RAW 264.7 cells, and therefore, affect the amount of nitric oxide production). In

general, both *L. reuteri* DPC16 supernatant and heat-killed cells showed a significant stimulation on the nitric oxide production in these experiments (*P value* <0.0001).



A B

Figure 5.7 The effect of *L. reuteri* DPC16 cells and cell-free supernatants on nitric oxide production by RAW 264.7 cells

Nitric oxide (NO) production was measured in triplicate using Griess reaction assays after RAW 264.7 cells were treated with *L. reuteri* DPC16 supernatant (A, a repesentative of two independent experiments) or heat-killed cells (B, a repesentative of three independent experiments) at 37 °C for 24 h. [X: controls and treatment Y: nitric oxide production (µg/mL). RPMI medium alone was the negative control (NC) and 1 µg/mL of LPS treatment was the positive control. The error bars are SD. LPS (1 µg/mL) and some of the concentrations of *L. reuteri* DPC16 supernatant (> 1.56% v/v) and HK cells (> 3.13 x10^6 cells/mL) significantly induced NO production (P value <0.001, marked as "***") compared to NC. The statistical analysis is shown in *Appendix II*.]

Next, the effects of bovine lactoferrin on nitric oxide production by the RAW 264.7 cell system were determined. Analysis of the results showed that the residuals were reasonably normally distributed. Subsequent analysis by a One–way ANOVA and Tukey's Multiple Comparison Test showed that LPS (1 µg/mL) significantly induced nitric oxide production (*P value* <0.001) compared to the negative control and bovine lactoferrin alone did not influence murine macrophage nitric oxide production at any concentrations tested (*P value* >0.05) (**Fig. 5.8)**.

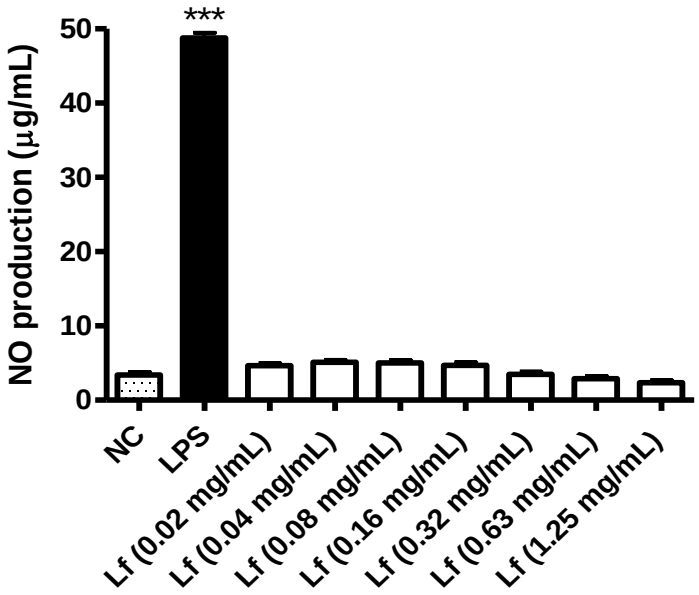


Figure 5.8 The effect of BLf on nitric oxide production by RAW 264.7 cells

Nitric oxide (NO) production was measured in triplicate using Griess reaction assays after RAW 264.7 cells were treated with bovine lactoferrin alone (the graph is a repesentative of two independent experiments) at 37 °C for 24 h. [X: controls and treatment Y: nitric oxide production (µg/mL). RPMI medium alone was the negative control (NC) and 1 µg/mL of LPS treatment was the positive control. The error bars are SD. 1 µg/mL of LPS treatment showed a significant induction of NO (*P value* <0.001, marked as "***"), while no concentrations of BLf tested were significantly different fromNC. The statistical analysis is shown in *Appendix II*.]

5.3.1.3 Effect of bovine lactoferrin on LPS-induced nitric oxide production and cell proliferation in RAW 264.7 cells

To investigate the reports that lactoferrin was able to regulate inflammation in the respiratory and gastrointestinal tracts (Baveye *et al.* 1999; Conneely 2001), experiments were designed to determine the effect of lactoferrin on LPS-induced nitric oxide production by RAW 264.7 cells. During these experiments, the murine macrophage cell morphology was observed using microscopy (Olympus, model CKX41, Japan). The microscope images (**Fig. 5.9**) revealed that the presence of LPS, at 1 µg/mL, resulted in altered cell morphology with increased pseudopod formation and reduced cell numbers, but this effect was blocked by addition of lactoferrin, at 1.25 mg/mL. Therefore, in the experiments, the cell proliferation ability was measured using the MTT cell proliferation assay after nitric oxide production had been determined.

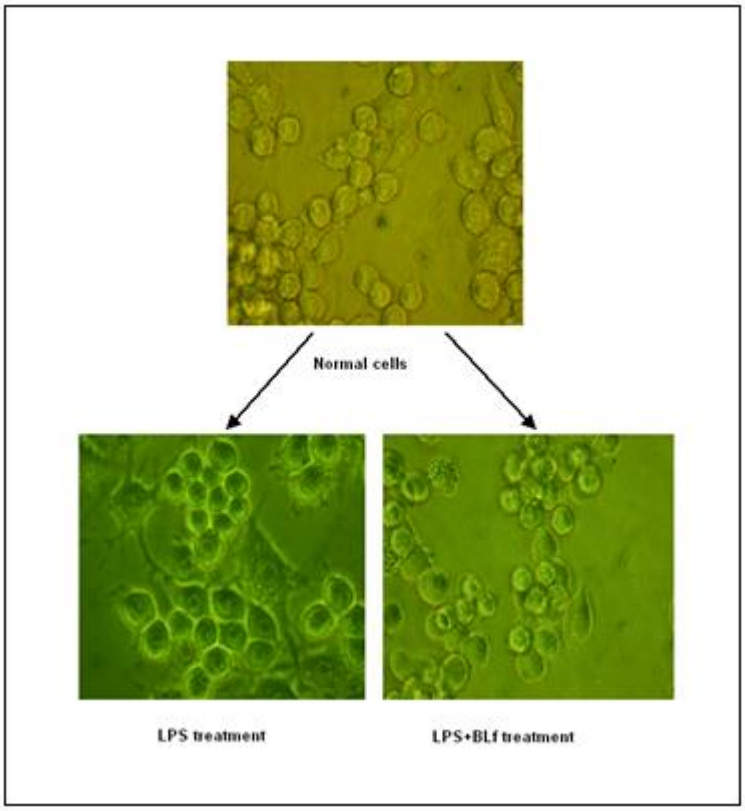


Figure 5.9 Microscope images showing the effects of 24 h treatment with LPS (1 µg/mL) alone and LPS plus lactoferrin (1.25 mg/mL) on the morphology of murine macrophage RAW 264.7 cells

The murine macrophages RAW 264.7 morphological images were taken using a digital camera (Nikon, Coolpix 995) attached to a microscope (Olympus, model CKX41, Japan, at 400x magnification).

The data collected from the Griess reaction and MTT assay were fitted to a sigmoidal concentration-response (variable slope) model. The curve and regression analysis showed that the data for bovine lactoferrin in combination with LPS fitted to a sigmoidal concentration-response model. Thus, BLf gave protection against LPS-induced murine macrophage cell stress (green curve) and nitric oxide production (red curve) in a concentration-dependent manner. The changes in cell proliferation and nitric oxide demonstrated a negative correlation, the former curve shows uphill (Hillslope=1.7) while the latter shows downhill (Hillslope=-5.0) (**Fig. 5.10**).

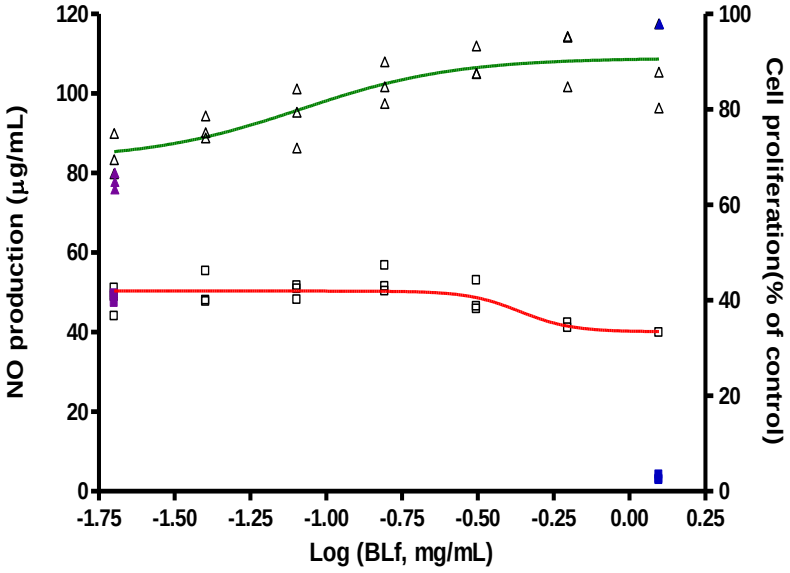


Figure 5.10 Effects of bovine lactoferrin (BLf) on LPS-induced nitric oxide production and cell proliferation in RAW 264.7 cells

Nitric oxide (squares) and cell proliferation (triangles) were determined in triplicate using the Griess reaction assay MTT assay, respectively, after BLf in the presence of LPS was incubated with RAW 264.7 cells at 37 °C for 24 h. [X: Log concentration of BLf (mg/mL) Left Y: nitric oxide production (µg/mL), Right Y: cell proliferation (% of control). RPMI medium alone was the negative control (NC, NO and cell proliferation marked with "blue" square and triangle, respectively) and 1 µg/mL of LPS treatment was the positive control (NO and cell proliferation marked with "purple" square and triangle, respectively). The statistical analysis is shown in *Appendix II.*]

5.3.1.4 Effects of *L. reuteri* DPC16 bacterial cells and cell-free supernatants on LPS-induced nitric oxide production by RAW 264.7 cells

The possibility that probiotics may play a role in prevention and treatment of inflammatory diseases such as IBD has been suggested (Tlaskalova-Hogenova *et al.* 2004). Experiments were, therefore, performed to study the effects of *L. reuteri* DPC16 on LPS-induced nitric oxide production in this murine cell system. The supernatant (12.5% v/v, pH 7.0) and heat-killed cells (10^7 cells/mL) of *L. reuteri* DPC16, were tested for their effects on nitric oxide production by RAW 264.7 cells in the presence of LPS (1 µg/mL). The residuals of data appeared reasonably normally distributed and the results were analysed using a One-way ANOVA followed by Tukey's Multiple Comparison Test. As shown in **Fig. 5.11,** the nitric oxide production in the presence of LPS (1 µg/mL) or LPS (1 µg/mL) together with 10^7 cells/mL of *L. reuteri* DPC16 heat-killed cells was significantly higher than that of negative control (*P value* <0.01). However, both of *L. reuteri* DPC16 supernatant and the heat-killed cells, at the concentrations tested, showed no significant effects (*P value* >0.05) on LPS (1 µg/mL)-

induced nitric oxide production by RAW 264.7 cells.

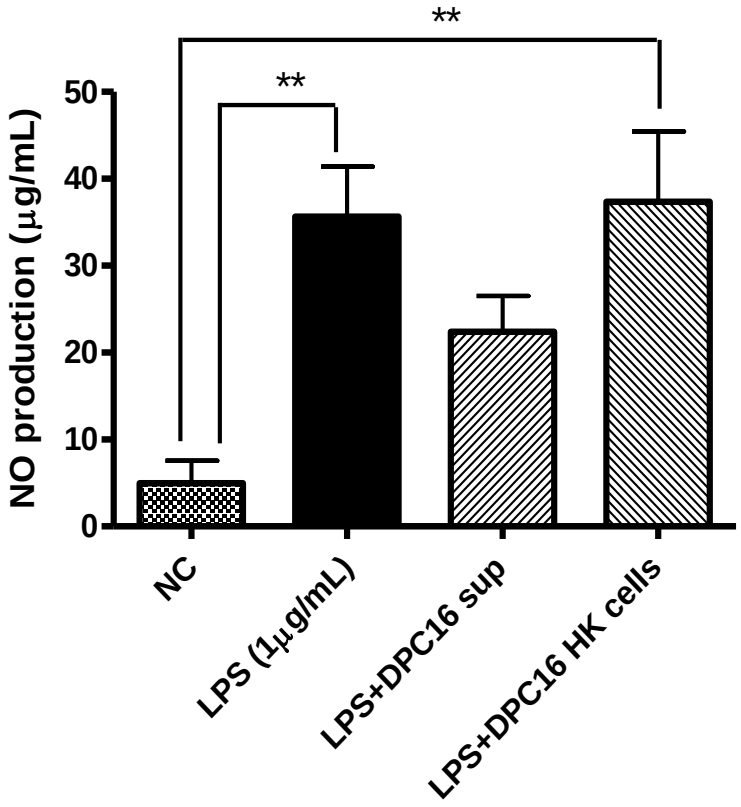


Figure 5.11 Effects of *L. reuteri* DPC16 supernatant and heat-killed cells on LPS-induced nitric oxide production by RAW 264.7 cells

Nitric oxide was determined using the Griess reaction assay (n=3) after *L. reuteri* DPC16 supernatant (sup, 12.5% v/v, pH 7) or heat-killed cells (HK cells, 10^7 cells/mL) were incubated in the presence of LPS, with RAW 264.7 cells at 37 °C for 24 h. [X: control and treatment Y: nitric oxide production (μg/mL), RPMI medium alone was the negative control (NC) and 1 μg/mL of LPS treatment was the positive control, the error bars are SD. The positive control and LPS (1 μg/mL) in combination with *L. reuteri* DPC16 HK cells (10^7 cells/mL) significantly induced NO production (*P value* < 0.01, marked as "**") compared to NC. *L. reuteri* DPC16 supernatant and the heat-killed cells, at the concentrations tested, showed no significant effects (*P value* >0.05) compared to the positive control. The statistical analysis is shown in *Appendix II*.]

These results generated using a murine RAW 264.7 model have confirmed that endotoxin-LPS can stimulate nitric oxide production by RAW 264.7 cells (Figs 5.6 and 5.10). The heat-killed cells of *L. reuteri* DPC16, at the higher concentrations tested, had a similar effect to that of LPS, while the *L. reuteri* DPC16 supernatants showed less significant effects at the concentrations tested. Therefore, the cell wall materials of *L. reuteri* DPC16 are likely to play the role of stimulating nitric oxide production by RAW 264.7 cells. However, neither the heat-killed cells nor the cell-free supernatant showed any significant protective or stimulatory effects on the LPS-induced response. This lack of effect could possibly be due to the lack of statistical power in these experiments or

due to the concentration of LPS (1 μg/mL) used to stimulate the number of RAW cells in the experiments being too high, with the result that nitric oxide production was at a saturated level, Thus, when the cells/supernatant were also present, any effects could not be detected. In the future, a lower concentration of LPS should be considered to use in similar experiments.

Finally, bovine lactoferrin showed a potential effect on down-regulation of the inflammatory response by protecting against LPS-induced nitric oxide production and cell stress. To confirm this finding, a human macrophage-like cell model (THP-1 model) was recruited to further study this event.

5.3.2 Establishment of the THP-1 cell model system

The human acute monocytic leukemia THP-1 cell line was grown as described in **Section 5.2.3.** Differentiation of the THP-1 cells into macrophage-like cells was induced by exposure of the cells ($1x10^6$ cells/mL) to phorbol myristrate acetate (PMA) (Cheng *et al.* 2005). After PMA activation, the differentiated THP-1 cells became adherent and stopped proliferating. The cells were then stimulated with LPS (Park *et al.* 2007) for 4 h, and TNF-α was measured in the supernatants using ELISA assays.

5.3.2.1 Conditions for differentiation of THP-1 cells

During PMA activation, morphological changes of the monocytic THP-1 cells were observed using microscopy (Olympus, model CKX41, Japan). As shown in **Fig. 5.12**, THP-1 cells started to differentiate from floating cells into attached macrophage-like cells after 48 h and were entirely differentiated from 72 h onwards. Therefore, 72 h activation was selected as one of the conditions for the THP-1 model system.

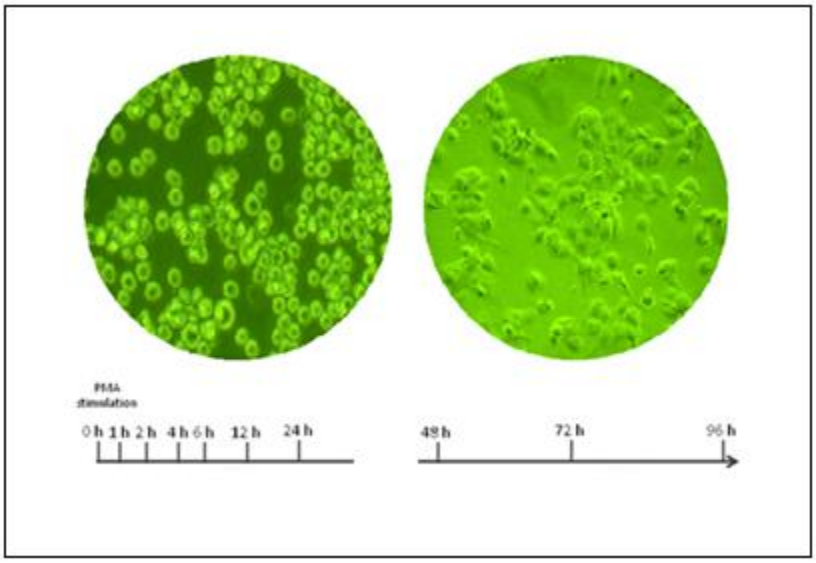


Figure 5.12 PMA differentiated THP-1 cells

THP-1 cells were differentiated from monocytes into macrophage-like cells in the presence of 10 nM PMA for 96 h, observed microscopically (Olympus, modeCKX41, Japan) at 100x magnification.

Two concentrations of PMA (10 nM and 50 nM) were selected to differentiate the THP-1 cells. The results (not shown) demonstrated that both concentrations could induce THP-1 differentiation. However, the higher concentration (50 nM) also induced a cytokine (TNF-α) response within 72 h. To avoid this potentially high background result, the lower concentration of PMA (10 nM) was selected for subsequent THP-1 culture differentiation and to allow study of the inflammatory response by stimulation with LPS and other substances.

5.3.2.2 Selection of the LPS concentration for the THP-1 system

After THP-1 cells were differentiated using 10 nM PMA, the cells were stimulated using different concentrations of LPS in the range of 0-500 ng/mL at 37 °C for 4 h. The amounts of TNF-α produced by the THP-1 cells reflect the degree of LPS stimulation. The concentrations of LPS tested in the experiments stimulated TNF-α release from THP-1 cells in a concentration-dependent manner (results not shown). A concentration of 100 ng/mL was chosen for subsequent experiments using the THP-1 model system as this concentration gave an amount of TNF-α that fell in the middle of the TNF-α ELISA standard curve.

Hence, 10 nM PMA, with 72 h activation was used as an appropriate condition for cell differentiation and attachment in the THP-1 model system, and LPS at 100 ng/mL was chosen as a suitable single concentration for subsequent work.

5.3.3 Effects of bovine lactoferrin on LPS-induced TNF-α production in THP-1 cells

After the conditions were selected in the THP-1 system, this human immune cellular model system was ready for use to assess the effects of bovine lactoferrin on LPS-induced TNF-α production in the system.

Cytotoxicity and pre-inflammatory assessments were performed and indicated that none of the concentrations of bovine lactoferrin or bovine serum albumin (as a control), at concentrations from 0.1 mg/mL to 2 mg/mL, were cytotoxic to THP-1 cells and did not induce TNF-α production when incubated alone in the THP-1 system for 4 h at 37^{o}C (results not shown). Subsequently, the above solutions were tested for the effects on inflammation induced by LPS in this human immune THP-1 model. TNF-α production from each treatment was determined using the ELISA assay.

The results (**Fig. 5.13)** were analysed by performing a curve and regression analysis. The global curve fitting showed that BLf (green curve) significantly reduced pro-inflammatory cytokine TNF-α release from LPS-induced THP-1 cells in a concentration-dependent manner, and the effects were significantly greater than that of BSA (red curve) by the curves sharing different parameters (*P value* <0.001) although the data sets of BSA were not as well fitted to the sigmoidal concentration-response (variable slope) model as that of BLf. Nevertheless, the results indicated that these two bovine proteins act in different ways in modulating TNF-α production in THP-1 cells.

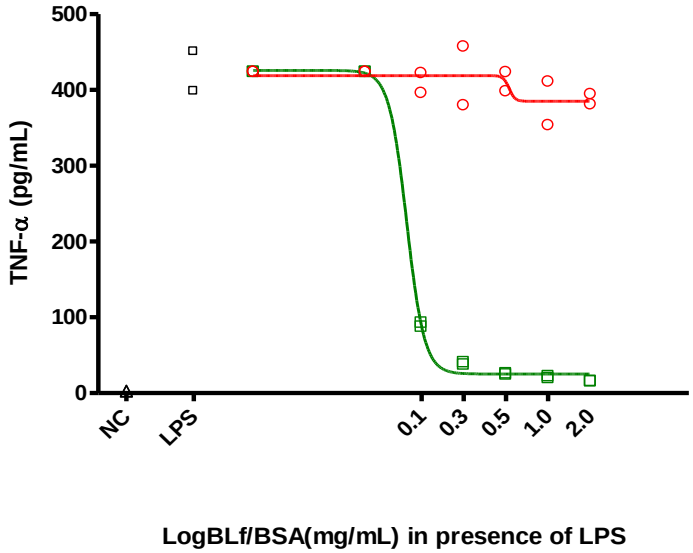


Figure 5.13 Anti-inflammatory effects of bovine lactoferrin

TNF-α release from LPS-challenged THP-1 cells, in the presence of BLf or BSA (as a control) at concentrations from 0.1 mg/mL to 2 mg/mL, was determined in duplicate by ELISA assay after 4 h incubation at 37 °C. [X: control and treatment (log form) Y: TNF-α production (pg/mL). PMA-activated THP-1 cells were the negative control while the activated cells treated with 100 ng/mL of LPS were the positive control. The result was obtained from a single experiment. The statistical analysis is shown in *Appendix II.*]

An experiment was subsequently performed to investigate whether BLf acts by blocking LPS receptors on THP-1 cells or by detoxification of LPS by binding it directly. THP-1 cells were pre-treated with BLf (1 mg/mL) for 1 h, after which the BLf-containing medium was removed prior to challenge with LPS (100 ng/mL). TNF-α production by these cells was compared with that where the same concentration of BLf was added concurrently with LPS. The data showed that a decrease in TNF-α production occurred during the BLf and LPS co-treatment but not during the BLf pre-treatment (**Fig. 5.14).** However, whether the reaction mediated by BLf is on the THP-1 receptors, or it acts by neutralization of LPS is not clear based on these data. Further experiments and statistical analysis are needed to understand the mechanisms.

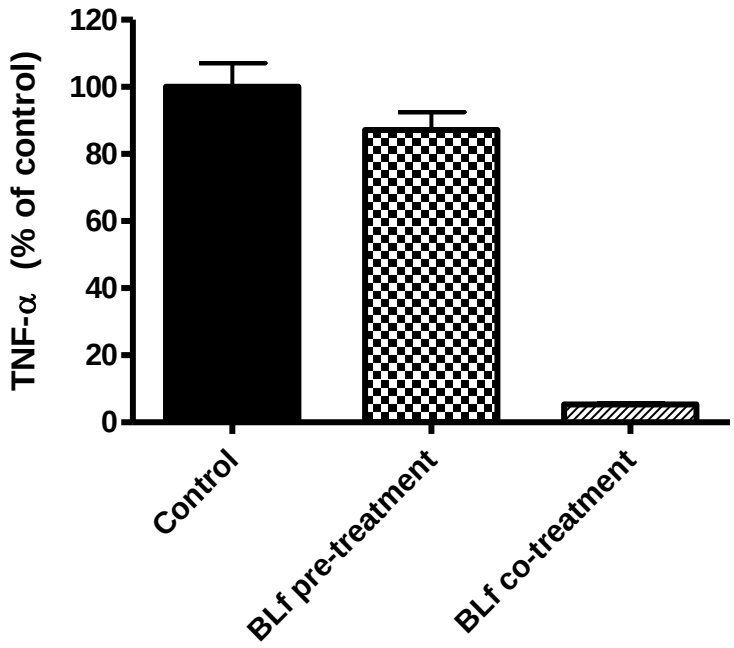


Figure 5.14 The effect of bovine lactoferrin treatments on TNF-α production by THP-1 cells

TNF-α release from THP-1 cells was measured in duplicate by ELISA after pre-treatment or co-treatment of BLf (1 mg/mL) with LPS (100 ng/mL) for 4 h at 37° C. [X: treatment Y: TNF-α production (pg/mL). LPS (100 ng/mL) only - treated THP-1 cells were the control. The data were obtained from a single experiment; the error bars are SD.]

The THP-1 cell model above was used in an attempt to add further information regarding the possible anti-inflammatory activities of lactoferrin. In this system, bovine lactoferrin did reduce LPS-induced TNF-α production. In contrast, bovine serum albumin did not provide such reduction; supporting the view that lactoferrin may have anti-inflammatory properties. Further, the mechanism by which lactoferrin acts appears to need further work.

5.3.4 Establishment of the Caco2/THP-1 co-culture model system

The transwell system described in **Fig. 5.3** allowed measurement of any interruption to the intestinal epithelial permeability caused by differentiated monocytes (THP-1). This was done by measuring the transepithelial electrical resistance (TEER, as described in **Section 5.2.10)** of the intestinal enterocytic monolayer (Caco-2 cells) following pro-inflammatory cytokine production by the THP-1 cells.

5.3.4.1 A study of the inflammation-associated interruption to the intestinal permeability barrier in the co-culture system

As shown in **Fig. 5.12,** the monocytic cell line (THP-1) can be activated and differentiated into macrophage-like cells by PMA in a certain period of time. According to previous studies of a co-culture system (Watanabe *et al.* 2004; Satsu *et al.* 2006), 200 nM PMA was suggested for differentiation of THP-1 cells, which were then able to reduce the transepithelial electrical resistance (TEER) of the Caco-2 monolayer. In the present work, multiple conditions were employed in order to achieve highly differentiated THP-1 cells (TNF-α as the activation biomarker), with 10 nM of PMA treatment in the presence or absence of 500 ng /mL of LPS, or 200 nM PMA, for 96 h. The conditions were then assessed for their ability to reduce the Caco-2 monolayer tight junction. The experimental data were analysed by a One-way ANOVA and Tukey's Multiple Comparison Test after the normality of data distribution was viewed. Significant changes in TEER were shown using both 10 nM PMA (*P value* <0.001*)* and 10 nM PMA+500 ng/mL LPS (*P value* <0.01) treatments, but not 200 nM PMA treatment (*P value* >0.05*),* compared with non-differentiated THP-1 cells (**Fig. 5.15**). Neither LPS nor a cytokine combination of TNF-α and IFN-γ without THP-1 cells induced a TEER reduction. These results indicate that an increase in Caco-2 monolayer permeability was caused only in the presence of activated THP-1 cells. However, the TEER reduction was not correlated to the concentration of PMA or the amount of TNF-α produced (results not shown).

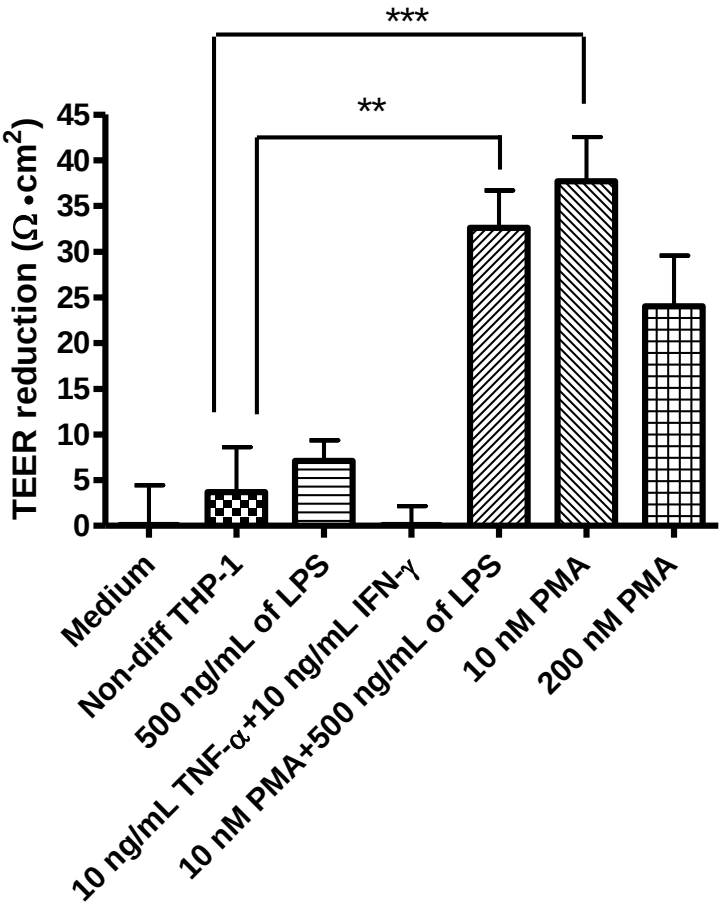


Figure 5.15 Changes in epithelial barrier integrity of the Caco-2 monolayer

The transepithelial electrical resistance (TEER) was measured in triplicate after the Caco-2 monolayer was incubated (basolateral side, 48 h at 37 °C) with differentiated THP-1 cells [PMA at 10 nM and 200 nM, and a mixture of PMA (10 nM) plus LPS (500 ng/mL)], undifferentiated THP-1 cells, LPS (500 ng/mL), and a mixture of cytokines (10 ng/mL TNF-α and 10 ng/mL IFN-γ). [X: control and treatment Y: TEER reduction ($\Omega \cdot cm^2$) Non-differentiated THP-1 was the negative control. The error bars are SD. "***" and "**" indicate that the TEER reductions were significantly different in the treatment groups of 10 nM PMA (*p value* <0.001) and 10 nM PMA+500 ng/mL, (*p value* <0.01) compared to the negative control. The data were collected from a single experiment. The statistical analysis is shown in *Appendix II.*]

In the LDH cell death assay, a One-way ANOVA and Tukey's Multiple Comparison Test was performed after the normality of data distribution was viewed. The statistical results showed that there was no significant Caco-2 monolayer cell death in any of the treatment groups (**Fig. 5.16**).

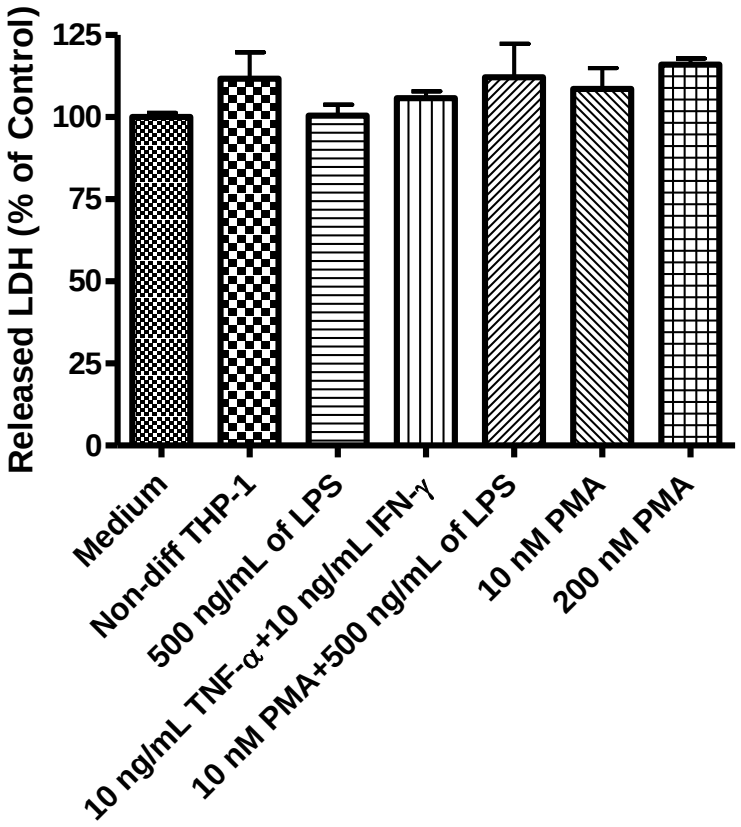


Figure 5.16 Lactate dehydrogenase (LDH) determination on the Caco-2 monolayer under the treatment conditions

LDH released in the apical medium from the Caco-2 monolayer cells was measured in triplicate transwells after the Caco-2 monolayer was incubated (basolateral side, 48 h at 37 °C) with differentiated THP-1 cells [PMA at 10 nM and 200 nM, and a mixture of PMA (10 nM) plus LPS (500 ng/mL)], undifferentiated THP-1 cells, LPS (500 ng/mL), and a mixture of cytokines (10 ng/mL TNF-α and 10 ng/mL IFN-γ). [X: control and treatments Y: LDH (% of control) Non-differentiated THP-1 was the negative control. The error bars are SD. The data were collected from a single experiment. The statistical analysis is shown in *Appendix II.*]

A possible reason why the high concentration of PMA (200 nM) did not cause maximum TEER reduction of the Caco-2 monolayer in the treatment group was that the cell activity or cell numbers of THP-1 may not have been sufficient to be activated by such a high concentration of the cell stimulator, PMA. Therefore, after some trials using different concentrations of THP-1 cells, a modification was made to the THP-1 activation step by seeding $1x10^6$ cells/mL in the 24-well plates with the volume of 1 mL/well, instead of $1.5x10^5$ cells/mL, followed by challenge with 200 nM PMA. As shown in **Fig. 5.17**, THP-1 cells (at a concentration of $1x10^6$ cells/mL), differentiated with 200 nM PMA and then co-cultivated with a Caco-2 monolayer in the transwell system, significantly reduced the TEER value compared with non-differentiated THP-1 cells at the same concentration (*p value* <0.001). However, the non-differentiated THP-

1 cells at this concentration also reduced the TEER significantly (*p value* <0.001) when compared with the control (medium only). The results were analysed by performing a One-way ANOVA followed by Tukey's Multiple Comparison Test.

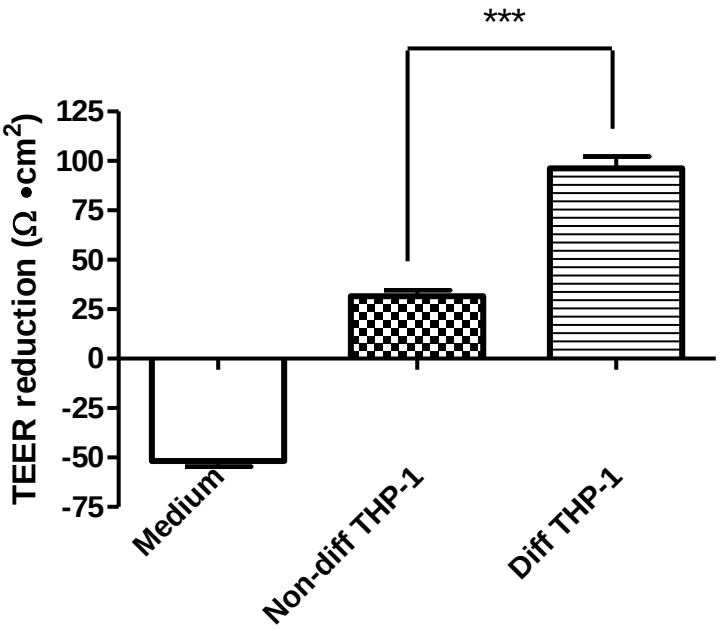


Figure 5.17 The TEER reduction of a Caco2 monolayer during co-cultivation with 200 nM PMA-differentiated THP-1 cells at an increased cell density

TEER was measured after the Caco-2 monolayer was incubated with 200 nM PMA-differentiated THP-1 cells (at a concentration of $1x10^6$ cells/mL), and compared to that of non-differentiated THP-1 cells, after 48 h incubation at 37 °C. [X: control and treatment Y: TEER reduction ($\Omega \cdot cm^2$) Caco-2 monolayer incubated with medium only was the negative control. Each treatment was conducted n ≥ 3, the error bars are SD. The TEER changes observed with 200 nM PMA-differentiated THP-1 cells and non-differentiated THP-1 cells were significantly different (*p value* <0.001, marked as "***"). The statistical analysis is shown in *Appendix II.*]

5.3.4.2 The effects of *L. reuteri* DPC16 and lactoferrin on the inflammation-associated interruption to the intestinal permeability barrier in the co-culture system

Using the modified conditions described above, test samples at a non-toxic level (based on the pre-assessment in the toxicity assays) to Caco-2 cells such as DPC16 supernatant (1%, pH 7) and lactoferrin (2 mg/mL) were added to the apical surfaces of the Caco-2 monolayer as soon as the two compartments of the transwell were grown together. MRS medium (1%, pH 7) and a mixture of short chain fatty acids (4 mM lactate plus 1.2 mM acetate, pH 7, according to the DPC16 supernatant analysis in **Section 2.3.3**) were

included as controls. After co-cultivation for 48 h at 37 °C, the TEER values were measured. The results, analysed by a One-way ANOVA followed by Tukey's Multiple Comparison Test (**Fig. 5.18),** show that none of the tested materials prevented the reduction in TEER that was caused by the differentiated THP-1 cells, i.e. there were no significant (*p value* >0.05) differences observed.

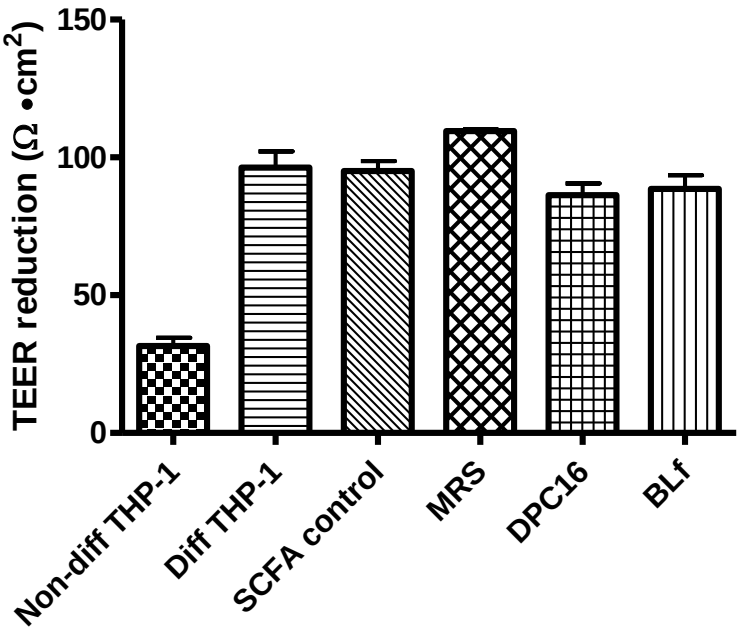


Figure 5.18 The TEER reduction of a Caco-2 monolayer THP-1 coculture following treatment with DPC16 supernatant and bovine lactoferrin

The effects of DPC16 supernatant (1%, pH 7), bovine lactoferrin (BLf, 2 mg/mL), MRS medium (1%, pH 7) and a mixture of lactate (4 mM) and acetate (1.2 mM) (SCFA control), pH 7, on the reduction of the TEER values in Caco2 monolayers caused by the co-cultivation of the monolayer with differentiated THP-1 cells, during 48 h incubation at 37 °C. [X: control and treatment Y: TEER reduction ($\Omega \cdot cm^2$) Caco-2 incubation with non-differentiated THP-1 cells was the negative control. Each treatment was conducted $n \geq 3$, the error bars are SD. There were no significant differences on TEER reduction between the differentiated THP-1 treatment and the other treatments (p value > 0.05). The statistical analysis is shown in *Appendix II*.]

Considerable difficulty was experienced during the establishment of the co-culture system, but conditions were eventually found that allowed an experimental system for the observation of an increase in the permeability of the Caco-2 monolayer *via* measurement of the TEER values. Thus, a large increase in permeability caused by differentiated THP-1 cells was demonstrated. However, high concentrations of non-differentiated THP-1 cells also showed some effects on the integrity of the Caco-2 monolayer which means that the conditions and cell densities of THP-1 applied in the

experiment need further study to minimise these effects **(Fig. 5.17)**. Additionally, it is not clear which factor may have caused the permeability increase since neither LPS nor a mixture of cytokines were able to reproduce the effect **(Fig. 5.15).**

The purpose of developing the co-culture system was to investigate if the DPC16 supernatant or lactoferrin could prevent the increase in monolayer permeability. The present results indicated no such prevention, but further development of this experimental system is required before further studies can be done.

5.4 Discussion

In this chapter, a cellular model approach has been used to assess the effects of *Lactobacillus reuteri* DPC16 and bovine lactoferrin on gut inflammatory responses. Human and murine cellular immune system models were designed to study the production of pro-inflammatory mediators, such as cytokines and nitric oxide, with and without exposure to endotoxin, so that the effects of probiotics and lactoferrin could be investigated.

In the immune system, nitric oxide is an important molecule mediating cytotoxic actions. Some phagocytes respond to activation by cytokines with increased synthesis of an inducible form of the enzyme iNOS. Although the nitric oxide produced may mediate some of the tumoricidal or antimicrobial effects of phagocytes (Brewington *et al.* 2001; Acharya & Tripathi 2003), its over-production can lead to vasodilatation, and hypotension in sepsis. Over-production of pro-inflammatory cytokines, such as TNF-α, can result in persistent inflammation and tissue damage (Kennedy *et al.* 2002). The involvement of commensal microflora and their components in a variety of diseases, including inflammatory bowel disease, and colon cancer has been recently suggested (Tlaskalova-Hogenova *et al.* 2004). Hence, regulation of the microflora composition using probiotics offers the possibility to influence the development of mucosal and systemic immunity and prevent and treat some diseases. In addition, there is some evidence to show that lactoferrin is able to regulate inflammation in the respiratory and gastrointestinal tracts (Baveye *et al.* 1999; Conneely 2001).

First, *L. reuteri* DPC16 strain was tested for its inflammatory effects in a murine

immune cell system, and compared to LPS. In the murine system, *L. reuteri* DPC16 cells at some higher concentrations stimulated nitric oxide production, and so resembled LPS in having pro-inflammatory activity. It is known that LPS is recognized by the Toll-Like Receptor 4 (TLR4), and forms a CD14/TLR4/MD2 receptor complex (Hao *et al.* 2009) which promotes the secretion of pro-inflammatory cytokines in many cell types (**Fig. 5.1**). It may be suggested that probiotics are able to be recognized by certain Toll-Like Receptors (such as TLR2 and TLR9). One recent report demonstrated that lactobacilli were unable to stimulate TLR4 production at the mRNA level; however, TLR2 and TLR9 transcription levels, as measured by quantitative PCR, were up-regulated when HT29 cells were incubated with lactobacilli (Vizoso Pinto *et al.* 2009). In another study, inflammatory responses of mast cells were suppressed by stimulation through TLR2 by probiotics, exerting potential anti-allergic effects, at least in part, through direct effects on mast cells (Kasakura *et al.* 2009). In this study, the pro-inflammatory property of *L. reuteri* DPC16 cells was observed with the murine cell system, but the actual mechanism is unknown. Also, whether or not *L. reuteri* DPC16 can reduce the inflammatory effect caused by LPS is not clear at this stage, although some researchers have suggested that the anti-inflammatory effects of probiotics may be mediated by metabolic products, and may be exerted by suppression of the LPS/TLR4 signalling pathway (Watanabe *et al.* 2009). Unfortunately, in the present study, the supernatant of *L. reuteri* DPC16 did not demonstrate a clear effect due to the experimental variation, but it showed a possible stimulation effect in each single experiment. In addition, neither the supernatant nor the cells of *L. reuteri* DPC16, at the concentration tested, were able to inhibit LPS (1 μg/mL) -stimulated nitric oxide production. Further work need to support this finding. In contrast, in the same system, lactoferrin was able to protect against LPS-induced nitric oxide production, suggesting that it may possess anti-inflammatory activity.

Secondly, using the human cell system (THP-1 cells), bovine lactoferrin, but not bovine serum albumin, did repress LPS-induced TNF-α production, providing support for the concept that this glycoprotein possesses anti-inflammatory activity. In the present work, the result obtained using lactoferrin suggests that there is a signalling inactivation between LPS and THP-1 cells caused by lactoferrin. Appelmelk *et al.* (1994) demonstrated, using *in vitro*-binding studies, that lactoferrin can bind directly to isolated lipid A and intact LPS, and this was suggested as the mechanism whereby lactoferrin

exerts its antibacterial activity. This binding may involve the special cationic protein structure of lactoferrin (Baker & Baker 2009), which may bind to negative charges on the LPS on cell membrane (**Fig. 5.19)**. However, the way in which lactoferrin blocks the immune signals between LPS and THP-1 cell receptors was not clear from preliminary experiments of BLf and LPS co-treatment and BLf pre-treatment (**Fig.5.14**). Further experimental work or *in vivo* study is required to answer this question.

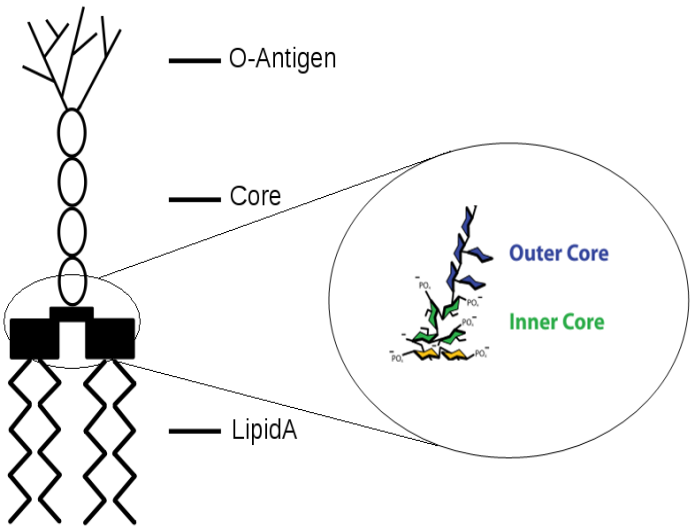


Figure 5.19 The negative charges on lipopolysaccharide (LPS) that may be a binding site for cationic lactoferrin

Lastly, the results obtained using the co-culture system were inconclusive, in that neither the *L. reuteri* DPC16 supernatant nor lactoferrin were able to prevent an increase in permeability of the cell monolayer that was caused by differentiated THP-1 cells. Nevertheless, co-culture cell models are increasingly gaining recognition in science, as they mimic normal organ, tissue and physiological organization in human organism. It has proved to be a powerful *in vitro* tool in unravelling the importance of cellular interactions during normal physiology, homeostasis, repair and regeneration (Hendriks *et al.* 2007). It is believed this system would be useful in future probiotics and intestinal immune studies.

Chapter 6
General Discussion

6.1 Discussion

Nutrition- and physiology-related diseases are leading to increased health problems that are related to our modern lifestyle. New Zealand has a high incidence of diet-related diseases (Ferguson & Philpott 2003; Ferguson *et al.* 2004; Gibson 2007; Ferguson & Philpott 2008). Dietary manipulation has the potential to significantly affect health and disease-related outcomes in humans. Some foods offer the solution of modifying the nutritive properties and modulating the physiological functions of the body, and are called "functional foods". This concept was first established in Japan in the early 1980s (Shimizu 2010). Foods that contain probiotic bacteria, or foods that contain bioactives, have become accepted as a potential means to control or overcome some of these diseases, particularly of the GI tract (Tuomola & Salminen 1998; O'Sullivan 2001; Parvez *et al.* 2006). Among a large number of dietary intervention studies designed to identify potential components that may modulate the microbial activities and their metabolites in the GI tract, pro/prebiotics have provided some promising results in some human clinical trials (Rosenfeldt *et al.* 2002b; Gratz *et al.* 2011). Hence, it is necessary to understand the underlying mechanisms by which pro/prebiotics may contribute to gut health.

Usually animal studies or human studies provide a general idea as to whether a proposed treatment works. However, due to the high cost to perform additional *in vivo* studies and the difficulty of controlling the complicated physiological system, using a real system to explain each single detail of mechanism of action becomes unfeasible. It is believed that the *in vitro* cellular model is suitable to answer some specific questions in some simplified conditions although it has some limitations when drawing conclusions. Therefore, *in vitro* assays and cellular models are commonly employed to screen treatment conditions, and to characterize and develop some essential model systems to mimic the whole/specific compartment under controlled parameters (Prakash

et al. 2011). Usually, the outcomes can propose some underlying mechanisms, discover the challenges and guide the directions of further *in vivo* studies. The *in vitro* approach is a complementary means that is used in combination with animal studies and human studies to help draw a clear conclusion in research.

Based on this concept and the previous *in vitro* and *in vivo* explorations on *L. reuteri* DPC16 and bovine lactoferrin, the present project has focused on investigating the efficacy and mechanisms of action of a novel commercial probiotic, *L. reuteri* DPC16, alone and in combination with bovine lactoferrin by using several simulated human gastrointestinal conditions and intestinal epithelia models. The study has centred on gut health by assessing the effects of this combination on the GI microflora, and their use in controlling inflammatory insult and genotoxicity, and aims to provide justification for further *in vivo* work.

6.1.1 The applications of 16S rRNA gene extraction and identification

The present study using DNA sequence analysis and chemical analyses confirmed that the DPC16 strain belongs to the *L. reuteri* species. Direct sequencing of the 16S rRNA gene has been proven as a powerful molecular approach to identify bacterial species, and has been suggested as an alternative technique to investigate microbial density within the mammalian gut instead of the traditional bacterial cultivation techniques (Ley *et al.* 2006). Therefore, the 16S rRNA based molecular microbiological methodologies such as DGGE (denaturing gradient gel electrophoresis) are preferable to analyze microbiota composition in human faeces (Mai *et al.* 2009; De Filippo *et al.* 2010; Windey *et al.* 2012b). The present study not only confirmed the strain identity of *L. reuteri* DPC16 but also validated the accuracy of 16S rRNA extraction and amplification using universal primers. It is hoped that 16S rRNA extraction, in combination with the DGGE technique, could be used in a future *in vivo* study to monitor *L. reuteri* DPC16 bacterial numbers in faecal samples during and after administration. The resulting faecal microbiota profiles detected by these molecular means could provide solid evidence on the efficacy of *L. reuteri* DPC16 and lactoferrin in relation to a healthy microbiota biodiversity and activity in the gut.

6.1.2 The probiotic properties of *L. reuteri* DPC16

The present study developed some conditions to simulate the human gastrointestinal physiological environment. *L. reuteri* DPC16 was found to be relatively tolerant of the harsh conditions that exist in the upper region of the gastrointestinal tract, and that the cells can be protected by the presence of nutrients. However, the viability of *L. reuteri* DPC16 was significantly reduced by some bile acid challenges that mimic the small intestinal conditions. The results indicated that achieving a sufficient number of viable *L. reuteri* DPC16 cells to reach the lower region of the GI tract (colon) is one of the major challenges in the administration of a probiotic strain. Nevertheless, the results show that ingestion of this strain with food would have a protective effect on its viability. A recent *in vitro* study employing a microencapsulation technology to protect the bacterial cells has demonstrated the additional protective affect of incorporating skim milk within a calcium alginate matrix (Zhao *et al.* 2011). Importantly, the metabolic pathways and the fermentation products with significant antibacterial activity that reflect the glycerol dehydratase gene expression in *L. reuteri* DPC16 strain (Lu 2007) were confirmed by chemical analysis and microbiological assays in the present study. This specific characteristic in conducting either homofermentative or heterofermentative pathway, enables this strain to utilize either glucose or glucose and glycerol, as growth substrates, which are derived from carbohydrates and lipids fermentation, and are available in any of three enterotypes human intestine. Under the homofermentative pathway, this strain most likely uses glucose to produce short-chain fatty acids, but under heterofermentative pathway, this strain is able to use both glucose and glycerol to produce short-chain fatty acids and reuterin. The latter fermentation appears to be responsible for the stronger antibacterial effects against the pathogens *Listeria monocytogenes, Staphylococcus aureus, Salmonella typhimurium* and *Escherichia coli* O157:H7 that were clearly demonstrated by the results in this study. The effects of its end products on itself or other *Lactobacillus* species are much weaker than those on the pathogens. Hence, as other researchers have suggested, *L. reuteri* can secrete sufficient amounts of reuterin to cause the desired anti-microbial effects since about 4-5 times the amount of reuterin is needed to kill "good" gut bacteria (i.e. *L. reuteri* and other *Lactobacillus* species) as "bad". This could allow *L. reuteri* to remove gut invaders while keeping normal gut flora intact, as previously suggested (Casas & Dobrogosz 2000). Although this possibility was demonstrated very clearly in the *in vitro* experiments, it is not guaranteed to be seen in a real *in vivo* situation because the

key question for *L. reuteri* DPC16 strain is whether this novel *L. reuteri* strain can colonise the host gut. The GI microflora can be categorised by their transition behaviours in the gut (Savage 1977) into two types: autochthonous flora (indigenous flora) and allochthonous flora (transient flora). Most pathogens are allochthonous microorganisms. Lactobacilli are one of the numerically dominant organisms in the human gut; however species such as *L. acidophilus*, *L. casei*, *L. paracasei*, *L. rhamnosus*, *L. delbrueckii*, *L. brevis*, *L. plantarum*, and *L. fermentum* have not been reported to form stable populations in the gut and, thus, are likely to be allochthonous. In contrast, *L. reuteri* seems to be present almost universally throughout the animal kingdom such as in pigs (Naito *et al.* 1995) and rodents (Molin *et al.* 1992). Therefore it may be called a "universal" gut organism. The strains closely related to *L. reuteri* and *L. johnsonii* are reported to be autochthonous to rodent and porcine gut (Walter 2008). Studies have shown that some *L. reuteri* strains are transmitted from mothers to newborn animals or infants during birth and the nursing process, mainly *via* the mammary duct (Casas & Dobrogosz 2000), and are thus considered to be one of the few true autochthonous (indigenous) *Lactobacillus* species in the human GI tract (Reuter 2001). Although *L. reuteri* occurs naturally in humans, it is not found in all individuals, and it probably has a host-specificity. *L. reuteri* DPC16 strain was originally isolated from humans (Shu & Liu 2008), but whether it belongs to the autochthonous flora and is able to re-colonise the human GI tract is unknown.

Based on food GI transit studies, the average food transit time is 2.5 hours after food intake to reach the small intestine and up to 40 hours to transit through the colon, although there is a great deal of variability among individuals and within the same person at different times and after different meals. If a probiotic strain can colonise the GI tract, then this strain could proliferate and produce metabolites by fermenting the food matrix in the intestine. Thus, there could be a balance of cells lost by being wrapped in the mucus and food residuals and passed out of the GI tract, and new cell production by replication. If a strain is not able to colonise the GI tract, it has less time stay in the gut and less chance to interact with the food matrix to produce enough metabolites to modulate the activities in the gut. Thus, the only way to maintain the numbers of beneficial bacteria is to regularly consume sufficient numbers of bacterial cells to maintain their beneficial functions in the gut as stated in the definition of probiotics.

The most popular *in vitro* models for the study of bacterial colonisation or adhesion to the human intestinal epithelium use Caco-2 and HT29 cell lines due to their typical entercytic differentiation (Tuomola & Salminen 1998; Gopal *et al.* 2001; Riedel *et al.* 2006; Tallon *et al.* 2007). The HT29-MTX cell line (a stable mucus-secreting subpopulation of HT29 cells created by adapting the cell line to methotrexate) is the ideal cell line (Hao & Lee 2004) to mimic the GI mucosal environment for bacterial adhesion. However, due to the unavailability of this cell line, the human intestinal epithelial Caco-2 cell line was used in this study as suggested by others (Tuomola & Salminen 1998; Gopal *et al.* 2001; Riedel *et al.* 2006; Tallon *et al.* 2007). *L. reuteri* DPC16 cells were observed to adhere to a Caco-2 epithelial monolayer, to the same extent as some other probiotic bacteria (some of which are probably allochthonous lactobacilli strains e.g. *L. acidophilus*, *L. plantarum* and *L. rhamnosus)*. However, by carefully considering of the theory of bacterial colonisation, the human Caco-2 epithelial monolayer cannot represent the real case of bacterial colonisation in the gut because the first step of colonisation occurs between bacterial cells and mucosa in the GI tract, and may have some "communication" with epithelial cells mediated by mucin expression (Mack *et al.* 1999). Without the presence of mucus on the Caco-2 epithelial monolayer, the result has less link to the reality of bacterial colonisation. Thus, the question of whether *L. reuteri* DPC16 is able to colonise the human GI tract as an ingestible probiotic remains unanswered. In addition, *L. reuteri* DPC16 has shown no mucin degradation characteristics *in vitro,* similar to two commercial reference strains [(*Bifidobacterium lactis* HN019 (DR10) and *L. rhamnosus* HN001 (DR20)]. Further, the mucin degradation assays should be repeated using human, rather than porcine, mucin to provide a more valuable answer. In summary, the *in vitro* adhesion and mucin degradation assays are good experimental and critical exercises to deepen the knowledge. However, the results provide insufficient information regarding the safety and colonisation of *L. reuteri* DPD16 strain in the human gut, reinforcing the suggestion that the safety and colonisation of probiotics must be tested in real *in vivo* systems. So far, no conclusion can be draw as to whether *L. reuteri* DPC16 is able to re-colonise the human gut.

A faecal genotoxin-induced colon epithelial DNA damage (HT29 cell line) model was developed in this study. This model in conjunction with a Comet assay has been recognized and widely used in faecal genotoxicity and colon cancer research. The

results in the present study have shown the potential of *L. reuteri* DPC16 to mitigate against faecal genotoxin-induced DNA damage in colonic epithelia. Possible mechanisms involving cell binding or degradation of the carcinogens or their metabolites have been proposed. Further to this observation, some additional results have also confirmed that *L. reuteri* DPC16 has no link to the production of a faecal metabolite, indole, which is found in faecal water samples and can be produced by some bacteria such as *E. coli.* Further, the study showed that *L. reuteri* DPC16 is capable of removing indole from faecal water, although the mechanism appears unclear. Subsequently, some results indicated that indole alone has no genotoxic effects on HT29 cells at the concentrations tested, although the exact identity of the toxins that cause DNA damage in the colonic HT29 cells is still unclear. Indole, as a biomarker in faecal water, provided evidence of the potential of *L. reuteri* DPC16 to remove faecal toxins.

6.1.3 Lactoferrin and *L. reuteri* DPC16

From the nutraceutical point of view, lactoferrin in combination with *L. reuteri* DPC16 demonstrated more promising effects than either alone. The most important contribution in the present study is the finding of the synergistic augmentation of bovine lactoferrin (BLf, NZMP7100) with *L. reuteri* DPC16 supernatant in the antibacterial activities against pathogens, with much less effect on the growth of probiotics, *in vitro* (Tian *et al.* 2010b). Subsequently, bovine lactoferrin was found, as a glycoprotein, to significantly reduce faecal genotoxin-induced DNA breakage (Tian *et al.* 2010a) and may act to reduce the risk of colon cancer as suggested by previous authors (Tsuda *et al.* 2000). Interestingly, using both the human and murine immunological studies, lactoferrin was shown to present a clear modulation of the inflammatory process, mainly by preventing release of cytokines and nitric oxide from both human monocytes and murine macrophages (Tian *et al.* 2010a). However, it is uncertain at this stage whether strain DPC16 can inhibit inflammation. The action of lactoferrin is probably achieved by regulation of the proliferation of immune cells *via* blocking/neutralization of endotoxin LPS. From the viewpoint of mechanism, the results from this study indicate that lactoferrin possibly modifies or blocks the necessary structures of LPS, which are able to be recognized by TLR4 on immune cells. Overall, the results support the concept that lactoferrin modulates the inflammatory process *via* its cationic protein structure (Baker

& Baker 2009), interacting with the negative charges of LPS and competing with LPS Binding Protein to prevent the transfer of LPS to CD14 present on the surface of immune cells (Baveye *et al.* 1999). This supports the proposal of Appelmelk (1994) that "lactoferrin binds directly to isolated lipid A and intact LPS", as well as the antibacterial mechanism proposed in **Fig. 3.10**.

6.2 Future work and challenges

In recent years, the beneficial effects of lactoferrin containing probiotic products on health have been shown in clinical and animal studies (Tomita *et al.* 2009). However, the concept that the regulation of the intestinal microflora composition by probiotics and lactoferrin offers the possibility to influence the development of mucosal and systemic immunity, and prevention and treatment of some diseases, is still short of entire evidence. The human dietary intervention studies to investigate the hypothetical model shown in **Fig. 6.1.** appear very challenging.

6.2.1 Extended work based on the present study

The results in the present thesis have been supported by a recent study regarding the relative resistance of lactic acid bacteria to bovine lactoferrin and hydrolysed bovine lactoferrin (Chen *et al.* 2013). The special cell wall structures of probiotics or the protective materials related to their thick cell walls may contribute to this property. Further research may require observation of probiotic cell membranes using Atomic Force Microscopy (AFM) (Francius *et al.* 2008) and other appropriate technologies. On the other hand, the present study provided an indication that the selective antibacterial activity of *L. reuteri* DPC16 and its synergistic augmentation by bovine lactoferrin may have potential in the food industry for food preservation purposes. Much more work can be attempted in these applications.

Although the intestinal co-culture (Caco2/THP-1) model described in this thesis is complicated, the knowledge obtained from the initial attempts has provided useful information for future work. This model seems to be applicable and could reveal more insight into inflammatory bowel diseases. It may be a practical tool to bridge the gap between an *in vitro* study and an *in vivo* study in gut health research. The system is

believed to be an ideal approach to reveal the mechanisms of inflammation related to intestinal barrier dysfunction, and possibly lead to the discovery of functional food solutions after further development and optimisation of the system. In addition, the discovery and use of stable DNA damage-inducers rather than the use of faecal water in general would be another goal to achieve a stable *in vitro* colon carcinogenesis model. Finally, *in vivo* studies are suggested to explore the safety and colonisation properties of *L. reuteri* DPC16 in human subjects.

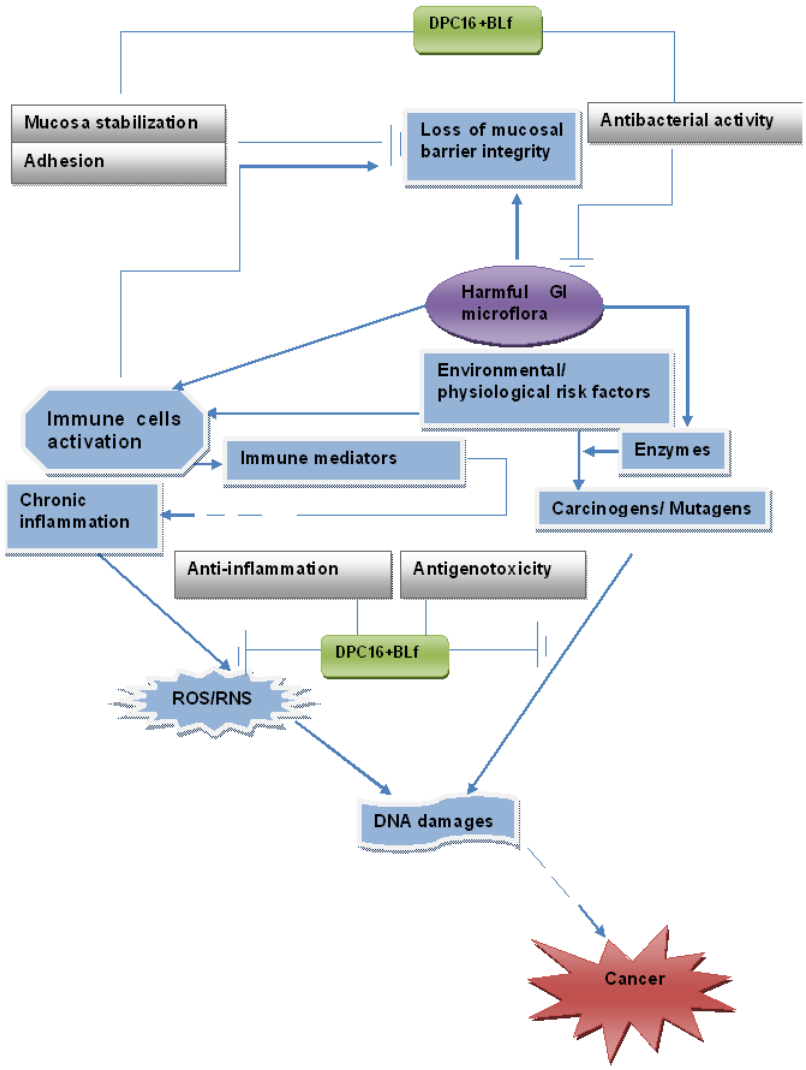


Figure 6.1 A hypothetical model of DPC16 + lactoferrin on intestinal disease prevention

6.2.2 Probiotic formulae in human GI intervention

Probiotic research is an interesting topic; studies in this field help understand the behaviour of GI microbiota in the human gut. However, it appears very challeging and

too early to know how and why probiotics modulate or benefit the gut functions. The knowledge on human GI microbiota is not sufficient and it is still in the accumulating stage. Some new publications in the last three years start to answer the questions of "who is there' and 'what are they doing there", and inadvertently arrive at the inquiry 'how and why' the GI microbiota functions (Korecka & Arulampalam 2012). Therefore, The major effects should be directed towards development of *in vitro* and *in vivo* models to understand the human gut microbiota and explore development of biotherapeutic possibility and methods (Prakash *et al.* 2011).

The present study has indicated that viable cells of *L. reuteri* DPC16 in the lower intestine may be reduced significantly due to the transit through the GI harsh digestion conditions and also there is a debateable point as to whether administered *L. reuteri* DPC16 cells are able to replicate and colonise *in vivo*. The composition of the human gut microbiota, as shown by the examination of faecal samples, has a remarkable stability (Savage 1977; Stebbings *et al.* 2002). Studies have shown that dietary supplementation is needed to introduce and maintain high levels of *L. reuteri* in hosts. Oral intake of *L. reuteri* has shown that faecal *L. reuteri* numbers increased rapidly within days of ingestion (Wolf *et al.* 1998). However, the question still remains as to whether its antimicrobial substances are able to suppress the growth of, and interfere with the adhesion of, pathogens *in vivo.* Similarly, with the other functions of antigenoxicity and immumodulation. Secondly, there is no doubt that the microbial profiles during the course of early childhood depend on the surrounding environmental exposure to both microbial and beneficial factors through breast milk (Palmer *et al.* 2007). Comparing to the stability of a core microbial community in the adult gut (Rajilic-Stojanovic *et al.* 2012), shaping the immature gut microbiota in infants could be less challenging. Thus, the outcomes of probiotic formulae intervention can be expected to be different in newborn and adult human models. Under the same principle, shaping the gut microbiota using a probiotic formula in a diseased human model could be different to that using a healthy human model. Hence, a carefully designed microencapsulated *L. reuteri* DPC16 and bovine lactoferrin combined formula is suggested to provide an opportunity to maintain higher bioavailability of both components and release within different timeframes to act synergistically in the gut. The microencapsulation studies should be extended to investigate not only how to maintain viable *L. reuteri* DPC16 cells in the target area but also how to keep them

active and stay longer, ideally to develop live probiotic cell *in situ* "factories" that could deliver live bacteria or continually release antibacterial and bioactive substances to maintain a healthy colon. The human clinical trials should be properly designed to achieve long-term randomised controlled studies by selecting proper biomarkers for the changes of GI microbiota composition, GI metabolites and GI functions as the primary outcomes of gut health improvement and well-being. Notably, even when the results from randomised, placebo-controlled trials support the beneficial effects of a particular probiotic for a specific indication, the benefits are generally not translatable to other probiotic formulations (Fontana *et al.* 2013).

6.3 Concluding remarks

The present study has demonstrated that *L. reuteri* DPC16 meets some criteria of probiotics *in vitro*, and that a relatively high number of viable cells could survive passage through the stomach and small intestine when they are ingested with food. The non-inhibitory nature of lactoferrin on probiotic bacteria, in comparison with the inhibitory effect on pathogens, has demonstrated a potential means of modification of the intestinal microflora synergistically and partially explains why the ingestion of colostrum results in the predominant pattern of bifidobacteria and lactobacilli in the breast-fed infant intestinal system. Finally, the combination of lactoferrin and probiotic *L. reuteri* DPC16 might be a means of preventing intestinal diseases through controlling pathogen invasion and endotoxin neutralization activities, as well as reducing the effects of harmful intestinal carcinogens.

Several significant properties of strain DPC16 and bovine lactoferrin have been demonstrated in the present study, including the antibacterial, antigenotoxic, and anti-inflammatory activities, and some possible mechanisms of action have been proposed. It has opened new avenues for exploration of the effects of their combination on disease prevention in the host. Unfortunately, the high cost of animal studies and time consumption did not allow testing of these hypotheses in this project. The limitation of *in vitro* models of intestinal wellness and disease is that they can not easily mimic the complex interactions between foods, microbiota and human cells that occur within the intestine; therefore they commonly exclude some of the important interactions. Thus, real system studies are strongly recommended to gain a better understanding of the

effects of probiotics and food bioactives on intestinal health for the future work. It remains possible that the observed *in vitro* effects may not be apparent or significant in the *in vivo* situation.

In summary, a major conclusion of this thesis refers to the **combination** of the probiotic properties of *L. reuteri* DPC16 and the bioactivities of bovine lactoferrin in the regulation of intestinal microflora and the prevention of intestinal inflammation and genotoxin-induced carcinogenesis. The results provide justification for conducting animal and clinical studies to assess the therapeutic.

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Publications

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MASSEY UNIVERSITY
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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Hong Tian

Name/Title of Principal Supervisor: Professor Ian Maddox

Name of Published Research Output and full reference:

Tian H., Maddox I.S., Ferguson L.R. & Shu Q. (2010) Influence of bovine lactoferrin on selected probiotic bacteria and intestinal pathogens. Biometals 23, 593-6.

In which Chapter is the Published Work: Chapter 3

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate completed all the work contained in the manuscript, while the co-authors have acted in a supervisory role and assisted in editing.

Candidate's Signature — Date: 20/08/2013

Principal Supervisor's signature — Date: 20/8/13

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Influence of bovine lactoferrin on selected probiotic bacteria and intestinal pathogens

Hong Tian · Ian S. Maddox · Lynnette R. Ferguson · Quan Shu

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Abstract This study investigated the effects of bovine lactoferrin (BLf) on the growth of different groups of bacteria in vitro. BLf showed a significant inhibitory effect on the growth of selected pathogens but not probiotics. BLf, in combination with probiotics, has the potential to influence the composition of the gut microflora via inhibition of intestinal pathogens with no significant effect on probiotic bacteria.

Keywords Bovine lactoferrin · Probiotics · Pathogens · Antibacterial activity

All authors contributed to this work.

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Introduction

Lactoferrin (Lf) is an iron-binding glycoprotein that is secreted in most mammalian external fluids. It acts as a first line defense agent against infections in the body (Levay and Viljoen 1995). It has been suggested that lactoferrin in breast milk plays a major role to maintain a predominance of bifidobacteria and lactobacilli in the infant intestinal system (Artym and Zimecki 2005; Coppa et al. 2006). This evidence suggests that the presence of Lf in the human gastrointestinal (GI) tract may contribute to re-structure the composition of the intestinal microflora. The bacteriostatic or bactericidal activity of lactoferrin is exhibited against a number of G^+ and G^- bacteria (Bullen 1976; Murdock et al. 2007). However, the reason why numbers of lactic acid bacteria, such as bifidobacteria and lactobacilli, in the digestive system remain high during breast-feeding of infants and in lactoferrin-supplemented animal model systems remains unclear (Artym and Zimecki 2005; Coppa et al. 2006; Tang et al. 2009). The effects of BLf on the growth of lactic acid probiotic bacteria appear not to have been studied. In the present study, the effects of BLf on the growth of different bacteria were tested, as well as the effect on pathogen growth of a combination of BLf and a selected probiotic bacteria strain.

Materials and methods

Bovine lactoferrin

Bovine lactoferrin (NZMP7100) was kindly supplied by Fonterra Co-operative Group, New Zealand. In this product the lactoferrin content is >90% of protein, and iron saturation is 10–20%. The pH value (5% solution at 20°C) is 5.5–6.5. Bacterial analysis is negative.

Bacteria strains

Probiotic bacteria *Lactobacillus acidophilus* DPC201, *Lactobacillus plantarum* DPC206, *Pediococcus acidilactici* DPC209, *Lactobacillus reuteri* DPC16, *Bifidobacterium lactis* HN019 and *Lactobacillus rhamnosus* HN001 strains and pathogen strains *Listeria monocytogenes* Scott-A ATCC49594, *Staphloccocus aureus* ATCC 25932, *Salmonella enterica* serovar Typhimurium ATCC 1772 and *Escherichia coli* O157:H7 strain 2988 were provided by Bioactives Research New Zealand (BRNZ), Auckland, New Zealand.

Culture media and conditions

The probiotic bacteria were grown in deMan-Rogosa-Sharpe (MRS) (Difco, Michigan, USA) broth containing cysteine (0.5 g/l) or on MRS agar plates for 18 h in an anaerobic jar at 37°C (Clayson Incubator, New Zealand). The pathogens were cultured in Brain Heart Infusion (BHI) (BBL, USA) broth or on BHI agar plates aerobically at 37°C for 24 h. Bovine Lactoferrin was filter-sterilized and added to autoclaved media as required.

Spectrophotometric turbidity bioassay

Bacterial cultures were diluted tenfold and 25 µl were transferred into a 96-well culture plate containing 225 µl of lactoferrin-supplemented medium. The growth response of each bacterial strain was monitored by determining the optical density at 620 nm using an ELISA plate reader (Model Multiskan EX, Thermo Electron Corporation) (Rosendale et al. 2008). Double strength bacterial culture medium was used in the experiments where cell-free supernatants of probiotic bacteria were tested for their inhibitory effects on the growth of pathogens and probiotics.

Bacteria standard drop plate count assay

After incubation, each bacterial culture was diluted in series using peptone saline water in 96-well culture. Appropriate concentrations were placed on agar plates using the standard drop plate count method (Chen et al. 2003). The viable colonies of bacteria were counted and reported as Log CFU/ml.

Statistical analysis

Data were processed using Excel spreadsheets. Results were expressed as means ± SD, and the significance of differences was determined using ANOVA.

Results and discussion

Effects of bovine lactoferrin on the growth of some pathogens and probiotics

The effects of bovine lactoferrin on the growth of pathogens, including G^+ and G^- strains, were determined by measuring the cell optical density. Figure 1a shows that bovine lactoferrin inhibited the growth of these pathogens in a dose-dependent manner. No significant inhibition was observed on the growth of probiotic strains (Fig. 1b).

As a comparison, bovine serum albumen (BSA), at the same concentrations as those used for lactoferrin, exhibited no inhibitory effects on the growth of either the pathogens or the probiotics. Figure 2 shows the colonies of *Escherichia coli* O157:H7 on BHI agar plates after it was incubated in the BLf- and BSA-supplemented growth medium at 37°C for 24 h.

Effects of polymyxin on growth of pathogens and probiotics

Polymyxin B is an antibiotic that is used for controlling G^- bacteria, but which has less effect on G^+ bacteria. Polymyxins are cationic proteins that bind to the bacterial cell membrane and alter its structure making it more permeable (Cardoso et al. 2007). In this study, it was hypothesized that the mechanism of action of the cationic protein lactoferrin against bacteria may be similar to that of polymyxin B. Thus, the effect of polymyxin on the growth of the selected pathogens and probiotics was studied. Figure 3a shows that

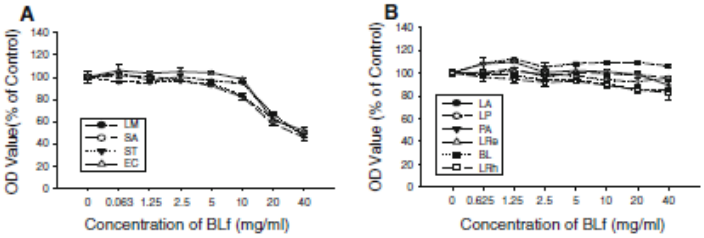

Fig. 1 **a** Dose responses of bovine lactoferrin (BLf) on growth of pathogens *Listeria monocytogenes* (LM), *Staphlococcus aureus* (SA), *Salmonella typhimurium* (ST) and *Escherichia coli* (EC). **b** Dose responses of bovine lactoferrin on probiotics *L. acidophilus* (LA), *L. plantarum* (LP), *P. acidilactici* (PA), *L. reuteri* (LRe), *B. lactis* (BL) and *L. rhamnosus* (LRh)

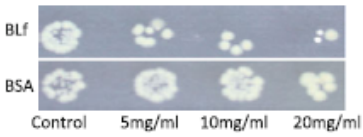

Fig. 2 The effects of BLf and BSA on the growth of EC tested by the standard drop plate count method

polymyxin B strongly suppressed the growth of the G^- bacteria ST and EC. A concentration of 60 μg/ml was required before significant inhibition of the G^+ bacteria LM and SA was observed. In contrast, Fig. 3b shows that polymyxin B did not inhibit any probiotics, even at the highest concentration tested.

Effects of bovine lactoferrin in combination with a probiotic cell-free supernatant on the growth of pathogens and probiotics

It is known that the probiotic supernatant contains compounds such as reuterin in *L. reuteri* culture that are inhibitory to the pathogens (Arques et al. 2004; Talarico et al. 1988). In the present study, the supernatant produced by *L. reuteri* DPC16 was examined for its effect on the growth of the pathogens, in the presence and absence of bovine lactoferrin. The results, shown in Fig. 4, reveal that the combination of lactoferrin (10 mg/ml) and *L. reuteri* DPC16 supernatant at a concentration of 0.125 (v/v) showed additional inhibition of the pathogens (Fig. 4a), but no effect on the probiotic bacteria (Fig. 4b), during growth for 24 h at 37°C.

In summary, the results demonstrated that Lf inhibited the growth of all tested G^+ and G^- pathogens but not the probiotics, at the concentrations tested. In contrast, BSA, tested as a protein control, inhibited neither the pathogens nor the probiotics. It is concluded, therefore, that lactoferrin is a special protein that possesses antibacterial capabilities to some bacteria, but not all. We propose that the special cell structures or metabolic substances of probiotics may protect the cells from the activities of cationic proteins

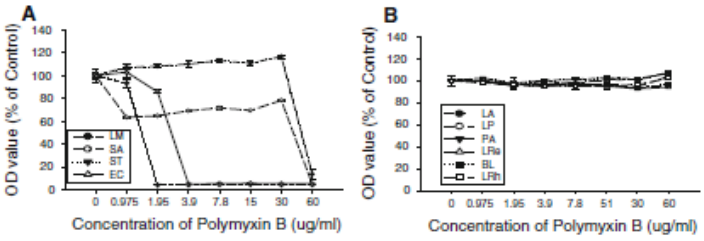

Fig. 3 **a** Dose response of polymyxin B against pathogens. **b** Dose response of polymyxin B against the probiotic bacteria. Symbols as for Fig. 1b

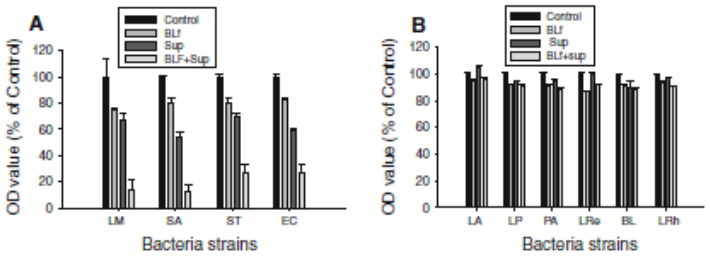

Fig. 4 **a** The effect of a combination of BLf and *L. reuteri* DPC16 supernatant on growth of pathogens. **b** The effect of a combination of BLf and *L. reuteri* DPC16 supernatant on growth of probiotic bacteria

such as lactoferrin and polymyxin B. Our results also show that a probiotic cell-free supernatant can augment the antibacterial activity of lactoferrin against pathogens without affecting the growth of the probiotics. These data provide more support to the suggestion that consumption of bovine lactoferrin with probiotics may contribute to a beneficial microflora balance in the GI tract.

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Hong Tian

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Name of Published Research Output and full reference:

Tian H., Maddox I.S., Ferguson L.R. & Shu Q. (2010) Evaluation of the cytoprotective effects of bovine lactoferrin against intestinal toxins using cellular model systems. Biometals 23, 589-92.

In which Chapter is the Published Work: Chapter 4 and Chapter 5

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate completed all the work contained in the manuscript, while the co-authors have acted in a supervisory role and assisted in editing.

Hong Tian
Candidate's Signature

20/03/2013
Date

Principal Supervisor's signature

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Date

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Evaluation of the cytoprotective effects of bovine lactoferrin against intestinal toxins using cellular model systems

Hong Tian · Ian S. Maddox · Lynnette R. Ferguson · Quan Shu

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Abstract Lactoferrin is an iron-binding glycoprotein that exhibits a range of health benefits including immune regulation and disease prevention derived from its structural properties. The present study employed immune cell models and a colon epithelial cell model to investigate the protective effects of bovine lactoferrin (BLf) on both immune cells and colon epithelium cells. BLf caused significant reduction of faecal genotoxin-induced DNA damage in HT29 cells, and down-regulation of lipopolysaccharide (LPS)-induced macrophage cell stress and endotoxic response, in an infection status.

Keywords Bovine lactoferrin · Cellular model system · DNA damage · Lipopolysaccharide · Faecal water

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Introduction

Lactoferrin is a multifunctional glycoprotein which may have beneficial preventive and therapeutic effects on infection, inflammation, and cancer as well as enhancing the iron status and growth of vulnerable groups (Lonnerdal 2009).

Lactoferrin has demonstrated an endotoxin-neutralizing capability in vitro and in vivo (Zhang et al. 1999). In addition, the preventive effects of BLf on colon carcinogenesis have been observed in animal models (Sekine et al. 1997; Tsuda et al. 2002). However, there are still many gaps in our knowledge of the detailed molecular mechanisms involved, including the binding interaction of Lf to intestinal toxins such as genotoxins and endotoxins, and on the locations of receptors associated with the immune response.

In light of these questions on the mechanisms of lactoferrin biological activity, the human and animal cellular model approach was considered appropriate to study the immunomodulatory and antitumor activities of BLf, with specific focus on the cellular level reduction of DNA damage and immune cell stress.

Materials and methods

Bovine lactoferrin (BLf)

BLf (NZMP7100) was kindly supplied by Fonterra Co-operative Group, New Zealand. In this product

the lactoferrin content is >90% of protein, and iron saturation is 10–20%. The pH value (5% solution at 20°C) is 5.5–6.5. Bacterial analysis is negative.

Immune cell models

Murine macrophage RAW 264.7 cell line was provided by Bioactives Research New Zealand (Auckland, New Zealand). Cells were incubated with 1 μg/ml of LPS with and without BLf for 24 h at 37°C in a 5% CO_2 humidified atmosphere (Cell culture CO_2 incubator: *SANYO*, Japan). This model was used to evaluate any relationships between inflammatory mediators and cell growth responses in the presence of LPS (from *E.coli* O127:B8, Sigma, USA) and BLf.

Human acute monocytic leukemia THP-1 cell line was provided by Plant and Food Research Ltd. (Auckland, New Zealand). Cells in concentration of 1 × 10^6 cells/ml were differentiated using 10 nM Phorbol myristrate acetate (PMA) (Sigma, USA) for 72 h. The differentiated THP-1 cells were then stimulated with 100 ng/ml of LPS at 37°C in a 5% CO_2 humidified atmosphere for 4 h. This model was used to test the inflammation responses of LPS during incubation with and without BLf. The TNF-α production was determined using Enzyme-linked immunosorbent assay (ELISA) (Park et al. 2007).

Colon carcinogenesis model

The human colon carcinoma cell line HT29 was obtained from the School of Medical Sciences (The University of Auckland, Auckland, New Zealand). HT29 cells were harvested and exposed to a faecal water sample in the presence of BLf. The Comet assay (Glei et al. 2006; Venturi et al. 1997) was performed to measure the antigenotoxic activity of lactoferrin on faecal water-induced DNA damage in HT29 cells.

Human faecal water preparation

Faecal samples were obtained from a healthy non-vegetarian, non-smoking female with no history of gastrointestinal disease, consuming a mixed diet. Faecal water was prepared as described elsewhere (Klinder et al. 2004).

Statistical analysis

Data were processed on an Excel spreadsheet. Results were expressed as means ± SD, the significance of differences was determined using ANOVA.

Results and discussion

Protective effects of bovine lactoferrin on LPS-induced murine macrophage cell stress

Cell proliferation and nitric oxide production were determined using MTT and Griess assays in the RAW 264.7 cell model. The results (Fig. 1) showed that bovine lactoferrin alone (1.25 mg/ml) did not influence murine macrophage cell stress or production of nitric oxide. In contrast, exposure of RAW 264.7 cells to LPS (1 μg/ml) at 37°C for 24 h significantly increased nitric oxide production and reduced cell proliferation (Fig. 1a). However, BLf at the concentration of 0.02–1.25 mg/ml significantly ($P < 0.05$) down-regulated nitric oxide production, or protected against LPS-induced immune cell stress, in a dose response manner (Fig. 1b).

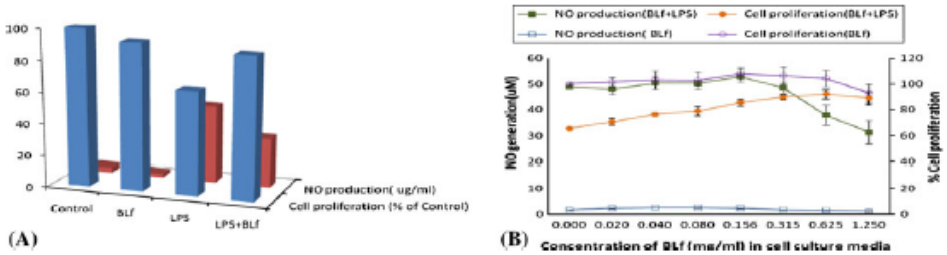

Fig. 1 **a** The effects of BLf or LPS and their combination on macrophage cell proliferation and nitric oxide production. **b** Dose responses of BLf on reduction of LPS-induced nitric oxide production and cell proliferation in macrophages

Effects of bovine lactoferrin on TNF-α production in LPS-induced THP-1 cells

To evaluate the endotoxin-neutralizing capability of lactoferrin by regulating production of pro-inflammatory cytokine TNF-α, BLf or bovine serum albumin (BSA), at concentrations of 0.125–2 mg/ml, were added to LPS-challenged (100 ng/ml) differentiated THP-1 cells. BLf, but not BSA, significantly reduced TNF-α release from LPS-activated THP-1 cells dose-dependently (Fig. 2a). Furthermore, to judge whether BLf blocks LPS receptors on THP-1 cells or detoxifies LPS by binding it directly, pre-treated THP-1 cells that were incubated with BLf for 1 h prior to challenge with LPS for 4 h were compared with BLf and LPS co-treatment for 4 h at 37°C in a 5% CO_2 humidified incubator. Figure 2b shows that a significant reduction in TNF-α production occurred during BLf plus LPS co-treatment rather than during BLf pre-treatment. These results confirm that the key reaction mediated by BLf was not directly on the THP-1 receptors; rather, the anti-inflammation actions were mediated by neutralization of endotoxin LPS.

Antigenotoxic effects of bovine lactoferrin on faecal water-induced colon epethelium DNA damage

The possibility of lactoferrin having antigentoxic properties on faecal water-induced DNA damage in HT29 cells was determined by measuring the DNA tail moment in the Comet assay. BLf (0.02–20 mg/ml) was incubated with faecal water at 37°C for 30 min prior to exposure to HT29 cells. The Comet images (Fig. 4) showed that lactoferrin, at 20 mg/ml and 10 mg/ml, significantly reduced faecal genotoxins-

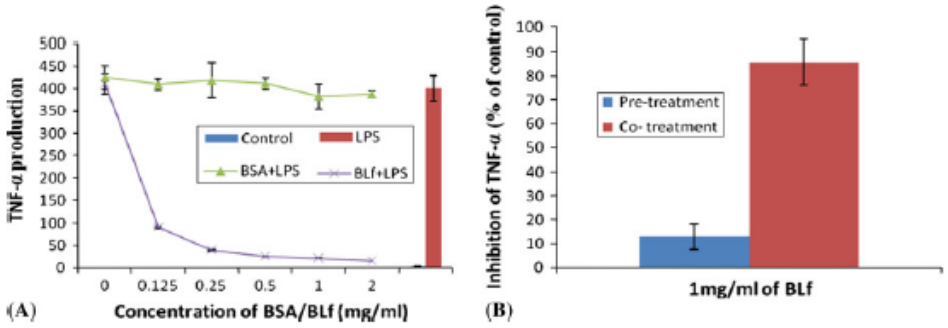

Fig. 2 **a** The effects of BLf and BSA on LPS-induced TNF-α production in THP-1 cell supernatant, tested using the ELISA method. **b** The comparison of TNF-α reduction in THP-1 cells during BLf plus LPS co-treatment and during BLf pre-treatment

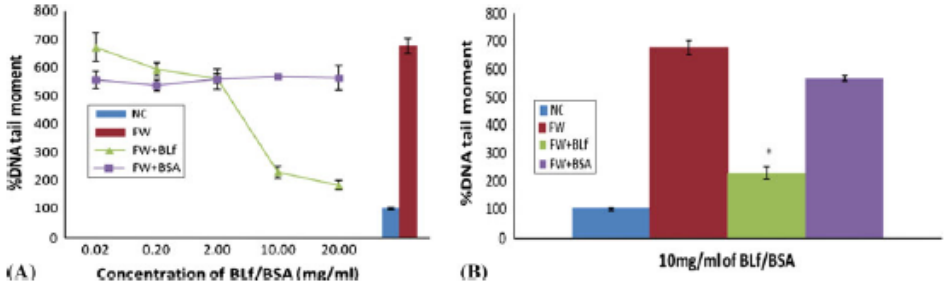

Fig. 3 **a** The dose responses of BLf and BSA on faecal water-induced HT29 cell DNA damage. **b** The reductive effect of BLf on faecal water-induced HT29 cell DNA damage ($P < 0.05$)

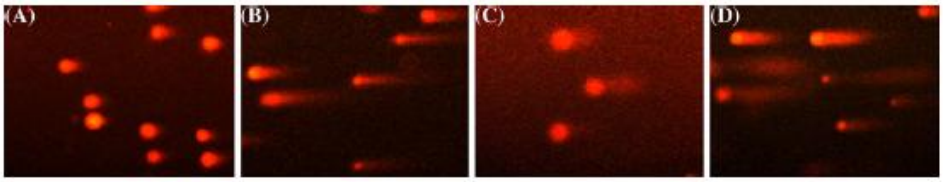

Fig. 4 Comet images are shown as negative control (NC, image **a**), positive control (faecal water, FW, image **b**) faecal water plus BLf (image **c**), faecal water plus BSA (image **d**)

induced DNA breakage (Fig. 3b). In contrast, bovine BSA, at same range of concentration as BLf, did not demonstrate any protective effects (Fig. 3a).

This study, using a cellular model approach, has demonstrated that BLf modulates the inflammatory process, mainly by preventing the release of cytokines and reactive nitro species from monocytes and macrophages, and by regulating the proliferation of immune cells via neutralization of endotoxin LPS. In terms of mechanism, these results not only confirm the effects of Lf on reduction of pro-inflammatory cytokines, but also partially explain the action of Lf involving a signaling pathway on immune receptors recognizing the pathogen-associated molecular pattern. Lf possibly modifies the necessary structures of LPS, which are able to be recognized by the CD14 and Toll-Like Receptors (TLRs) on immune cells. Taken as a whole, the present results and previous observations (Appelmelk et al. 1994) strongly suggest that lactoferrin is one of the key molecules which defend against pathogenesis and modulate the inflammatory response. These antibacterial and endotoxin-reduction activities are related to the ability of lactoferrin to bind lipopolysaccharides (LPS) with high affinity. Additionally, the results have shown that BLf is able to protect against epithelium DNA damage induced by genotoxic faecal water, and, thus, possibly reduce the risk of colon cancer. These results provide additional biological mechanism evidence to support the previous Lf anticancer activities in animal studies (Iigo et al. 2009; Tsuda et al. 2000).

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