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Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials

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Abstract

The enteric flora comprise approximately 95% of the total number of cells in the human body and are capable of eliciting immune responses while also protecting against microbial pathogens. However, the resident bacterial flora of the gastrointestinal tract (GIT) may also be implicated in the pathogenesis of several chronic conditions such as inflammatory bowel disease (IBD). The University College Cork-based Probiotic Research Group has successfully isolated and identified lactic acid bacteria (LAB) which exhibit beneficial probiotic traits. These characteristics include the demonstration of bile tolerance; acid resistance; adherence to host epithelial tissue; and *in vitro* antagonism of potentially-pathogenic micro-organisms or those which have been implicated in promoting inflammation. The primary objective of this report is to describe the strategy adopted for the selection of potentially effective probiotic bacteria. The study further describes the evaluation of two members of the resulting panel of micro-organisms (*Lactobacillus salivarius* subsp. *salivarius* UCC118 and *Bifidobacterium longum infantis* 35624) under *in vitro* conditions and throughout *in vivo* murine and human feeding trials. Specifically, an initial feeding study completed in Balb/c mice focused upon (i) effective delivery of the probiotic micro-organisms to the GIT and evaluation of the ability of the introduced strains to survive transit through, and possibly colonise, the murine GIT; (ii) accepting the complexity of the hostile GIT and faecal environments, development of a method of enumerating the introduced bacterial strains using conventional microbiological techniques; and (iii) assessment of the effects of administered bacterial strains on the numbers of specific recoverable indigenous bacteria in the murine GIT and faeces. Additional research, exploiting the availability of murine models of inflammatory bowel disease, demonstrated the beneficial effects of administering probiotic combinations of *Lactobacillus salivarius* UCC118 and *Bifidobacterium longum infantis* 35624 in prevention of illness-related weight loss. A further ethically-approved feeding trial, successfully conducted in 80 healthy volunteers, demonstrated that yoghurt can be used as a vehicle for delivery of *Lactobacillus salivarius* strain UCC118 to the human GIT with considerable efficacy in influencing gut flora and colonisation.

Introduction

Criteria for the selection of probiotic micro-organisms

Diet, stress, and modern medical practices have been implicated as factors capable of exerting an influence on human health and nutrition. As a result of these and other factors, the incidence of illnesses which may be induced by deficient or compromised microflora (e.g., gastrointestinal tract infections; constipation; irritable bowel syndrome (IBS); inflammatory bowel disease (IBD) – Crohn's disease and ulcerative colitis; food allergies; antibiotic-induced diarrhoea;

cardiovascular disease; and certain cancers) is increasing (Salminen et al. 1995, 1998a; Schaafsma 1995). Probiotics have been defined as live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance (Fuller 1989), or more broadly, as 'living micro-organisms, which upon ingestion in certain numbers, exert health affects beyond inherent basic nutrition' (Guarner & Schaafsma 1998). Combinations of various micro-organisms, particularly species of *Lactobacillus* and *Streptococcus*, have traditionally been used in fermented dairy products to promote human health through the influences they may exert on the microbial ecology of the host, lactose intolerance, incidence of diarrhoea, mucosal immune response, levels of blood cholesterol, and cancer (Salminen et al. 1998a,b). Indeed, a number of probiotic bacteria are now being successfully exploited commercially (e.g., *Lactobacillus rhamnosus* GG (Saxelin 1997), *L. casei* Shirota (Aso & Akazan 1992), and *L. acidophilus* LA-1 (Bernet et al. 1994)). However, many consumers, consumer organisations, and members of the scientific community remain sceptical of such products and their associated probiotic claims. As a result, the food industry (in collaboration with academic scientists, clinicians, and workshops such as The Lactic Acid Bacteria Industrial Platform (LABIP) and PROBDEMO (FAIR CT-96 1028) (Mattila-Sandholm 1997) supported by European Union-funded programmes) aims to promote the generation and dissemination of consensus opinions obtained through the direct interaction of research institutions and universities with industry.

Such a consensus was reached when the participants involved in the LABIP workshop on probiotics concluded that 'probiotics may be consumed either as a food component or as a non-food preparation'. The LABIP participants further supported criteria outlined by others for the selection and assessment of probiotic LAB (Guarner & Schaafsma 1998). In summary, to fulfil these criteria (Marteau & Rambaud 1993; Huis in't Veld & Shortt 1996; Salminen et al. 1996; Tannock 1997; Brassart et al. 1998; Guarner & Schaafsma 1998) probiotic micro-organisms should:

- Be of human origin.
- Demonstrate non-pathogenic behaviour.
- Exhibit resistance to technological processes (i.e., viability and activity in delivery vehicles).
- Prove resistant to gastric acid and bile.
- Adhere to gut epithelial tissue.
- Be able to persist, albeit for short periods, in the gastrointestinal tract.
- Produce antimicrobial substances.
- Modulate immune responses.
- Have the ability to influence metabolic activities (e.g., cholesterol assimilation, lactase activity, vitamin production).

In addition, these requirements were further expanded by Berg (Berg 1998) and Salminen et al. (Salminen et al. 1996, 1998a) who stated that:

a) each potential probiotic strain should be documented and assessed independently.
b) extrapolation of data from closely related strains is not acceptable.
c) only well defined strains, products and study populations should be used in trials.
d) where possible all human studies should be randomised, double-blind, and placebo-controlled.
e) results should be confirmed by independent research groups.
f) preferentially, the study should be published in a peer-reviewed journal.

Isolation of potential probiotic bacteria

In the development of probiotic foods intended for human consumption, strains of lactic acid bacteria such as *Lactobacillus*, *Bifidobacterium*, *Enterococcus* have been most commonly used. This is primarily due to the perception that they are desirable members of the intestinal microflora (Table 1) (Goldin & Gorbach 1992; Berg 1998). In addition, these bacteria have traditionally been used in the production of fermented dairy products and have 'GRAS: generally regarded as safe' status (O'Sullivan et al. 1992). However, a subset of the probiotic micro-organisms currently employed in the dairy-food industry are not of human origin and, therefore, do not meet the criteria for the selection of probiotic microbes acceptable for human consumption, as outlined above (Daly & Davis 1998).

The enteric flora, including greater than 500 bacterial species, comprise approximately 95% of the total number of cells in the human body and contribute significantly to the host's resistance to infectious disease. Furthermore, changes in the composition of the intestinal flora are often associated with disease and may, in some cases, be a causative factor (Morotomi et al. 1975). At University College Cork, it was recognised that while lactobacilli isolated from human faeces have been relatively well characterised (Kandler & Weiss 1986)few attempts had been made to isolate potential adherent probiotic bacteria directly

Table 1. Probiotic bacteria and their reported properties

Strain	Reported effects in clinical studies	Selected references
Lactobacillus acidophilus LC1	Immune enhancing; vaccine adjuvant; adherence to human intestinal cells; balancing of intestinal microflora	(Bernet et al. 1994)
Lactobacillus acidophilus NCFO1748	Lowering of faecal enzymes; prevention of radiotherapy -related diarrhoea; treatment of constipation	(Lidbeck et al. 1992)
Lactobacillus rhamnosus GG	Prevention of antibiotic associated diarrhoea; treatment and prevention of rota-virus diarrhoea; treatment of relapsing *Clostridium difficile* diarrhoea; prevention of acute diarrhoea; alleviation of Crohn's disease; antagonistic against anticarcinogenic bacteria	(Salminen et al. 1993)
Lactobacillus casei Shirota	Prevention of intestinal disturbances; balancing of intestinal bacteria; lowering of faecal enzymes; inhibition of superficial bladder cancer	(Aso & Akazan 1992)
Lactobacillus gasseri	Faecal enzyme reduction; survival in the intestinal tract.	(Pedrosa et al. 1995)
Bifidobacterium bifidum	Treatment of rota-virus diarrhoea; balancing of intestinal microflora; treatment of viral diarrhoea	(Marteau et al. 1990)

(Adapted from Lee & Salminen (1995)).

from the human intestinal mucosa (the environment into which they may subsequently be re-introduced and required to function). Therefore, bacteria were isolated from resected human terminal ileum (the small intestine consists of the duodenum, jejunum, and ileum) obtained from patients undergoing urinary tract reconstructive surgery. Approximately 1500 catalase negative bacterial isolates were chosen and further characterised. Of these, greater than 60% were Gram positive, homofermentative cocci; approximately 18% proved to be Gram negative rods and heterofermentative coccobacilli; while 22% were predominantly homofermentative coccobacilli. Of the latter group, thirty-eight Gram-positive isolates were further characterised by physiological and biochemical means. Of these micro-organisms, 28 were subsequently found to belong to the *Lactobacillus* genus (mainly *L. paracasei* and *L. salivarius*) (Thornton 1996). *Lactobacillus acidophilus* has previously been recovered in relatively high numbers from the GIT. However, additional reports have demonstrated that other *Lactobacillus* groups (*L. crispatus*, *L. gasseri*, *L. salivarius*, and *L. reuteri*) can also be isolated at similar levels (Molin et al. 1993), indicating the lack of a single dominant species.

In addition to lactobacilli, presumptive strains of *Bifidobacterium* were isolated from ileum tissue samples which had been homogenised. Using molecular analysis, these bifidobacteria were classified as *B. infantis* (O'Riordan & Fitzgerald 1997). Interestingly, these results differ significantly from previous observations that adults do not possess detectable levels of *B. infantis* but rather *B. longum* and *B. bifidum* (Mitsouka 1984).

Assessment of potential probiotic bacteria *in vitro*

A selection of the bacterial strains isolated from the surgically-removed segments of human ileum were chosen for *in vitro* evaluation, within the parameters outlined above (Marteau & Rambaud 1993; Huis in't Veld & Shortt 1996; Salminen et al. 1996; Tannock 1997; Brassart et al. 1998; Collins et al. 1998; Guarner & Schaafsma 1998).

Resistance to gastric acid

Prior to reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach where the secretion of gastric acid constitutes a primary defence mechanism against the majority of ingested micro-organisms. Therefore, preliminary experiments were performed to determine the level of acid resistance exhibited by a number of both *Lactobacillus* and *Bifidobacterium* strains isolated from the human ileum. Survival of the bacterial strains was initially assessed by adding approximately 10^8 cfu/ml and 10^6 cfu/ml lactobacilli and bifidobacteria, respectively, to MRS medium (De Man et al. 1960) amended with HCL to between pH2.0 and pH3.4. However, survival

of bacterial strains in human gastric juice is a more accurate indication of the ability to survive passage through the stomach. For this reason, human gastric juice was obtained from healthy subjects by aspiration through a nasogastric tube. As the pH of the stomach is known to fluctuate (e.g., when an individual is fasting the stomach pH may be as low as 1.5 (Draser et al. 1969)) the pH of the obtained samples were quantified prior to use. The gastric juice was then added to MRS medium as described above. The observed results indicated that the lactobacilli isolates (including *Lactobacillus salivarius* UCC118*)* have the potential to successfully transit the human stomach and may possess the ability to reach the intestinal environment in which they may function effectively. However, when exposed to human gastric juice the bifidobacteria strains proved significantly less acid resistant than the lactobacilli (Thornton 1996).

Bile acid resistance

Whilst evaluating the potential of utilising lactic acid bacteria as effective probiotics it is generally considered necessary to evaluate their ability to resist the effects of bile acids (Lee & Salminen 1995). Bile acids are synthesised in the liver from cholesterol and are secreted from the gall-bladder into the duodenum in the conjugated form (500-700 ml/day) (Hoffman et al. 1983). These acids then undergo extensive chemical modifications (deconjugation, dehydroxylation, dehydrogenation, and deglucuronidation) in the colon due almost solely to microbial activity (Hill & Draser 1968; Shimada et al. 1969; Hylemon & Glass 1983). Both conjugated and deconjugated bile acids exhibit anti-bacterial activity inhibiting the growth of *E. coli* strains, *Klebsiella* sp., and *Enterococcus* sp. *in vitro* (Lewis & Gorbach 1972; Stewart et al. 1986). However, the deconjugated forms are more inhibitory (Floch et al. 1972; Percy-Robb & Collee 1972; Stewart et al. 1986), while Gram positive bacteria are found to be more sensitive than Gram negative (Floch et al. 1972).

In these preliminary studies, the objective was to determine the level of bile acid resistance exhibited by a number of both *Lactobacillus* and *Bifidobacterium* strains isolated from the human ileum. In short, solid media were supplemented with bovine bile (Sigma Chemicals Co. Ltd., Poole, UK), porcine bile (Sigma), and human bile (obtained at laproscopic cholecystectomy from human gall-bladders) to final concentrations of between 0.3 and 7.5%. These plates were then incubated at 37 °C under anaerobic conditions and growth was recorded after 24–48 hours (Thornton 1996). Following analysis, it was apparent that while the tested lactobacilli and *Bifidobacterium* strains exhibited resistance to the bovine bile used, the porcine bile employed in these assays proved significantly more inhibitory to both of the bacterial groups (Thornton 1996). However, in relation to the assessment of probiotic strains intended for human consumption, the most relevant determination is that of their ability to grow in the presence of human bile. Interestingly, regardless of the resistance patterns observed in the presence of either bovine or porcine bile, all of the assayed bacteria were capable of growth in physiologically relevant concentrations of human bile (approximately 0.3%) (Thornton 1996).

Adherence of bacterial isolates to HT-29 and Caco-2 cell-lines

As outlined above, the consensus opinion of the LABIP participants in relation to the selection of probiotic bacterial strains is that they be capable of adhesion to gut epithelial tissue and possess the ability to colonise the gastrointestinal tract.

HT-29 and Caco-2 cells are human intestinal cell-lines expressing morphological and physiological characteristics of normal human enterocytes (Brassart et al. 1998). These cell-lines have been previously exploited to elucidate the mechanisms mediating enteropathogen adhesion (Neeser et al. 1989; Kerneis et al. 1991; Bernet et al. 1994). In more recent studies, however, they have been employed in order to select for, and subsequently assess, lactic acid bacteria on the basis of their adherence properties (Coconnier et al. 1992; Bernet et al. 1993; Greene & Klaenhammer 1994; Crociani et al. 1995; Sarem et al. 1996; Tuomola & Salminen 1998). In our studies, completed using both HT-29 and Caco-2 cell-lines, the observed adherence of the *Lactobacillus* strains (including *Lactobacillus salivarius* UCC118) compared well with that of the well-characterised adherent strain *Lactobacillus rhamnosus* GG (Dunne et al. 1999). However, significantly lower levels of adherence were observed when the *Bifidobacterium* strains were assessed, regardless of the cell-line used (Dunne et al. 1999).

Antimicrobial activity

Several metabolic compounds produced by lactic acid bacteria demonstrate antimicrobial effects, including

organic acids, fatty acids, and hydrogen peroxide (Ouwehand 1998). However, bacteriocins or proteinaceous substances with specific inhibitory activity against closely related species are perhaps the most extensively studied (Abee et al. 1994; McAuliffe et al. 1998; Ouwehand 1998). Of these, nisin which is produced by some *L. lactis* subsp. *lactis* strains is the only purified bacteriocin approved for use in products intended for human consumption (Dodd & Gasson 1994; Jack et al. 1995).

In vitro *antagonism of selected micro-organisms by* Lb. salivarius *UCC118 or its antimicrobial product, ABP118*

In our studies, the lactobacilli and bifidobacteria isolated from the human ileum were assayed for antimicrobial activity against a range of indicator bacteria including strains of *Listeria*, *Bacillus*, *Enterococcus*, *Staphylococcus*, *Clostridium*, *Pseudomonas*, *E. coli*, *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, and *Lactococcus*. Antimicrobial activity of *Lb. salivarius* UCC118 was detected using the method described by Tagg et al. (1971). In summary, strain UCC118 was incubated anaerobically in MRS broth for 12–16 h at 37 °C. Following centrifugation at 3000 *g* and washing in quarter-strength Ringer's solution, strain UCC118 was resuspended in an equal volume of quarter-strength Ringer's solution. *Lb. salivarius* UCC118 was then inoculated (100 μl) onto solid MRS medium (supplemented with 2% ß-glycerophosphate (Sigma Chemicals, Poole)) and incubated anaerobically overnight at 37° C. The grown cultures were overlaid with the test micro-organisms in sloppy agar, incubated overnight and zones of inhibition (greater than 1 mm in radius) were recorded. To determine whether the observed antimicrobial activity of *Lb. salivarius* UCC118 was secretory in nature, aliquots (500 μl) of the 12–16 h UCC118 culture were filter-sterilised and the cell-free supernatant subsequently assayed for the ability to inhibit the growth of selected potential pathogens, as described above.

Both *Lb. salivarius* UCC118 and its cell-free supernatant were found capable of significantly inhibiting the *in vitro* growth of a range of both Gram positive and Gram negative bacterial cultures (Table 2). Significantly, neither *Lb. salivarius* UCC118 nor its supernatant were found capable of impairing the *in vitro* growth of closely related lactic acid bacteria (such as *Lb. acidophilus* or *Lb. reuteri*) with the exception of *Lb. fermentum* KLD (Table 2). The growth of strains

Table 2. Inhibition of the growth of selected micro-organisms by *Lactobacillus salivarius* subsp. *salivarius* strain UCC118 or its derived antimicrobial factor, ABP118, under *in vitro* conditions

Assayed micro-organisms	*In vitro* inhibition of growth by *Lb. salivarius* UCC118	*In vitro* inhibition of growth by *Lb. salivarius* UCC118-derived ABP118
Lactobacillus acidophilus	–	–
Lactobacillus bulgaricus	–	–
Lactobacillus rhamnosus GG	–	–
Lactobacillus fermentum KLD	+	+
Lactobacillus plantarum	–	–
Streptococcus thermophilus	–	–
Streptococcus mutans	–	–
Bifidobacterium longum	+/–	–
Bifidobacterium bifidum	+/–	–
Bifidobacterium breve	+/–	–
Bifidobacterium infantis	+/–	–
Bacillus subtilus	+	+
Bacillus cereus	+	+
Bacillus thuringiensis	+	+
Bacillus coagulans	+	–
Enterococcus faecalis	+	+
Enterococcus faecium	+	+
Escherichia coli	+	–
Listeria monocytogenes	+	+
Pseudomonas putida	+	–
Pseudomonas aeruginosa	+	–
Pseudomonas fluorescens	+	+
Salmonella typhimurium	+	–
Salmonella enteriditis	+	–
Staphylococcus aureus (non-methicillin resistant)	+	–
Staphylococcus aureus (methicillin resistant)	+	+
Candida albicans	–	–

of *Leuconostoc*, *Lactococcus*, and *Pediococccus* was also uninhibited (data not shown).

Purification and partial sequencing of the Lb. salivarius *UCC118-derived proteinaceous antimicrobial factor ABP118*

Lb. salivarius UCC118 was incubated anaerobically in 2 L of liquid MRS medium at 37 °C for approximately 6–8 h. Following centrifugation at 3000 *g*, the *Lb. salivarius* UCC118-produced antimicrobial agent, ABP118, was purified to homogeneity in a sequential process of ammonium sulphate precipitation, hydrophobic interaction chromatography, cation exchange chromatography, and C_2/C_{18} reverse-phase chromatography. Antimicrobial activity of the purified factor was confirmed using the method described above (Table 2).

The purified antimicrobial factor was hydrolysed and analysed on an amino acid analyser, as described previously (Fykse et al. 1988). The amino acid sequence was also analysed by Edman degradation (Cornwell et al. 1988). The C-terminal sequence of ABP118 was determined following cleavage of the antimicrobial factor with cyanogen bromide (CnBr) as described previously (Sletten & Husby 1974).

Strain UCC118-derived ABP118 was found to be heat stable, resistant over a wide pH range, and resistant to treatment with a number of detergents and organic solvents (data not shown). On the basis of the amino acid composition the ABP118 peptide has a molecular weight of between 4.8 and 5.2 kDa. Although, the N-terminus was blocked for sequencing by Edman degradation, following cyanogen bromide cleavage residues (K-R-G-P-N) were sequenced at the C-terminus. Similarities in the non-redundant protein databases at the National Centre for Biological Information (NCBI) were searched for using the BLAST algorithm (Altschul et al. 1990). However, the sequenced residues did not demonstrate any significant homology with other antimicrobial factors in the databases.

Probiotic modulation of host immune responses

Both immunological and non-immunological defence mechanisms protect the human gastrointestinal tract, considered to be the largest immune organ in the body due to the extensive surface area of the small bowel, from colonisation by intestinal bacteria (McCracken & Gaskins 1999). Innate defence mechanisms include the low pH of the stomach, bile salts, peristalsis, mucin layers and anti-microbial compounds such as lysozyme (Savage 1997). Immunological mechanisms include specialised lymphoid aggregates termed Peyers patches which are distributed throughout the small intestine and colon. Luminal antigens presented at these sites can result in the stimulation of appropriate T and B cells, the establishment of cytokine networks, and the secretion of antibodies into the gastrointestinal tract (Neutra & Kraehenbuhl 1996). Notably however, as the gastrointestinal mucosa is the largest surface at which the host interacts with the external environment, specific control mechanisms must be in place to regulate immune responsiveness to the 100 tons of food handled by the gastrointestinal tract over an average lifetime (Shanahan 1994). Furthermore, as the gastrointestinal tract is colonised by over 500 species of bacteria (numbering 10^{11}–10^{12}/g in the colon) these control mechanisms must be capable of distinguishing non-pathogenic adherent bacteria from invasive pathogens potentially capable of causing significant damage to the host.

Immune education

The enteric flora are an important aspect of the development and appropriate function of the intestinal immune system. In the absence of an enteric flora, the intestinal immune system is underdeveloped, as demonstrated in germ free animal models, and certain functional parameters are diminished (e.g., macrophage phagocytic ability and immunoglobulin production) (Wostman 1996). Furthermore, the importance of the gut flora in stimulation of non-damaging immune responses is becoming more evident. In short, the increases in observed incidence and severity of allergies (and conditions such as inflammatory bowel disease – Crohn's disease and ulcerative colitis) in the Western world has been linked with increases in standards of hygiene and sanitation, concomitant with a decrease in the number and range of infectious challenges encountered by the growing and developing host. This lack of immune education may allow the host to over-react to non-pathogenic antigen-containing commensal flora, resulting in inflammatory damage and/or allergy and autoimmunity.

The deliberate consumption of non-pathogenic bacteria, such as probiotic bacteria, may provide a health promoting immune-educating challenge to the host. The promotion of broadspectrum immuno-

globulin secretion into the lumen, following consumption of probiotic bacteria, may result in binding of allergens and prevention of their transmucosal uptake by the host. In addition, interaction with certain intraepithelial lymphocyte subsets may suppress immune responses to allergens within the gastrointestinal tract possibly resulting in tolerance to perceived antigens. Thus, the deliberate consumption of probiotic bacteria in order to replace immune stimuli artificially is being researched extensively.

Oral vaccination using recombinant probiotic bacteria

The majority of pathogenic organisms gain entry via mucosal surfaces. Efficient vaccination of these sites may protect against invasion by particular infectious agents. Oral vaccination strategies have concentrated, to date, on the use of attenuated live pathogenic organisms or purified encapsulated antigens. However, lactic acid bacteria, modified to deliver antigens derived from infectious agents, may provide an attractive alternative as these bacteria are considered to be safe for human consumption (GRAS status). The European Commission-sponsored 'LABVAC' programme (BIO4-CT96-0542: representing the collaboration of 10 research groups from France, Belgium, the Netherlands, Ireland, Italy, England, and Australia) is specifically focused upon the development of lactic acid bacteria as non-pathogenic viable antigen delivery systems. Notably, within this programme, particular emphasis has been placed on the immunological evaluation of such bacteria as potential vectors for the production and local delivery of protective antigens in the gastrointestinal tract.

In vivo assessment of potential probiotic bacterial strains

To date, the benefits of probiotics have predominantly been demonstrated under defined and well-controlled laboratory conditions. In response, the LABIP workshop participants, amongst others, have stated that while 'several *in vitro* assays or animal studies ... are very useful in the preselection of (probiotic) bacterial strains ... the proof of efficacy in humans should be granted by at least one well-designed human study (Berg 1998; Collins et al. 1998; Guarner & Schaafsma 1998). Recently, several prospective studies have demonstrated the efficacy of lactic bacteria administration for both prophylactic and therapeutic use against diarrhoea in premature infants (Millar et al. 1993), new-borns (Sepp et al. 1993), children (Isolauri et al. 1995) and in the therapy of antibiotic-related diarrhoea (Siitonen et al. 1990), and traveller's diarrhoea (Oksanen et al. 1990).

Murine feeding studies

As described above, the criteria used in our studies for the selection and assessment of potential probiotic bacterial strains resulted in the identification of a small number of *Lactobacillus* and *Bifidobacterium* strains capable of resisting the effects of bile and low pH, of adhering to human epithelial cell-lines, and of exhibiting antimicrobial activity *in vitro* (Thornton 1996). Of these bacterial isolates, *Lb. salivarius* UCC118 and *Bifidobacterium longum infantis* UCC35624 were selected for assessment of their ability to survive transit through the murine gastrointestinal tract.

Feeding trial in Balb/c mice

Due to the fact that the gastrointestinal tract is a complex and hostile environment, it appears unlikely that a single probiotic bacterial strain will be capable of significantly influencing the microbial ecology of the host. However, for any micro-organism to effectively fulfill a prophylactic role, it must be capable of surviving and colonising this environment at least transiently. For instance, while *Lb. rhamnosus* GG is easily enumerated, it shows limited persistence in faeces after termination of feeding and was found to cause no significant changes in the population of lactobacilli over a five week study (Goldin et al. 1992). Recently, however, Alander et al. (1997) have shown that *Lb. rhamnosus* GG is recoverable from colonic biopsies obtained from humans administered the probiotic strain, indicating that faecal enumeration is not truly reflective of what occurs in the human gut.

The LABIP workshop participants, amongst others, have further stated that *in vitro* assays or animal studies are useful in the preselection of (probiotic) bacterial strains (Berg 1998; Collins et al. 1998; Guarner & Schaafsma 1998). Therefore, the objectives of this study completed in pathogen-free mice focused upon (i) effective delivery of the probiotic micro-organisms to the GIT and evaluation of the ability of the strains to survive transit through, and possibly colonise, the murine GIT; (ii) accepting the complexity of the hostile GIT and faecal environments, development of a method of enumerating the

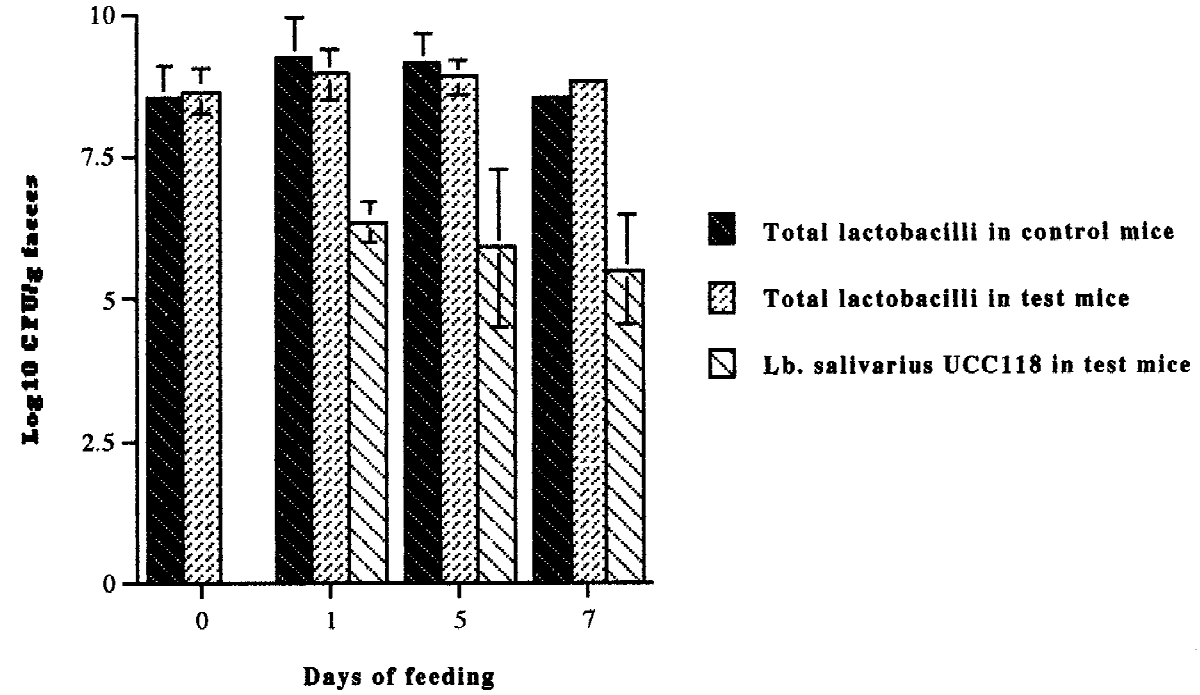

Figure 1. Enumeration of total lactobacilli and *Lb. salivarius* subsp. *salivarius* strain UCC118 in mice during feeding trials. Results are expressed as $\log_{10}$ CFU/g faeces.

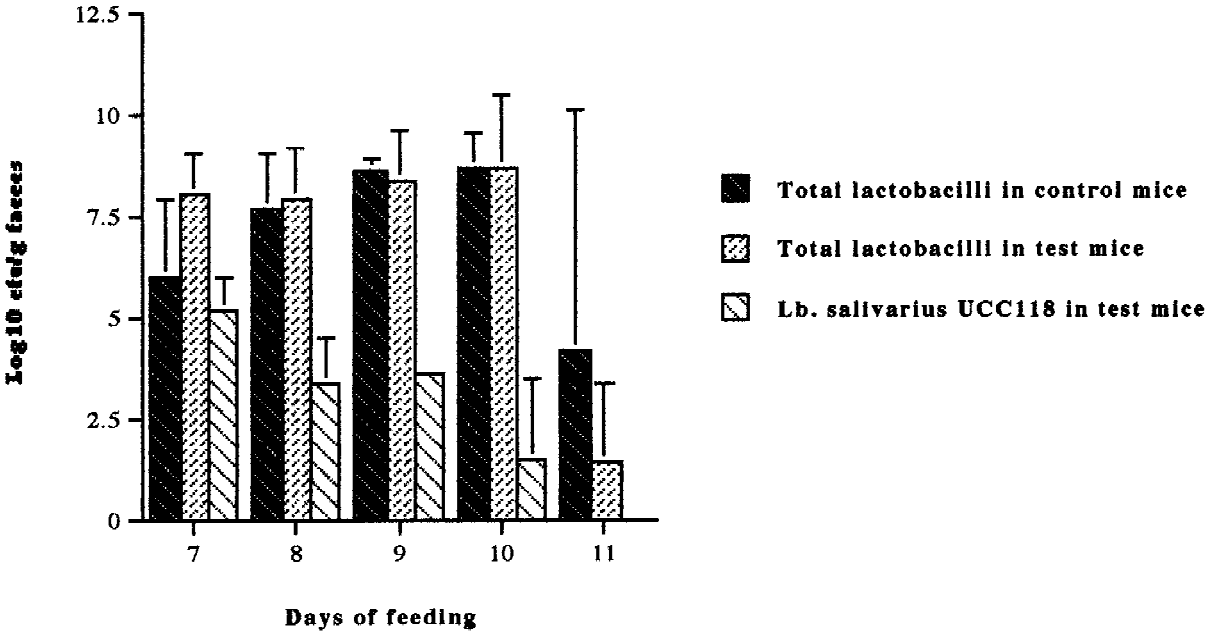

Figure 2. Enumeration of total lactobacilli and *Lb. salivarius* subsp. *salivarius* strain UCC118 following cessation of feeding at day 7 of the experiment. Results are expressed as $\log_{10}$ CFU/g faeces.

introduced bacterial strains using conventional microbiological techniques; and (iii) assessment of their effect on the numbers of indigenous bacteria in the murine GIT and faeces. *Lb. salivarius* strain UCC118 was administered (4×10^9 CFU/day) to Balb/c mice in skim milk diluted with sterile drinking water and enumerated from collected faeces (Figure 1) due to their previously-generated resistance to rifampicin. Importantly, examination of the survival of strain UCC118 indicated that rifampicin resistance is a suitable non-genetic method of enumerating introduced bacterial strains and that skim milk proved an effective delivery vehicle. A further objective of this study was to investigate the length of time administered *Lb. salivarius* UCC118 would persist in faeces following termination of feeding. In this study, it was found that the probiotic strain could be recovered (with no oral supplementation) from faeces for up to three days after cessation of feeding (Figure 2). Strain UCC118(LM2) was no longer recoverable from faeces after 4 days (Figure 2). These results suggest that *Lb. salivarius* strain UCC118(LM2) is capable of persistence in the mouse gut, at least for a limited period. This observation is supported by the results of a study completed by Goldin et al. (Goldin et al. 1992) where it was demonstrated that 60–80% of individuals consuming *Lb. rhamnosus* GG excreted this strain for 3–4 days, but only 33% of the population after 7 days. Therefore, it appears likely that daily administration of the preferred strain is necessary for maintenance of high probiotic levels.

It is not difficult to understand how a single bacterial strain, introduced into an environment such as the human GIT containing greater than 500 bacterial species, may be unable to effectively compete and become established. Fonty et al. (Fonty et al. 1993), suggested that the establishment of an introduced strain is governed, not only by the mode of administration of the strain, but also by the interaction of micro-organisms within the gut environment. Throughout this study, the levels of indigenous enterococci, bifidobacteria, coliforms, and *Bacteroides* culturable from mouse faeces were not significantly modified by administration of *Lb. salivarius* UCC118.

Feeding trial in a murine model of inflammatory bowel disease

Inflammatory Bowel Disease (IBD) – ulcerative colitis and Crohn's disease are immune mediated diseases of the intestine where inappropriate T cell reactivity to constituents of the normal intestinal flora may be of etiological significance. Although no specific bacterial species have been directly associated with the pathogenesis of human IBD, analysis of the luminal enteric flora has revealed alterations in the composition of the bacterial species compared to healthy individuals. In Crohn's disease, concentrations of bacteroides, Eubacteria and *Peptostreptococcus* are increased, whereas bifidobacteria numbers are significantly reduced. Furthermore, in ulcerative colitis concentrations of facultative anaerobic bacteria are increased. These results indicate that in the setting of intestinal inflammation, bacterial populations can be significantly altered and that there may be reductions in the numbers of bifidobacteria or lactobacilli which are considered healthy or potentially beneficial components of the normal flora.

In this study, the potential benefits of administering a combination of the probiotic strains *Lb. salivarius* UCC118 and *Bifidobacterium longum infantis* UCC35624 in modifying the effects of IBD were evaluated in a laboratory model. This model was developed through the transfer of spleen- or lymph node-derived $CD4^+$ T lymphocytes, expressing high levels of $CD45RB^{high}$, from normal mice into immunodeficient SCID (Severe Combined ImmunoDeficient) mice. The recipient mice subsequently suffer from a wasting disease of chronic intestinal inflammation, most severe in the colon (Morrissey et al. 1993; Aranda et al. 1997).

The results of this study demonstrated that oral administration of *Bifidobacterium* strain UCC35624 combined with *Lb. salivarius* UCC118 significantly reduced disease severity as manifested by reduced weight loss (Figure 3), improvement of colon pathology and markedly improved appearance of the mice over a six week period. All the control mice developed a chronic wasting disease which was also observed in the mice administered a non-probiotic dairy product (Figure 3). The significance of these results lie in the fact that while bifidobacteria are one of several predominant culturable bacteria present in the colonic microflora, their functions in the colon have not been completely elucidated. However, it has recently been found that patients suffering active Crohn's disease have significantly less recoverable bifidobacteria in their faeces compared to healthy individuals and that this reduction in bifidobacteria numbers was observed to be directly correlated with decreased levels of β-D-galactosidase (produced by bifidobacteria) activity (Favier et al. 1997). Therefore, the data presented

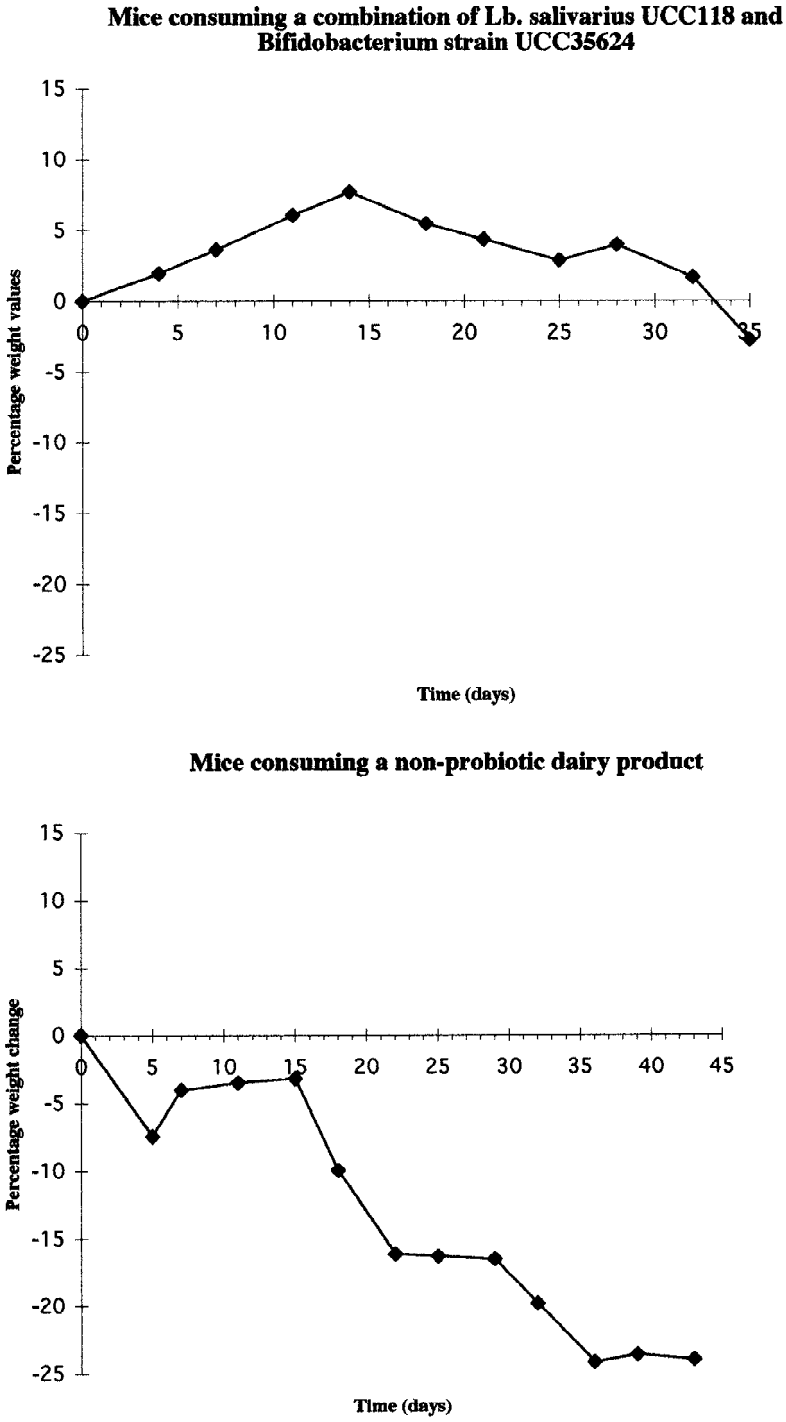

Figure 3. Assessment of weight loss in CD45RB^{high} SCID (Severe Combined ImmunoDeficient) mice consuming a probiotic combination of *Lb. salivarius* subsp. *salivarius* UCC118 and *Bifidobacterium longum infantis* UCC35624 compared with those fed a non-probiotic dairy product.

here support suggestions proposed in other studies (Bartram et al. 1994) that strains of bifidobacteria may play important roles in maintaining a balanced healthy intestinal microflora.

Feeding studies involving healthy human adults

Following the successful completion of the mouse feeding trial (Murphy et al. submitted), an eighty-volunteer, double-blinded, placebo-controlled, ethically approved feeding trial was performed which compared the efficacy of two standard food systems (fermented milk and fresh milk) in delivering *Lactobacillus salivarius* strain UCC118 to the human gastrointestinal tract. The results of this trial have indicated that both fresh milk and yoghurt are effective vehicles for the bacterial strain (Morrissey et al. unpublished observations). In addition, in a proportion of volunteers (<10%) significant numbers of strain UCC118 were still detectable in faeces 3 weeks after cessation of its administration, indicating that the strain was capable of persisting within the human GIT (Morrissey et al. unpublished observations). Interestingly, strain UCC118 did not obviously influence the numbers of other lactobacilli in the gut, but did cause significant alterations in the numbers of excreted clostridia and enterococci. Notably, these results correlate well with those observed by Spanhaak et al. (1998) upon completion of a study assessing the survival of the commercially-available probiotic strain *Lactobacillus casei* Shirota during passage through the gastrointestinal tract of healthy human males. However, while the *Lb. casei* Shirota strain was also found capable of surviving transit through the human intestinal environment, in this feeding study a significant increase was observed in the numbers of excreted faecal-borne bifidobacteria following four weeks of probiotic intake. In addition, it was found that the numbers of *Clostridium* and *Enterococcus* were not significantly influenced by consumption of the *Lb. casei* Shirota probiotic product (Spanhaak et al. 1998).

Several immunological parameters were assessed during the completion of both of the described human feeding studies. During the administration of *Lb. casei* Shirota there were no distinct effects on assessed immune responses (which included natural killer cell activity; phagocyte functions; delayed-type hypersensitivity; and cytokine and humoral parameters) (Spanhaak et al. 1998). Analysis of immune parameters in the *Lb. salivarius* UCC118 feeding study demonstrated that macrophage phagocytic activity also remained unchanged over the period of probiotic consumption, while granulocyte phagocytic activity significantly increased in those volunteers consuming yoghurt-borne strain UCC118. Systemic antibody titres (both total antibody levels and antibodies specific to strain UCC118) remained constant. In addition, levels of systemic IL-1α, IL-1β, IL-2 soluble receptor, IL-6, TNFα and IFNγ remained unaltered throughout the trial period, indicating that consumption of *Lb. salivarius* strain UCC118 did not induce significant inflammatory responses in healthy subjects. Mucosal IgA antibodies titres, specific to the probiotic strain, increased significantly in volunteers consuming the probiotic yoghurt product, while total IgA levels remained unchanged. Thus, consumption of strain UCC118 in a fermented dairy product resulted in stimulation of the mucosal immune system, but did not appear to induce systemic responses.

Conclusions

In this report, we have attempted to describe the logical criteria adopted for *in vitro* selection of probiotic bacteria. However, given that the human gastrointestinal tract is a complex and hostile environment, we acknowledge that it is unlikely that a single probiotic bacterial strain will have the capacity to influence the microbial ecology of the host and, by doing so, beneficially affect lactose intolerance, the incidence of diarrhoea, mucosal immune responses, levels of blood cholesterol, and the induction of cancer. More likely, we feel that these effects will require the introduction of combinations of strains, such as those described above in relation to the prevention of weight-loss in diseased mice. Furthermore, it is essential that these probiotic strains are not developed as individual entities but rather as the active ingredients of the food products which are ultimately intended for human consumption.

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